Pattern Recognition Receptors, Immune Proteins, and NF-κB Signaling Regulate Behaviors Associated With Aging Phenotypes

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PATTERN RECOGNITION RECEPTORS, IMMUNE PROTEINS, AND NF-κB SIGNALING REGULATE BEHAVIORS ASSOCIATED WITH AGING PHENOTYPES

by

Nicholas William DeKorver

A DISSERTATION

Presented to the Faculty of the University of Nebraska Medical Center Graduate College in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Pharmacology and Experimental Neuroscience Graduate Program

Under the Supervision of Professor Stephen J. Bonasera

University of Nebraska Medical Center
Omaha, NE

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Abstract

ROLES FOR PATTERN RECOGNITION RECEPTORS, IMMUNE PROTEINS, AND NF-κB SIGNALING IN REGULATING BEHAVIORS ASSOCIATED WITH AGING PHENOTYPES

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University of Nebraska Medical Center, 2017
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The aging process is accompanied by functional impairments, including reduced locomotor function, fragmentation of active states, and alterations in energy balance. Our lab has demonstrated that immune proteins are increased in specific regions of the mouse brain that correlate with strain specific deficits. These immune proteins include toll-like receptors (Tlr), class I major histocompatibility complex proteins (MHC I), and complement proteins. There is an increasing appreciation for the role of immune proteins in neurodevelopment; however, their involvement in age-associated deficits is poorly understood. Here, we present data demonstrating that 1) activation of a specific immune receptor (Tlr2) leads to changes in cell signaling that may underlie age related functional deficits, and 2) loss of specific immune proteins (Tlr2 and C3) and mutation of MHC I H2-Kb lead to impairments in behaviors commonly effected by aging.

First, we present data demonstrating that activation of toll-like receptor 2 by a synthetic agonists leads to activation of NF-κB signaling in cerebellar granule cells. Our data, among others, suggests that NF-κB signaling is dysregulated in the aging brain. Additionally, we suggest that accumulation of amyloid β in the aging brain may act as an endogenous agonist for Tlr2 activation. This data reveals that a less appreciated pathway, neuronal immune receptor signaling, may play a role in age related NF-κB dysregulation.
Second, analysis of mice lacking specific immune proteins revealed that loss of complement protein 3 leads to deficits in locomotor function characterized by reduced gait speed and markers of gait ataxia. Additionally, loss of complement 3 leads to changes in cerebellar granule cell synapse density and excitability in vitro. Loss of toll-like receptor 2 results in consolidation of active and inactive states in young mice, suggesting that toll-like receptor 2 may play a role in hypothalamic functions. Mutation of major histocompatibility complex isoform H2-Kb leads to a progressive obesity phenotype characterized by reduced activity and deficits in orexin neuron function. Collectively, this data demonstrates the involvement of immune proteins in regulating behaviors often disrupted in aging, including locomotor function, active state regulation, and energy balance.
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Overall Introduction

Aging is characterized by functional deficits across multiple organ systems that lead to significant impairments in normal function. Deficits in several central nervous system (CNS) mediated functions including locomotion, metabolism, and cognition are associated with aging even in the absence of explicit neurodegenerative disease. Functional deficits in mobility are linked to increased institutionalization and death in community dwelling adults\(^1\). It was found that disturbances in mobility defined by abnormal gait effect upwards of 35% of adults between 70 and 99 years of age with roughly half described as neurologic gait disturbances\(^1\). Despite the large clinical burden, a definitive neurologic and/or molecular mechanism underlying age related gait and mobility disturbances has not been identified.

Similarly, processes associated with aging can influence an individual’s maintenance of energy balance resulting in unintentional weight loss or weight gain. Roughly 15% of older adults experience unintentional weight loss in the absence of cancer, gastrointestinal disease, or stroke\(^2\). Unintentional weight loss and the inability to maintain healthy weight are key characteristics of frailty; a geriatric condition associated with increased health care complications and poorer survival rates\(^3\). On the opposite side of the spectrum, the prevalence of obesity in older demographics of both sexes and all races is increasing\(^4,5\). Obesity leads to increased risk of heart disease, diabetes, coronary artery disease, and even certain forms of cancer\(^6-8\). The relationship between obesity and mortality in the elderly population remains controversial, but an independent association between obesity and all-cause mortality has been shown in adults\(^9\). Much of the debate around mortality risk and obesity results from arguments on how to define obesity and stratify risk factors in the complex nature of aging physiology. What the data does clearly demonstrate is that changes in weight, either gains or losses are a strong predictor of mortality\(^10\). A 15 year follow up study of males aged 40-59 at the start of the
study, demonstrated reduced mortality rates for individuals that maintained a steady weight compared to those with weight gain or fluctuation\textsuperscript{11}. Additionally, an independent study demonstrated that even modest weight loss in elderly adults increases mortality risk\textsuperscript{12}. While peripheral mechanisms, diet, and lifestyle play large roles in energy balance, changes in CNS function underlying energy balance maintenance likely contribute heavily to these phenotypes.

Our lab has previously demonstrated that aged mice recapitulate many of the deficits that occur in human aging, and that these deficits are tied to distinct genetic backgrounds\textsuperscript{13}. Specifically, we tested cohorts of young (2-3 mo.), middle aged (12-13 mo.), and aged mice (21-24 mo.) from two strains (C57BL/6J and BALBc) generated from distinct breeding lineages\textsuperscript{14} to assess age-related functional impairments. Behavioral assessment was completed using our custom state-of-the-art home cage monitoring system (HCM). The HCM system functions to automate the quantitative characterization of mouse behaviors within their home cage environment with high spatial (within 0.5 cm) and temporal (within 1 ms) resolution for extended periods of time within no direct human interaction. Using this system we classify bouts of feeding, drinking, forward locomotion, and non-forward locomotion into active and inactive states throughout the circadian light and dark cycles. Following classification, we can assess for genotypic differences in up to 665 distinct measures of feeding, drinking, motor, and circadian activities. Furthermore, we can determine time budgets for control and mutant animals to assess the percentage of time spent in active or inactive behaviors during an average day. The HCM system has been employed in multiple institutions and behavioral data gathered for C57BL/6J controls are highly similar regardless of location or investigator, demonstrating the reproducibility of this platform. Specific methods for
the HCM system and data analysis can be found in the subsequent chapters, and the full methodology can be found in Goulding et al., 2008\textsuperscript{15}.

Our assessment of young (2-3 mo.), middle aged (12-13 mo.), and aged mice (21-24 mo.) C57BL/6J and BALBc mice using the HCM demonstrated remarkable strain specific function deficits compared to young mice of the same strain. Aged C57BL/6J mice have significant deficits in locomotor function evident by reduced overall daily locomotion, reduced locomotor bout onsets, increased bout duration, and characteristics of gait ataxia\textsuperscript{13}. C57BL/6J mice were independently found to exhibit age-dependent motor dysfunction evident by significantly reduced performance on wire hang task, reduced latency to fall on rotarod assessment, decreased locomotor activity in open field, and slower moving speeds\textsuperscript{16}. Interestingly, the oldest group of mice in the previous study was considered middle aged (8-12 mo.) in our analysis suggesting that with increasing age impairments become even more pronounced. Balance beam testing of C57BL/6J mice also demonstrates age-dependent locomotor dysfunctions both with and without vestibular stimulation evident by increased foot slips and total time to traverse the beam in aged mice (27-28 mo.)\textsuperscript{17}. It can be concluded from this data that C57BL/6J mice demonstrate age-associated deficits in locomotion, indicating potential disruption of CNS pathways responsible for organization and completion of motor functions.

Aged BALBc mice did not demonstrate any deficits associated with locomotor function. Conversely, aged BALBc mice exhibited significant dysregulation of energy balance. Normal aging is characterized by maintenance of body weight, and decreased basal metabolic rate, activity, and food consumption. Both C57BL/6J and BALBc mice experienced age-associated weight loss when compared to middle aged controls. However, each strain responds to the weight loss differently. C57BL/6J mice have reduced activity levels and increased chow consumption, likely in effort to regulate body
mass. Whereas, BALBc mice show no change in activity and reduced chow consumption, which may underlie significant weight loss observed with aging. Overall, it can be concluded from this data that aged BALBc mice fail to compensate for age related weight loss, signifying potential deficits in pathways that control energy balance.

In efforts to elucidate potential mechanisms underlying the age related functional deficits observed in C57BL/6J and BALBc mice through unbiased means, our lab performed microarray analysis of tissues associated with motor and metabolic functions. Microarray analysis of whole cerebellar and hypothalamic tissues in young, middle aged, and aged C57BL/6J and BALBc mice demonstrated strain and brain region specific changes. Within the C57BL/6J cerebellum and BALBc hypothalamus (regions associated with observed deficits) there was significant upregulation (p < 0.001 for both regions) in transcripts important for immune/defense functions when characterized by ontology analysis (Figure 1). Further ontological sub classification demonstrated that these transcripts belong to specific functional categories including classical and atypical major histocompatibility complex I proteins (MHC I), pattern recognition receptors (PRRs), complement, and cell adhesion molecules with significant overlap between strains and regions (Figure 1). PRRs are classically known for their role in innate immune defense and recognition of pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs). PRRs include several subclasses including toll-like receptors, Nod-like receptors, RIG-like receptors, AIM2-like receptors, dectin receptors, and C-type lectin receptors among others. Analysis of aged human cerebellar tissue demonstrated similar upregulation of transcripts analogous to those increased in the C57BL/6J cerebellum with aging\textsuperscript{13}. Microarray analysis of the C57BL/6J hypothalamus and BALBc cerebellum demonstrated the site-specific nature of increased immune/defense transcripts. Ontology analysis of these regions showed increases in
Figure 1. Age-associated increase in C57BL6 cerebellum and BALB hypothalamus PRR expression. Top: Differential gene expression in the male BALB hypothalamus, n=7 young (2-3 mo), n=6 aged (21-24 mo) mice. Bottom: Differential gene expression in the male C57BL6 cerebellum, n=4 young, n=4 aged mice. In the hypothalamus young-to-aged comparison, 153 DEGs identified, 113 upregulated in aged mice. In the cerebellum young-to-aged comparison, 101 DEGs identified, 100 upregulated in aged mice. P values reflect multiple comparison corrections across all ontologies. RNA purified by standard methods, samples with A260/A280 ratio < 1.8 or 18S peak larger than 28S peak (per BioAnalyzer 2100) discarded secondary to contamination/degradation. Gene expression measured by Agilent whole mouse genome 4x44k microarray, candidate genes confirmed by RT-qPCR SyberGreen. DEGs determined by B statistics. Ontologies per OntoExpress (Draghichi et al., 2003). Panels colored in violet correspond to BALB hypothalamus, panels colored in yellow correspond to C57BL6 cerebellum, loci in red are common between the two regions.
transcripts related to signal transduction, development, and neuronal processes. Several independent studies have also demonstrated increases in immune/defense transcripts within different brain regions with increasing age\textsuperscript{18-21}. While data demonstrate clear increases in immune genes within the aging brain, the CNS roles of the proteins encoded by these transcripts are poorly understood. This may be partly due to the relative infancy of the field.

The historical perspective that the central nervous system resides in an immune privileged state, walled off from the peripheral immune system by a tightly regulated blood brain barrier, has been repeatedly challenged and proven to be false. Acceptance of this fact has led to vast growth in the knowledge of immune protein functions in the CNS, but a large portion of this work is focused on neurodevelopment and neurodegeneration. Our data, along with select others suggests that immune proteins and pattern recognition receptors are increased in specific regions of the aging brain and play a role in the development of age-associated functional deficits. Many of the immune proteins upregulated in the aging brain signal through common pathways, many involving nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Activation of NF-κB can alter CNS functions though its impact on synapse and cytokine regulation. Therefore, much of the mechanistic work presented in this dissertation focuses on NF-κB signaling.

The works presented in this dissertation are the culmination of our attempts, so far, to elucidate the role of specific immune proteins (complement 3, toll-like receptor 2, and MHC Class I isoform H2-Kb) in the regulation of behaviors disrupted by the aging process (metabolism, locomotion, and active/inactive state organization). If behavioral phenotypes were identified, we performed follow up experiments in attempts to understand potential mechanisms underlying these changes. Chapter 1 will outline in
detail our work to determine how PRR activation can lead to signaling changes in neurons that may impact functional outcomes. Chapters 2-4 will each focus on a specific immune protein, the behavioral workup for animals deficient in that protein, and potential mechanisms for the behavioral findings. Additional immune proteins have been tested, but as much of this work is in its infancy, only the more mature projects are being presented as part of this dissertation.
References


Chapter 1: Increased Expression and Signaling of Pattern Recognition Receptors: Potential Mechanisms of age related functional deficits.

Introduction

Age-Associated Deficits in Mobility

The Administration on Aging (AOA) estimates the number of Americans greater than 65 years of age will increase to 72.1 million by 2030 making up 19% of the U.S.A population\(^1\). Functional impairments that accompany aging have complex etiologies spanning multiple organ systems. In humans, these declines significantly alter an individual’s capacity for independent living, leading to increased need for caregivers and decreased quality of life. In particular, mobility deficits are linked to increased institutionalization and death in community dwelling adults\(^2\). Slow gait speed (< 1 m/s) in community-dwelling older adults (age ≥70) increased self-reported difficulties in home-based activities including walking inside home, climbing and descending stairs, as well as in several gait-independent motor tasks\(^3\). Additionally, slower gait speeds in older adults (age ≥ 65) have been associated with lower survival rates\(^4\). Deficits in mobility can arise from complications within multiple organ systems including the musculoskeletal system, peripheral nervous system, and central nervous system. However, over half of reported gait disturbances that plague one third of all adults between the ages of 70 and 99 are deemed neurogenic\(^2\). Neurologic gait impairments are extremely difficult to treat, as limited interventions exist. With inadequate interventions, this clinical burden will continue to increase as the elderly population grows. Due to the large clinical impact of mobility disturbances in elderly individuals and the high percentage categorized as having CNS involvement; we set out to investigate molecular mechanisms of age-related mobility impairments.
Our lab previously established a mouse model of age-related locomotor deficits characterized by decreased overall locomotor activity, reduced gait speed, increased locomotor bout duration, and markers of gait ataxia\(^5\) (General Introduction Figure 1). Proper locomotor function is dependent on several CNS pathways primarily involving the cortex, basal ganglia, brainstem, spinal cord, and cerebellum. CNS lesion studies have aided in understanding the roles of these specific regions. In the context of aging, disturbances in balance, gait, and coordination of movements indicate deficits in cerebellar function.

**Cerebellar Anatomy, Function, and Age-Associated Changes**

The cerebellum resides in the posterior fossa, comprising only 10% of the CNS volume, but containing over 50% of all neurons. Grossly, the cerebellum consists of a tightly folded cortical surface with underlying white matter, several deep nuclei, and a central ventricle. The anatomy of the cerebellum can be broken down by phylogenetic criteria into the vestibulocerebellum (eye movements and balance), spinocerebellum (body and limb movement), and cerebrocerebellum (movement planning and sensory evaluation). On the cellular level, the cytoarchitecture of the cerebellum is organized into a laminar structure. The outermost molecular layer contains the axons of cerebellar granule cells, dendrites of Purkinje cells, and two types of inhibitory interneurons, basket cells and stellate cells. The single cell thick Purkinje cell layer lies between the molecular layer and the granule cell layer and contains the cell bodies of large, highly arborized Purkinje cells. Deep to the Purkinje cell layer is a small population of Lugaro cells. The innermost cerebellar granule cell layer contains densely packed cell bodies of cerebellar granule cells, unipolar brush cells, and Golgi cells. Finally, imbedded in the white matter tracks are the deep cerebellar nuclei. Unlike many other regions of the CNS, cerebellar connectivity is organized on a fixed set of rules around the granule cell, Purkinje cell,
deep nucleus loop. Incoming mossy and climbing fibers, carrying sensorimotor information, form excitatory synapses with cerebellar granule cells and Purkinje cells, respectively. Additionally, both of these projections synapse with neurons in the deep cerebellar nuclei. Cerebellar granule cells project axons to the molecular layer where they bifurcate and extend in opposite directions running parallel to one another forming excitatory synapses with Purkinje cell dendrites. Purkinje cells, the sole output for the cerebellum, form inhibitory connections with the deep cerebellar nuclei, which project out to premotor areas.

Historically, the cerebellum has been described for its role in the maintenance of balance and posture, and the coordination of voluntary movements. However, over the past several decades the cerebellum has also been shown to play functional roles in motor learning, cognition, emotion, and behavior. While the cerebellum is not necessary for the initiation of movement events, damage to the cerebellum leads to dysmetria, or erratic movements that vary in size and direction. Furthermore, lesions to the cerebellum do not often lead to overt loss of motor function, but instead result in deficits in specific aspects of motor function including loss of coordination, balance, tremor, and gait ataxia. An ataxic gait is characterized by veering movement paths, irregular foot placement and trajectories, as well as discoordination of joint movements.

Mechanisms underlying gait disturbances in the elderly are poorly understood. Overall, the aging process results in minor morphological changes in the majority of cerebellar regions in humans, predominately characterized by reductions in white matter and Purkinje cell volumes with no overall changes in cell number. However, within cerebellar subregions, specifically within the anterior lobe, a selective loss of cerebellar granule cells and Purkinje cells has been noted with increasing age. Within rodent models, there is no measured loss of cerebellar granule cell density with aging. The
absence of large anatomical disruption suggests that age related mobility deficits might occur as a result of alterations at the level of cellular function. Functional changes may be a result of white matter or cell density changes undetectable with current imaging modalities or changes at the level of the synapse.

**Age-Associated Changes in Synaptic Function**

On the neuronal level, deficits in synaptic density, function, and/or regulation can all negatively impact downstream signaling and result in functional deficits. Aging has been associated with synaptic changes in multiple regions of the brain, including the cerebellum. These synaptic changes include increased, but likely less functional vesicular glutamate 1 (Vglut1) positive puncta in the cerebellar granule cell layer (Bonasera, et al., 2017). It has also been found that loss of boutons on Purkinje cell dendritic networks accompany age-associated Purkinje cell involution and cell loss. Regeneration of neuronal function and synaptic connectivity is coordinated by complex signaling pathways, and dysregulation within those pathways has profound effects on downstream function. Several pathways including NF-κB, mTOR, SIRT1, IGF-1, and P53 have been implicated in the aging process. However, a cross species study using motif module mapping in promoters of genes upregulated with aging demonstrated that NF-κB gene regulation is the most widely conserved pathway underlying mammalian aging. NF-κB signaling is involved in both synaptic and immune regulation. Analysis of our aging gene expression data demonstrated that 16% of the cerebellar genes differentially expressed between young and old C57BL/6 mice with locomotor impairment are known NF-κB targets; this value rises to 33% by including NF-κB target predictions by TRANSFAC (ver 7.0 Public 2005). Therefore, we chose to further assess NF-κB signaling in cerebellar neurons as a potential mechanism for age-related functional deficits.
**NF-κB Signaling and Functions within the CNS**

NF-κB signaling proteins are present in the neuronal soma and synapse, and have an extended number of roles important for CNS functions\(^{21}\). NF-κB exists as hetero- or homo-dimers of Rel-family proteins (c-Rel, p52, p50, RelB, and RelA/p65). Each protein contains a Rel homology domain, a prototypical amino-terminal sequence of roughly 300 amino acids that mediate cytoplasmic retention, dimerization, nuclear translocation, and DNA binding\(^{17, 22, 23}\). The most characterized, ‘canonical’ combination is a heterodimer of p65 and p50\(^{24}\). While the majority of NF-κB signaling proteins are present in neurons, it was demonstrated that mice genetically lacking p65 fail to express other subunits at the synapse\(^{25-27}\). This suggests that p65 is the predominant driver of synaptic NF-κB expression. In a non-activated state p65/p50 is held inactive in the cytoplasm by inhibitory κB regulatory proteins (IκBs)\(^{28}\). IκBα prevents NF-κB translocation by physically masking p65 nuclear localization signals. NF-κB pathway activation, by one of over 150 inducers, causes IκB phosphorylation and degradation, allowing p65/p50 to translocate to the nucleus and regulate transcription\(^{28, 29}\). An additional level of NF-κB regulation occurs through post-translational modifications to p65, p50, and IκB, primarily through phosphorylation. Phosphorylation of NF-κB family proteins can act to either stabilize inactive or active forms, thereby regulating both activation and repression of NF-κB signaling\(^{30}\). The majority of NF-κB regulation studies have been performed in cells other than neurons; therefore the specific mechanisms guiding the selectivity and duration of activation in the CNS are not completely understood.

Early studies of NF-κB signaling in neurons provided evidence for high basal (constitutive) activity based on antibody staining and reporter assays of glutamatergic neurons in the hippocampus and cortex\(^{25}\). These findings were later extended to other brain regions including the amygdala, hypothalamus, olfactory bulbs, and cerebellum\(^{31, 32}\).
Several studies demonstrated that constitutive neuronal NF-κB activity is likely a result of glutamate signaling and excitatory stimulation\textsuperscript{27, 33, 34}. Inducible NF-κB activity in neurons has been historically debated. However, inducible NF-κB proteins have been identified at the synapse, where they traffic back to the nucleus in response to neuronal activity from glutamate stimulation and regulate activity dependent transcription\textsuperscript{27, 35}. Accordingly, NF-κB family proteins c-Rel\textsuperscript{36-38}, RelA/p65\textsuperscript{27}, and p50\textsuperscript{39} have all been implicated in learning and memory. Genes with NF-κB transcriptional response elements are selectively enhanced in response to learning tasks in mouse models, suggesting NF-κB mediated transcriptional regulation is critical for synaptic changes that underlie plasticity\textsuperscript{37, 40}. Studies modulating NF-κB activity through exogenous expression or genetic deletion of NF-κB proteins have revealed marked synaptic changes. Exogenous expression of NF-κB within hippocampal pyramidal neuron cultures results in an increase in excitatory synapse density, but no change in inhibitory synapses\textsuperscript{41}. Loss of p65 expression leads to a reduction in dendritic spines in early hippocampal development. Accordingly, NF-κB activity is the highest before and during rapid synapse formation and decreases following synapse formation\textsuperscript{41}. However, mature neurons can increase NF-κB signaling in response to demand for synaptic plasticity. Mature hippocampal neurons stimulated with bicuculline or 17β-estradiol show increased dendritic spine and synapse numbers similar to experience dependent plasticity in mammals\textsuperscript{42}. In the absence of p65 this response is completely absent, demonstrating the necessity of NF-κB signaling in synapse remodeling within adult neurons\textsuperscript{41}. Much of the work on NF-κB stems from studies of hippocampal and cortical neurons. The expression pattern and functions of NF-κB signaling in cerebellar neurons share some similarities, but also appear to have some distinction.

Cerebellar NF-κB signaling shows temporal differences in activity and induction that appear to differ from other brain regions. In rats, constitutive NF-κB is expressed in the
early developing cerebellum at post-natal day 5 (P5), but constitutive expression is lost by P7, where it shifts to inducible expression controlled by glutamate. In mice, cerebellar granule cell cultures generated from older mice (8-10 days old) show constitutive activity, whereas cultures generated from younger mice (2-3 days old) show glutamate inducible NF-κB expression. Therefore, inducible activation appears to be temporally regulated during development, as glutamate also fails to activate NF-κB in cerebellar granule cells in adult mice. Functionally, NF-κB activation in cerebellar granule cells is important for cell survival. Overexpression of NF-κB subunit p65 protects cerebellar granule cells from apoptosis induced by low potassium levels. In parallel, increased expression of IκBα leads to increased apoptosis even in high potassium conditions. Other studies have revealed that long lasting NF-κB activation by exposure to sub-toxic levels of amyloid β protect cerebellar granule cells from apoptosis upon exposure to higher doses of toxins. The role for NF-κB in regulating cell survival mechanisms has been extended to protection against excitotoxicity and oxidative stress in other neuronal cell types. While there are clear protective roles of NF-κB activity, dysregulation of NF-κB activation has been described in neuroinflammation, Alzheimer’s disease, Parkinson’s disease, and age-associated hypothalamic decline. This argues that tight regulation of NF-κB signaling is critical for proper function.

**Regulation of NF-κB Signaling**

Regulation of NF-κB signaling is extraordinarily complex, due to the vast array of activating pathways. In the CNS, NF-κB can be activated by inflammatory cytokines (TNF-α) in response to tissue injury, nerve growth factor (NGF), Ca²⁺, methyl-D-aspartate (NMDA), reactive oxygen species (ROS), β-amyloid, and insulin-like growth factor (IGF-1) among others. Additionally, pattern recognition receptors (PRRs) and immune protein activation can lead to increased NF-κB activation, yet the
understanding of this mode of activation in neurons is poorly understood. In regards to activation by immune signaling, the mechanisms of regulation become even more complicated as many PRRs and immune proteins are transcriptionally regulated by NF-κB activity creating the potential for the synthesis of further modes of NF-κB activation. The primary mechanism for regulating NF-κB activity is through autoregulation by IκB proteins. Activation of NF-κB leads to the transcription of IκB, which functions to shuttle p65 out of the nucleus and hold it inactive in the cytoplasm, thereby resolving activation. Further regulation occurs at the level of post-translational modifications to NF-κB family proteins including phosphorylation, acetylation, glycosylation, ubiquitination, and sumoylation. Specific post-translational modifications relevant to our studies will be discussed in the results and discussion sections.

**Dysregulation of NF-κB Signaling in the Aged Cerebellum**

Multiple lines of evidence point to NF-κB dysregulation in the aging cerebellum, including (1) increased immune gene expression, (2) increased granule cell layer excitatory synaptic density, (3) reduced global excitatory function, (4) the absence of overt cell loss, and (5) functional impairments. To confirm potential dysregulation of NF-κB signaling in the aging cerebellum, we performed an unbiased phosphorylated protein array to assess protein and phosphoprotein levels (1318 site-specific antibodies) from greater than 30 signaling pathways. Additionally, we generated a protocol for the isolation and culture of neurons, enriched for primary cerebellar granule cells (pCGCs). This culture system was then characterized for cell purity, the expression of NF-κB signaling molecules, PRRs, and immune molecules, as well as for synaptic development. With the goal of detailing a signaling pathway from PRR activation to NF-κB signaling in cerebellar neurons, we needed to select a single PRR to serve as the initiation point for our pathway. Toll-like receptor 2 was chose for the following reasons, (1) Tlr2 expression is increased in
the aged cerebellum, (2) Tlr2 has a well characterized, commercially available, and specific synthetic agonist (Pam3CSK4), (3) our lab had access to Tlr2 deficient mice to serve as controls. Therefore, we stimulated pCGC cultures with Pam3CSK4 and measured induction of NF-κB activity. Measuring activation of NF-κB signaling can be achieved at multiple levels using current techniques that investigate independent stages of activation from DNA binding, transcriptional control, translocation, and signaling protein phosphorylation. In efforts to provide the most detailed assessment of NF-κB activation in response to PRR stimulation, we employed multiple methods listed above including measuring translocation, NF-κB protein phosphorylation, NF-κB reporter assay, and quantifying downstream transcriptional products. The works presented in this chapter are the summary of our investigation into mechanisms that may underlie age-related dysregulation of NF-κB signaling in the cerebellum.

Methods and Materials

Animals

All studies were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Breeder triads (2♀ and 1♂) of wild-type C57BL/6J mice were purchased from Jackson labs (Stock #000664) and mice generated from these breeders were used for all studies, with exception of phosphoprotein arrays. Breeder triads were replaced with a new cohort purchased from Jackson after roughly 1 year. For phosphoprotein arrays adult (12 month) and aged (20 month) C57BL/6J mice were obtained from Hilltop Labs (C57BL/6Hla®CVF®). Hilltop Labs was a new vendor, so per University of Nebraska Medical Center policy, the mice were quarantined for 2 months until adult mice were 14 months of age and aged mice were 22 months of age. At this time mice were processed for downstream analysis. For all other mice, upon arrival at UNMC, mice were housed (< 5 per cage) in microisolator cages in rooms with a 12:12
lighting cycle (lights on 0600 CST), provided with chow (Envigo Teklad #7012) and water ad libitum. Mice purchased for breeding were allowed to acclimate for at least one week before being transferred to cages for breeding. To test specificity of Pam3CSK4 → Tlr2 → NF-κB signaling, mice lacking Tlr2 receptor (Tlr2<sup>tm1Aki</sup>) were used. Mice were housed and bred using same detailed strategy as above (For full details on these mice, see Chapter 3 Methods).

**Phospho Explorer Antibody Array**

Aging frequently impacts cell signaling pathways, which are vital to proper neuronal function. The components of these signaling pathways, their alterations in specific groups of neurons, and role in cellular aberrations are of significance, yet remain unclear. Many proteins in cell signaling are regulated by phosphorylation via kinases that either inhibit or enhance activity. Therefore, assessing protein phosphorylation status within a given pathway can offer insight regarding its activation state. Our previous data suggests dysregulation of NF-κB signaling based on unbiased microarray analysis of the cerebellar transcriptome. However, as aging affects multiple pathways, we felt it necessary and more informative to take a similar unbiased approach when assessing signaling pathway activation. To assess for age related changes in protein phosphorylation we purchased Phospho Explorer protein microarrays from Full Moon Biosciences (Sunnyvale CA, Catalog # PEX100). Manufacturer array validation suggests 92% reactivity for mouse proteins across 1318 site-specific and phosphospecific antibodies from greater than 30 signaling pathways. Each slide contains 2 technical replicates per Ab, with positive (GAPDH, β-actin) and negative controls. We obtained samples for 3 biological replicates per cohort of old (22-month) and adult (14-month) C57BL/6 mice.
To isolate cerebellar tissues, mice were euthanized with CO$_2$ followed by rapid decapitation and removal of the brain. The cerebellum and meninges were removed under a dissection microscope with fine microdissection forceps in ice cold phosphate buffered saline (PBS) (Hyclone #SH30256). Dissected cerebellar tissue was then flash frozen in small conical tubes in a dry-ice and ethanol slurry. All samples were stored at -80° C until further processing. To prepare cerebellar lystate for protein arrays, samples were thawed on ice and 20 mg of cerebellar tissue was removed for lystate preparation. Remaining tissue was once again flash frozen and stored at -80° C for future use. Thawed tissues were washed by vortexing in ice cold PBS five times. Protein lysates were extracted using the buffer provided in the kit with the addition of phosphatase inhibitors (ThermoFisher #78420) and protease inhibitors (Sigma #P8340) at 1:100 dilution. Briefly, 100 µl of ice-cold extraction buffer was added to tissue along with one vial of metal beads. To disrupt tissues, samples were taken though 5 cycles consisting of 30 second vortexing followed by 10 minute incubation on ice. Following disruption, lysates were centrifuged at 10,000 G for 5 minutes at 4° C. The supernatant was transferred to a new tube and centrifuged again at 18,000 G for 15 minutes at 4° C. The resulting supernatant was then collected in a clean tube and 100 µl of the lysate was added to a pre-prepared purification spin column. Columns were centrifuged at 750 G for 2 minutes at 4° C and the remaining lysate was assessed for quality and concentration (by spectrophotometry at A280). Low quality, cloudy, or unclear lysates can result in low labeling efficiency and high background. High quality and clarity lysates show two clearly separated peaks, one between 200-230 nm and a second between 240-270 nm. All samples used for protein arrays had two distinct peaks and no noticeable opaqueness. Lysate concentration was determined by bicinechinonic acid (BCA) assay (Pierce Catalog # 23225) according to manufacturer protocol. After quality control and quantification lysates were frozen on dry ice and ethanol and stored at -80° C until further use.
Lysates were allowed to thaw on ice, and 70 µg of lysate was biotinylated using biotin reagent and N,N-Dimethylformamide provided in the kit. Antibody array slides were brought to room temperature, dried, and blocked for 45 minutes in provided blocking buffer. Slides were rinsed extensively with ultrapure water obtained from a Milli-Q system. Protein lysate was then coupled to antibody array slides for 2 hours at room temperature. Slides were washed extensively again. For the detection of proteins, slides were incubated with Cy-3 streptavidin (Sigma #56402) at 0.5 mg/ml in detection buffer for 20 minutes at room temperature. Slides were washed extensively again, and quickly dried using bench top air outlet with an inline flow regulator. All slides were then sent to Full Moon Biosystems for image collection. Images received from Full Moon Biosystems were then analyzed using GenePix Pro Microarray Analysis Software (GenePix 4000B, Molecular Devices, Sunnyvale CA), graciously supplied by the University of Nebraska Medical Center Genomics Core facility to generate fluorescent intensity values for each spot.

**Phospho Explorer Antibody Array Data Analysis**

To normalize intensity values across multiple slides, all values were divided by the median fluorescence value for all spots on the corresponding slide, per manufacturer’s recommendation. In addition to total and phosphorylated protein levels, we wanted to assess the ratio of phosphorylated to total protein for each target. Therefore, we divided the total phosphorylated protein value by the total protein values to generate a ratio for each phosphorylation site-specific antibody. The resulting normalized values for total, phosphorylated, and the phosphorylated protein ratios were analyzed using by two-tailed student t-tests assuming unequal variance with an alpha of 0.05. We used this data to generate volcano plots of differentially expressed proteins between middle-aged adult and aged cerebellar samples for total, phosphorylated, and phosphorylation ratios for
each protein plotted as significance ($-\log_{10}(p\text{-value})$) versus fold change ($\log_2(\text{ratio})$). Proteins with an alpha of $<0.05$ and a greater than 33% change from adult cerebellar expression are considered significant in volcano plot analysis. To determine biological themes present in the differentially expressed protein, phosphoprotein, and phosphoprotein to total protein ratios, we analyzed differentially expressed protein lists with Panther (version 11.1)$^{60}$.

**Cerebellar Granule Cell Cultures**

To prepare cerebellar neuron cultures, post-natal mice (P0/1-P9) were rapidly decapitated, and the brain was removed and placed into cold calcium-magnesium free PBS in a petri dish on ice. For the majority of studies, P4-P6 mouse pups were used, as they yielded the most cells and best viability. For some early validation studies other ages were used and noted in the results. Brains were then transferred to a new petri dish with fresh ice cold PBS and the cerebellum was removed under a dissecting microscope. The white matter in the center of the cerebellum containing the deep cerebellar nuclei was removed leaving predominately cerebellar folia with a small amount of white matter connecting them. Meninges were removed in strips by carefully pulling meningeal tissue laterally along the surface of the brain using fine dissection forceps. Cerebellar tissue was then transferred to a new petri dish with ice-cold PBS (Hyclone #SH30256). This process was then repeated for additional brains with a maximum of 12 per procedure in efforts to minimize time from dissection to culture. Once all brains were dissected, each was segmented into small relatively uniform (1-2 mm) pieces using micro dissection scissors under a dissecting microscope. Using a 25 mL pipette the tissue pieces were transferred to separate 15 mL conical tubes holding 10 mL of cold PBS. As a wash step dissected tissues were allowed to sink to the bottom of the conical tubes. PBS was then aspirated from the tubes and 1 mL of a trypsin
solution (1200 µL of 2.5% Trypsin (Worthington Labs #3707), 33 µL 30% glucose (Fisher #D16), and 1767 µL of BME stock solution (240 mL Earls Balanced Salt Solution (EBSS), 2.5 mL Basal Medium Eagle vitamins (Sigma-Aldrich #B6891), 2.5 mL Basal Medium Eagle amino acid solution (Sigma-Aldrich #B6766), and 1 M NaHCO₃ to pH of 7.2-7.4) was added. Tissue was incubated at room temperature in trypsin solution for 3 minutes with occasional manual mixing to evenly distribute tissues in solution. Following incubation, trypsin solution was removed and 1 mL of DNase solution (150 µL of 1% DNase (Sigma #DN25), 183 µL 30% D-glucose, and 2817 µL of BME stock solution) was added to degrade any DNA released for cells damaged during dissection. The addition of DNase solution aids in downstream trituration, as it reduces tissue clumping. Using a P1000 pipette set to 750 µL the tissue was triturated until a single cell suspension was formed (trituration maximum of 15 repetitions). The single cell suspension was then centrifuged at 700G for 5 minutes at 4 °C. The DNase solution was then removed and 2 mL of cold PBS with 500 µL of DNase solution was added, followed by resuspension using a P1000 pipette as previously described. The single cell suspension in each tube was then filtered through a 40 µm nyl cell strainer (BD Falcon #352340) into new 15 mL conical tubes to remove any aggregated cells or tissue that failed to breakdown. After filtering, cells were pelleted by centrifugation at 700G for 5 minutes at 4 °C. The PBS/DNase solution was removed and the pellet re-suspended in 1 mL pre-warmed serum containing plating media (81.5 mL BME stock solution, 5 mL fetal bovine serum (Gibco #10100-139), 2.5 mL horse serum (Sigma-Aldrich #H1138), 50 µL pen-strep solution (Gibco #15140-122), 500 µL L-glutamine (Gibco #25030-081), 800 µl 30% glucose D-glucose (Sigma #G8270), and 50 µL of 1% DNAse solution. Cells density was quantified using a hemocytometer. For immunocytochemistry studies cells were plated at a density of 200,000 cells per 14 mm onto glass insert petri dishes (MatTek #P35G-0-14-C) or 24 well plastic bottom dishes (BD Falcon #351147).
western blot or RNA analysis cells were plated at 1,500,000 cells per well in 6 well plates (BD Falcon #353046). All plating surfaces were coated with 100 µM poly-D-lysine (Sigma #P7886 or #P6407) for 4-6 hours before cell isolation and washed twice with sterile culture grade water (Hycone #SH30529) before plating. Cells were incubated for 4-6 hours at 37 °C and 5% CO₂ to facilitate adherence. Serum containing media was removed and each coverslip or petri dish was gently rinsed with pre-warm serum free media (92.4 mL Low-Glucose DMEM (Life #11965-092), 2 mL of N2 supplement (Gibco #17502-048), 2 mL B27 supplement (Gibco #17504-044), 1.6 µl 20% sucrose (Fisher #BP22-01), 1 mL L-glutamine, and 100 µl Pen-Strep). Fresh pre-warmed serum-free media (2 mL) was then added to each well. After two days in culture, 5 µM AraC (Sigma #C1768) was added to inhibit the growth of dividing cells, primarily glia and endothelial cells. One half of the media was replaced with fresh serum free media on day 4 and then every half of the media was changed every three days.

**Pam3CSK4 Treatment of Cerebellar Granule Cell Cultures**

Cerebellar granule cell cultures were treated with Pam3CSK4 (molecular formula C₈₁H₁₅₆N₁₀O₁₃S), a synthetic triacylated lipoprotein that mimics the acylated amino terminus of bacterial lipoproteins. Pam3CSK4 (Invivogen #tlrl-pms) was diluted in sterile cell culture grade water (Hyclone #SH30529) at a concentration of 0.5 mg/mL and stored at -20 °C until further use. Pam3CSK4 has been shown to be a potent activator of NF-κB signaling through ligation and activation of Tlr1/2 heterodimers in multiple cell types. Cerebellar granule cell cultures were treated at a concentration of 1 µg per ml of media. All cultures were treated on day in vitro 6 (DIV6), but the duration of treatment varied depending on downstream analysis. To assess NF-κB activation pCGCs were treated at DIV4 and RNA was collected after 2, 8, 24, and 72 hours to quantify NF-κB transcriptional products. To demonstrate that downstream transcriptional products are
the result of Tlr2 induced NF-κB activity, pCGC cultures were treated with pyrrolidine dithiocarbamate (PDTC), a potent inhibitor of NF-κB activation. PDTC is thought to inhibit NF-κB activity by reversibly blocking phosphorylation and degradation of the IkBα inhibitory subunit, thereby preventing translocation of NF-κB subunits (p50/p65)\(^{61}\). pCGC cultures were pre-treated with 300 uM PDTC (Sigma #P8765, reconstituted in sterile water) for 30 minutes, followed by Pam3CSK4 treatment as detailed above. For Western blot analysis of NF-κB phosphorylation, pCGC cultures were treated with or without PDTC pretreatment and protein was collected after 2 hours post treatment. To determine the level of NF-κB activation by p65-GFP translocation, pCGCs were transfected and treated for 1 hour and processed for immunocytochemistry. NF-κB activation was also assessed using a fluorescent p65 reporter assay after 48 hours of Pam3CSK4 treatment.

**Cerebellar Granule Cell Culture Transfection**

In order to assess NF-κB activation by translocation of p65 or p65 reporter assay, pCGC cultures were transfected with corresponding plasmids. Transfection of pCGC cultures were carried out at DIV2 using lipofectamine 2000 (ThermoFisher #11668027) with 1.0 µg p65-GFP (Addgene #23255) or 1.0 µg p65-GFP-rpt (generous gift from Dongsheg Cai Laboratory\(^{62}\)) and 0.5 µg Tdtomato using manufacturers recommended protocol. Briefly, in one tube plasmid DNA was combined with DMEM (Life #11965-092) at the concentrations above to reach 25 µL per well. In a second tube, 2 µL lipofectamine per well was combined with 23 µL per well of DMEM. The tubes were mixed, incubated at room temperature for 15 minutes, and 50 µL of the mixture added to each well. TdTomato transfection served as a counterstain for unbiased identification of transfected cells.

**Immunocytochemistry**
Primary cerebellar neuron cultures were fixed 4% Paraformaldehyde/4% sucrose solution pre-warmed to 37 °C for 10 minutes at room temperature. Cultures were rinsed 3 times in PBS (Hyclone). To allow antibody access to intracellular protein epitopes, cell cultures were permeabilized with 0.1% triton X-100 for 10 minutes at room temperature. Following permeabilization cells were rinsed 3 times for 10 minutes in PBS then blocked with 5% BSA in PBS for 1 hour at room temperature. After blocking, primary antibodies were diluted in 1% BSA in PBS and incubated overnight at 4 °C. A primary and secondary antibody list containing the target, company, catalog number, and dilution for antibodies used in Chapter 1 is provided in Table 1. After primary incubation cells were rinsed 3 times in PBS, and incubated with secondary antibodies diluted in 1% BSA in PBS for 1 hour at room temperature. Cells were then washed 3 times with PBS for 10 minutes each. Nuclear counterstaining was completed with 4',6-diamidino-2-phenylindole (DAPI) in PBS for 1 minute. Cells were rinsed twice with PBS and then cover slipped using Prolong Gold or Diamond Antifade Reagent (Invitrogen P36934 or P36961). Mounting media was allowed to dry overnight in the dark and then samples were stored at -20°C until imaging.

**Primary Cerebellar Granule Cell RNA Extraction and Quality Assessment**

RNA was isolated from cerebellar granule cell cultures using an RNeasy minikit (Qiagen #74104). Briefly, wells were rinsed once in sterile PBS and 350 µL RLT Lysis Buffer was added. Wells were repeatedly rinsed with lysis buffer to ensure even cell disruption. Lysis buffer was then collected into an Eppendorf tube and manually disrupted using a tuberculin syringe by drawing buffer in and out of the syringe five times. The remainder of the protocol was performed as described in manufacturer protocols. RNA samples for developmental expression of PRRs and immune proteins were not processed with DNase digestion step. However, for samples from treated
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Table 1. List of antibodies used for Chapter 1 experiments
cerebellar granule cell cultures the DNase digestion step was added. We did not notice any quantifiable difference in RNA quality obtained from either process. RNA purity was assessed using a spectrophotometry (NanoDrop II, Thermo, Waltham MA) to measure A260/230 and A260/280 ratios. RNA degradation was assessed by micro gel electrophoresis (BioAnalyzer 2100, Agilent Technologies). Only RNA samples meeting minimum purity and quality were used for downstream analysis. Specifically, any samples with A260/A280 ratios less than 1.8 were deemed unsuitable for analysis secondary to RNA contamination. Additionally, any samples with larger 18S instead of 28S peaks, or RNA integrity numbers (RINs) < 7.0 were deemed unsuitable for analysis secondary to RNA degradation.

**Real-Time PCR Analysis**

One-Step RT-PCR was performed using Verso SYBR Green 1-Step kits (Thermo Scientific Catalog #AB4105) according to manufacture protocols with all samples run in triplicate. All reactions were processed with no enzyme and no template controls. All primers used were commercially available Qiagen Quantitect primers. A full list, including catalog numbers, for primers used in the work presented in Chapter 1 are provided in Table 2. RT-PCR data was analyzed for relative expression using the $2^{(\Delta\Delta CT)}$ method with normalization to Gusb as a housekeeping gene using Microsoft Excel. To quantify differences between culture days, one-way analysis of variance testing with Bonferroni post-hoc analysis was performed. For analysis of transcript expression with Tlr2 stimulation, a one-way ANOVA was performed for the 2-hour and 8 hour treatment groups. For 24, 48, and 72 hours two-tailed student t-tests were performed. All statistical comparisons were made using IBM SPSS Statistics 22 or Microsoft Excel 2010.
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*Table 2. List of primers used for Chapter 1 RT-PCR experiments*
**Primary Cerebellar Granule Cell Culture Protein Isolation**

To analyze the activation state of NF-κB signaling and protein expression lysates were collected from pCGC cultures in control or treated conditions. Primary neuronal cultures were washed with twice with ice cold PBS, followed by incubation with RIPA buffer (1% Triton X-100, 0.1% SDS, 50mM Tris (pH 7.4), 107 mM NaCl, Halt Phosphatase Inhibitor (ThermoFisher #78420, 1:100), Protease Inhibitor (Sigma #P8340, 1:100), 5 mM EGTA, and 5 mM EDTA) for 15 minutes on a rotary shaker set to 100-140 rpm. Wells were scraped using a rubber cell scraper and the lysate collected, sonicated, and centrifuged at 14,000 G for 15 minutes at 4°C. Protein concentration was then quantified by BCA assay (Pierce Catalog # 23225) according to manufacturer protocol.

**Western Blot Analysis**

Lysate was run on 4-12% SDS-Page gels. Protein was transferred to PVDF membranes, blocked in 5% non-fat milk for total protein antibodies or 5% BSA for phospho-specific antibodies overnight at 4°C. Blots were then rinsed in TBST and probed with species appropriate secondary antibodies for 1 hour at room temperature. For a complete list of antibodies used in this chapter, please refer to Table 1. Some blots were stripped for multiple rounds of antibody staining using Restore™ Plus Western Blot Stripping Buffer (ThermoFisher #46430) for 10 to 15 minutes at room temperature on a rocker. If blots were to be stripped, they were always probed with phosphorylation specific antibodies first. All blots were imaged using SuperSignal West Extended Duration chemiluminescence HRP substrate (ThermoFisher #34075) and analyzed using AlphaView software (Version 3.4.0.0, ProteinSimple). Data was loaded into Excel to generate graphics. For comparison of two groups, two-tail student t-tests
were used. For comparisons of more than two groups, data was analyzed by one-way ANOVA with Bonferroni post hoc analysis using IBM SPSS Statistics 22.

**Confocal Microscopy**

All images were collected using a Zeiss LSM 700 scanning confocal microscope using either 10x (Zeiss EC Pan-Neofluar 10x/0.3 NA, 1.11 μm resolution, #440330-9902-000), 20x (Zeiss EC Plan-Neofluar 20x/0.50 NA, 0.67 μm resolution, #420350-9900-000), or 40x (Zeiss EC Plan-Apochromat 40x/1.3 NA, 0.26 μm resolution, #420762-9800-799) objectives. All data was collected using settings optimized for each experiment and the settings were maintained across all samples. Confocal imaging was restricted to a maximum of 4 channels using 405 nm (5 mW), 488 nm (10 mW), 555 nm (10 mW), and 639 nm (5 mW) diode lasers, with each set to collect on independent channel for minimal bleed through. For pCGC culture cell type analysis, images were collected using the 20x objective with 0.5x digital zoom to increase the field of view. Images for translocation studies were collected at 40x with a 4x digital zoom. To assess NF-κB reporter assay expression z-stack images were collected at 40x with 0.5x digital zoom. For PRR and NF-κB signaling protein expression in pCGC cultures, images were collected using a 40x objective with 2x digital zoom.

**pCGC Culture Cell Type Analysis**

To determine the relative distribution of cell types to the cultured cell population, pCGC cultures were fixed and immunostained for markers of neurons (NeuN), astrocytes (GFAP), and microglia (CD11b) at DIV6. This process was performed on cultures generated from P3, P5, P7, and P9 C57BL6/J pups to assess if post-natal age effects cell distribution. Images were collected by confocal microscopy and analyzed using analyze particles feature in ImageJ software. A minimum of 8 fields (technical replicates) per sample were imaged across multiple experimental (biological) replicates.
Images were uploaded as .ism files and the global scale was set based on imaging perimeters. For the DAPI* and NeuN* cell quantification, a macro was used to automate the counting procedure. First, the batch threshold was set manually where fainter cells were quantifiable while the brighter cells remained distinct from surrounding cells. Images were then smoothed to ensure detection of cell borders and converted to binary, where the watershed function was used to separate closely opposed cells. Cells were quantified using the analyze particles function controlling for size and circularity. To validate count accuracy, the masks feature was used and overlaid on the original image. For quantification of the non-nuclear immunoreactivity of GFAP and CD11b, the positive cells were counted manually using the cell counter application of ImageJ.

**pCGC Culture Apoptosis Analysis**

It was clear from cell type quantification that a small percentage of DAPI positive cells were not NeuN, GFAP, or CD11b positive, suggesting the presence of other cell types or apoptotic cells that have lost protein expression. Additionally, we felt it necessary to characterize the level of cell death in pCGC cultures in control conditions. Therefore, pCGC cultures generated from P5, 7 and 9 mice were fixed for quantification of cleaved caspase-3 positive cells, as a marker of cell apoptosis. Images were collected at 20x and quantified using ImageJ analyze particle functions as described previously. The number of cleaved caspase-3 positive cells and DAPI positive cells were used to quantify the percentage of apoptotic cells in pCGC cultures at DIV6. For this validation study we captured a minimum of 8 images per well across multiple wells for each biological sample (P5 n = 2, P7 n = 2, P9 n = 2). Statistics were not performed due to the limited number of biological samples assessed.

**Quantification of p65-GFP Translocation**
For downstream transcriptional regulation by NF-κB signaling to occur, NF-κB subunits must translocate from the cytoplasm to the nucleus. As a method of quantifying NF-κB activation, we assessed translocation of GFP tagged p65 in pCGC cultures stimulated with a PRR agonist. pCGCs were transfected with p65-GFP and TdTomato constructs at DIV2 and treated with 1.0 µg / mL of Pam3CSK4 at DIV6. Following 1 hour of stimulation with Pam3CSK4, cells were fixed, immunostained for IκB, and imaged using confocal microscopy. To assess for nuclear translocation of p65-GFP, the cytoplasmic and nuclear fluorescent intensity of transfected primary cerebellar neurons was calculated using ImageJ software. The nuclear region of each cell was traced using the DAPI channel, and the outline of the cell body was traced using counter transfected TdTomato. The GFP fluorescence of the nuclear region was then measured, followed by the GFP fluorescence of the cytoplasm after subtracting the nucleus from the cell. The average nuclear to cytoplasmic ratio and the number of cells with a nuclear to cytoplasmic ratio greater than 1 were calculated. Similarly, the fluorescent intensity of IκBα immunostaining in the cytoplasm and nucleus was quantified using ImageJ. We imaged over 40 cells per condition (60 control, 47 Pam3CSK4) distributed across 3 biological replicates. Data was analyzed by multiway analysis of variance (MANOVA) using IBM SPSS Statistics 22 controlling for sample, treatment, and sample*treatment interactions.

Quantification of p65 Reporter

Primary cerebellar granule cell cultures were transfected with p65-GFP reporter and tdTomato at DIV2. On DIV4 cells were treated for 48 hours with 1.0 µg Pam3CSK4 and fixed on DIV6. Blinded samples of fixed pCGC cultures were imaged using confocal microscopy to assess the level of GFP fluorescence in transfected neurons. Cells to be imaged were identified by Tdtomato expression to avoid selection of only the brightest
GFP cells. Using ImageJ software, the TdTomato signal was used to create a cell mask for each transfected neurons and the GFP signaling intensity and integrated density was measured for each cell. Using this method we analyzed 243 control cells and 190 Pam3CSK4 cells, distributed across 4 biologic replicates (Sample 1 140; Sample 2 108, Sample 3 104; Sample 4 81). Data was imported into IBM SPSS Statistics 22 and a multiway analysis of variance (MANOVA) was performed to determine if sample, treatment, or sample*treatment interactions had an effect on characteristics of GFP fluorescence (Mean, Median, Max, Integrated Density).

Results

*Increased NF-κB Related Protein Phosphorylation in the Aging C57Bl6/J Cerebellum*

Our previous data demonstrates a significant upregulation of immune protein transcripts associated with in NF-κB signaling in the cerebellum of aged compared to young mice. Transcriptional data provides an excellent view into potential dysregulated cellular processes, but it fails to provide information on the activation state of specific pathways. As many signaling pathways are regulated by kinases, investigating the protein phosphorylation state can provide critical knowledge regarding the activation of specific signaling modalities. In efforts to obtain unbiased data on which signaling pathways may be dysregulated in the aging cerebellum, we examined differences in protein expression and phosphorylation status between middle-aged adult (14 mo.) and aged (22 mo.) male C57BL/6J mice. We assessed levels of phosphorylated proteins within over 30 signaling pathways, and use ontology analysis to determine over represented pathways. Differences at individual protein loci were first determined by two-sided, unequal variance t-test. Within the aged brain, we detected 31 differentially expressed total proteins, 19 phosphorylated proteins, and 20 phosphorylated
protein/total protein ratios (Table 3). Further analysis was performed to generate volcano plots presenting the data as a function of significance versus fold change. Using this method, we identified 11 total proteins (Figure 1A), 3 phosphorylated proteins (Figure 2A), and 12 phosphorylated protein/total protein ratios (Figure 3A) with greater than a 33% change between adult and aged mice. Next, we processed each of the differentially expressed protein lists through ontology analysis with Panther (version 11.1) to determine biological themes present within each category.

Ontological analysis of differentially expressed total proteins demonstrated age-associated enrichment in several pathways pertaining to reactive oxygen species generation and response, cell aging, positive regulation of neuron death, response to calcium, cell responses to damage (gamma and UV radiation), and transmembrane receptor tyrosine protein tyrosine kinase signaling, among others (Figure 1B). Phosphorylated proteins in the aged cerebellum correspond to pathways involved in neurotrophin signaling, positive regulation of superoxide anion generation, negative and positive regulation of apoptotic signaling, negative regulation of kinase activity, and several pathway associated with cell stress responses, among others (Figure 2B). Ontological analysis of protein/phosphoprotein ratios demonstrated age-associated enrichment for pathways involved in MAPK signaling, negative regulation of protein kinase signaling, apoptotic processes, and several CNS specific pathways including neuron differentiation and neurogenesis (Figure 3B). Of particular interest to our hypothesis, several proteins differentially expressed in the aged cerebellum interact with spleen tyrosine kinase (Syk), a kinase upstream of NF-κB activation. These interacting proteins include Src, Cd5, Zap70, Vav1, Shc1, Pla2g4a, Raf1, and Hsp27. Additionally, c-Pla2, Fos, Bim, Beta-amyloid A4, PKC delta, and EGFR are all increased in the aged brain and have κB binding sites within the promotor or are linked to NF-κB activation. This data demonstrates that cerebellar aging is associated with a more global dysregulation
Table 3. Differentially expressed protein results from phospho protein array sorted by fold change for total protein, phosphorylated protein, and phosphoprotein ratios.

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Figure 1. Protein expression data from array and ontology analysis. Continued on the next page...
Figure 1. Total protein expression data from array and ontology analysis continued... Volcano plot generated to demonstrate total protein expression in middle aged and aged cerebellar tissue versus the statistical significance of the observed difference. The fold change ratio values are represented on the x-axis (ratio) with the statistical significance on the y-axis (p-values). Proteins with a 33% or greater increase in aged mice are represented in the upper left corner. Proteins with a 33% or greater decrease in aged mice are represented in the upper right. The top center region contains proteins that were significantly different (p < 0.05) between adult and aged cerebellum, but were less than a 33% change. All proteins below the center horizontal line failed to reach significance. Differentially expressed proteins were processed through ontology analysis using PANTHER (11.1) to determine enriched biological themes.
Figure 2. Phosphorylated expression data from array and ontology analysis. Volcano plot generated to demonstrate phosphoprotein expression in middle aged and aged cerebellar tissue versus the statistical significance of the observed difference. The fold change ratio values are represented on the x-axis (ratio) with the statistical significance on the y-axis (p-values). Phosphoproteins with a 33% or greater increase in aged mice are represented in the upper left corner. Phosphoproteins with a 33% or greater decrease in aged mice are represented in the upper right. The top center region contains phosphoproteins that were significantly different (p < 0.05) between adult and aged cerebellum, but were less than a 33% change. All phosphoproteins below the center horizontal line failed to reach significance. Differentially expressed phosphoproteins were processed through ontology analysis using PANTHER (11.1) to determine enriched biological themes.
Figure 3. Phosphorylated protein to total protein ratio expression data from array and ontology analysis. Volcano plot generated to demonstrate the ratio (adult/aged) of the total protein to phosphoprotein ratios based on specific antibody pairs versus the statistical significance of the observed difference. The fold change ratio values are represented on the x-axis (ratio) with the statistical significance on the y-axis (p-values). Protein ratios with a 33% or greater increase in aged mice are represented in the upper left corner. Protein ratios with a 33% or greater decrease in aged mice are represented in the upper right. The top center region contains protein ratios that were significantly different (p < 0.05) between adult and aged cerebellum, but were less than a 33% change. All protein ratios below the center horizontal line failed to reach significance. Differentially expressed protein ratios were processed through ontology analysis using PANTHER (11.1) to determine enriched biological themes.
of proteins whose expression and activation regulate NF-κB. These studies further validate the hypothesis that NF-κB dysregulation may play a role in aging cerebellar phenotypes. In efforts to detail a potential mechanism for this dysregulation we generated and characterized an in vitro cerebellar neuron model.

**Establishment and Characterization of a Reduced Systems Culture Model**

Primary cerebellar neuron cultures have been used as a reduced system to study processes associated with neuronal function for over 30 years\(^7\)\(^4\). As many differences exist in protocols for isolation and enrichment of cerebellar granule cells for culture, we deemed it necessary to characterize and validate our culture system. Key tasks in characterization were to (1) determine optimal post-natal day to harvest cerebellum for the highest neuronal yield and viability, (2) determine the percentage and type of neuronal and non-neuronal cells within the culture, (3) assess neuronal morphology and gene expression to confirm cerebellar granule cell lineage. Cerebellar neuron cultures generated for these studies are predominately neuronal with a very small population of astrocytes and microglia when harvested from post-natal mice between the ages of P3 and P9. Quantification of cell types at DIV6 in cultures generated from different aged mice demonstrated that greater than 75% of cells within pCGC cultures are NeuN expressing neurons (P3 77.60 ± 2.82%; P5 80.03 ± 1.13%; P7 81.23 ± 3.04%, P9 78.84 ± 1.56%), with less than 2% GFAP expressing astrocytes (P3 1.53 ± 1.19%; P5 1.14 ± 0.70%; P7 0.87± 0.17%; P9 0.35 ± 0.11%), and less than 1% CD11b microglia (P3 0.44 ± 0.06%; P5 0.53 ±0.12%; P7 0.37 ± 0.11%; P9 0.37 ± 0.06%) (Figure 4A,B). After quantification it was evident that a percentage of DAPI positive cells were not NeuN, GFAP, or CD11b positive, suggesting the presence of other cell types or cells that had lost protein expression. While NeuN is expressed in the majority of neuronal cell types, there are subpopulations of neurons that lack NeuN expression. These include Purkinje
cells, inferior olive neurons, γ-motor neurons of the spinal cord, olfactory bulb mitral cells, and retinal photoreceptors\textsuperscript{75}. Additionally, loss of NeuN protein expression has been associated with apoptotic pathway activation and cell death\textsuperscript{76}. Data also demonstrates that NeuN expression can be reduced in neurons due to damage without loss of cell viability\textsuperscript{77}. To determine if the discrepancy in cell percentages were a result of cell death, pCGC cultures were immunostained for cleaved caspase-3, a marker of apoptotic signaling. Quantification of cleaved caspase-3 in DIV cultures generated from P5, P7, and P9 mice showed that less than 15% of cells in pCGC cultures were positive for activated caspase-3 (P5 7.52 ± 0.20%; P7 11.31 ± 4.27%; P9 12.5 ± 1.11%) (Figure 4C). This data demonstrates that the discrepancies in cell percentages are partially explained by the presence of a small population of apoptotic cells within pCGC cultures. To further validate the neuronal identity of cells in culture and to validate the presence of a large population of cerebellar granule cells, we transfected pCGC cultures at DIV2 using a DNA vector with GFP under the CMV promoter. This allowed for the identification of individual neurons. We stained transfected cultures with NeuN and Tau to identify neurons and their processes. pCGC cultures demonstrated strong Tau immunoreactivity grossly, and on the level of individually transfected neurons (Figure 5A). It is important to note that transfected neurons morphologically appear to be granule cells, evident by a small cell body with little cytoplasm, few dendrites, and a long thin axon. In efforts to confirm the presence of cerebellar granule cells, we isolated RNA from pCGC cultures at DIV3, DIV6, and DIV9 and assessed the level of GABA(A) receptor alpha 6 subunit (GABAR alpha 6), a marker of mature cerebellar granule cells\textsuperscript{78,79}. RT-PCR analysis demonstrated a significant (p < 0.01) increase in GABAR alpha 6 expression as pCGC cultures matured (DIV3 1.01 ± 0.21; DIV6 14.47 ± 5.04; DIV9 112.25 ± 3.82, data represented as fold change from DIV3) (Figure 5B). pCGC cultures contain predominantly excitatory synapses immunopositive for vesicular glutamate
Primary cerebellar granule cell cultures were generated from post-natal mice at various stages (P3, P5, P7, and P9) of cerebellar development to determine if developmental age affected the resulting cell distribution. (A) Representative image of cell types for pCGC cultures generated from C57BL/6J mice at day in vitro 6. Images were captured at 20x with a 0.5x digital zoom (scale bar = 100 mm). (B) Quantification of immunocytochemistry for markers of neuronal (NeuN+), astrocytic (GFAP+), and microglia (CD11b+) populations proved that pCGC cultures are enriched for neuronal cells, independent of the age of mouse between P3 and P9. NeuN+ neurons composed >75% of the cell population (P3 77.60 ± 2.82%; P5 80.03 ± 1.13%; P7 81.23 ± 3.04%; P9 78.84 ± 1.56%) at all ages, with < 2% GFAP+ astrocytes (P3 1.53 ± 1.19%; P5 1.14 ± 0.70%; P7 0.87 ± 0.17%; P9 0.35 ± 0.11%) and < 1% CD11b+ microglia (P3 0.44 ± 0.06%; P5 0.53 ± 0.12%; P7 0.37 ± 0.11%; P9 0.37 ± 0.06%). For cell type quantification > 10 image were collected for each sample well across multiple biological replicates for each age (P3 n = 3; P5 n = 4; P7 n = 2; P9 n = 3). (C) pCGC cultures were immunostained for cleaved caspase-3 to assess levels of apoptosis. Apoptotic cells were found to represent less than 15% of all cells in pCGC cultures independent of the age of pup from P5 to P9 (P5 7.52 ± 0.20%; P7 11.31 ± 4.27%; P9 12.5 ± 1.11%). For apoptosis analysis a minimum of 8 images per well across multiple wells generated from individual biological replicates were collected (P5 n = 2; P7 n = 2; P9 n = 2).
Figure 5. **Primary cerebellar neuron cultures are enriched for cerebellar granule cells.** To validate the enrichment of cerebellar granule cells, primary neuronal cultures were transfected with pGFP at DIV2 and immunostained for neuronal proteins NeuN and Tau at DIV6. (A) pCGC cultures contain pGFP transfected neurons that morphologically resemble cerebellar granule cells in vivo, with a small cell body, few dendrites, and a long thin axon. Transfected cells are also positive for neuronal markers NeuN and Tau. Images were obtained at 20x. Scale bar represents 50 mm. Mature cerebellar granule cells can be identified by the expression of GABA receptor subunit alpha 6 (GABAR alpha 6). (B) RT-PCR analysis of RNA isolated from DIV3, 6, and 9 pCGC cultures demonstrates a significant increase in GABAR alpha 6 expression as cultures mature (DIV3 1.01 ± 0.21; DIV6 14.47 ± 5.04; DIV9 112.25 ± 3.82, data represented as fold change from DIV3. * = p < 0.05, **** = p < 0.0001; n = 3 per time point.
transporter 1 (vGlut1) and with increasing density from DIV3 to DIV6 (Data not shown, see WT synapse data in Chapter 2 Figure 7 and 8). Additionally, a small number of inhibitory synapses immunopositive for GAD65 are present, likely resulting from a small percentage of inhibitory interneurons within pCGC cultures (Data not shown, see WT synapse data in Chapter 2 Figure 7 and 8). Cerebellar granule cells are still dividing and migrating from the external granule cell layer to the inner granule cell layer in the post-natal brain. A higher number of granule cells can be isolated from later post-natal stage brains, but with this increase we also noted increased apoptosis and poorer overall cell appearance (data not shown). Therefore, using the information gained from the characterization studies, we chose to use P4-6 C57BL/6 pups for the all downstream pCGC culture work, as they had the lowest level of apoptosis rates, generated an acceptable number of cells per pup, and appeared healthy throughout the duration of the experimental timeline.

**Primary Cerebellar Granule Cells in Culture Express Pattern Recognition**

**Receptors and Immune proteins**

The expression of immune proteins and PRRs in neurons has been historically debated. Recent data demonstrates that neurons express proteins belonging to multiple classes of immune signaling proteins including complement\(^\text{80-82}\), major histocompatibility complex\(^\text{1}\)\(^\text{83-86}\), toll-like receptors\(^\text{87, 88}\), Fc receptors\(^\text{89}\), and paired immunoglobulin like receptors\(^\text{89}\). However, there is limited data on cerebellar granule cell expression of these proteins. Therefore, we felt it was necessary to assess the expression patterns of immune proteins with pCGC cultures. RNA was harvested at multiple stages of culture maturation and RT-PCR was performed to determine developmental patterns of transcript expression for proteins belonging to the complement (C3, C1q), MHC Class I (MHC I H2-Q1), toll-like receptor (Tlr2, Tlr4), Leukocyte Immunoglobulin Like Receptor
(Lilrb3), Triggering Receptor Expressed On Myeloid Cells (Trem2), Fc Receptor (FceGr), and C-Type Lectin Domain (Clec7a) family proteins. Transcript expression for all immune proteins tested increased with culture maturation, many with significant 3-5 fold increases from DIV0 to DIV9 in culture (Figure 6). The increase in expression of PRRs and immune protein transcripts does not correlate with increases in immune cells, as Cytarabine (AraC) is added to cultures at DIV2 to prevent expansion of the glial population. Additionally, DIV6 glial cells make up less than 3% of the culture population. Therefore, our data confirms the more recent literature demonstrating the expression of immune protein transcripts in neuronal cells.

**Primary Cerebellar Granule Cells in Culture Express NF-κB Signaling Transcripts and Proteins**

Cerebellar granule cell NF-κB signaling is important for early cell survival, but other functions are poorly understood. Furthermore, there is debate on the level of induced NF-κB activity with cerebellar granule cells. As our model hypothesizes dysregulated NF-κB activity in response to PRR stimulation, we deemed it critical to first confirm the presence of signaling molecules associated with NF-κB activation in our reduced systems model on both the transcript and protein level. RT-PCR was performed on RNA isolated from pCGC cultures at DIV0, 3, 6, and 9 and analyzed for the expression of two functionally nonredundant IκB kinases, IκB Kinase alpha (IKK-α) and IκB Kinase β (IKK-β), which are necessary for phosphorylation of p65, p50, and IκBα. Transcript levels of NFκB1, the precursor of p50 were also measured. Additionally, we measured spleen tyrosine kinase (Syk), a kinase downstream of multiple PRRs and potential integration point in dysregulated signaling through NF-κB. pCGC cultures expressed all of the transcripts measured, and demonstrated slight increases as cultures matured from DIV0
Figure 6. Primary cerebellar granule cell cultures express transcripts for pattern recognition receptors and immune protein. RNA was isolated from pCGC cultures immediately after isolation or after 3, 6, or 9 days in vitro. RT-PCR analysis demonstrates increasing expression of transcripts for PRRs and immune proteins throughout culture maturation. a = significantly different from DIV 0, b = significantly different from P3, c = significantly different from DIV 6. Data is representative of 3 biological replicates for DIV 0 and 2 replicates for DIV 3, 6 and 9. Significant differences were determined by one-way ANOVA with Bonferroni post-hoc testing. Error bars represent standard deviation of the mean.
to DIV 9 (Figure 7A). We noted a significant increase in both kinases responsible for phosphorylation of NF-κB signaling molecules and trending increases in Syk and NFκB1.

As this was an initial characterization screening, the sample sizes were small. We would anticipate that with larger sample sizes, these increases would reach significance. However, these data clearly demonstrate the presence of NF-κB signaling associated transcripts in pCGC cultures. To confirm protein expression of NF-κB related proteins, pCGC cultures transfected with pGFP to visualize individual neurons were immunostained for IKK-α, IKK-β, and Syk. Visual analysis clearly demonstrates the presence of all three NF-κB related proteins in their expected cytoplasmic distribution at DIV6 within cerebellar granule cells (Figure 7B). These data collectively confirm that cerebellar granule cells express immune proteins, PRRs, and signaling proteins necessary for the activation of NF-κB pathway. However, whether or not NF-κB activity can be induced by PRR ligation remained unclear. In efforts to answer this question, we choose to investigate the effect of PRR ligation on NF-κB signaling in pCGC cultures.

To assess if PRR activation leads to increased NF-κB activity in cerebellar granule cells we need to select and a PRR and agonist to serve as our model system. The Tlr2 receptor was selected for our modeling of PRR induced NF-κB signaling pathway analysis, because there is commercially available synthetic agonist (Pam3CSK4) that is selective for Tlr2. Many other PRRs agonists are poorly defined in terms of selectivity. Additionally, we had obtained Tlr2 knock-out mice (see Chapter 3) that could be used as to validate pathway specificity.

**Activation of Tlr2 in pCGC Cultures Increases NF-κB Signaling Pathway**

**Phosphorylation**

In the inactive state, NF-κB is sequestered in the cytoplasm by inhibitory IkB family inhibitors (IkBa). Upon activation of immune receptors, upstream kinases phosphorylate
Figure 7. Primary cerebellar granule cells express NFκB signaling molecules IKK-α, IKK-β, and Syk. (A) RT-PCR analysis demonstrates transcript expression of NFκB pathway associated molecules Syk, IKK-α, IKK-β, and NFκB1 during culture maturation from day in vitro 0 to 9. (B) Immunocytochemistry validates protein expression of Syk, IKK-α and IKK-β in cerebellar granule cells at day in vitro 6. n=3 for DIV 0, n=2 for DIV 3, DIV 6, DIV 9. a = significantly different from DIV 0, b from DIV 3, c from DIV 6. Analysis was performed by one-way ANOVA with Bonferroni post-hoc tests. Error bars represent standard error of the mean.
IKK complexes, which in turn phosphorylate downstream proteins including IκBα, p65, and p50. Phosphorylation of IκBα leads to polyubiquitination and subsequent degradation. Phosphorylation of p65 and p50 are indicative of activation and effect DNA binding, translocation, stability, and direct downstream responses depending on the specific site of phosphorylation. Each NF-κB subunit has multiple phosphorylation sites that dictate downstream functions. NF-κB subunit p65 contains 8 serine residues identified as phosphorylation sites (Ser20590, Ser27691-97, Ser28190, 98, Ser31199-102, Ser316103, Ser486104, Ser52999-102, and Ser536105-109, as well as 3 threonine residues T254110, 111, T435112, 113, and T505114-116 each targeted by select upstream kinases30. Of particular interest to our studies, p65 ser536 is phosphorylated by a multitude of kinases including IKKβ and IKKa and leads to transactivation of p65105, 106, 108, 109. As both are present within pCGC cultures, we chose to assess the phosphorylation state of p65 ser536 upon stimulation of Tlr2 with Pam3CSK4. Protein was isolated from pCGC DIV6 cultures after 2 hour treatment with 1.0 µg Pam3CSK4 or vehicle. Tlr2 stimulation resulted in a greater than 2-fold increase in phosphorylated (ser536) p65 (p-p65) compared to vehicle treated controls (Figure 8). This increase in phosphorylated p65 is attenuated with 30 minute pretreatment with PDTC, an NF-κB inhibitor that prevents IκBα degradation (Figure 8). While phosphorylation of p65 suggests pathway activation, it is not definitive of activation. Therefore, we chose to measure translocation of p65 protein upon Tlr2 ligation.

**Activation of Tlr2 in pCGC Cultures Increases NF-κB Translocation**

Degradation of IκBα and translocation of NF-κB subunits are a requisite for DNA binding and transcriptional regulation in the canonical signaling pathway. To determine if Tlr2 activation leads to increased translocation of p65, we transfected DIV2 cerebellar granule cells with p65-GFP and TdTomato vectors. Validation pilot studies were
Figure 8. Activation of Tlr2 receptors in pCGC cultures results in increased phosphorylation of p65 ser536. Phosphorylation of NF-κB signaling proteins occurs with pathway activation by upstream kinases. 2 hour treatment with 1.0 µg Pam3CSK4 or PDTC + Pam3CSK4 do not significantly alter total p65 protein levels (Control 1.0 ± 0.04; Pam3CSK4 1.10 ± 0.12; PDTC + Pam3CSK4 1.08 ± 0.34). Pam3CSK4 treatment significantly increases phosphorylated p65 (Ser536) compared to controls (Control 1.00 ± 0.39; Pam3CSK4 2.17 ± 0.33, one-way ANOVA p < 0.01). This increase is attenuated by pretreatment with PDTC (PDTC + Pam3CSK4 1.45 ± 0.06, one-way ANOVA, PDTC + Pam3CSK4 vs Control p > 0.05, PDTC + Pam3CSK4 vs Pam3CSK4, p < 0.05). Data is representative of 3 biological replicates per group. Error bars represent standard deviation of the mean.
performed to characterize p65-GFP transfection and to determine an adequate timeline
to assess translocation. Within control pCGC cultures, cerebellar granule cells existed in
both activated (high nuclear fluorescent intensity) and inactivated (high cytoplasmic
fluorescent intensity) states, further confirming the necessity for active NF-κB signaling
in pCGCs (Figure 9A). Live cell confocal microscopy was performed on a small number
of cells to measure translocation in response to Tlr2 activation (Figure 9B). From these
initial pilot experiments we decided measure p65-GFP translocation after 1 hour of
stimulation with Pam3CSK4. For a larger quantitative study, pCGC cultures were treated
with 1.0 μg Pam3CSK4 at DIV6 for 1 hour, fixed, and immunostained for IκBα (Figure
10A). Transfected pCGCs were identified using the TdTomato channel and imaged by
confocal microscopy for downstream calculation of cytoplasmic and nuclear p65-GFP
and IκBα fluorescent intensities. pCGC cultures were created so each well is generated
from an individual mouse. Upon quantification, we noted differing levels of baseline
nuclear and cytoplasmic intensities in cultures generated from different mice. As NF-κB
activity is critical for cerebellar granule cell survival, seeing some cells with activated
NF-κB in culture was not surprising, but it complicated analysis. Therefore, we used a
multivariate analysis of variance (MANOVA) to assess for significant changes due to
Pam3CSK4 treatment as well as a sample by treatment interaction. Tlr2 stimulation
leads a significant (p > 0.05) increase in p65-GFP translocation evident by increased
nuclear to cytoplasmic ratios in all Pam3CSK4 treated samples (Figure 10B).
Quantifying the number of cells with a nuclear to cytoplasmic p65 ratio greater than 1
demonstrated an increase in p65 translocation with Pam3CSK4 treatment and Tlr2
activation (Figure 10C). Quantification of IκBα fluorescent intensity showed a trending,
but non-significant reduction (data not shown). IκBα degradation is necessary for
activation, but is also a product of NF-κB activation functioning as a form of
autoregulation. Therefore, our timeline may not have allowed us to detect significant
**Figure 9.** *NF-κB localization in pCGC cultures and translocation timeline.* (A) Preliminary experiments demonstrate p65-GFP subcellular localization is heterogeneous under control conditions evident by the presence of cells with both high cytoplasmic / low nuclear and low cytoplasmic / high nuclear expression. (B) Translocation of p65-GFP in response to Tlr2 stimulation appears to occur within 60 minutes treatment, based on preliminary live cell time-lapse imaging trials.
Figure 10. *Trl2 stimulation increases nuclear localization of p65-GFP in pCGC culture*. Translocation of p65 into the nucleus is indicative of activation of NF-κB signaling. Using a p65-GFP vector, the effect of Tlr2 stimulation of p65 translocation was assessed by pCGC treatment with Pam3CSK4. (A) Representative images for control and Pam3CSK4 treated cells transfected with p65-GFP demonstrating an increased nuclear to cytoplasmic ratio in treated cells. Scale bar = 10 mm. IκBα florescence was also measured, but showed only trending differences. (B) Quantification of images demonstrated a significant treatment effect for Pam3CSK4 in increasing the mean nuclear to cytoplasmic ratio of p65-GFP fluorescence (Control 1.21 ± 0.09; Pam3CSK4 1.58 ± 0.93; p = 0.005 for treatment interaction by multi-way ANOVA. Data represented for individual samples with greater than 15 cells imaged per sample. (C) Graphic representation of the number of cells that have a nuclear to cytoplasmic ration greater than or less than 1. Higher nuclear/cytoplasmic ratios represent p65 translocation and activation. Pam3CSK4 treated cells have higher numbers of activated cells compared to controls. Data represents 60 control cells and 47 Pam3CSK4 treated cells across 3 biological replicates.
degradation, due to generation of new IκBα. Translocation of p65 is indicative of pathway activation, but it fails to confirm the next step in the sequence of activation, DNA binding and transcriptional regulation.

**Activation of Tlr2 in pCGC Cultures Increases NF-κB Reporter Activity**

Upon translocation to the nucleus, NF-κB proteins function as transcriptional regulators through DNA binding mediated by Rel homology domains and transcriptional activation through transcription activating domains (TAD). NF-κB proteins recognize IκB motifs, a specific DNA sequence characterized as 5’-GGGRNNYYYCC-3’ in which R is a purine, Y a pyrimidine, and N is any nucleotide. For a gene to be regulated by NF-κB signaling, the presence of an IκB motif is necessary, but not sufficient to ensure transcriptional activation. Further levels of regulation by post-translational modifications, chromatin modifications, and non-Rel family proteins are important in directing specific transcriptional outcomes. However, as all NF-κB regulated transcripts contain IκB motifs, identifying when these motifs are bound provides indisputable evidence for NF-κB activation. Therefore, we obtained a p65-GFP reporter with a GFP open reading frame controlled by a DNA cassette with 5 tandem repeats of NF-κB transcriptional response element (TRE), a generous gift from the Dongsheng Cai Laboratory at The Albert Einstein College of Medicine. Primary cerebellar granule cell cultures were co-transfected at DIV2 with the p65-GFP reporter and TdTomato for cell visualization and treated for 48 hours with Pam3CSK4 starting at DIV4. Similar to translocation studies, as NF-κB is necessary for granule cell survival with a baseline level of activity and a tightly regulated dynamic range, we anticipated only a small change in activity and some variability between samples. Therefore, data was analyzed using a MANOVA to determine interactions for sample, treatment, and sample*treatment that effect characteristics of GFP fluorescence. Quantification and analysis of reporter intensity
after 48 hours of Tlr2 stimulation demonstrated a significant treatment effect for mean GFP intensity (Control 30.5 ± 1.1; Pam3CSK4 34.5 ± 1.2; \( p = 0.015 \)), median GFP intensity (Control 28.2 ± 1.1; Pam3CSK4 31.6 ± 1.1; \( p = 0.03 \)), and GFP integrated density (Control 2217.7 ± 107.5; Pam3CSK4 2608.7 ± 113.9; \( p = 0.013 \)) (Figure 11). Significant sample by treatment interactions were also noted for these measurements demonstrating variability in the degree to which each sample responded to Pam3CSK4. However, even with these differences Pam3CSK4 leads to a significant increase in p65-GFP reporter expression, further validating that PRR ligation in pCGC cultures can increase NF-\( \kappa \)B activity. While DNA binding of NF-\( \kappa \)B is functionally critical for transcriptional regulation, we wanted to validate increased activity of this pathway through quantification of downstream transcriptional products.

**Activation of Tlr2 in pCGC Cultures Increases Transcriptional Expression of NF-\( \kappa \)B regulated products**

There are an estimated \( 1.4 \times 10^4 \) potential \( \kappa \)B domains in the human genome where NF-\( \kappa \)B proteins can bind and regulate transcription\(^2^3\). When and where these proteins actually bind is tightly regulated by a complex network of post-translational modifications, chromatin modifications, and protein interactions\(^1^1^8\),\(^1^1^9\). Due to the massive number of potential targets available to assess NF-\( \kappa \)B activation, we had to be selective in our approach. Thus, we narrowed targets down to those believed to be involved in age related functional deficits. We chose to quantify transcript levels of a set of genes with known \( \kappa \)B motifs spanning multiple protein classes that are upregulated in the aged cerebellum\(^5\). pCGC cultures were treated on DIV6 with 1.0 \( \mu \)g Pam3CSK4, vehicle control, or PDTC pre-treatment followed by 1.0 \( \mu \)g Pam3CSK4. RNA was isolated after 2 hours, 8 hours, 24 hours, 48 hours, and 72 hours of treatment from each treatment group and RT-PCR was performed. Of note, PDTC pretreatment leads to significant cell
Figure 11. Tlr2 stimulation increases NF-κB activity in pCGC. (A) Representative image of control and Pam3CSK4 treated pCGC cultures transfected with p65-GFP reporter demonstrating increased GFP fluorescence with Tlr2 stimulation. (B) Quantification of p65-GFP reporter intensity. 48 hour Tlr2 stimulation with Pam3CSK4 leads to a significant treatment effect for mean GFP intensity (Control 30.5 ± 1.1; Pam3CSK4 34.5 ± 1.2; p = 0.015), median GFP intensity (Control 28.2 ± 1.1; Pam3CSK4 31.6 ± 1.1; p < 0.03), and GFP integrated density (Control 2217.7 ± 107.5; Pam3CSK4 2608.7 ± 113.9; p = 0.013). Additionally, significant sample*treatment effects were noted for all measurements, demonstrating the variability between samples. Data represents means for 243 control cells and 190 Pam3CSK4 cells, distributed across 4 biologic replicates (Sample 1 140; Sample 2 108, Sample 3 104; Sample 4 81). Error bars equal standard error of the mean (SEM). * p < 0.05, ns = not significant for treatment effect by multi-way ANOVA with treatment, sample, and sample*treatment interactions measured.
death after 8 hours (data not shown), likely due to strong inhibition of NF-κB activity. Therefore, we did not assess transcript levels in PDTC + Pam3CSK4 treated cells past the 8 hour time point. IκBα is a direct product of NF-κB activation, forming an autoregulatory loop where increased IκBα competes with κB motifs in the nucleus to bind p65 and shuttle it back to the cytoplasm. As a further validation of NF-κB activation, transcript levels of IκBα were measured in each condition. Tlr2 stimulation with Pam3CSK4 leads to a time dependent increase in IκBα transcript. At 2 hours we noted no significant increase in treated pCGCs (Control 1.05 ± 0.39; Pam3CSK4 0.96 ± 0.39; p = 1.00). However, at 8 hours Pam3CSK4 treatment resulted in a significant increase in IκBα transcript compared to controls (Control 1.01 ± 0.15; Pam3CSK4 1.97 ± 0.49, p = 0.0053). This increase is attenuated by pretreatment with PDTC (PDTC + Pam3CSK4 0.86 ± 0.18; p = 0.002 compared to Pam3CSK4; p = 1.00 compared to Control). Increased IκBα is still present after 24 hours of Tlr2 stimulation (Control 1.04 ± 0.29; Pam3CK4 2.02 ± 0.19, p = 0.0013) (Figure 12).

RT-PCR analysis showed that transcripts belonging to immune proteins and PRRs were also increased upon Tlr2 stimulation, but to varying degrees and after different durations of activation. Transcript levels of C3 were not increased after 2 hours (Control 1.09 ± 0.52; Pam3CSK4 0.69 ± 0.18; p = 0.406), but were significantly increased after both 8 (Control 1.04 ± 0.33; Pam3CSK4 2.52 ± 0.20; p = 0.00031) and 24 hours of treatment (Control 1.04 ± 0.35; Pam3CSK4 4.03 ± 1.22; p = 0.0034). Pretreatment with PDTC only partially attenuated this increase at 8 hours (PDTC + Pam3CSK4 1.72 ± 0.12; p = 0.0086 compared to control, p = 0.0032 compared to Pam3CSK4) (Figure 13A). Transcript for High affinity Immunoglobulin Gamma Fc receptor (Fcgr1) was increased after 24 hour Pam3CSK4 exposure (Control 1.03 ± 0.29; Pam3CSK4 2.56 ± 0.26; p = 0.00024) (Figure 13B). Additionally, increases in β2-microglobulin, a protein
related to MHC class I signaling was not increased at 2, 8, or 24 hours, but after 72 hours of Pam3CSK4 treatment β2-microgloblin transcript was significantly higher than controls (Control 1.02 ± 0.23; Pam3CSK4 1.60 ± 0.26; p = 0.016) (Figure 13C).

The final group of transcripts we assessed belonged to toll-like receptor family proteins. Activation of NF-κB yields transcription of Tlr2\textsuperscript{79, 120} and Tlr9\textsuperscript{121}, but Tlr4 does not appear to be NF-κB regulated. To prove specificity of NF-κB regulated transcription, and not a general inflammatory response we assessed the levels of Tlr2 and Tlr4 transcript upon Tlr2 stimulation. As Tlr9 is not increased in the aging cerebellum and is unlikely to be stimulated by extracellular agonists (endogenous or exogenous) due to its intracellular location, we did not assess Tlr9 levels. Pam3CSK4 treatment of pCGCs results in a robust increase in Tlr2 transcript at 2 hours (Control 1.01 ± 0.16; Pam3CSK4 3.60 ± 0.60; p = 0.00006). This increase persists after 8 hours (Control 1.02 ± 0.24; Pam3CSK4 3.01 ± 0.18; p = 0.0008), 24 hours (Control 1.01 ± 0.15; Pam3CSK4 4.35 ± 0.85; p = 0.0002), and 72 hours (Control 1.05 ± 0.38; Pam3CSK4 4.57 ± 0.27; p = 5.6 x 10\textsuperscript{-6}) of agonist exposure. Pretreatment with PDTC attenuates this increase at both the 2 hour (PDTC + Pam3CSK4 0.90 ± 0.47; p = 0.00004 compared to Pam3CSK4; p = 1.00 compared to Control) and 8 hour (PDTC + Pam3CSK4 0.72 ± 0.79; p = 0.0003 compared to Pam3CSK4; p = 1.00 compared to Control) time points (Figure 14A). Pam3CSK4 treatment does not lead to a significant increase in Tl4 transcript expression at 2 hours or 8 hours of exposure, demonstrating specific activation of NF-κB transcriptional regulation (Figure 14B). Furthermore, we felt it was imperative to confirm this response was not due to a gross activation of glial cells and was specific to activation of Tlr2 by Pam3CSK4. Therefore, we measured transcripts levels of astrocytic Gfap and microglial Aif1 and revealing that neither was significantly elevated after 8 hours of Tlr2 agonist exposure (Figure 15).
Figure 12. **Pam3CSK4 treatment of pCGC cultures increases NF-κB activation and transcription of autoregulatory IκBβ.** pCGC cultures were treated for 2, 8, or 24 hours with vehicle control, 1.0 μg Pam3CSK4, or a 30 minute pretreatment with PDTC followed by Pam3CSK4. RNA was isolated and RT-PCR performed to quantify NF-κB regulated transcriptional products. At 2 hours no significant increase in IκBβ was found between treated and control samples (Control 1.05 ± 0.39; Pam3CSK4 0.96 ± 0.39; p = 1.00). However, at 8 hours Pam3CSK4 treatment leads to a significant increase in IκBβ transcript compared to controls (Control 1.01 ± 0.15; Pam3CK4 1.97 ± 0.49, p = 0.0053). This increase is attenuated pretreatment with PDTC (PDTC + Pam3CSK4 0.86 ± 0.18; p = 0.002 compared to Pam3CSK4; p = 1.00 compared to Control). Increased IκBβ is still present after 24 hours of Trl2 stimulation (Control 1.04 ± 0.29; Pam3CK4 2.02 ± 0.19, p = 0.0013). Each time point with 3 groups was analyzed by one-way ANOVA and Bonferroni post hoc testing of data normalized to control expression. For time points with 2 groups, a two-tailed Student t-test was performed. n = 4 for each sample at all time points. Error bars represented standard deviation, a = p < 0.01 compared to control, b = p < 0.01 compared to Pam3CSK4, c = p < 0.01 compared to PDTC + Pam3CSK4.
Figure 13. Pam3CSK4 treatment of pCGC cultures increases NF-κB activation and transcription of multiple classes of immune proteins and PRRs. pCGC cultures were treated for 2, 8, or 24 hours with vehicle control, 1.0 μg Pam3CSK4, or a 30 minute pretreatment with PDTC followed by Pam3CSK4. RNA was isolated and RT-PCR performed to quantify NF-κB regulated transcriptional products. (A) Expression of C3 is not increased after 2 hours (Control 1.09 ± 0.52; Pam3CSK4 0.69 ± 0.18; p = 0.406), but is significantly increased after both 8 (Control 1.04 ± 0.33; Pam3CSK4 2.52 ± 0.20; p = 0.000031) and 24 hours of treatment (Control 1.04 ± 0.35; Pam3CSK4 4.03 ± 1.22; p = 0.0034). Pretreatment with PDTC only partially attenuates this increase at 8 hours (PDTC + Pam3CSK4 1.72 ± 0.12; p = 0.0086 compared to control, p = 0.0032 compared to Pam3CSK4). (B) Transcript for High affinity Immunoglobulin Gamma Fc receptor (FcgR1) was increased after 24 hour Pam3CSK4 exposure (Control 1.03 ± 0.29; Pam3CSK4 2.56 ± 0.26; p = 0.00024). (C) β2-microglobulin, a protein related to MHC class I signaling was not increased at 2, 8, or 24 hours, but after 72 hours of Pam3CSK4 treatment β2-microglobulin transcript was significantly higher than controls (Control 1.02 ± 0.23; Pam3CSK4 1.60 ± 0.26; p = 0.016). Each time point was analyzed by one-way ANOVA and Bonferroni post hoc testing of data normalized to control expression. For time points with 2 groups, a two-tailed Student t-test was performed. n = 4 for each sample at all time points. Error bars represented standard deviation, a = p < 0.01 compared to control, b = p < 0.01 compared to Pam3CSK4, c = p < 0.01 compared to PDTC + Pam3CSK4.
Figure 14. Pam3CSK4 treatment of pCGC cultures selectively increases NF-κB regulated toll-like receptor transcripts. pCGC cultures were treated for 2, 8, or 24 hours with vehicle control, 1.0 ug Pam3CSK4, or a 30 minute pretreatment with PDTC followed by Pam3CSK4. RNA was isolated and RT-PCR performed to quantify NF-κB regulated transcriptional products. (A) Pam3CSK4 treatment produces a robust increase in Tlr2 transcript at 2 hours (Control 1.01 ± 0.16; Pam3CSK4 3.60 ± 0.60; \( p = 0.00006 \)). This increase persists after 8 hours (Control 1.02 ± 0.24; Pam3CSK4 3.01 ± 0.18; \( p = 0.0008 \)), 24 hours (Control 1.01 ± 0.15; Pam3CSK4 4.35 ± 0.85; \( p = 0.0002 \)), and 72 hours (Control 1.05 ± 0.38; Pam3CSK4 4.57 ± 0.27; \( p = 5.6 \times 10^{-6} \)) of agonist exposure. Pretreatment with PDTC attenuates this increase at both the 2 hour (PDTC + Pam3CSK4 0.90 ± 0.47; \( p = 0.00004 \) compared to Pam3CSK4; \( p = 1.00 \) compared to Control) and 8 hour (PDTC + Pam3CSK4 0.72 ± 0.79; \( p = 0.0003 \) compared to Pam3CSK4; \( p = 1.00 \) compared to Control) time points. (B) Pam3CSK4 treatment does not lead to a significant increase in Tlr4 transcript expression at 2 hours or 8 hours of exposure, demonstrating specific activation of NF-κB transcriptional regulation. Each time point was analyzed by one-way ANOVA and Bonferroni post hoc testing of data normalized to control expression. For time points with 2 groups, a two-tailed Student t-test was performed. \( n = 4 \) for each sample at all time points. Error bars represented standard deviation, \( a = p < 0.01 \) compared to control, \( b = p < 0.01 \) compared to Pam3CSK4, \( c = p < 0.01 \) compared to PDTC + Pam3CSK4.
Figure 15. *Pam3CSK4 treatment of pCGC cultures does not increase transcript expression of glial markers*. pCGC cultures were treated for 8 hours with vehicle control, 1.0 ug Pam3CSK4, or a 30 minute pretreatment with PDTC followed by Pam3CSK4. RNA was isolated and RT-PCR performed to quantify NF-κB regulated transcriptional products. Pam3CSK4 treatment did not increase levels of Gfap or Aif1 transcript expression in culture, demonstrating the absence of a large glial response. Each time transcript was analyzed by one-way ANOVA and Bonferroni post hoc testing of data normalized to control expression. n = 4 for each sample at all time points. Error bars represented standard deviation. No significant differences were detected.
To prove specificity, we repeated the above experiment for select transcripts (C3, β2-microglobulin, Fcgr1, and IκBα) using pCGC cultures generated from mice lacking Tlr2 receptors (For details on mice, see Chapter 3 methods). Quantification of these transcripts at 8 and 24 hours revealed no significant differences between control and Pam3CSK4 treated samples (Figure 16, 8 hour data not shown). These data prove that increased levels of NF-κB regulated transcripts are a definitive result of Pam3CSK4 activation of Tlr2 and subsequent activation of NF-κB signaling, and not a result of off target effects.

Potential Activators of Tlr2 Signaling in the Aged Cerebellum

The work presented in this chapter details a potential mechanism for NF-κB activation in the cerebellum, particularly in cerebellar granule cells. Our data demonstrates that (1) PRRs, immune proteins, and NF-κB signaling proteins are expressed in cerebellar granule cells and (2) stimulation of a single specific PRR (Tlr2) can result in p65 translocation, p65 phosphorylation, DNA binding, and transcriptional activation resulting in the generation of more immune proteins that is abolished in neuronal cultures lacking Tlr2. This data clearly displays that PRR to NF-κB signaling is present in pCGCs and is inducible. One major caveat to activation within this model is the reliance on a synthetic agonist. While Pam3CSK4 was an excellent tool for activating this pathway, it does not mimic the human condition. Pam3CSK4 is absent in the aging brain and its biological counterpart would only be present during infection with specific bacterial strains. Therefore, if this mechanism does contribute to NF-κB dysregulation in the aging brain, an endogenous agonist would be necessary to initiate the process. Reexamination of our protein array data suggests a possible candidate that may similarly activate Tlr2 signaling within the aging brain.
Increased PRR and immune protein transcripts in pCGC culture are specific to Tlr2 activation by Pam3CSK4. Tlr2 knock-out pCGC cultures were treated for 24 hours with vehicle control or 1.0 ug Pam3CSK4. RNA was isolated and RT-PCR was performed to quantify NF-κB regulated transcriptional products. In the absence of Tlr2 receptors, Pam3CSK4 treatment does not increase the transcript level of C3, β2-microglobulin (β2M), Fcer1, or IκBα after 24 hours of agonist exposure. This demonstrates that increased PRR and immune protein transcripts in WT pCGC cultures are a specific result of Tlr2 ligation and not off target effects. Error bars represent standard deviation. Two-tailed Student’s t-tests between treatment and control for each transcript demonstrated no significant differences between treated and control samples. Data is representative of 4 biological replicates per group. Error bars represent standard deviation from the mean.
Specifically, we noted that aged cerebellar tissue expresses significantly higher levels of β-amyloid and tau related proteins compared to adult controls. We noted a greater than 2-fold increase in the expression of amyloid β A4, also known as amyloid precursor protein (APP) (Adult 1.00 ± 0.22; Aged 2.13 ± 0.50; p = 0.04). Aged cerebellar samples were also enriched for total tau (Adult 1.0 ± 0.05; Aged 1.15 ± 0.03; p = 0.028) and phosphorylated tau (S422: Adult 1.0 ± 0.17; Aged 1.42 ± 0.12; p =0.027) (Table 3). Other independent studies have demonstrated that Aβ42, a key molecule associated with Alzheimer’s disease pathogenesis, increases in the aging brain and is a known activator of PRRs in vivo and in vitro. Amyloid β oligomers can activate several classes of PRRs and immune proteins including toll-like receptors and complement. However, the research has been focused on glial activation and inflammatory responses, often in the framework of Alzheimer’s disease. In the context of glial cells, activation of Tlr2 by amyloid β increases its clearance by promoting phagocytosis. Loss of Tlr2 leads to a reduction in phagocytosis and increased accumulation of Aβ42 deposits. The role for amyloid β induced neuronal PRR activation is much less understood. Aside from amyloid, PRRs have other endogenous ligands that are present in the aging brain, often classified as damage associated molecular patterns (DAMPs). These include endogenous Tlr2 agonists: heat shock proteins (HSPs) and HMGB1. In support of our hypothesis that dysregulation of NF-κB occurs in distinct brain regions, endogenous PRR ligands appear to be enriched in select brain regions and cell types within the aging brain.

Conclusion and Discussion

Proposed Mechanism

In this chapter, we described the presence of inducible of NF-κB signaling in cerebellar granule cells evident by increased translocation, phosphorylation, DNA
binding, and transcriptional activation in response to stimulation of Tlr2. Our previous data suggests that NF-κB dysregulation may contribute to age-related cerebellar granule cell deficits and subsequent locomotor dysfunction. We have also described the accumulation of β amyloid in the aged C57BL/6 cerebellum, which can act as an endogenous ligand to PRRs. Based on our data we propose that long term, compounding activation through multiple inputs leads to dysregulation of NF-κB activity. Specifically, we propose that (1) increased expression of amyloid β, HSPs, HMGB1, and other endogenous PRR agonists activate neuronal PRRs, (2) PRR stimulation leads to increased NF-κB activity, (3) activated NF-κB upregulates transcription of κB regulated gene products including those for synaptic regulation, PRRs, and immune proteins, (4) increased expression of immune proteins leads to further NF-κB activation through a positive feedback loop, (5) continued activation leads to increased expression of PRRs, immune genes, and synaptic components observed in the aging cerebellum, (6) increased synaptic proteins lead to cellular dysfunction and downstream functional deficits (Figure 17). This hypothesis is intentionally broad, as the details of NF-κB signaling in the aging CNS are limited and there is still much to learn about mechanisms controlling NF-κB activation. The following paragraphs will discuss some these topics in relation to our results and general mechanisms for age related cerebellar dysfunction.

**Modes of NF-κB Signaling Activation in the Aging Brain**

We chose to focus our analysis on a single mode of activation as a proof of principle that immune receptor stimulation can lead to changes in NF-κB activity in cerebellar granule cells. The availability of a well-defined commercial agonist, age-associated increases in expression, and accumulation of potential endogenous agonists made Tlr2 a logical target. However there are several other receptors that are upregulated with aging that can initiate NF-κB activity and therefore contribute to dysregulation. NF-κB
Figure 17. Proposed mechanism for NF-κB dysregulation in the aged cerebellum. (A) NF-κB activity is present at a basal state in young and adult cerebellar neurons. Basal signaling for cell survival and response to injury leads to activation of NF-κB signaling through phosphorylation and degradation of inhibitory IκB. NF-κB proteins translocate to the nucleus and regulate transcription. Transcriptional products for PRRs, synaptic proteins, etc. are generated for maintenance of normal cell health. IκB is generated, competes with DNA to bind NF-κB proteins, and regulates the level and duration of activation. (B) As aging progresses, DAMPs and β-amyloid slowly accumulate and initiate aberrant NF-κB signaling. Increased input through PRRs, leads to increased NF-κB activity (red arrows), but compensation occurs through increased regulatory activity (IκB) to limit signaling. Increased PRR transcription leads to higher levels of surface expression. (C) Further increases in DAMPs and β-amyloid result in higher levels of PRR activation and NF-κB activity to a point of dysregulation. Products of NF-κB regulated transcription increase leading to altered expression of synaptic proteins, immune molecules, PRRs, and markers of cell stress, among others. Changes in expression results in neuronal dysfunction and age related deficits in the absence of significant cell death.
signaling initiators increased in aging including dectins\textsuperscript{132,133}, complement\textsuperscript{134,135}, and Fc receptors\textsuperscript{136}, among others\textsuperscript{5}.

We also speculate that amyloid \( \beta \) acts as a driver of NF-\( \kappa \)B activity based on our observation of increased levels in the aging cerebellum. Amyloid \( \beta \) exposure has been shown to activate NF-\( \kappa \)B activity in cerebellar granule cells, but the effect appears dose dependent. At low, sub-toxic doses exposure to amyloid \( \beta \) leads to increased NF-\( \kappa \)B activity that protects neurons against subsequent higher dose exposure\textsuperscript{46}. Our Western blot data shows a significant increase in the deposition of amyloid \( \beta \), using an antibody that detects both cleaved forms and APP, which was increased in phosphoarray analysis (data not shown). One cleavage product, A\( \beta \)42 is considered the most pathological form of amyloid, as it is the most likely oligomerize and form fibrils\textsuperscript{137-139}. It is important to note that while A\( \beta \)42 is often associated with Alzheimer’s disease, studies repeatedly find amyloid deposition in the brains of elderly subjects in the absence of neurodegeneration or dementia\textsuperscript{140-142}. The gradual accumulation of amyloid \( \beta \) in the aging cerebellum may function to inhibit its toxic effects. This is in agreement with our previous findings that increased PRR expression and synaptic alterations are not associated with cell death. Furthermore, we observed increased levels of Bcl-2 transcript, a known anti-apoptotic factor in the aged cerebellum\textsuperscript{5}. In the aging brain, it is unlikely that amyloid \( \beta \) is the sole driver of NF-\( \kappa \)B dysregulation, as aging is associated with increases in several endogenous activators of immune proteins. A more likely mechanism would involve the accumulation of PRR activators including A\( \beta \)42\textsuperscript{122,123}, mitochondrial components\textsuperscript{143}, heat shock proteins (HSPs)\textsuperscript{127-129}, and HMGB1\textsuperscript{130,131}, coupled with reduced microglial clearance\textsuperscript{144} resulting in a microenvironment increasing cell susceptibility to NF-\( \kappa \)B activation via multiple immune receptors. The resulting NF-\( \kappa \)B activation may overwhelm compensatory mechanisms leading to dysregulation of transcriptional activity. Future
studies will need to assess the regulatory mechanisms of NF-κB signaling within the aging brain from initiation to resolution in order validate this hypothesis.

**NF-κB Signaling Cascades and Regulation**

Our data clearly demonstrates induction of NF-κB activity in response to Tlr2 stimulation, but the mechanism and roles of intermediary kinases responsible for activation were not determined. We did demonstrate that several kinases upstream from NF-κB are present in pCGC cultures including IKK-α, IKK-β, and Syk, but we have not fully detailed the activation of each of these signaling molecules in response to Tlr2 ligation. Previous work from our laboratory suggests age-related increases in phosphorylation of Syk at several key sites responsible for promoting long-term activation (unpublished observations). Syk can serve as an integration site for several upstream immune pathways, which suggests it may be a high value target for future studies. In concordance with this data, our protein phosphorylation array revealed increased expression of numerous Syk interacting proteins including including Src, Cd5, Zap70, Vav1, Shc1, Pla2g4a, Raf1, and Hsp27. Investigating regulation of Syk activity in response to aging and PRR stimulation may offer novel insight into mechanisms of age related CNS decline. Additionally, direct inhibition of NF-κB signaling is acutely toxic to neurons, and constitutive NF-κB activity is important for cell survival. Therefore, NF-κB cannot serve as a therapeutic target. However, targeting upstream activators or mechanisms of specific gene regulation may offer potential therapeutic benefits.

Post-translational modifications, primarily phosphorylation, are critical players in the regulation of NF-κB activity. Collectively, there are 11 known phosphorylation sites in the p65 protein, each with roles in NF-κB regulation. The two most studied sites are serine residues 536 and 276. Our analysis of Tlr2 stimulated cerebellar granule cells
demonstrated increased phosphorylation of S536 after 2 hours of stimulation. This increase can be attenuated by pretreatment with PDTC, an inhibitor of NF-κB activity. S536 is most commonly phosphorylated by several kinases, IKK-α and IKK-β that are expressed in cerebellar neurons based on our data. The site is located in the C-terminal transactivating domain and the effect of phosphorylation appears to be context dependent. The main effect is enhanced transactivation by increasing CBP/p300 binding and p65 acetylation\textsuperscript{145}. However, S536 phosphorylation has also been shown to increase p65 turnover\textsuperscript{107} and reduce nuclear import\textsuperscript{146}. In the context of Tlr signaling, S536 phosphorylation has been shown to lead to proteosomal degradation of p65 and therefore attenuation of NF-κB activity\textsuperscript{107}. Selectivity of gene expression has also been linked to S536 phosphorylation and interactions with IκBα. Nuclear IκBα can prevent p65 binding to specific promoters of TNF-α, IL-1β, and IL-6, but when phosphorylated at S536 it does not inhibit binding to the IL-8 promoter. In summary, this data suggests that S536 phosphorylated p65 may act independently from IκBα in the nucleus to regulate specific subsets of gene transcription\textsuperscript{147}. While we did not evaluate S276 phosphorylation, we feel it is worth noting the general understanding of its functional implications. Protein kinase A activity phosphorylates p65 at S276 leading to increased NF-κB activity by increasing p65 binding with CBP/p300\textsuperscript{147}. Additionally, p65 phosphorylated at S276 functions in epigenetic repression through interaction with DNA (cytosine-5)-methyltransferase \textsuperscript{148}, selective regulation of gene expression though cyclin dependent kinase 9 / cyclin T1 complexes\textsuperscript{149}, and regulating other post-translational modifications (ie. acetylation)\textsuperscript{145}. There are several other sites of phosphorylation for p65, and numerous other NF-κB family proteins contain phosphorylation sites including p50, p100, c-Rel, RelB, and p105 that we did not assess.
Evaluation of each would add value to generating a mechanistic understanding of PRR mediated NF-κB signaling in cerebellar neurons.

While phosphorylation of NF-κB proteins are the most studied, other modifications are also critical in guiding activity including acetylation, glycosylation, sumoylation, and ubiquitination. Acetylation of p65 K310 promotes p65 interactions with CBP/p300 thereby increasing activity. Ubiquitination of NF-κB protein will often lead to protein degradation by the proteasome, which can either activate or regulate NF-κB activity. Ubiquitination of IκBα leads to its degradation freeing p65 to translocate and activate transcription. However, ubiquitination and degradation of DNA bound NF-κB has been identified as an important IκBα independent mechanism of regulation. Glycosylation can negatively regulate p65 phosphorylation at S536 thereby indirectly regulating activity. Similar to ubiquitination, sumoylation functions as a negative regulator of NF-κB activation through its actions on both p65 and IκBα.

**Potential Roles for Non-canonical Signaling**

The majority of research, including the work presented in this chapter has focused on the canonical NF-κB signaling pathway (p50/p65 heterodimer). Data supports the presence and function of canonical signaling in the CNS. However non-canonical NF-κB activity may also contribute to dysregulation. Non-canonical signaling involves activation of the p52/RelB complex and is dependent on the inducible processing of p100 and NF-κB-inducing kinase (NIK). Activation of receptor activator of nuclear factor kappa-B ligand (RANKL), CD40 ligand (CD40L), B cell-activating factor (BAFF), lymphotoxin beta (LTβ), TNF-like weak inducer of apoptosis (TWEAK), or LIGHT (a member of the TNF ligand superfamily) activates NIK which phosphorylates and activates IKK-α. Activated IKK-α phosphorylates p100 triggering ubiquitination and partial degradation of p100 to generate p52 that can function to activate specific target
genes though dimerizing with RelB\textsuperscript{154, 155}. Our data demonstrated activation of canonical signaling (p65 translocation and phosphorylation) with stimulation of Tlr2, but it is possible that additional non-canonical signaling occurs in response to Tlr2 ligation. Much less is known about non-canonical signaling with the CNS. The transcriptional products we assessed are only a small snapshot of those regulated by NF-κB activity; therefore it is possible that non-canonical signaling also plays a role in age-related dysregulation. Future work to detail non-canonical NF-κB activity in response to immune receptor ligation in neurons may offer novel insights beneficial for directing future therapies.

**Selectivity of NF-κB Gene Regulation**

The human genome contains an estimated 1.4 x 10\textsuperscript{4} potential κB binding domains; yet, activation of NF-κB appears to yield specific transcriptional outcomes. Within our studies, pCGC cultures were exposed to a constant concentration (1.0 μg/mL) of Tlr2 agonist for a given duration of time, after which we measured NF-κB activity. We measured translocation at 1 hour, phosphorylation at 2 hours, reporter levels at 48 hours and transcriptional products at 2, 8, 24, 48, and 72 hours. Interestingly, we noted early increases in select PRRs and immune protein transcripts, later increases in others, and no change in others (Lyz, data not shown). This demonstrates a selective transcriptional response to Tlr2 stimulation. The selectivity of NF-κB responses is dependent on multiple factors including chromatin structure, phosphorylation, the presence of binding partners, and duration of activation. The duration of NF-κB activity is controlled by IκBα dependent export and nuclear degradation of NF-κB signaling proteins\textsuperscript{156}. Our analysis of transcriptional products demonstrated a significant increase in IκBα as after 8 hours of Tlr2 stimulation. Additionally we noted a trend towards increased IκBα fluorescent intensity in the translocation experiments at 1 hour, suggesting regulatory mechanisms were activated. However, increased levels of NF-κB regulated transcripts persisted
through all time points, suggesting counter-regulatory mechanisms may have been attenuated by chronic stimulation. Understanding mechanisms of response to chronic activation may be a critical when treating conditions characterized by increased NF-κB activation. Contextually, this supports the idea for chronic activation by amyloid β in the aging brain, where early activation leads to cell survival mechanisms that are then overridden by continued input. Amyloid β deposition in the brain is a gradual process of accumulation, where increasing levels may drive further stimulation of PRRs. Additionally, the potential for the generation of positive feedback loops through the production of PRRs and immune proteins would lead to even greater sites for potential stimulation. A more thorough understanding of the activation cascade and post-translational status of NF-κB proteins will help inform transcriptional selectivity of NF-κB activation. Furthermore, with the advent of more cost-effective single cell sequencing platforms, the full status of transcriptional expression in response to PRR stimulation could be assessed. This would allow one to determine if there are cell type or subpopulation specific responses to NF-κB activation. Understanding these mechanisms on the cellular level would inform future research on the downstream effects on neuronal structure and function.

**Role of NF-κB Dysregulation in the Aging Brain**

NF-κB dysfunction is involved in many neurodegenerative conditions including Alzheimer’s disease, Parkinson’s disease, and aging both systemically and within the CNS. Over 500 NF-κB regulated genes have been linked to pathological conditions, but there is not a single clinically approved NF-κB inhibitor, as global inhibition leads to many side effects. Inhibition of NF-κB in cerebellar granule cells cultures results in apoptosis, and our unpublished observations). Research on other cell types, primarily hippocampal neurons, demonstrates that NF-κB activity promotes...
dendritic spine and synapse formation, and is involved in long-term potentiation and synaptic plasticity\textsuperscript{26, 41, 161, 162}. However, it has also been shown that long-term activation of NF-κB by TNF-α treatment in cultured hippocampal neurons impairs generation of synaptic currents by modulation of calcium channels and glutamate receptors\textsuperscript{163}. Therefore, the effect of NF-κB activation on synapses appears context dependent. Our future research will be geared toward understanding how NF-κB activation through PRR stimulation affects neuronal structure and function. Upon inhibition of NF-κB signaling by PDTC, we noted a decrease in Vglut1 mRNA, but as PDTC treatment leads to cell death after 24 hours it was not clear if this was a direct effect on synapses or a result of apoptotic processes (data not shown). Synaptic densities were also measured after stimulation of Tlr2, but we did not observe differences that reached statistical significance. Our model is based on chronic dysregulation through multiple inputs, therefore we hypothesis that with a more chronic or combined NF-κB activation model we would observe synaptic differences in either density or function.

Overall, our data confirms the presence of inducible NF-κB signaling in cerebellar granule cells, and begins the process of detailing potential mechanisms of dysregulation in the aging brain. Additional research is required to identify molecular mechanisms underlying NF-κB activation and regulation in the aging brain. As direct NF-κB inhibition is not a viable therapeutic option due to toxicity, determining upstream kinases and regulatory mechanisms may provide more selective targets to specifically modulate outcomes of NF-κB activation and therefore functional deficits.
References


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Chapter 2: Complement Protein C3 Loss leads to Significant Locomotor Deficits and Cerebellar Synaptic Deficits

Introduction

Complement Pathway Signaling and Regulation

The complement pathway has been well studied for over one hundred years and primarily known for its function within the innate immune system. The first description of complement, historically called ‘alexin’ (Greek for ‘to ward off’), came from Buchner and his colleagues in 1891 when they discovered a heat labile factor in blood that aided in the killing of bacteria. Over the next 100 years several major discoveries led to our current understanding of complement. First, in the early 1900’s Ferrata and Brand concluded that complement is not a single factor, as they were able to separate complement into two fractions each necessary for bactericidal function. Additional work demonstrated even more complement components, including the discovery of the heat-stable complement factor C3. Complement was originally thought to be a lipid complex, but advances in techniques for protein isolation and characterization revealed that complement factors were in fact proteins. Over the succeeding decades critical works demonstrated the array of complement proteins including C4, C5, C6, C7, C8, and C9. It was through the discovery of each of these components that the first details of the classical complement activation cascade was elucidated with sequential binding of C1 followed by C4, C2, C3, C5, C6, C7, C8, C9. Since these early discoveries much progress has been made on the understanding of complement function and different complement activation pathways.

Within the classical complement activation cascade, IgM or IgG antibody/antigen complexes bind to C1q, leading to activation of C1r, and consequentially cleavage of C1s. C1s then cleaves both C4 and C2, leading to the generation of C4b2a (C3
convertase). C4b2a cleaves C3 into C3a and C3b, which possess distinct biological functions. C3a, a 10 kD protein fragment functions primarily as an anaphylatoxin to promote local inflammatory responses via activation of pro-inflammatory signaling pathways. C3b, in the classical activation pathway binds to the C4b2a complex generating C5 convertase that in turn cleaves C5 into C5a and C5b. C5b initiates the formation of the membrane attack complex (MAC) through association with C6, C7, C8, and C9. This complex creates pores in invading cells and leads to the death of the targeted pathogen. Over fifty years after the discovery of the classical complement pathway Pillemre et al. proposed an alternative pathway of activation.

Now well described, the alternative complement activation pathway occurs in the absence of antibody/antigen interactions when C3 is spontaneously cleaved into a C3b like molecule known as C3-(H2O). This spontaneous cleavage of C3 is due to a highly reactive internal thioester bond. C3-(H2O) can then deposit onto an invading pathogen, bind to complement factor B, which is then cleaved by factor D to generate C3bBb. The C3bBb complex is stabilized on the bacterial surface by properdin (Factor P), which is enriched on the microbial membrane. This stabilized complex functions as a convertase generating more C3b, and incorporation of an additional C3b into C3bBb generates C5 convertase and the downstream formation of the membrane attack complex. Importantly, circulating C3b that binds to a surface also functions an opsonin, thereby tagging that surface whether it be a microbe, cell debris, extracellular protein, etc. for targeted phagocytosis.

Roughly four decades after the description of the alternative pathway, a third mode of complement activation was discovered and described as the Lectin pathway. Like the alternative pathway of complement activation, the Lectin pathway does not require an antibody antigen interaction to initiate the cascade. For the Lectin pathway, initiation
of the complement cascade occurs when mannose-binding lectin (MBL) or ficolin interact with specific carbohydrate residues less common in the host, leading to MBL-associated serine protease (MASP) activation. MASP proteins then cleave C2 and C4 to form C3 convertase (MASP-2) or directly cleave C3 (MASP-1)\(^{14, 15}\). Ficolin proteins can also interact with MASP proteins to active complexes capable of complement protein cleavage\(^{16}\).

Complement can also be activated through serine proteases that are not part of the traditional three cascades. For example, coagulation factors Xa, Xia, and plasmin are capable of cleaving C3 and C5\(^{17}\). Additionally, von Willebrand factor and factor VIII can activate lectins leading to complement cascade activation\(^{18}\). While complement activation is critical for proper innate immune responses in the host, it can also be detrimental if activation goes unchecked. Therefore, there are a host of complement regulatory proteins to reduce the risk of complement-mediated damage to autologous tissues.

Complement activation is predominately controlled through regulatory proteins that exist as membrane bound or plasmatic proteins. Initiation of classical and lectin pathway activation is regulated by plasmatic C1 inhibitor (C1INH or SERPING1) through irreversible binding and inactivation C1 subunits C1r and C1s, as well as MASP proteins\(^{19-21}\). Activation of the alternative pathway is controlled by complement factor H (CFH) and complement factor I (CFI) where CFI functions as a serine protease to permanently inactivate C3b and CFH is a cofactor for proper CFI function\(^{22}\). Furthermore, CFH competes with complement factor B for binding to C3b and destabilizes C3bBb preventing C5a convertase formation, which also restricts alternative complement pathway activity\(^{20, 21, 23}\). CFH is also able to regulate complement activation at the cellular membrane by binding to heparin salts and the C3d portion of C3b
preventing complement-mediated damage to host cells\textsuperscript{24-27}. Additional membrane bound regulators of complement activation include decay-accelerating factor (DAF/CD55), membrane cofactor protein (MCP/CD46), and CR1 (CD35). DAF functions to accelerate the dissociation of C3 convertase in alternate pathway activation, MCP functions as a cofactor for CFI and CR1 has multiple roles including serving as a CFI cofactor and accelerating the dissociation of C3 convertase in all three pathways\textsuperscript{28-30}. Membrane bound complement regulators are not expressed in all cells and their expression level varies across tissues, therefore the coordination of both plasmatic and membrane bound complement regulators is necessary to reduce damage to host cells.

The majority of complement proteins and plasmatic regulators are primarily synthesized in the liver, with hepatocytes contributing over 90% of serum complement proteins\textsuperscript{31}. However, there is a wealth of primary data demonstrating synthesis in other tissues. Interestingly, this includes both tissues/cells that are exposed to peripheral complement like parenchymal kidney cells and immune cells, as well as areas that are not normally exposed to complement proteins from peripheral sources like the CNS\textsuperscript{32-35}. In peripheral systems complement activation results in the destruction of pathogens through inflammatory and phagocytic responses, and if left unregulated it results in damage to self-tissues through those same pathways. While the role of complement in the immune system is well established, much less is known for the role of complement proteins within the nervous system. This lack of knowledge stems partially from the long-standing, but incorrect belief that the central nervous system is immune privileged. Interestingly, within the CNS, complement proteins have maintained some aspects of peripheral roles, but also function in distinct CNS processes like neuronal migration, neuronal stem cell maturation, synaptic pruning, and cell survival.

\textit{Complement Proteins in the Central Nervous System}
Despite the large breadth of knowledge on complement pathway activation and functions in the periphery, less in known on the roles that complement proteins play within the CNS. Complement proteins were first identified in the CNS in human samples, but concern over the specificity of synthesis or entry through leakage in the blood brain barrier limited the impact of this observation. For complement proteins to have functional roles in the CNS, local synthesis is required as the circulating complement proteins are excluded from CNS entry by the blood brain barrier. Therefore, complement proteins in the CNS were not heavily studied until in situ hybridization confirmed the local CNS synthesis of complement proteins\textsuperscript{36-38}. Through numerous other studies it was demonstrated that the majority of complement proteins and receptors are expressed in microglia, astrocytes, and neurons throughout the majority of brain regions\textsuperscript{33, 39-43}. Initial studies concluded that complement proteins are expressed and synthesized in the CNS, but the expression was considered to be primarily a result of injury or inflammatory signaling\textsuperscript{44, 45}. However, additional studies demonstrated the presence of complement proteins in the non-pathological state in several glial subtypes and neurons.

**Complement Expression in the CNS**

The primary producers of complement proteins in the CNS are glial cells including astrocytes, microglia, and oligodendrocytes. Synthesis of many years of research concluded that astrocytes are able to produce complement proteins within the classical and alternative pathways including C1q, C1r, C1s, C2, C3, C4, factor B and factor D\textsuperscript{36, 41, 46-51}. Astrocytes synthesize terminal complement components C5-C8 and express complement receptors C1qR, CR2, C3aR, C5aR. As far as complement regulatory proteins, astrocytes produce both soluble factors including C1INH, factor H, and factor I, and membrane bound CD59, DAF, MCP, and CR1\textsuperscript{52}. Similarly, microglia are able to synthesize complement proteins of both classical and alternative signaling pathways and
may be the predominate producer of C1q in the CNS\textsuperscript{36, 49}. Oligodendrocytes and oligodendrocyte precursor cells have been shown to produce numerous complement proteins including C1q, C1s, C2, C3, C4, C5, C6, C7, C8, and C9, but lack expression of many complement regulatory proteins\textsuperscript{53}. There is increasing evidence for complement production in other cell types within the nervous system including pericytes\textsuperscript{54}, endothelial cells\textsuperscript{55}, and ependymal cells\textsuperscript{56}, but the level of contributions of complement to the CNS from these cell types is poorly understood. Aside from glial, endothelial, and epithelial cells, neurons also produce complement proteins. In situ hybridization studies have demonstrated de novo synthesis of C1q, C2, C3, C4, C5, C6, C7, C8, and C9 in both the temporal cortex and hippocampus\textsuperscript{57}. Constitutive expression of complement proteins C4 and C1q, as well as inducible expression of C3 has also been observed in cerebellar granule cells\textsuperscript{58}. Neurons express complement regulatory proteins MCP and CD59 at low levels, and completely lack DAF and CR1, which may partially explain their susceptibility to complement mediated cell damage\textsuperscript{59}.

Newly developed techniques for single cell sequencing have greatly increased the ability to determine complement protein expression in subtypes of neuronal cells with higher accuracy and efficacy than \textit{in situ} hybridization. As this technology is just becoming popularized there is limited data, but initial studies offer promise for providing a more detailed description of complement expression within discrete neuronal and glial subtypes. One recent study using the drop-seq single cell-sequencing platform set out to characterize transcript expression of cells in the arcuate nucleus of the hypothalamus and median eminence. While the primary output for this study was to identify distinct cell populations, it also offers great insight to single cell expression of complement proteins. Astrocytes, microglia, and neurons in this study were shown to expresses variable levels of RNA for complement proteins and receptors\textsuperscript{60}. A more detailed analysis of data sets
from future single cell studies will provide novel insights into complement expression in the CNS.

**Complement Functions in the CNS**

Complement proteins in the CNS have functional importance in both the healthy and pathological setting with roles both analogous and distinct to those in the periphery. Similar to the periphery, complement proteins can function as opsonins and tag cellular debris for phagocytosis by microglia, the resident CNS phagocytic cell. However, there are numerous non-immunological roles for complement proteins. Complement proteins regulate neuronal migration, proliferation, adult neurogenesis, and synaptic regulation. Loss of the receptor for C3a (C3aR) leads to reduced neurogenesis and decreased numbers of migrating newly formed neurons in response to ischemic injury. Additionally, stimulation of C3aR through sub-dural delivery of a C3aR agonist results in a transiently reduced thickness of the cerebellar external granule cell layer (EGCL) and increased thickness of the cerebellar internal granule cell layer (IGCL). Conversely, stimulation of C5aR results in the opposite phenotype with increased thickness of the EGCL and a thinner IGCL, suggesting that C3 and C5 play opposing roles in regulating cerebellar granule cell proliferation and migration. Interestingly, there are no long-term gross cerebellar abnormalities in mice lacking C3, C5, C3aR, or C5aR suggesting that complement protein expression may serve as a rescue mechanism in the event of developmental inflammation or the existence of compensatory mechanisms to ensure proper gross cerebellar development. Complement proteins interactions have also been shown to regulate the differentiation and migration of neuroprogenitor cell in vitro.

Aside from roles in differentiation, proliferation, and migration complement proteins can function in neuroprotection by reducing apoptosis through downstream signaling. C5 deficient mice are more susceptible to glutamate-mediated toxicity, demonstrated by
increased hippocampal neurodegeneration due to increased AMPA binding capacity in response to Ca2+ incubation\textsuperscript{65}. C5a has been shown to reduce apoptosis through ERK1/2 inhibition of caspase-3 and down regulation of glutamate receptor subunit 2\textsuperscript{66-68}. C5a activation has also been shown to lead to rapid activation of protein kinase c and NF-κB in neuroblastoma cells, further suggesting potential roles in neuroinflammatory regulation\textsuperscript{69}. Additionally, C3a can protect neurons from N-methyl-D-Aspartate (NMDA) induced injury; however, a direct mechanism has yet to be determined\textsuperscript{70}.

Finally, complement proteins are important for axon growth and synaptic development, pruning, and maintenance. C1q regulates axonal growth by direct interaction with myelin-associated glycoprotein to reduce the activation of growth inhibitory signaling, thereby enhancing axon growth properties\textsuperscript{71}. Both C1q and C3 have been shown to deposit and mark or opsonize synapses for targeted pruning by microglia in the developing brain\textsuperscript{33, 72}. Supporting this role for complement proteins, C1q and C3 knockout mice show significant impairments in synaptic elimination in the lateral geniculate nucleus of the visual system leading to non-segregated eye-specific territories and retention of multi-innervated relay neurons\textsuperscript{33}. C1q knockout animals also show increased presynaptic terminals in layer V pyramidal neurons and increased evoked and spontaneous epileptiform field potentials, signifying increased excitatory function\textsuperscript{72}. It is believed that increased synaptic density in complement knockout animals is a result of reduced engulfment of synapses by microglia. Supporting this hypothesis, loss of C3 or its receptor leads to a significant impairment in microglial ability to engulf synaptic proteins in the retinogeniculate system\textsuperscript{73}. While loss of complement clearly leads to profound changes in synaptic pruning, mice lacking complement proteins still undergo significant pruning during development suggesting the importance of multiple pathways in these processes\textsuperscript{33}. In fact, many other immune protein pathways have been shown to
play critical roles in synaptic regulation, including toll-like receptor signaling (Chapter 1 and 3) and Class I MHC I proteins (Chapter 4). In addition to the role of complement proteins during development, there is increasing evidence describing complement proteins functions in aging and neurodegenerative disease\textsuperscript{74-76}.

Increased NF-κB signaling and complement expression are considered hallmarks of Alzheimer's disease pathology in both humans and murine models. Recent data has shown that Beta-amyloid exposure can increased NF-κB signaling in astrocytes leading to increased C3 production, which can act to disrupt neuronal dendrite morphology and synaptic function mediated through neuronal C3aR\textsuperscript{77}. Loss of C1q, C3, or antagonism of C3a in an AD model results in reduced phagocytic microglia and synapse loss further demonstrating functions for complement in regulating synaptic disruption in neurodegenerative conditions\textsuperscript{78}. Aside from AD pathology, mutations in proteins involved in the alternative pathway of complement activation have been linked with an increased risk of age-related macular degeneration\textsuperscript{79}. Additionally, human complement 4 has been shown to localize to multiple components of neurons including synapses and variation of the gene encoding for complement 4 has been linked to an increased risk of schizophrenia\textsuperscript{80}. Complement proteins C1q, and C3 among others have also been shown to increase in normal aging in both mouse and human samples\textsuperscript{75,76}. Interestingly, up regulation of complement proteins appear to occur in distinct brain regions that correlate with functional declines commonly observed in aging.

**Complement Proteins and Functional Deficits Associated with Aging**

Aging is associated with cognitive, metabolic, and locomotor disruptions, which lead to functional impairments that significantly lower quality of life and increase risk of health complications. Age-related functional deficits are often associated with CNS disruptions within specific brain regions including the hippocampus and cortex for cognitive decline,
the hypothalamus for metabolic disturbances, and the cerebellum for locomotor impairments. Interestingly, complement proteins are increased in a region specific manner in the aged brain. Complement protein C1q is upregulated in areas often associated with cognition in mice and humans including the hippocampus and piriform cortex. Additionally, C1q knockout mice are partially protected against age-related hippocampus-dependent cognitive decline as assessed by a battery of behavioral assays thought to measure hippocampal function including the Morris Water Maze (MWM) and Y-Maze. Similar to C1q, loss of C3 can protect against age related cell and synapse loss in the hippocampus and increase synaptic plasticity at 12 months of age. The majority of research investigating the role for complement proteins is based in cognitive research and neurodegenerative disease.

Aside from hippocampal and cortical areas, complement proteins are increased in other regions of the brain affected by aging and contribute to aging phenotypes. Our lab has demonstrated that complement proteins are increased in the cerebellum and hypothalamus of mouse strains with locomotor and metabolic phenotypes, respectively. Importantly, these changes are not isolated to mouse models as C3 and C4 transcripts were also found to be elevated in the human cerebellum with increasing age. However, the roles of complement in the development of functional deficits are poorly understood. While complement proteins are commonly associated with glial cell release and glial function, complement increases in the aged mouse cerebellum and hypothalamus can only be partially explained by microglia. When glial cells were isolated from these regions, only increases in C3 in the aged C57BL6/J cerebellum could be explained by increased transcripts in microglial cells. This data suggests that other cells, including neurons have increased complement expression with aging. While the cognitive effects of complement deletion have been well described, the roles of
complement within other systems are poorly detailed. Therefore, we set out to determine
genotypic differences between wild-type C57BL6/J control mice and mice deficient in
complement protein C3 (C3 KO) using a custom homecage monitoring system to assess
daily locomotor, eating, and drinking behaviors. Additionally, neuronal cultures were
assessed for differences in synaptic phenotypes to offer cellular mechanisms for any
underlying deficits found during behavioral assessment.

**Methods and Materials**

**Mice and Animal Husbandry**

Cohorts of 8 male C57BL/J6 (stock # 000664) and 8 male B6;129S4-C3\textsuperscript{tm1Crr}/J (C3
KO, stock #003641) mice were acquired from Jackson Laboratory. Prior to behavioral
assessment mice were allowed to acclimate to the UNMC vivarium for 1-2 weeks, in
efforts to reduce stress associated with delivery that may alter normal behaviors. For this
acclimation period, mice were singly housed in standard mouse cages on microisolator
racks (Lab Products Inc., Seaford DE). Cages contained a layer of ground corn bedding,
and mice were provided with a nestlet and ad libitum access to chow (#7012 Envigo) and
water. Following acclimation mice were moved into the home-cage monitoring system
(HCM) for a total of 21 days. Within the HCM, each mouse was housed in a low profile
cage (Allentown PC10196HT, 48×26×15 cm). Within each cage were a nestlet, niche,
corn bedding, and Alpha-dri Plus\textsuperscript{®} tabs (Shepherd Specialty Papers, Watertown, TN) to
reduce ammonia levels. While in the HCM mice had ad libitum access to milled chow
(#5058 PicoLab) and water. The health of each animal, as well as the level of food and
water available was visually checked daily. Food and water was changed twice weekly,
and the entering and exit weights were used to calculate daily consumption of food and
water. The first 5 days the mice are in the cage are considered acclimation days and are
therefore not analyzed. The last 16 days are data collection days and analyzed for genotypic differences in homecage behaviors.

*Homecage Monitoring System*

The homecage monitoring system was designed to characterize freely behaving mice with minimal human contact over an extended period of time with high spatial and temporal resolution. Using this system, activity patterns organized around circadian time are elucidated. Mouse position in the cage was calculated using three load cells positioned in a triangle at the base of the cage. Load cell voltage shares a linear relationship with torque; mouse positions can thus be determined by solving the three dimensional equations relating torque, force, and moment arm length. Load cell voltages are sampled at 1 KHz permitting temporal resolution of 1 ms. Feeding was measured by breaking of a photobeam when mice descend their snout into the feeder. Water intake was measured through capacitance changes in the lick spout when mice make contact to drink. For WT and C3 KO mice, feeding, drinking, and movement event data was collected using Labview 2012 (National Instruments) and a PXI-8109 (National Instruments) for roughly 24 hours per day for 16 days. Once the data collection was complete, it was processed using MATLAB 2011b (Mathworks) and a series of behavioral classification and analysis algorithms. Behavioral data undergoes a brief user-guided quality control assessment to identify any gross data quality issues (blocked photobeam, leaking lick spout, excessive position drifts). If identified, these data are removed from further analysis. Data loss from these errors is typically less than 1%. Next, automated classification of event data is transformed into measures of feeding, drinking, and movement. The HCM system can classify over 700 behavioral characteristics. Using false discovery rate analysis, a list of differentially expressed
behaviors is generated. For full characterization of this system, please refer to Goulding, et al. 2008.

**Assessment of locomotor function**

Mobility is measured using a machine learning algorithm that examines gait speed and turning angle characteristics of all locomotor events not involving either the feeder or licker, and uses the distribution of these measures to determine if movements best conform to forward locomotion, movement in place, or no movement. Data from all locomotor states can then be further analyzed for duration and circadian timing for each event. We assessed for gait ataxia in WT and C3 KO mice by using minimum bounding rectangle (MBR) analysis to quantify the straightness of individual locomotor paths. Differences in MBRs were quantified by repeated measures ANOVA using genotype as treatment factor, replicated for each animal over all days of data collection, and individual mice as covariates.

**Metabolic Assays**

To test for genotypic differences in body composition or metabolic function, WT and C3 KO mice were analyzed using Dual energy X-ray absorptiometry (DEXA) and indirect calorimetry. DEXA studies were performed by standard protocol (modified from mutant mouse phenotyping center www.mmpc.org/shared/showFile.aspx?doctypeid=3&docid=104 and Whitelabs www.whitelabs.org/Lab%20Protocols/live%20animal%20protocols/DEXA%20scanning%20protocol.htm) using inhalational isoflurane anesthesia, a PIXImus scanner (GE Lunar, Inside/Outside Inc., WI), and Piximus 2.10 software. The abdomen just below the diaphragm was chosen as the region of interest, focusing our assessment on central adiposity. Bonferroni-corrected two-tailed t-tests were used to assess for differences in DEXA parameters including bone mineral density (BMD), bone mineral content (BMC),
bone area (BArea), tissue area (TArea), ratio of soft tissue attenuation (RST), total tissue mass (TTM), percent adiposity, and body weight. Indirect calorimetry was performed using an open circuit system (Oxymax Equal Flow, Columbus Instruments, OH) composed of an air pump, CO₂ sensor (range 0%-0.8%; 0.002% resolution; drift < 20ppm CO₂ per hour), paramagnetic O₂ sensor (range 0 – 100%; 0.002% of specified range resolution; drift < 0.06% of specified range per 24 hours), air dryer, controller, 8 hermetically sealed indirect calorimetry chambers (2.1 x 10.2 x 12.7 cm³, #760M-D8, Columbus Instruments), chamber photocell bracket (1.27 cm spacing between photocells), photocell controller (Opto M3, Columbus Instruments), and Oxymax for Windows 4.49 running on dedicated hardware. Mice were fasted from roughly 5:00 PM until testing at 11:00 AM. Prior to running indirect calorimetry the system was allowed to equilibrate prior to calibration. Calibration gasses consisted of 100% N₂ and a mixture of 0.5% CO₂ / 20% O₂ / 79.5% N₂ (span gas). Indirect calorimetry data was collected for 20 intervals, each with 2 minute duration epochs. Therefore, 40 minutes of data was collected for each mouse over a period of roughly 5.5 hours. Basal metabolic rates were calculated by averaging values from three epochs where the individual mouse demonstrated the least activity. Activity-associated metabolic rates were calculated by averaging values from the three epochs where the mouse had the most activity. ANCOVA analysis was performed to identify significant differences in metabolic parameters (VO₂, DO₂, O₂-out, VCO₂, DCO₂, CO₂-out, and heat) between genotypes as a function of adiposity.

**Primary Cerebellar Granule Cell Culture**

In efforts to determine if deficits in locomotion observed in C3 KO mice were a result of anomalies in cerebellar neuron structure or function, cerebellar neuron cultures
enriched for granule cells were generated from postnatal day 5 C57BL6/J or B6;129S4-C3tm1Crr/J for downstream analysis as detailed in the methods section of Chapter 1.

**Transfection**

Primary cerebellar granule cell cultures were transfected with a plasmid carrying GFP under the CMV promoter as described in the methods section of Chapter 1. Briefly, pCGC cultures at DIV 2 were transfected with 1.0 μg of plasmid DNA per ml of media using Lipofectamine 2000, according to manufacturer protocols. Using this method we were able to achieve low-density transfection suitable for analysis of individual cerebellar granule cell synaptic densities.

**Immunocytochemistry**

To assess for differences in cell type, apoptosis, or synaptic proteins primary cerebellar neuron cultures were processed for immunocytochemistry at either day *in vitro* 3 or 6, as detailed in Chapter 1 Methods. Briefly, cultures were fixed with pre-warmed (37°C) 4% Paraformaldehyde/4% sucrose in PBS for 10 minutes, followed by 3 rinses in PBS. To allow for antibody access to intracellular epitopes fixation was followed by permeabilization with 0.1% triton X-100 for 10 minutes. Cells were again rinsed 3 times for 10 minutes in PBS, followed by blocking with 5% BSA in PBS for 1 hour at room temperature. After blocking, primary antibodies were diluted in 0.5 % BSA in PBS and incubated overnight at 4°C (**Table 1**). After primary incubation cells were rinsed 3 times in PBS followed by incubation for with species specific secondary antibodies in 0.5% BSA/PBS for 1 hour at room temperature. Cells were again washed 3 times with PBS for 10 minutes to remove residual secondary antibodies and DAPI counterstained for 1 minute. Following DAPI counterstaining, cells were rinsed a final time and glass coverslips were placed on top of cellular monolayers using prolong gold or prolong diamond mounting media (ThermoFisher, #P36961). Samples were allowed to dry
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Table 1. Antibody list and concentrations used for Chapter 2 experiments.
overnight at room temperature in the dark, and then stored at -20°C or -80°C until further processing.

**Confocal Imaging of pCGC Cultures**

Synaptic densities, cell types, and caspase-3 activation in pCGC cultures were all analyzed using confocal microscopy under blinded conditions. Images were taken on a Zeiss LSM 710 configured as previously described in Chapter 1 Methods. For *in vitro* synaptic density analysis, z stack images were taken using a 40x objective at 1024 x 1024 resolution (160 µm x 160µm x 4.58 µm; 0.352 µm z-step). For cell type and cleaved caspase-3 measurements images were collected using a 20x objective with 0.5x digital zoom at 1024 x 1024 resolution (640 µm x 640 µm) with focus set to the brightest DAPI plane.

**Analysis of Cell Types and Apoptosis**

Blinded confocal images were analyzed as described in Chapter 1 Methods with modification for image intensity and background. Cell type analysis for C3 KO and WT pCGC cultures was performed using the detect spots feature in IMARIS (Bitplane, South Windsor CT) controlling for fluorescent signal intensity and quality. Statistics for activated caspase-3 staining was performed in Microsoft Excel, using a two-tailed student t-test to compare genotypes. Statistics were not performed on cell type analysis, as we only used 2 biological replicates for WT controls. However, the data concurs with that generated for original culture characterization.

**Analysis of pCGC Synaptic Density**

Gross synaptic densities were quantified in blinded samples using detect spots function in IMARIS software on blinded confocal images. Settings for puncta size and quality were optimized for the data set and remained uniform for all samples. Vglut1 and
GAD65 puncta counts were quantified, along with the number of DAPI positive cells per field. Data was presented as gross synaptic counts per field, as well as counts normalized to the number of cells per field. For cell specific synaptic densities per µm of surface area, pCGC transfected with pGFP were imaged and analyzed. Using the create surfaces function in IMARIS the structure of the cell was generated. Next, detect spots was used to identify excitatory and inhibitor synaptic puncta. We then used the split into surface objects function to identify synaptic puncta in contact with the generated surface. The number of puncta detected was then normalized to surface area of the individual cell. All comparisons were performed using two-tailed student t-tests in Microsoft Excel.

**Calcium Imaging and Analysis**

To assess functional properties of cerebellar granule cells generated from WT and C3 KO mice, we performed live cell calcium imaging using a calcium indicator dye that is permeable to cell membranes (Fluo-4AM, ThermoFisher #F14201). pCGC cultures were loaded with 1 µM Fluo-4AM and pluritonic acid for stabilization and imaged using an upright flourscent microscope. Cultures were imaged in artificial cerebrospinal fluid (mM: 124 NaCl, 26 NaHCO₃, 3 KCl, 2 MgCl₂, 2 CaCl₂, 1.25 NaH₂PO₄, 10 glucose, 0.5 ascorbic acid, 1.5 Na-pyruvate, 1 thiourea; pH 7.4, maintained by carbogen [95% O₂ and 5% CO₂] bubbling at room temperature. pCGC cultures were imaged every 5 seconds, but for 7 minutes with 25 mM KCL added on the 15th cycle to induce depolarization. Images were then analyzed using Axiovision Software (Version 4.8.2.0). A total of 60 WT cells and 90 C3 KO cells were analyzed from samples generated from 2 and 3 biological replicates respectively. A third WT sample was lost due to complications with imaging autofocus parameters. For analysis, baseline fluorescent intensity for each cell was calculated as the average GFP intensity of the first minute of imaging (a total of 12 cycles with 5 seconds between cycles). The GFP intensity of each cell at each time
point was then normalized to individual cell baseline to generate a fold change from baseline. Fold change data was analyzed by two-way repeated measures ANOVA controlling for genotype, time, and sample. Significant interactions (p < 0.001) included GFP intensity by time and genotype*time.

**Transcardial Perfusion and Tissue Processing**

C57BL6/J or B6;129S4-C3tm1Crr/J male mice at 1 and 3 months of age were deeply anesthetized with 17 µl/g bodyweight of 2.5% Avertin (2,2,2-Tribromoethanol 97%, Sigma #T48402). Transcardial perfusion was performed to preserve tissue architecture for downstream histological analysis. Once mice were deeply under anesthesia, they were sprayed with ethanol and the chest cavity was opened using surgical scissors. The diaphragm was dissected away to expose the apex of the heart. A 25G butterfly needle (BD Biosciences, #367298) connected to a transcardial perfusion pump (Manostat® Vera) was placed into the left ventricle near the apex of the heart. Immediately the pump was started and the right atrium was opened with a dissection scissors. Mice were perfused with PBS for 3 minutes to clear blood, followed by 5 minutes with 4% PFA/PBS (32% Paraformaldehyde Stock, Electron Microscopy Sciences, #15714). Following perfusion, the brain was collected and post fixed in 4% PFA/PBS for 24 hours at 4 °C. To preserve cell architecture and prevent crystal formation upon freezing, brains were cyroprotected with 30% sucrose at 4 °C until the tissues sank, roughly 24 hours. Tissues were then embedded in Sakura ® Tissue-Tek ® O.C.T. (VWR, #4583), and frozen in dry ice and ethanol slurry. Tissue blocks were stored at -80°C for further processing. For sectioning, OCT tissue blocks were allowed to equilibrate to the cryostat temperature of -20 °C for 30 minutes. Tissues were sectioned on a cryostat (Leica Cyrostar NX50) in either the sagittal or coronal plane at 14 µm and
directly mounted on glass slides (Fisher Scientific, #12-550-15). Tissues mounted on slides were stored at -80 °C until staining.

**Immunohistochemistry**

To assess for genotypic differences between wildtype and C3KO mice in vivo, tissue sections were immunostained with antibodies targeting specific synaptic proteins. For a full list of antibodies used in the experiments presented in Chapter 2, please refer to Table 1. Tissue sections were allowed to warm to room temperature for 30 minutes prior to processing. Slides were washed 3 times in PBS to remove excess OCT, followed by blocking in 5% bovine serum albumin (BSA) in PBS. To promote antibody access to intracellular epitopes, the blocking, primary, and secondary antibody buffers contained 0.2 % Triton-X 100. After blocking, slides were incubated overnight at 4 °C in antibody solution (0.5% BSA in PBS with 0.2 % Triton) containing primary antibodies for excitatory and inhibitory synaptic proteins. Following overnight incubation, slides were washed 3 times in PBS. The slides were then covered with antibody solution containing species-specific secondary antibodies for 2 hours at room temperature in the dark. Following secondary antibody staining, slides were washed 3 times, counterstained with DAPI, washed again, and cover slipped with Prolong Diamond anti-fade mounting media. Slides were allowed to dry overnight in the dark at room temperature and then stored at -20 °C until imaging.

**In Vivo Synaptic Density Confocal Microscopy**

Densities of excitatory (Vglut1, Vglut2) and inhibitory (GAD65) synaptic proteins in wildtype and C3 KO mice were assessed using confocal microscopy. To assess synaptic density in the molecular layer confocal z-stack images from 3 month old mice were acquired using 40x objective and 2x zoom on a Zeiss LSM 700 scanning confocal
microscope. Images were taken at 1024 x 1024 resolution over a 160 μm x 160μm x 12.04 μm window with a z-step of 0.430 μm. Synaptic formations in the cerebellar granule cell layer were imaged under the same objective and settings, but with modified gain and laser intensity.

**Analysis of In Vivo Synaptic Density**

The primary vesicular protein associated with molecular layer excitatory synapses is Vglut1 arising from parallel fibers of cerebellar granule cells. Vglut1 puncta in the molecular layer are very dense and uniform in size. To detect each puncta, we employed the detect spots feature in IMARIS software controlling for quality of fluorescent signal. GAD65 positive and Vglut2 positive puncta were assessed using the same feature but as they are less uniform in size. We also quantified average puncta volume using the ‘Detect spots of varying sizes’ feature with local contrast settings to define sizes. Output from this analysis included the number of puncta, average puncta mean intensity, average puncta median intensity, and for Vglut2 and GAD65 the average puncta volume.

Input to cerebellar granule cells occurs through a specialized glomerular synaptic structure. Thus, individual synaptic puncta are not well defined. For measuring Vglut1 and Vglut2 in the cerebellar granule cell layer, the ‘Create surfaces’ function was used to map the structure of the excitatory synaptic glomerulus. GAD65 puncta, which form an inhibitory ring around each glomeruli were identified using the ‘Detect spots’ feature. The output for this analysis included the number of glomeruli, average glomerulus surface area and volume, fluorescent intensity mean and median of glomeruli, and the number, size and intensity of GAD65 puncta. Genotypic differences in these metrics were assessed by two-sided t-tests assuming equal variance as implemented in Microsoft Excel.
Results

*Loss of C3 leads to locomotor functional deficits characterized by decreased gait speed, increased locomotor bout duration*

Complement protein C3 functions in the CNS to opsonize targets for phagocytosis, either for immunological clearance or synaptic pruning. C3 is important for pruning within the cortex and hippocampus, and therefore studies interrogating behavior in C3 knockout mice are often narrowly focused on cognitive functions. A small number of studies have also included measures of anxiety and gross locomotor function\(^{81}\). However, the effect of C3 loss on daily behaviors in freely moving mice has not been assessed. Therefore, we set out to determine if there are any genotypic differences in behavior between wild type control and mice lacking C3. 1-month old B6;129S4-C3tm1Crr/J mice constitutively lacking complement protein C3 (C3 KO) and age-matched C57BL/6J controls (WT) were singly housed in a custom state-of-the-art system for homecage monitoring (HCM) for 21 days. Following quality control and automated data analysis, 565 behavioral characteristics were measured in WT and C3 KO mice. Using false discovery rate (FDR) analysis, we identified 143 behaviors differentially expressed between WT and C3 KO mice. Differences were observed in aggregate measures of intake and movement, time budget analysis, state analysis, feeding bouts, drinking bouts, and movement bouts. Volcano plots were generated from differential behaviors lists to generate plots of significance versus fold change from WT. This analysis yielded 53 with a greater than 2 fold change from controls (Figure 1A). Of the 53 behaviors, 25 were significantly decreased and 28 significantly increased (Figure 1B). Within this data set are several behavioral differences that correspond to functional aspects of locomotion, bout probabilities, and feeding and drinking properties.
Figure 1. Differentially expressed behaviors in C3 KO mice. (A) Volcano plot diagram depicting 53 differentially expressed behaviors between WT and C3 KO mice plotted as significance vs ratio (fold change). Behaviors in the upper left show a greater than two-fold reduction in C3 KO mice (Red). Behaviors in the upper right indicated a two-fold or greater increase in C3 KO mice (Green). Behaviors that failed to reach significance fall below the horizontal dashed line. Behaviors that were significant, but showed a lower than two-fold difference between genotypes fall into the upper center segment. (B) Table of differentially expressed behaviors that are decreased or increased in C3 KO mice. TBA = time budget analysis, BA = bout analysis, SA = state analysis, BD = bout dominance analysis, PE = photobeam event, LE = lick event, ME = movement event, PE = photobeam (feeding) event, IP = inactive phase, LC = light cycle, DC = dark cycle, DCLC = collective dark and light cycle.
Of particular interest toward determining mechanisms of age related locomotor deficits, analysis of locomotor function in C3 KO mice demonstrated several marked impairments. C3 KO mice showed no difference in overall locomotor movement (cm/hr) across the light and dark cycles either combined or individually (Figure 2A). Additionally, there was no difference in the number of locomotor bout onsets per hour or in bout probability (Figure 2B,C). However, C3 KO mice show a significant increase in the duration of locomotor bouts across the combined light and dark cycles (WT 786.35 ± 263.1, C3 KO 1305.54 ± 370.83 ms, FDR t-test p = 0.0192). This difference holds when the light cycle and dark cycle are assessed independently as well (Figure 2D). Increased bout duration is likely a result of a significantly reduced gait speed for forward locomotion in C3 KO mice (WT 23.73 ± 4.50, C3 KO 15.43 ± 3.04 cm/s, FDR t-test p = 0.0067). This difference holds though both the dark and light cycle individually as well as combined (Figure 2E). The deficits in locomotor gait speed and increased duration did not lead to a significant impairment in the distance traveled per locomotor bout (Figure 2F). These data collectively demonstrate that C3 KO mice have deficits in functional aspects of locomotion, but motivation for movement appears to be spared, as the number of onsets, probability, and total movement are equal to controls. Therefore, we chose to further assess functional aspects of locomotion, primarily characteristics of locomotor paths in WT and C3 KO mice.

**Loss of C3 leads to Markers of Gait Ataxia**

To determine if there are differences in the way WT and C3 KO mice move, we measured locomotor path straightness using minimum bounding box areas fit to motor paths, a reliable means to predict characteristics of gait ataxia. As previously mentioned, gait ataxia and reduced gait speeds are correlated with poorer health outcomes in the elderly. Therefore, determining mechanisms of gait disturbances is imperative for future
Figure 2. C3 CKO mice display slower gait speeds and increased locomotor bout duration. (A) Over a 24 hour period C3 CKO and WT mice move the same overall distance throughout both the light and dark cycles. (B) The number of forward locomotor bouts across the light and dark cycles is unchanged in C3 CKO mice. (C) There is a trending increase in the probability of locomotor bouts in C3 CKO mice, but this difference fails to reach significance. (D) C3 CKO mice show a significant increase in the duration of locomotor bouts across both the light and dark cycles. (E) Forward locomotor bout speed is significantly reduced in C3 CKO mice, but there is no difference in the distances traveled per bout (F). * indicate $p<0.01$, Bonferroni corrected; error bars are ± 1 standard error of the mean.
therapies. Analysis of locomotor paths in WT and C3 KO mice revealed a significant increase in average bounding box area in C3 KO mice (Median Minimum Bounding Box Area: WT 3.65 cm$^2$, C3 KO 7.91 cm$^2$, $p = 1.64 \times 10^{-29}$), which is indicative of less straight locomotor paths (Figure 3). This finding further validates that C3 KO mice had significant deficits in the functional aspects of locomotion.

**Loss of C3 does not impair metabolism or lead to significant changes in body composition**

Complement proteins are expressed throughout the nervous system, including in the hypothalamus. As we noted increased complement in the hypothalamus of BALBc mice with aging, we wanted to determine if loss of C3 leads to any gross changes body composition or metabolism. To assess body composition, dual-energy X-ray absorptiometry (DEXA) scans were performed on WT and C3 KO mice at 2-3 months of age. Analysis of these scans demonstrated minimal differences between genotypes. There was no significant change in bone mineral density (BMD), bone mineral content (BMC), total bone area, soft tissue ratio (RST), % fat, total tissue mass, or weight (Figure 4A,B,C,E,F,G,H respectively). The only significant difference was a slight change in total tissue area (WT 8.45 ± 0.48, C3 KO 90.5 ± 0.60, $p = 0.044$) (Figure 4D). Analysis of resting and active metabolism was performed by indirect calorimetry. Loss of C3 had no effect on any metrics of basal (Figure 5) or active (Figure 6) state metabolism when normalized for adiposity. These results suggest that loss of C3 leads to minimal changes in metabolic properties or energy balance phenotypes in the C57Bl6 background. As there were no major differences, we chose to focus the rest of our analysis on the locomotor deficits observed in C3 KO mice and working towards identifying mechanisms underlying dysfunction.
Figure 3. C3 KO mice display characteristics of gait ataxia. (A) Representative diagram of direct and indirect locomotor paths (solid line) and the bounding box area necessary to enclose each (shaded rectangle). Larger minimum bounding box areas are indicative of a less straight or weaving path. (B) Frequency distribution curve of minimum bounding box rectangle (MBR) areas for WT (blue) and C3 KO (red) mice demonstrates a significant increase in the median MBR (MBRs; cut off at 10 to better show small rectangle areas).
Figure 4. Body composition analysis of C3 KO mice revealed minimal differences. DEXA scan analysis of WT and C3 KO mice demonstrated no genotypic differences in bone mineral density, bone mineral content, bone area, soft tissue ratio, % fat, total tissue mass, or mouse weight. The only difference appreciated was a small increase in tissue area of C3 KO mice (WT 8.45 ± 0.48, C3 KO 90.5 ± 0.60, p = 0.044). Data representative of 8 animals per genotype. Comparisons made by Bonferroni-corrected two-tailed t-tests. * indicates p < 0.05. Error bars represent standard deviation of the mean.
Figure 5. **C3 KO mice show no deficits in basal metabolic function.** C3 KO and WT mice were assessed by indirect calorimetry to determine genotypic differences in measures of metabolic function. No differences were noted between genotypes for VO$_2$, O$_2$out, DO$_2$, VCO$_2$, CO$_2$out, or calculated heat during resting periods (basal state). Data analyzed by multiway ANOVA controlling for genotype and adiposity.
Figure 6. C3 KO mice show no deficits in activity associated metabolic function. C3 KO and WT mice were assessed by indirect calorimetry to determine genotypic differences in measures of metabolic function. No differences were noted between genotypes for VO$_2$, O$_2$ out, DO$_2$, VCO$_2$, CO$_2$ out, or calculated heat during periods of mouse activity. Data analyzed by multiway ANOVA controlling for genotype and adiposity.
**Loss of C3 leads to increased Vglut1+ synaptic puncta in cerebellar granule cell cultures**

Complement proteins have been shown to tag synapses for pruning by microglia in multiple brain regions. Loss of complement expression has been linked to increased synaptic density in the cortex and hippocampus, but less is known about this process in the cerebellum\textsuperscript{33, 72, 81}. The majority of studies have linked complement proteins to changes in cell proliferation, migration, and survival\textsuperscript{63, 64}. As we observed significant functional impairments in C3 KO mice, we chose to measure synaptic density in a reduced model of cerebellar neurons. To assess for synaptic alterations in cerebellar granule cells, primary neuron cultures enriched for granule cells were isolated from postnatal day 5 mice and matured in culture (For full characterization see Chapter 1). WT and C3 KO granule cell cultures were fixed at multiple stages of synaptic maturation and immunostained for excitatory and inhibitory synaptic proteins. At the early stages of synaptic development (day \textit{in vitro} 3) there was no significant differences in gross excitatory (WT 367 ± 112; C3 KO 522 ± 98 Vglut1\textsuperscript{+} puncta per field; \( p = 0.145 \)) or inhibitory (WT 27 ± 17; C3 KO 40 ± 1 VGAT puncta per field, \( p = 0.257 \)) synaptic density (Figure 7A,B). To ensure that cell density was not a factor, synaptic puncta counts were normalized to DAPI\textsuperscript{+} cells per field and there was still no significant difference in synaptic counts at DIV3 (Figure 7C). When cerebellar neuron cultures were analyzed at a later stage in synaptic maturation (day \textit{in vitro} 6), C3 KO cultures showed a significant increase in excitatory and inhibitory synaptic densities compared to wild-type controls. This increase in excitatory synapses was consistent across several methods of quantification and normalization. When assessed as total puncta per field, C3 KO cerebellar granule cell cultures show a greater than two fold increase in excitatory synaptic puncta (WT 1090 ± 373; C3 KO 3066 ± 254; \( p = 0.0016 \)) (Figure 8A,B). There
was no significant difference in the number of cells per field, but to account for any
difference in synaptic number due to variability in cell density, we normalized synaptic
counts to cells per field as above. C3 KO cerebellar granule cells still showed a
significant increase in excitatory synaptic puncta (WT 23 ± 5; C3 KO 46 ± 7; p = 0.0107)
(Figure 8C). To assess inhibitory synaptic density, VGAT+ puncta per field were
quantified and C3 KO mice show an increase in inhibitory synaptic puncta (WT 179 ± 89;
C3 KO 392 ± 8; p = 0.0149) (Figure 8AB). This difference falls just below significance
when normalized to cell number per field (WT 3.72 ± 1.2; C3KO 6.01 ± 0.76; p = 0.052)
(Figure 8C).

While gross synaptic densities in culture systems provide a general understanding
of synaptic changes, we wanted to assess how synapses are changed on the single cell
level. To determine how excitatory and inhibitory input onto individual cells differs
between WT and C3 KO mice, cerebellar granule cell cultures were transfected with a
GFP vector to identify individual cerebellar neurons and processes. Cerebellar neuron
cultures were then fixed and stained for excitatory and inhibitory synaptic markers at day
3 and 6 in vitro. Individual cell synaptic input was then analyzed per 100 µm² of pGFP
cell surface area using create surface, detect spots, and split spots into surface objects
functions in IMARIS, see methods for parameters. Analysis using this method confirmed
our previous finding that C3 KO cultures had a significant increase in Vglut1+ synaptic
puncta compared to WT controls at DIV 6 (WT 2.75 ± 0.49; C3 KO 5.42 ± 0.78; p =
0.0075). When inhibitory VGAT puncta were analyzed, C3 KO cerebellar neurons had
an increased number VGAT+ puncta per 100 µm² of GFP cell surface area (WT 0.51 ±
0.159; C3 KO 0.81 ± 0.088; p =0.047) (Figure 9A,B). Cultures analyzed at DIV3,
showed a trending, but non-significant increase in Vglut1+ puncta per 100 µm² of GFP
surface area (WT 1.02 ± 0.21; C3KO 1.60 ± 0.39; p = 0.085) (Figure 10A,B).
Additionally, there was no significant difference in total surface area per cell for either DIV3 (Figure 10C) or DIV6 (Figure 9C). However, quantification of primary dendrite number demonstrated no significant difference at DIV3 (Figure 10D), but a significant increase at DIV6 (WT 6.03 ± 0.15; C3 C3 KO 6.83 ± 0.31; p = 0.015) (Figure 9D).

Complement proteins and receptors have been previously described to regulate cell migration, proliferation, and apoptosis. To assess whether synaptic differences seen in C3 KO compared to WT cultures were a result of differences in cell percentages, inflammatory signaling, or changes in apoptosis we performed immunohistochemistry for cell specific markers including neurons (NeuN), astrocytes (GFAP), and microglia (CD11b), as well as cleaved caspase 3. Importantly, loss of complement protein C3 does not lead to any significant changes in cell type distribution, cell apoptosis, or inflammatory signaling in cerebellar neuron cultures that may alter synaptic density (Figure 11). Levels of other transcripts relating to inflammatory NF-κB signaling were also assessed by immunohistochemistry with no differences seen in expression of p105, IKK-α, Syk, or p65 (data not shown). Additionally, pCGC cultures are enriched for neurons, with low levels of glial cells. Taken together, our data suggests that synaptic alterations are a result of neuronal complement 3 loss and not a secondary effect from changes in cell distribution or glial response. Next, we wanted to determine in increased synaptic density led to any changes in pCGC function that may underlie locomotor deficits.

**Loss of C3 leads to increased responsiveness to KCL induced depolarization**

Neuronal activity leads to increased intracellular calcium levels by Ca$^{2+}$ entry through L-type voltage-dependent calcium channels and ligand gated channels, including N-methyl-D-aspartate receptors (NMDARs) and α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate receptors (AMPARs) that respond to glutamate. Additionally,
Figure 7. *Excitatory and inhibitory synaptic densities are unchanged at early stages of granule cell culture maturation.* Primary cerebellar granule cell cultures generated from WT and C3 KO mice were assessed for changes in synapse density at early stages of culture maturation. (A) Representative images of WT and C3 KO cultures at DIV3 stained with antibodies targeting excitatory Vglut1 and inhibitory VGAT. Scale bar equals 20 mm. (B) Quantification of excitatory (WT 367 ± 112; C3 KO 522 ± 98 Vglut1+ puncta per field; p = 0.145) and inhibitory (WT 27 ± 17; C3 KO 40 ± 1 VGAT puncta per field, p = 0.257) synaptic puncta per field revealed no significant differences between genotypes. (C) Normalization of puncta numbers to the number of cells per field also demonstrated no differences. Data representative of cultures generated from 3 biological replicates per condition. Comparisons between genotypes made by two-tailed student t-tests. Error bars represent standard deviation.
Figure 8. Increased excitatory and inhibitory synapse density in cerebellar granule cell cultures generated from C3 KO mice. Primary cerebellar granule cell cultures generated from WT and C3 KO mice were assessed for changes in synapse density in mature cultures. (A) Representative images of WT and C3 KO cultures at DIV6 stained with antibodies targeting excitatory Vglut1 and inhibitory VGAT. A clear increase in puncta density is observed in both Vglut1 and VGAT staining. Scale bar equals 20 mm. (B) Quantification of excitatory synaptic puncta reveal a significant increase in puncta density in C3 KO mice (WT 1090 ± 373; C3 KO 3066 ± 254; p = 0.0016). An increase in inhibitory VGAT puncta density is also noted (WT 179 ± 89; C3 KO 392 ± 8; p = 0.0149). There were no significant differences in cell densities between genotypes. However, to ensure that synapse densities were not a result of small changes in cell density, the data was normalized to cell number. (C) Even with normalization to cell density, C3 KO cerebellar granule cell cultures have an increased excitatory synapse density compared to controls (WT 23 ± 5; C3 KO 46 ± 7; p = 0.0107). Differences in inhibitory synapses just failed to reach significance when normalized to cell number (WT 3.72 ± 1.2; C3 KO 6.01 ± 0.76; p = 0.052). Data representative of cultures generated from 3 biological replicates per condition. Comparisons between genotypes made by two-tailed student t-tests. Error bars represent standard deviation.
Figure 9. **C3 KO cerebellar granule cells have increased excitatory and inhibitory input on a single cell level.** Cell specific synapse densities were quantified by generating surfaces for individual pGFP transfected cerebellar granule cells. (A) Representative images of WT and C3 KO pGFP cerebellar granule cells at DIV6 stained for excitatory and inhibitory synaptic puncta. Far left panel is the maximum projection image from confocal z-stacks (raw), center panel shows the surface and spot detection, far right panel shows output of surfaces with contacting synaptic puncta with excitatory (Vglut1) in red and inhibitory (VGAT) in teal. Scale bar represents 20 mm. (B) Quantification of synaptic puncta per 100 mm of pGFP surface area revealed a significant increase in excitatory (WT 2.75 ± 0.49; C3 KO 5.42 ± 0.78; p = 0.0075) and inhibitory (WT 0.51 ± 0.159; C3 KO 0.81 ± 0.088; p =0.047) in C3 KO cells. (C) There were no significant differences in the total surface area of pCGCs between genotypes. (D) C3 KO pCGCs have an increased average number of primary dendrites compared to WT controls (WT 6.03 ± 0.15; C3 KO 6.83 ± 0.31; p = 0.015). Data representative of > 20 cells spread across 3 biological replicates per genotype. Differences were calculated by two-tailed student t-tests. Error bars represent standard deviation of the mean.
Figure 10. Excitatory and inhibitory input onto individual cerebellar granule cells is similar in WT and C3 KO cultures at DIV3. Cell specific synapse densities were quantified by generating surfaces for individual pGFP transfected cerebellar granule cells. (A) Representative images of WT and C3 KO pGFP cerebellar granule cells at DIV3 stained for excitatory and inhibitory synaptic puncta. Far left panel is the maximum projection image from confocal z-stacks (raw), center panel shows the surface and spot detection, far right panel shows output of surfaces with contacting synaptic puncta with excitatory (Vglut1) in red and inhibitory (VGAT) in teal. Scale bar represents 20 mm. (B) Quantification of synaptic puncta per 100 mm of pGFP surface area revealed a trending but not significant increase in excitatory synapse density (WT 1.02 ± 0.21; C3KO 1.60 ± 0.39; p = 0.085). (C) There were no significant differences in the total surface area of pCGCs between genotypes. (D) No differences in the number of primary dendrites were noted between genotypes. Data representative of > 20 cells spread across 3 biological replicates per genotype. Differences were calculated by two-tailed student t-tests. Error bars represent standard deviation of the mean.
Figure 11. Cerebellar neuron cultures generated from C3 KO mice resemble those from WT mice. To verify that synaptic changes observed in culture were not a reflection of changes in cell distribution, pCGC cultures were fixed and immunostained for cell specific markers of neurons (NeuN), astrocytes (GFAP), and microglia (CD11B). (A) Quantification of cell types demonstrated no obvious differences between WT and C3 KO cultures (NeuN: WT 82.6 ± 1.0, C3 KO 79.3 ± 1.8%; GFAP: WT 0.57 ± 0.36%, C3 KO 0.91 ± 0.23%; CD11B: WT 0.37 ± 0.04%, C3 KO 0.26 ± 0.04%). Statistics were not performed, as the number of biological replicates used to generate wells of technical replicates were small (WT n = 2, C3 KO n = 3). To ensure that apoptosis rates were not a factor in synaptic density differences, pCGC cultures were immunostained for cleaved caspase-3. (B) Quantification of activated caspase-3 demonstrated no significant differences between WT and C3 KO cultures (WT 17.9 ± 2.1%, C3 KO 19.9 ± 8.4%, p = 0.72). Finally, to validate that synaptic differences were not a result of increased inflammatory signaling, transcript expression of IL1-β and IkBa was measured. (C,D) Quantification transcripts indicative of inflammation showed no significant differences between WT and C3 KO cultures (IkBa: WT 1.12 ± 0.58, C3 KO 1.01 ± 0.32 fold change from control, p = 0.78; IL1-β: WT 1.02 ± 0.24, C3 KO 0.75 ± 0.8 fold change from control, p = 0.13). Comparison was made by two-tailed student t-test, n = 3 for each group. Error bars represent standard deviation.
Ca$^{2+}$ is released from the endoplasmic reticulum in response to activity$^{85-90}$. Downstream calcium signaling is important for a diverse set of cellular functions including activity dependent regulation of synapse size, number, and function$^{91-94}$. Measuring changes in intracellular calcium levels can serve as a method to assess neuronal response to activity inducing stimuli. Treatment of neurons with potassium chloride (KCl) results in robust depolarization by increasing the extracellular potassium concentration, which causes the opening of voltage-gated calcium channels and rapid Ca$^{2+}$ influx. Using a cell permeant calcium indicator dye (Fluo-4, AM) that exhibits fluorescence upon calcium binding, we assessed neuronal response to KCl induced depolarization in control and C3 KO pCGC cultures. Cerebellar granules cells isolated from C3 KO mice have an increased responsiveness to KCl depolarization and impaired washout response, taking longer to return towards baseline fluorescence (Figure 12). A significant genotype*time interaction for GFP intensity was noted by two-way repeated measures ANOVA. This data demonstrates potential functional differences between WT and C3 KO cerebellar granule cells.

**Loss of complement protein C3 does not alter cerebellar synaptic density in vivo**

Loss of complement proteins in animal models has been shown to result in increased excitatory synaptic density in select regions of the brain. Furthermore, our in vitro analysis of cerebellar granule cells revealed significant differences in both excitatory and inhibitory synaptic densities. We therefore hypothesized that C3 KO mice may have increased synaptic densities within selected regions of the cerebellum that underlie locomotor deficits. To quantify synaptic densities in vivo, we processed 1 month and 3 month old WT and C3 KO mice for immunohistochemistry and subsequent confocal microscopy of several cerebellar regions. The molecular layer contains the primary synaptic output from cerebellar granule cells, where projecting parallel fibers
Figure 12. C3 KO cerebellar granule cells have an increased calcium response to KCL depolarization in vitro. To determine if C3 KO cerebellar granule cells demonstrated functional differences compared to WT controls, calcium imaging was performed to measure cellular response to potassium chloride induced depolarization. (A) Representative images of WT and C3 KO cerebellar granule cell cultures at baseline and after KCL induced depolarization. Scale bar represents 20 mm in both image and inset. (B) Quantification of the fold change in fluorescent intensity from baseline for WT and C3 KO cerebellar granule cells before and after KCL induced depolarization. Baselines for individual cells were calculated as the average of the first 60 seconds (12 images spaced at 5 second intervals). Arrows indicate relative positions of representative images in A. Two-way repeated measures ANOVA indicated a significant (p < 0.001) interaction between genotype and time. Time was also significant, but sample and sample by time were not found to be significant. Data is representative of 60 cells for WT and 90 cells for C3 KO from 2 and 3 biological replicates, respectively. Error bars represent standard error of the mean.
synapse with the large dendritic trees of Purkinje cells. As we observed increased excitatory synaptic density in cultures, we hypothesized we would observe similar changes in the molecular layer. However, quantification of Vglut1+ excitatory synapses and GAD65 inhibitory synapses in the molecular layer yielded no significant differences in synaptic puncta number or mean intensity (Figure 13A,B,C). In addition to Vglut1 positive synapses from cerebellar granule cells, climbing fibers from the inferior olive form Vglut2 excitatory synapses in the molecular layer. Quantification of Vglut2 positive synapses also yielded no differences in density or intensity (Figure 14A,B). As we observed no differences in the molecular layer, we decided to assess the granule cell layer. Excitatory mossy fibers from the pons form excitatory synapses with cerebellar granule cells in a specialized synaptic structure surrounded by a ring of inhibitory synapses. Quantification of glomeruli number, intensity, and volume of Vglut1 positive glomeruli and the GAD65 inhibitory surrounding synapses demonstrated no differences in vivo (Figure 15). Quantification of Vglut2 positive glomeruli number, intensity, and volume also showed no differences (Figure 16). These findings were unexpected, especially given the large differences observed in culture. However, as we observed differences in synaptic function, it is possible there are differences in synaptic activity but a normal synaptic density. The implications of these findings will be discussed in further detail below.

Conclusion and Discussion

Here, we present data generated from an unbiased behavioral assessment of C3 KO mice, where we identified a novel locomotor phenotype characterized by reduced gait speed, increased locomotor bout duration, and evidence of gait ataxia. Additionally, we identified increased excitatory and inhibitory synaptic density and increased KCL excitability in cerebellar granule cell cultures generated from C3 KO mice. Intriguingly,
Figure 13. **C3 KO mice show no difference in excitatory or inhibitory synapse density in the molecular layer of the cerebellum compared to control mice.** 3-month old WT and C3 KO mice were processed and imaged for excitatory (Vglut1) and inhibitory (GAD65) synapses. (A) Representative images of WT and C3 KO molecular layer synapse densities. Scale bar represents 50 mm. (B) Quantification of the number of excitatory and inhibitory synaptic puncta in the molecular layer demonstrated no genotypic differences. (C) No differences in the average puncta fluorescent intensity were observed between groups. Data is representative of 4 WT and 3 C3 KO mice. Error bars represent standard deviation. Statistical analysis was performed using two-tailed student t-tests.
Figure 14. **C3 KO mice show no difference in excitatory synapse density in the molecular layer of the cerebellum compared to control mice.** 3-month old WT and C3 KO mice were processed and imaged for excitatory (Vglut2) synapses arising from climbing fibers. (A) Representative images of WT and C3 KO molecular layer synapse densities. Scale bar represents 50 μm. (B) Quantification of the number of excitatory synaptic puncta in the molecular layer demonstrated no genotypic differences. (C) No differences in the average puncta fluorescent intensity were observed between groups. Data is representative of 4 WT and 3 C3 KO mice. Error bars represent standard deviation. Statistical analysis was performed using two-tailed student t-tests.
Figure 15. C3 KO show no changes in excitatory or inhibitory synapses in the cerebellar granule cell layer. Cerebellar granule cells receive excitatory input from mossy fibers in a specialized synaptic glomerular structure surrounded by a ring of inhibitory synapses. (A) Representative images of WT and C3 KO cerebellar granule cell layer stained for Vglut1 and GAD65. Scale bar represents 50mm. (B) Quantification of several properties of excitatory and inhibitory synapses demonstrated no significant differences between genotypes. The number of Vglut1 glomeruli was lower in C3 KO mice, but this difference failed to reach significance when adjusted for multiple comparisons. Data represents 4 WT and 3 C3 KO mice. Error bars represent standard deviation. Statistical analysis was performed using two-tailed student t-tests adjusted for multiple comparisons.
Figure 16. C3 KO show no changes in excitatory synapses in the cerebellar granule cell layer. Cerebellar granule cells receive excitatory input from mossy fibers in a specialized synaptic glomerular structure surrounded by a ring of inhibitory synapses. (A) Representative images of WT and C3 KO cerebellar granule cell layer stained for Vglut2. Scale bar represents 50mm. (B) Quantification of several properties of excitatory synapses demonstrated no significant differences between genotypes. Data represents 4 WT and 3 C3 KO mice. Error bars represent standard deviation. Statistical analysis was performed using two-tailed student t-tests adjusted for multiple comparisons.
we did not identify any *in vivo* differences in synaptic density or puncta intensity in either the molecular layer or cerebellar granule cell layer of C3 KO mice.

This work stems from our initial observation of increased pattern recognition receptors and immune proteins in the cerebellum of aging mice with locomotor deficits. Identification of age-associated brain region specific changes in gene expression and synapse density, coupled with functional deficits relating to that particular brain region spurred the idea that immune proteins may have functional roles in regulating neuronal properties underlying aging deficits. Complement protein expression was amongst those upregulated in the aging cerebellum. As complement proteins had previously been identified to function in synaptic regulation and pruning, we chose to assess mice lacking a specific complement protein (C3) for alterations in behaviors compared to wild type controls. Much of the current knowledge for C3 in the CNS stems from research focused on neurodevelopment and pathological conditions. A small number of studies have investigated the role of complement proteins in aging from a behavioral perspective, but the focus was directed towards detecting deficits in cognitive functions. Data from these studies revealed that C1q protein is drastically increased in the aged brain, deposits in close proximity to synapses, and is correlated with declines in hippocampal dependent cognitive functions. C1q deficiency may protect against age-related hippocampal decline of function, but interestingly no differences were observed between 3-month-old mice lacking C1q and WT controls. Similarly, complement 3 deficiency has been shown to protect mice from age related deficits in hippocampal function. In Shi, et al., 2015 the authors robustly demonstrate that C3 deficiency protects against age related decreases in synaptic density in multiple regions of the hippocampus, decreases in hippocampal long-term potentiation, and neuron loss in CA3 of the hippocampus. Additionally, they demonstrate that C3 KO mice have improved
spatial and contextual memory at 16 months of age compared to WT controls and demonstrate an anxiolytic phenotype. In both studies the authors note no gross observable deficits in motor function in complement deficient mice. In Shi, et al., 2015 motor function was assessed by SHIRPA (SmithKline Beecham, Harwell, Imperial College and Royal London Hospital phenotype assessment) and rotarod with no significant impairments noted between WT controls and C3 KO mice. While this may appear to contradict the findings of our study, the sensitivity and measured aspects of function between the methods used varies greatly between studies.

The SHIRPA procedure was designed to provide a rapid assessment of mouse phenotyping in a high-throughput manner, mimicking many aspects of human neurological examination. The primary SHIRPA screen offers a general overview of physical and neurological health, as well as gross motor and sensory functions. However, the sensitivity of the SHIRPA screen has previously been questioned, especially in the context of mobility function. Primary SHIRPA examination of transgenic synphilin 1 mice showed no deficits compared to wildtype mice, despite marked deficits in rotarod and footprint testing. Similarly, deficits in exploratory behavior were missed in 129/SvJ and C57BL/6J mice developmentally deficient in vitamin D. One of the major pitfalls to this form of assessment is the reliance on observational measurements in real time. Rotarod testing is a well characterized assessment for identifying locomotor deficits. Interestingly, C3 KO mice demonstrated no significant impairments on rotarod testing at either 4 or 16 months of age. While this may appear contradictory to our findings of locomotor deficits in C3 KO mice, it is important to note the scale of deficits measured in each task. Our data indicates slowed gait speed and markers of gait ataxia that may not impair function in a forced locomotor task, but effect daily ambulatory activity. C3 KO mice in our analysis were subject to continuous
analysis, 24 hours per day for 16 days. Mice in the homecage system are freely moving within a habituated home space offering assessment of true daily functions in the absence of forced testing. Additionally, rotarod measures a very different aspect of motor function compared to homecage monitoring. Rotatrod testing requires balance/vestibular function to perform the task, while there is no dependence on balance in homecage monitoring. Quantification of daily ambulatory function, especially gait speed is a critical measure of overall health as reduced gait speeds in the elderly are associated with poor healthcare outcomes. Interestingly, the lack of deficits on rotarod assessment may suggest that C3 KO mice do not demonstrate impaired motor learning, but the exact methods of rotarod testing would need to be assessed to make that conclusion.

In efforts to elucidate a mechanism for altered locomotor function in C3 KO mice, we assessed synaptic density in cultures enriched for cerebellar granule cells. Our data demonstrated increased excitatory and inhibitory synapse density in C3 KO pCGC cultures after 6 days in vitro, but not at an earlier time point. Additionally, C3 KO cultures showed an increased response to KCL depolarization compared to controls. The mechanisms underlying increased synaptic density in these cultures are currently unknown. Previous research has shown increased synaptic density in C3 KO mice in other neuronal types, results from reduced microglial pruning stemming from the loss of complement 3 tagged synapses. While this is certainly a possibility, pCGC cultures in our studies are enriched for neurons with a low percentage of glial cells, both astrocytic (< 1.0%) and microglial (< 1.0%). Therefore, while it is possible that even in a system with reduced glial numbers synaptic aberrations appear from reduced pruning, it may also be indicative of neuronal driven processes of synaptic regulation. Recently, astroglial C3 release was shown to negatively affect neuronal dendritic complexity and
synaptic properties through binding to C3aR on the neuronal surface in culture conditions with low microglial numbers. Further analysis suggests that C3aR mediated dendritic and synaptic changes result from intraneuronal calcium signaling. The mice used in our study constitutively lack complement 3 expression from all cell types, therefore synaptic changes observed in pCGC cultures may be a result of lost C3 secretion from the small percentage of astrocytes in culture. Treatment of cortical cultures with recombinant C3 was shown to reduce dendritic arborization and synapse density in vitro. As we also noted an increase in the number of primary dendrites in C3 KO pCGCs, it is likely that C3 plays a role in regulating dendritic growth in addition to synapses in cerebellar granule cells as well. Our data demonstrating increased responsiveness to KCL induced depolarization also proposes that loss of C3 leads to changes in synaptic function. However, additional electrophysiology experimental validation is necessary to definitively prove changes in synaptic function in cerebellar granule cells of C3 KO mice. We feel it is important to note functions of other complement family proteins in cerebellar synaptic regulation, as they it may offer additional insight into neuron specific roles of complement proteins. There is a well-established set of complement like proteins that function in synaptic regulation through neuronal interactions. Cerebellin 1 (Cbln1) is a member of the cerebellin subfamily of the C1q family of proteins. Cbln1 is highly expressed in cerebellar granule cells and functions as a transneuronal regulator of synapse development. Specifically, Cbln1 functions in the formation and stabilization of the cerebellar granule cell Purkinje cell synapse, as well as in controlling synaptic plasticity though regulation of post-synaptic endocytosis of AMPA receptors. Whether similar mechanisms exist from complement 3 has yet to be determined. Future studies to identify cell specific roles of complement proteins will be greatly improved by current works to create cell specific complement deficient mouse lines.
Our *in vivo* analysis of C3 KO mice yielded the unexpected finding that synaptic densities in WT and C3 KO mice did not differ in either the molecular layer or the cerebellar granule cell layer. Based on our *in vitro* data we expected to observe increased synapse density in the molecular layer, the sole target region of cerebellar granule cell parallel fibers. It is possible that changes *in vitro* are due to the reduced culture conditions where glial cells are extremely low in number and within an *in vivo* system compensatory mechanisms are present. Cerebellar granule cells in culture synapse with other cerebellar granule cells and with a small population of inhibitory interneurons. A more physiological system and area of future study would be to use co-cultures of cerebellar granule cells and Purkinje cells. As C3 KO granule cells showed increased calcium response to KCL induced depolarization, it is possible that cerebellar granule cells synapses *in vivo* have altered functions that underlie deficits observed in homecage behavioral phenotyping. Decreased tonic inhibition of cerebellar granule cells was identified as a cause of gait ataxia in a mouse model of Angelman syndrome. Increased synaptic activity or number could result in increased activation of Purkinje cells. Proper Purkinje cell function is important for locomotor behavior. Purkinje cells are rhythmically modulated during locomotor function with high activity during transitions between ipsilateral stance and swing phases, and low activity at mid-stance. Altering Purkinje cell input could interfere with rhythmic signaling leading to impaired gait dynamics and ataxia.

It is also important to note that as complement proteins are expressed throughout the brain, alterations in other regions of the brain controlling locomotor function may also be involved. However, as C3 KO mice did not show overt loss of locomotor function, we do not expect significant involvement of primary motor circuits. Additionally, analysis of the primary motor cortex in 1 month old C3 KO mice did not reveal any differences in
synaptic density from WT controls (data not shown). Deficits observed appear to be more characteristic of cerebellar functional changes. A more detailed assessment of other behaviors controlled by cerebellar function (motor learning, balance, posture, eye movement) may reveal further deficits.

Our previous research demonstrated increased expression of complement proteins in the aged cerebellum in mice with increased excitatory synaptic density and locomotor deficits. Interestingly, loss of complement 3, one of transcripts increased in the aging brain also leads to locomotor deficits and synaptic alterations in vitro. As complement plays a role in regulating synapse density, it is possible that complement expression is increased as a compensatory mechanism for synaptic changes in the aging brain. Our data clearly demonstrates that loss of complement protein leads to impaired characteristics of locomotor function, but not an overt loss of locomotion, which is similar to many aging phenotypes. Identifying mechanisms underlying gait disturbances in the elderly is critical for the development of novel therapeutic strategies. Further analysis of cerebellar functions in C3 KO mice may offer insight into age related locomotor deficits and potential therapies.
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Chapter 3: Loss of Toll-like Receptor 2 results in significant consolidation of active states

Introduction

Age-Associated Deficits in Sleep and Introduction to Sickness Behaviors

Aging is accompanied by deficits in the ability to regulate transitions between active and inactive states according to circadian rhythm. Altered circadian rhythm in the elderly has been linked to increased napping frequency and duration\(^1\), higher fragmentation of rest and activity cycles\(^2\), earlier onset sleep\(^3\), and early awakening from sleep\(^4\). Additionally, sleep fragmentation characterized by multiple awakenings and arousals that disrupt normal sleep architecture is associated with advancing age. Sleep fragmentation is accompanied by significant clinical outcomes, including cognitive impairment\(^5,6\), falls\(^7\), decreased patient quality of life\(^8\), and social isolation\(^9\). In adults older than 65, insomnia and sleep disordered breathing (usually from obstructive sleep apnea) account for 60% and 40% of sleep fragmentation complaints, respectively\(^10\). Animal models of aging have also demonstrated fragmentation of activity with advancing age. One study of wheel running activity in adult (6-9 mo.) and aged (19-22 mo.) old C57BL/6J mice revealed increased fragmentation with no change in maximum running activity in aged mice. Fragmentation was characterized by shorter bouts of activity with longer rest periods in between bouts\(^11\). CNS aging\(^2,12,13\) and CNS neurodegeneration\(^14-16\) both contribute significantly to insomnia and active state fragmentation. Given the high prevalence and impact that sleep fragmentation has on older adults’ lives, it is not surprising that sleep disturbances are associated with greater future health care expenditures\(^17,18\).

Sleep is a highly complex physiological process organized over multiple sites within the CNS. Within the CNS, functional changes in hypothalamic regions, including the
suprachiasmatic nucleus and lateral hypothalamus have been proposed to underlie sleep fragmentation\textsuperscript{19, 20}. The lateral hypothalamus (LH) is a critical region for the generation and regulation of sleep behaviors. The LH contains a discrete neuronal population that expresses orexin, a 33/28 peptide hormone (orexin A/B, respectively) first described for its involvement in narcolepsy, an acquired sleep disorder\textsuperscript{21}. Mice genetically engineered to ablate prepro-orexin\textsuperscript{22}, orexin receptor-2\textsuperscript{22}, or orexin\textsuperscript{23} all exhibit severe sleep fragmentation. Electrophysiological studies demonstrate that resting membrane voltages in orexinergic neurons are intrinsically depolarized\textsuperscript{24}, making them exquisitely sensitive to excitatory synaptic input\textsuperscript{25-27}. Orexinergic neurons share reciprocal inhibitory projections with LH neurons expressing melanin-concentrating hormone (MCH). Data obtained from optogenetic approaches strongly support the concept that under basal conditions (\textit{e.g.}, without sleep pressure\textsuperscript{28}) joint activity of orexinergic and MCH neurons constitute a biological “switch” that toggles between wake (active) and sleep (inactive) states\textsuperscript{29, 30}. Therefore, to better understand sleep regulation and potential causes of CNS mediated age associated sleep fragmentation; it is critical to identify molecules that regulate orexinergic neuron function.

Changes in sleep patterns are often associated with infections, and suggest that immune signaling may play a role in sleep regulation. Sickness behaviors are a set of coordinated behavioral changes (including increased sleepiness, anorexia, and lethargy) that focus metabolic resources toward fighting infections. Sickness behaviors have been directly linked to the suppression of orexinergic signaling\textsuperscript{31}, thereby suggesting that immune proteins may play a role in the regulation of orexinergic neuron function. In fact, one particular group of immune proteins, hypothalamic toll-like receptors, has been strongly linked to sickness behaviors\textsuperscript{32, 35}. Specifically, toll-like receptor 2 (Tlr2), has been shown to play a prominent role in organizing sickness behavior\textsuperscript{34, 35}. 
**Toll-like Receptor 2 Signaling and Expression**

Tlr2 is an intrinsic membrane protein containing multiple extracellular tandem leucine-rich-repeat motifs that fold into a characteristic horseshoe shape\(^{36}\). Tlr2 forms heterodimers with similarly shaped partners such as toll-like receptor 1 (Tlr1), or toll-like receptor 6 (Tlr6); alternatively, Tlr2 forms homodimers with itself\(^ {37, 38}\). These dimers, when organized into larger structures containing Cd14 and Cd36, recognize extracellular diacylated and triacylated lipopeptide ligands\(^{39}\). Of all toll-like receptors, Tlr2 has been shown to recognize the largest array of pathogen associated molecular patterns from distinct bacteria, yeast, and viruses. Bacterial peptidoglycans, lipoproteins, atypical lipopolysaccharide, and glycosylphosphotidylinositol lipids can all serve as agonists. Additionally, yeast zymosan, human cytomegalovirus (hCMV) envelope proteins, and hCMV miRNA UL112-3p activate Tlr2\(^ {40-43}\). Aside from exogenous sources, numerous endogenous ligands activate Tlr2 receptors. These include biglycans\(^{44}\), endoplasm\(^{45}\), High Mobility Group Box Protein 1 (HMGB1)\(^ {46}\), Heat Shock Protein 60 (HSP60)\(^ {47}\), Heat Shock Protein 70\(^ {48, 49}\), human cardiac myosin\(^ {50}\), hyaluronan\(^ {51-53}\), monosodium urate crystals\(^ {54, 55}\), alpha synuclein\(^ {56}\), oxidative stress related carboxyalkylpyrroles\(^ {57}\), and necrotic cell products\(^ {58}\). Activation of Tlr2 dimers results in complex intracellular signaling cascades\(^ {59}\), but can be summarized by (1) Tlr2 ligand binding recruiting the adaptor protein MyD88, (2) MyD88 activation of pathways that phosphorylate IkB, causing (3) nuclear translocation of NF-κB\(^ {60}\). The effects of NF-κB activation are context dependent, where the duration and location of activation are important in determining the end outcome. Within neurons, NF-κB signaling plays a role in axon growth\(^ {61}\), dendritic arborization\(^ {62}\), synapse formation\(^ {63-65}\), plasticity\(^ {63, 64}\), and behavior\(^ {65}\).

Tlr2 is widely expressed throughout the CNS on multiple cell types including microglia, astrocytes, neurons, and oligodendrocytes\(^ {66, 67}\). Within the hypothalamus,
orexinergic neurons strongly express both Tlr2 transcript and protein\textsuperscript{68}. Additionally, hypothalamic expression of the putative Tlr2 endogenous agonist Hsp70 occurs under a variety of stressor conditions\textsuperscript{69, 70}. Changes in Tlr2 signaling may have an impact on regulation of orexinergic signaling and regulation of active states. Supporting this theory, NF-κB, the downstream signaling pathway of Tlr2 has been tied to regulation sleep behaviors. Injection of a cell permeable NF-κB inhibitor attenuates IL-1beta-induced sleep and febrile responses\textsuperscript{71}. This data suggests that Tlr2 signaling through NF-κB may function in regulating sleep behaviors by multiple mechanisms. This may include NF-κB mediated changes in neuron structure and function or through NF-κB mediated cytokine signaling. In efforts to determine the role of Tlr2 in the regulation of activity patterns and circadian rhythm, we tested control C57BL/6J (WT) and Tlr2\textsuperscript{tm1Aki} (Tlr2\textsuperscript{-/-}) male mice in our HCM system at two time points, 4-4.5 months and 8-8.5 months. Using this system we were able to assess the circadian nature of daily activity and inactivity and determine if there are any genotypic differences between mice lacking function Tlr2 signaling and WT controls.

**Methods and Materials**

**Mice and Animal Husbandry**

Tlr2\textsuperscript{tm1Aki} homozygous mutant mice (engineered with a constitutive mutation deleting a transmembrane domain and protein cytoplasmic tail)\textsuperscript{72} were obtained from a colleague’s colony and triad-mated to create cohorts of mutant mice. We confirmed presence of this genetic lesion by PCR of DNA obtained from tail biopsy of putative Tlr2\textsuperscript{-/-} mice. WT male and female (C57BL/6) mice were obtained from Jackson labs and triad-mated to create a WT colony. Since Tlr1, Tlr6, and Tlr10 do not form homodimers, Tlr2\textsuperscript{-/-} mice cannot transduce intracellular signals evoked by exogenous or endogenous Tlr2 ligands\textsuperscript{73, 74}, and do not have a dominant negative phenotype evoked from aberrant Tlr1,
Tlr6, or Tlr10 signaling. Prior to behavioral testing, mice were housed in the UNMC vivarium at a density of ≤5 per cage in a microisolator system (Lab Products Inc., Seaford DE), provided with chow (Envigo Teklad #7012) and water *ad libitum*, given environmental enrichment (Crinkle Paper Pouches, WF Fisher), and maintained on a 12:12 circadian lighting cycle (lights on 0600 CST). Vivarium temperatures ranged between 20-23 °C. Mice were separated by sex at weaning. All studies were performed in full concordance with both institutional and federal regulations regarding animal care and use. The protocol was approved by the UNMC Institutional Animal Care and Use Committee (IACUC).

**Body mass composition determination**

We performed a longitudinal assessment using dual emission x-ray absorptiometry (DEXA) for *in vivo* estimates of mouse adiposity. We evaluated one cohort of male WT (*n* = 8) and male Tlr2−/− (*n* = 8) mice at 4-4.5 months and 8-8.5 months of age. One mouse was lost by attrition from the 8-8.5 month old WT cohort. Before data collection, all instrumentation was calibrated to a phantom approximating mouse body composition characteristics. Briefly, mice were lightly anesthetized with isoflurane, and imaged (Piximus I, GE Lunar). Measures of body mass composition, including bone mass density (BMD), bone mineral content (BMC), bone area (BArea), tissue area (TArea), ratio of soft tissue attenuation (*R*<sub>ST</sub>), total tissue mass (TTM), and percent adiposity (% fat) were calculated from images using vendor provided software (Piximus 2.10). Differences in cohort DEXA and body weight values were determined by unpaired two-sided Student’s *t*-tests, with Bonferroni correction of the critical *p* to control the false positive rate.

**Metabolic rate determination**
We performed a longitudinal assessment of mouse metabolic parameters using indirect calorimetry with the mouse cohorts detailed above. Briefly, mice were fasted overnight prior to metabolic assay. Animals were placed individually in a calorimetry chamber (8 total; Oxymax, Columbus Instruments), and tested between 12:00 and 17:00 for one day. Measures of metabolic rate, including maximum oxygen uptake ($\dot{V}O_2$), global oxygen delivery ($DO_2$), oxygen output ($O_2$ out), maximum CO$_2$ production, ($\dot{V}CO_2$), global CO$_2$ removal ($DCO_2$), CO$_2$ output ($CO_2$ out), and heat generated were calculated using vendor provided software (Oxymax for Windows 4.49). These metabolic parameters were adjusted for mouse adiposity per ANCOVA. Full details regarding our system components and operation are provided in Bonasera et al. 2017.

**Home cage behavioral monitoring**

Using the mouse cohorts described above, we performed a longitudinal assessment of home cage behaviors including feeding, drinking, movement, and circadian rhythm. Briefly, mice were placed in a custom-designed home cage monitoring arena (32 total) with *ad libitum* access to milled chow (#5058, PicoLab) and water. This system provides high spatial (within 0.5 cm) and temporal (within 1 ms) precision of all mouse behaviors. Mice are habituated to the home cage environment for 5 days before the start of data collection, which then proceeds for at least 15 days (to ensure collection of at least 14 days of data for each mouse). The system collects data at all times (except brief intervals every 3-4 days for replacement of mouse food and water supplies). After the mouse is introduced to the home cage, it is not handled until the end of the experiment. Since mouse handling is a well-appreciated stressor known to alter many behaviors, we thus capture mouse home cage behaviors without imposing significant external stressors. Behavioral data obtained from this system describing C57BL/6 mice are highly similar regardless of system location or investigator (Tecott, Goulding, personal
communication). Data passing automated quality control procedures are then classified to determine mouse active/inactive states, mouse intake (of food and water) bouts, and mouse movement (locomotion and movement-in-place) bouts. Following classification, we determine up to 665 distinct measures of mouse feeding, drinking, movement, and circadian activity. We employ false discovery rate (FDR) statistics to minimize family-wise error rates; behaviors found significant in this manner are subjected to further analysis. Full details regarding our system hardware and software characteristics have been published\textsuperscript{76, 78, 79}.

We examined circadian periodicities using Lomb-Scargle analysis\textsuperscript{80, 81}; this method detects multiple periodicities within a time series and has been validated in the setting of incompletely sampled data streams. Feeding, drinking, and movement data were binned into 6-minute epochs, and significant periodicities (up to 60 hr duration) were calculated using an implementation described by Van Dongen et al., 1999\textsuperscript{82} and coded in MATLAB 2011b (MathWorks, Natick MA). We examined patterns of active state onset and duration using the comparison clustering algorithm described by Goulding et al. (2008) and coded in MATLAB 2011b. Briefly, active state onset and duration data from the WT and Tlr2\textsuperscript{-/-} groups are combined. Between 2-50 clusters were fit to this dataset using bivariate normal distributions. We then test the null hypothesis that WT and Tlr2\textsuperscript{-/-} mice are equally represented in a given cluster by calculating a $c^2$ statistic comparing the observed to predicted number of data points that WT and Tlr2\textsuperscript{-/-} contribute to this cluster. The overall difference between WT and Tlr2\textsuperscript{-/-} mice active state patterns is thus represented by the sum of $c^2$ values over all clusters.

The major limitations of our home cage behavioral monitoring approach include brief epochs of data loss secondary to blocked photobeams (e.g., chow pile, or resting mouse close enough to encroach on the beam radius), licker malfunction (in particular, drips),
mouse weight gain/loss during the experiment, and excessive rearing along the cage wall (both altering reported movement distances). All of these issues are identified during our data quality control phase, and removed from the dataset at that time. Since these issues occur infrequently and at random times, there are no statistical problems introduced by removing this small percentage of collected data during the quality control phase.

Open field assay

Mice were placed with their head facing into the same corner of an open field arena (49 cm long × 49 cm wide × 15 cm high, white walls and floor, custom fabricated by California Model and Design, San Francisco, CA). Locomotor activity was recorded at 30 frames/s to .mp4 format from a camera mounted directly above the arena; video files were analyzed using EthoVision XT8 (Noldus, Leesburg VA) to measure locomotor distance, center dwell time, center crossings, and thigmotaxis. We defined our thigmotaxis zone as extending 7 cm from the arena walls; the remainder of the arena was defined as the center zone. Mice were tested in 20 minute trials between 11:00 and 15:00 under room lighting. The arena was washed with dilute Clidox-S solution, rinsed with 70% ethanol, and dried between mouse trials. We compared total open field distance between WT and Tlr2−/− cohorts using unpaired, two-sided Student’s t-tests. Center crossings and center dwell times (normalized to locomotor distance) were compared using unpaired, two-sided Student’s t-tests. We performed Bonferroni adjustment of critical p value to account for 3 comparisons.

Elevated zero maze

Mice were placed in the center of the zero maze closed path (34 cm inner diameter, 46 cm outer diameter, on four-braced legs 58 cm off the ground; 12 cm high walls for closed path, white Plexiglas, California Model and Design, San Francisco). Behavior was
recorded at 30 frames/s to .mp4 format from a camera mounted directly above the arena. Video files were analyzed using EthoVision XT8 (Noldus) to determine locomotor distance within the arena, and assessed for zone crossings and zone dwell time by manual observation. Zone crossings were scored only when all four of the animal’s paws crossed the border between open and closed regions of the maze. Mice were tested in 6 minute trials between 11:00 and 15:00 under room lighting. Arena was washed with dilute Clidox-S solution, rinsed with 70% ethanol, and dried between mouse trials. We compared total zero maze locomotor distance between WT and Tlr2<sup>−/−</sup> cohorts using unpaired, two-sided Student’s t-tests. We compared zone crossings and zone dwell times (normalized to locomotor distance) using unpaired, two-sided Student’s t-tests. We performed Bonferroni adjustment of critical p value to account for 3 comparisons.

**Results**

*FDR identifies 73 differentially expressed behaviors between 4-4.5 mo old WT and Tlr2<sup>−/−</sup> mice, but none in the same mice at 8-8.5 months old*

Most of the genotypic differences between the WT and Tlr2<sup>−/−</sup> 4-4.5 mo old cohorts involve behaviors occurring during the circadian light cycle (46 of the 73 total). Tlr2<sup>−/−</sup> mice showed significantly less light cycle movement, feeding, and drinking, and budgeted significantly less time to perform these behaviors compared to WT controls. Bout metrics for light cycle intake/movement onsets, intake/movement durations, per bout intake/movement, bout intake/movement intensities, and bout intake rates/movement speeds all show large genotypic differences as a consequence of the overall paucity of Tlr2<sup>−/−</sup> light cycle behaviors. Inactive and active state properties (discussed in next section) were generally characterized by Tlr2<sup>−/−</sup> mice having fewer daily active/inactive states, lower active/inactive state transition rates, but longer active/inactive state durations. These findings are all consistent with the concept that
Tlr2\(^{-}\) mice have consolidated active and inactive states. Further analysis of behavioral data demonstrated 57 behaviors with a greater than two-fold change between Tlr2\(^{-}\) and WT mice at 4-4.5 months of age that reached statistical significance (Figure 1A). In this figure, 38 of the 57 behaviors identified as having genotypic differences were observed during the light cycle. Interestingly, at 8-8.5 months of age, these same mouse cohorts demonstrated far fewer genotypic differences in behavior. No differences were appreciated by FDR analysis; however significant differences in 6 behaviors were noted by volcano plot analysis (Figure 1B).

**4-4.5 mo old Tlr2\(^{-}\) mutant mice demonstrate greater active and inactive state stability**

As mentioned above, most of the behaviors differing between young WT and Tlr2\(^{-}\) cohorts reflect significant changes in how these two genotypes regulate active states. We therefore decided to examine mouse active and inactive state properties in greater detail. Over the combined dark and light cycles, Tlr2\(^{-}\) mice had fewer active states per day (Figure 2A, Table 1), fewer active states per hour (Figure 2B), and a shorter active phase (Figure 2C) compared to WT mice. Active state durations trended to be longer in Tlr2\(^{-}\) mice, but this finding did not achieve statistical significance. Similarly, over the combined dark and light cycles Tlr2\(^{-}\) mice had fewer inactive states per day (Figure 2D), fewer inactive states per hour (Figure 2E), a longer inactive phase (Figure 2F), and longer duration inactive states (Figure 2G) compared to WT mice. These findings are consistent with Tlr2\(^{-}\) mice experiencing fewer transitions between active and inactive states throughout the circadian day, and thus having increased state stability. Simply stated, when Tlr2\(^{-}\) are active they tend to remain active, and when inactive remain inactive.
<table>
<thead>
<tr>
<th>behavior</th>
<th>WT (mean ± sd)</th>
<th>Tlr2&lt;sup&gt;-/-&lt;/sup&gt; (mean ± sd)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>number daily active states</td>
<td>12.2 ± 2.1</td>
<td>8.3 ± 2.3</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>active states per hour</td>
<td>0.52 ± 0.09</td>
<td>0.36 ± 0.1</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>active phase duration (s)</td>
<td>45,652 ± 1,834</td>
<td>43,476 ± 813</td>
<td>&lt;0.0126</td>
</tr>
<tr>
<td>(hh:mm:ss)</td>
<td>12:40:53 ± 00:00:43</td>
<td>12:04:36 ± 00:13:33</td>
<td></td>
</tr>
<tr>
<td>number of daily inactive states</td>
<td>12.7 ± 1.9</td>
<td>9.1 ± 2.4</td>
<td>&lt;0.006</td>
</tr>
<tr>
<td>inactive states per hour</td>
<td>0.55 ± 0.08</td>
<td>0.39 ± 0.1</td>
<td>&lt;0.007</td>
</tr>
<tr>
<td>inactive phase duration (s)</td>
<td>37,904 ± 1,874</td>
<td>40,159 ± 648</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(hh:mm:ss)</td>
<td>10:31:44 ± 00:31:14</td>
<td>11:09:19 ± 00:10:48</td>
<td></td>
</tr>
<tr>
<td>inactive state duration (min)</td>
<td>64.9 ± 9.9</td>
<td>108.7 ± 35</td>
<td>&lt;0.004</td>
</tr>
</tbody>
</table>

**Table 1.** Comparison of overall active and inactive state properties between 4-4.5 mo old WT and Tlr2<sup>-/-</sup> mice.
Figure 1. Volcano plot of differentially expressed behaviors between wildtype and Tlr2/- cohorts. A. 4-4.5 month old cohort. Generally, Tlr2/- mice demonstrated fewer behaviors overall during the circadian light cycle, and had altered active/inactive state properties. Dashed vertical lines depict boundaries for two-fold decreases (left) and increases (right) in behavior (WT compared to Tlr2/-); dashed horizontal line depicts behavioral significance p<0.05. Behaviors between the left and right dashed vertical lines show no significant differences between WT and Tlr2/- mice. Descriptions of identified behaviors are provided in columns A-C of Supplemental Table 1. Abbreviations forming behavior names are as follows: AFL -> activity-feeding-licking overall averages; TBA -> time budget analysis; SA -> active/inactive state analysis; BA -> intake/movement bout analysis; BD -> bout dominance analysis; DCLC -> behaviors aggregated across both dark and light cycles; DC -> behaviors aggregated over dark cycle; LC -> behaviors aggregated over light cycle; PE -> photobeam event (feeding); LE -> lickometer event (drinking), ME -> movement event; AP -> mouse active phase; IP -> mouse inactive phase. Identified behaviors (per column A) are: (1) BA_ME_DCLC_Go_LC_ActProb, (2) TBA_DCLC_MeanPerAct_LC, (3) TBA_DCLC_MeanPerStop_LC, (4) BA_ME_DCLC_Other_LC_BoutTotDur_ms, (5) SA_IP_NumActStates_EatDrink, (6) BA_ME_DCLC_Other_LC_BoutTotMove_cm, (7) SA_NumActStates_SmallEatLargeDrink, (8) SA_IP_NumActStates_SmallEatLargeDrink, (9) BA_ME_DCLC_Stop_LC_BoutTotMove_cm, (10) BA_ME_DCLC_Small_LC_BoutRate_onperms, (11) BA_ME_DCLC_Small_LC_BoutNumber, (12) TBA_DCLC_MeanPerFeed_LC, (13) BA_ME_DCLC_Large_LC_MeanToralBoutDur_ms, (14) BA_ME_DCLC_Large_LC_BoutRate_onperms, (15) BA_ME_DCLC_Large_LC_BoutNumber, (16) AFL_avg_LC_Move_m, (17) BA_LE_DCLC_Large_LC_BoutRate_onperms, (18) BA_LE_DCLC_Large_LC_BoutNumber, (19) SA_PerActStates_SmallEatLargeDrink, (20) BA_ME_DCLC_Large_LC_MeanTotalBoutIntake_mg,…continued on next page
Figure 1 continued. (21) SA_AP_PerActiveStates_SmallEatSmallDrink, (22) BA_PE_DCLC_Small_LC_MeanTotalBoutIntake_mg, (23) AFL_avg_LC_Chow_gkg, (24) SA_AP_NumActiveStates_SmallEatSmallDrink, (25) BA_LE_DCLC_Large_LC_BoutRate_onperactms, (26) AFL_avg_LC_Liquid_g, (27) SA_AP_NumActStates_NoEatNoDrink, (28) SA_AP_NumActStates_SmallEatLargeDrink, (29) BA_PE_DCLC_Small_LC_MeanTotalBoutDur_ms, (30) BA_ME_DCLC_Other_LC_BoutRate_onperms, (31) BA_ME_DCLC_Other_LC_BoutMeanNumber, (32) SA_NumActStates_SmallEatSmallDrink, (33) BA_LE_DCLC_Small_LC_BoutProbability, (34) SA_AP_PerActStates_NoEatNoDrink, (35) TBA_DCLC_MeanPerStopAtOther_LC, (36) SA_IP_NumActStates_LargeEatLargeDrink, (37) SA_AP_PerActStates_SmallEatLargeDrink, (38) BA_PE_DCLC_Large_BoutRate_onperms, (39) BA_PE_DCLC_Large_BoutNumber, (40) BA_ME_DCLC_Stop_LC_BoutRate_onperms, (41) BA_ME_DCLC_Go_LC_BoutRate_onperms, (42) SA_PerActStates_SmallEatSmallDrink, (43) BA_ME_DCLC_Stop_LC_BoutMeanNumber, (44) BA_ME_DCLC_Go_LC_BoutMeanNumber, (45) BA_ME_DCLC_Go_LC_BoutMeanMove_cm, (46) BA_LE_DCLC_Small_LC_BoutRate_onperactms, (47) SA_IP_NumActStates_SmallEatSmallDrink, (48) SA_DCLC_LC_MeanInactMove_cm, (49) BA_LE_DCLC_Large_BoutSlopeIntensity_mgs, (50) BA_LE_DCLC_Large_DC_BoutSlopeIntensity_mgs, (51) SA_DCLC_LC_MeanInactDur_ms, (52) TBA_DCLC_MeanPerActShortStopAtHomeBase_LC, (53) SA_DCLC_MeanInactMove_cm, (54) BA_PE_DCLC_DC_BoutMeanDur_ms, (55) BA_PE_DCLC_Small_DC_BoutMeanSize_evtdur, (56) BA_PE_DCLC_Small_DC_BoutMeanSize_mg, (57) BA_PE_DCLC_Small_BoutMeanDur_ms. B. 8-8.5 month old cohort. Behaviors significantly differing between WT and Tlr2/-/- mice depicted per A. Supplemental Table 1 provides descriptions for each of these behaviors. Identified behaviors are: (1) BD_Chow_BtSizeRsq, (2) BD_Chow_BtSizePerRSq, (3) SA_NumActStates_SmallEatLargeDrink, (4) SA_AP_NumActStates_SmallEatLargeDrink, (5) BA PE DCLC_Small_TotalDur_ms, (6) BA PE DCLC_Small_PerCumIntake.
Figure 2. 4-4.5 mo old Tlr2^−/− mice have fewer, more consolidated active and inactive states throughout the day. (A) Boxplot of number of daily active states. Red line depicts group median, the box spans the first through third quartiles, the whiskers extend to the most extreme data points not identified as outliers. Lack of overlap between notches of adjacent box pairs suggests that the true medians differ at 95% confidence. For all boxplots, blue depicts values from WT mice, green depicts values from Tlr2^−/− mice. (B) Daily active state rate (per hour). (C) Daily active state phase duration. Axis on left depicts number of seconds × 10^4; axis on right depicts duration in hour-minute format. (D) Daily number of inactive states. (E) Daily inactive state rate (per hour). (F) Daily inactive state phase duration. Axes as given in (C). (G) Median duration for a single inactive state. Axes as given in (C).
This altered active/inactive state transition frequency is particularly evident during the circadian light cycle (Table 2). Compared to WT, Tlr2−/− mice expressed fewer active states (Figure 3A), had a lower active state rate (Figure 3B), and a shorter active state duration (Figure 3C) during the circadian light cycle. Similarly, Tlr2+/− mice had fewer inactive states (Figure 3D), a lower inactive state rate (Figure 3E), and a longer inactive state duration (Figure 3F) during the circadian light cycle. These values suggest that WT mice spend 13% of the light cycle in an active state, while Tlr2−/− mice spend 4% of the light cycle in an active state. Not surprisingly, decreased light cycle activity duration in Tlr2−/− mice was accompanied by decreased feeding, drinking, and movement compared to WT controls (Figure 3G,H,I,J respectively). Tlr2−/− mice clearly experience fewer transitions between active/inactive states during the circadian light cycle compared to WT.

This Tlr2−/− light cycle phenotype is well illustrated in Figure 4, which shows a dual plot actogram for a representative 4-4.5 month old WT and Tlr2−/− mouse over 16 days. Note that the Tlr2+/− mouse demonstrated decreased light cycle activity, and greater active state consolidation over this time period compared to WT (longer solid lies above bout markers). These findings extend to all the mice within the WT and Tlr2−/− cohorts (Figure 5A). For all mice within a cohort across all experiment days, Figure 5A depicts active state onset on the x axis and active state duration on the y axis. This data clearly demonstrates that Tlr2−/− mice express fewer active states during the light cycle compared to WT controls. This plot also demonstrates that short duration active states occurring throughout the circadian dark cycle are statistically underrepresented in Tlr2−/− mice.
Figure 3. 4-4.5 mo old Tlr2−/− mice demonstrate dramatic behavioral consolidation during the circadian light cycle. (A) Boxplot of the number of circadian light cycle active states. Red line depicts group median, the box spans the first through third quartiles, the whiskers extend to the most extreme data points not identified as outliers. Lack of overlap between notches of adjacent box pairs suggests that the true medians differ at 95% confidence. For all boxplots, blue depicts values from WT mice, green depicts values from Tlr2−/− mice. (B) Circadian light cycle active state rate. (C) Circadian light cycle active state duration. (D) Circadian light cycle number of inactive states. (E) Circadian light cycle inactive state rate. (F) Circadian light cycle inactive state duration. (G) Circadian light cycle chow consumption. (H) Circadian light cycle water consumption. (I) Circadian light cycle movement. (J) Total daily light cycle movement.
Figure 4. Dual plot actogram demonstrating dark cycle active state consolidation and light cycle inactive state consolidation of a representative Tlr2⁻/⁻ mouse in comparison to a representative WT mouse. X axis depicts military time (lights off at 12:00 and 36:00, lights on at 24:00 and 48:00) in hours. Y axis depicts experimental day. Note that the data shown in the last half of one daily trace is the first half of data shown in the following day (except for the last day of collection). Drinking, feeding, and movement events are depicted by blue, orange, and green hash lines, respectively. The empty rectangles at the top of tracings for each day represent duration of calculated active states. Dashed vertical lines show lighting transitions, grey background depicts dark cycle.
8-8.5 mo old Tlr2<sup>−/−</sup> mice demonstrate fewer differences in active and inactive states.

There were no genotypic differences between 8-8.5 mo old WT and Tlr2<sup>−/−</sup> mice in overall active/inactive state properties including (1) number of daily active states, (2) active state onset rates, (3) active phase durations, (4) number of daily inactive states, (5) inactive state onset rates, (6) inactive phase duration, or (7) inactive state duration. Similarly, there were no genotypic differences between 8-8.5 mo old WT and Tlr2<sup>−/−</sup> mice in light cycle active/inactive state properties including (1) number of light cycle active states, (2) light cycle active state onset rates, (3) light cycle active state duration, (4) number of light cycle inactive states, (5) light cycle inactive state onset rates, (6) light cycle inactive state duration, or (7) light cycle feeding, drinking, and movement. All of these metrics showed significant phenotypic differences between 4-4.5 mo old WT and Tlr2<sup>−/−</sup> mice.

However, 8-8.5 mo old Tlr2<sup>−/−</sup> mice continue to demonstrate a statistically significant underrepresentation in short duration active states occurring during the dark cycle (Figure 5B), with this finding further extending to short duration active states within the light cycle. However, this phenotype is less pronounced in the older mouse cohort apparent by comparison of similar time epochs in Figures 5A and 5B.

Tlr2<sup>−/−</sup> mice show less variability in circadian patterns of movement, feeding, and drinking compared to WT.

We performed Lomb-Scargle analysis of movement, feeding, and drinking time series (from 4-4.5 month and 8-8.5 month old WT and Tlr2<sup>−/−</sup> mice) to assess periodicity of these behaviors. In both 4-4.5 month old mice and 8-8.5 mo old mice, normalized power of the 24-hour spectral component is significantly greater in Tlr2<sup>−/−</sup> mice compared to WTs for movement, feeding, and drinking (Figure 6). The decrease in amplitudes of
Figure 5. Tlr2<sup>−/−</sup> mice have fewer short duration active states throughout the circadian day. (A) 4-4.5 mo old mice. Each point represents an active state from a WT (top left) or Tlr2<sup>−/−</sup> (bottom left) mouse within the cohort over the total experimental duration. x axis depicts active state onset time, y axis depicts active state duration (in log). Dashed lines depict onset (left) and offset (right) of the dark cycle, which is further highlighted in light grey. Active states highlighted in green depict regions where states with these specific onset/duration properties are statistically overrepresented in wildtype mice and underrepresented in Tlr2<sup>−/−</sup> mice. Note that for 4-4.5 mo old mice, these states include shorter duration active states throughout the circadian day, as well as all duration active states for the last half of the light cycle. (B) 8-8.5 mo old mice. Each point represents an active state from a WT (top right) or Tlr2<sup>−/−</sup> (bottom right) mouse. Plot annotations otherwise per (A). Active states highlighted in violet depict regions where states with these specific onset/duration properties are statistically overrepresented in wildtype mice and statistically underrepresented in Tlr2<sup>−/−</sup> mice. For 8-8.5 mo old mice, these states include shorter duration active states throughout the circadian day.
Figure 6. *Tlr2*\(^{-}\) mice have stronger 24-hour periodicities compared to WT mice. (A) Lomb-Scargle periodogram for movement, 4-4.5 mo old mice. WT periodogram in blue, Tlr2\(^{-}\) periodogram in green. (B) Observed movement for WT (blue) and Tlr2\(^{-}\) (green) cohorts, 4-4.5 mo old mice. Thin line with points depicts the mean values for each cohort. (C) Lomb-Scargle periodogram for feeding. 4-4.5 mo old mice. WT in blue, Tlr2\(^{-}\) in green. (D) Observed feeding for WT (blue) and Tlr2\(^{-}\) (green) cohorts. 4-4.5 mo old mice. (E) Lomb-Scargle periodogram for drinking. 4-4.5 mo old mice. WT in blue, Tlr2\(^{-}\) in green. (F) Observed drinking for WT (blue) and Tlr2\(^{-}\) (green) cohorts. 4-4.5 mo old mice. (G) Lomb-Scargle periodogram for movement, 8-8.5 mo old mice. WT periodogram in cyan, Tlr2\(^{-}\) periodogram in dark green. (H) Observed movement for WT (cyan) and Tlr2\(^{-}\) (dark green) cohorts. 8-8.5 mo old mice. Thin line with points depicts the mean values for each cohort. (I) Lomb-Scargle periodogram for feeding. 8-8.5 mo old mice. WT in cyan, Tlr2\(^{-}\) in dark green. (J) Observed feeding for WT (cyan) and Tlr2\(^{-}\) (dark green) cohorts. 8-8.5 mo old mice. (K) Lomb-Scargle periodogram for drinking. 8-8.5 mo old mice. WT in cyan, Tlr2\(^{-}\) in dark green. (L) Observed drinking for WT (cyan) and Tlr2\(^{-}\) (dark green) cohorts. 8-8.5 mo old mice. For A,C,E,G,I,K error bars are ± 1 standard deviation. No significant periodicities of longer than 24 hours obtained for any behaviors.
these 24-hour spectral components (as well as 12-hour and 8-hour ultradian spectral components) between 4-4.5 month old and 8-8.5 month old mice suggests that the older mice have greater variability in minute-to-minute and day-to-day movement, feeding, and drinking events compared with younger mice, and is consistent with our prior observations showing age-related decreases in the amplitude of these same spectral components in older C57BL/6 and BALB mice (unpublished data). Lomb-Scargle analysis suggests that Tlr2⁻/⁻ mice have less variability in movement, feeding, and drinking behaviors compared to WT mice, regardless of age. This data is supported for consolidated behavioral states in Tlr2⁻/⁻ mice, indicating less fluctuation in neuronal pathways driving state regulation.

**Tlr2⁻/⁻ mice have lower body weights than WT cohorts, but otherwise show no genotypic differences in metabolic parameters.**

WT mice were significantly heavier (WT 25.2 ± 0.8 g; Tlr2⁻/⁻ 23.1 ± 1.1 g; \( p < 0.0008 \) at 4-4.5 months; WT 29.0 ± 0.9 g; Tlr2⁻/⁻ 26.7 ± 1.7 g; \( p < 0.004 \) at 8-8.5 months), and had a greater total tissue mass (TTM: WT 21.3 ± 0.8 g; Tlr2⁻/⁻ 19.8 ± 1.0 g; \( p < 0.006 \) at 4-4.5 months; WT 25.4 ± 0.85 g; Tlr2⁻/⁻ 23.3 ± 1.5 g; \( p < 0.005 \) at 8-8.5 months) than Tlr2⁻/⁻ mice at both ages assessed. No significant differences in BMD, BMC, BArea, TArea, RST, or % adiposity were noted at either 4-4.5 or 8-8.5 months. We noted no significant differences in the metabolic parameters of \( \dot{V}O_2, O_2 \text{ out}, DO_2, \dot{V}CO_2, CO_2 \text{ out}, DCO_2, \) and heat either at rest (basal status) or with moderate activity.

**No difference between WT and Tlr2⁻/⁻ cohorts in both 4-4.5 and 8-8.5 mo old mouse cohorts for select behavioral measures**

Regarding home cage phenotypes, we noted no significant differences in overall food and water consumption, or overall daily movement. There were no differences in the 24 hour behavioral time budgets for inactivity, feeding, drinking, locomotor, or
nonlocomotor movements. Similarly, there were no differences in active state time budgets examining percentage of time devoted to feeding, drinking, locomotion, and nonlocomotor movements. Additionally, there were no differences in the probabilities of specific behaviors occurring after the start of new active states or preceding the finish of ongoing active states. We also found no significant differences in the dark cycle (4-4.5 month old) or dark and light cycle (8-8.5 month old) patterns of feeding, drinking, or movement bouts, including no differences in hourly bout onset rates, bout probabilities, bout speed/intensities, bout durations, or per-bout intake/movement.

Finally, we assessed anxiety-related behaviors by elevated zero maze and open field assays, and found that Tlr2<sup>−/−</sup> mice demonstrated increased anxiety related behaviors (increased thigmotaxis and decreased entries into open field center; Figure 7) compared to WT mice in the open field assay. Additionally, we note that 4-4.5 month old Tlr2<sup>−/−</sup> mice demonstrate significantly reduced locomotion in both the open field and elevated zero maze assays (data not shown). No genotypic differences were appreciated in zone transitions, time spent on open arm, or time spent on closed arm. These data suggest that loss of Tlr2 function in C57BL/6 mice is associated with decreased exploratory behavior and increased anxiety-related behaviors.

**Conclusions and Discussion**

We provide the first description of Tlr2<sup>−/−</sup> mouse behavior in a home cage environment assessed over an extended period of time. Most notably, we find that loss of Tlr2 function in 4-4.5 month old mice leads to a marked consolidation of both mouse active and inactive states, as determined by active/inactive state durations, onset frequencies, and a marked paucity of all behaviors occurring during the circadian light cycle. As a result of these changes, we also note that Tlr2<sup>−/−</sup> mice demonstrate stronger day-to-day periodicity of movement, feeding, and drinking behaviors as revealed by
Figure 7. 4-4.5 mo old Tlr2⁻/⁻ mice show decreased locomotion and increased thigmotaxis in an open field assay of exploratory behavior. Leftmost plot depicts open field total distance. White bars depict WT (n=8), grey bars depict Tlr2⁻/⁻ (n=8). Error bars are ± 1 standard deviation. ** p<0.01. Rightmost plot depicts open field center dwell time (left pair of bars, with corresponding left vertical axis) and number of center crossings (right pair of bars, with corresponding right vertical axis). Annotations per above. * p<0.05.
Lomb-Scargle analysis. Of note, nearly all of these genotypic differences were no longer observed upon repeat examination of these same mouse cohorts at 8-8.5 months of age, although there was still evidence of active/inactive state consolidation in older Tlr2<sup>−/−</sup> mice.

Behavioral phenotypes arising from Tlr2 loss remain unclear due to the limited scope of available data. Multiple studies suggest that Tlr2 functional loss is accompanied by altered energy balance and metabolism. For example, baseline hyperphagia accompanied by obesity and increased adiposity (on both regular and high fat diets), poor glucose tolerance, and increased respiratory ratio (RER) has been described in 4- and 7-month old Tlr2<sup>tm1Aki</sup> constitutive knockout mice bred to a C57BL/6 background<sup>83</sup>. This same study also noted increased light cycle (and unchanged dark cycle) activity in 12 month old Tlr2 knockout compared to wildtype controls. By contrast, mice with Tlr2 lesions derived from a different founder strain (Tlr2<sup>tm1Kir</sup>) demonstrated hyperphagia, enhanced glucose tolerance, decreased insulin resistance and decreased RER on a high fat diet compared to controls<sup>84</sup>. Similar resistance to the metabolic syndrome phenotype was noted in 3+ month old Tlr2<sup>tm1Aki</sup> mice bred to a C57BL6/Hsd background and receiving a high fat diet<sup>85</sup>. Three month old mice with constitutive knockout of both Tlr2 and Tlr4 (bred to a C3H/HeJ background) maintained on a high fat diet for 8 weeks were noted to have lower body weights, enhanced glucose tolerance, and increased dark cycle locomotor activity compared to control mice kept on the same high fat diet<sup>86</sup>.

Our study finds no evidence of hyperphagia, obesity, or increased adiposity in Tlr2<sup>−/−</sup> mice fed a regular diet compared to C57BL/6 controls; in fact, Tlr2<sup>−/−</sup> mice were significantly lighter at 4-4.5 and 8-8.5 month time points. These results differ from data presented by Shechter <i>et al.</i> (2013, their Figure 1B). We also did not observe any differences in metabolism between WT and Tlr2<sup>−/−</sup> mice. There are no obvious factors to
explain these discrepancies, which may be secondary to differences in mouse breeding strategy, housing (singly housed after HCM in our study), diet, or handling across different rodent colonies. Differences in how the two studies dispensed food (powdered vs pelleted chow) may also account for discrepancies in food intake.

Tlr2 functional loss has also been linked to sleep performance. EEG evaluation of sleep architecture revealed increased consolidation of both awake and sleeping states in Tlr2/Tlr4 double mutant mice, demonstrating significantly less NREM sleep during the dark cycle while simultaneously showing greater REM sleep during the light cycle compared to wildtype cohorts (Sartorius et al., 2012). Our observed phenotypes of dark cycle active state consolidation and light cycle inactive state consolidation are consistent with the sleep phenotypes reported by Sartorius and colleagues. Finally, there is evidence that Tlr2\textsuperscript{tm1Aki} mice have decreased anxiety-related behaviors (increased open field locomotion with decreased thigmotaxis, increased open arm dwell time on an elevated plus maze, decreased marble burying), impaired social behaviors (less recognition in a three-chamber social novelty assay, fewer contacts in a reciprocal social interaction test), increased aggression (more attacks in a cotton bud biting test), impaired sensorimotor gating (decreased prepulse inhibition of an otherwise normal acoustic startle response), and impaired cognition (diminished freezing to aversive context, diminished novelty seeking, poor performance in a Barnes maze task) compared to wildtype cohorts\textsuperscript{87, 88}. We noted increased anxiety-related behaviors as assessed by open field and elevated zero maze; similar factors as discussed above may explain these discrepancies.

Tlr2 signaling occurs when either Tlr1/Tlr2 heterodimers (in concert with Cd14) detect triacylated lipopeptides\textsuperscript{89}, or when Tlr2/Tlr6 heterodimers (in concert with Cd36/Cd14) detect diacylated lipopeptides\textsuperscript{90}. Tlr2 signaling in these contexts are early
events promoting inflammation\textsuperscript{91} and sickness behavior\textsuperscript{35}. Tlr2 regulation of active and inactive state lengths likely occurs either upon the orexinergic/MCH neuronal populations, or onto the interneuron network immediately upstream of these centers\textsuperscript{92, 93}. Orexinergic neurons strongly express both Tlr2 transcripts and protein, and are thus potential loci where integration of animal health status and animal alertness/activity may occur\textsuperscript{68}. Tlr2 expressed by orexinergic neurons is thus particularly well placed to integrate animal health status and animal activity, since orexinergic neurons receive excitatory inputs from multiple CNS regions signaling potential life-threatening conditions (\textit{e.g.} posterior hypothalamic nucleus\textsuperscript{94} (thermoregulation); ventrolateral preoptic nucleus\textsuperscript{94, 95} (overall arousal state); bed nucleus of stria terminalis\textsuperscript{95} (anxiogenic states); amygdalar CRF projections\textsuperscript{96} (stress responses); nucleus accumbens\textsuperscript{97, 98} (reward); locus coeruleus and dorsal raphe nuclei\textsuperscript{99, 100} (arousal states)).

Further, sickness behaviors have been directly linked to suppression of orexinergic signaling\textsuperscript{31}. Organization of CNS systems converging onto MCH neurons is not well-understood at this time; however, MCH neuronal activity can be modulated by neurotransmitters characteristic of the above systems, including glutamate, GABA, norepinephrine, and serotonin\textsuperscript{101}. Our data suggests that following Tlr2 activation, activity fragmentation will increase, active state durations will decrease, and affected individuals may thus limit time spent in social contact with conspecifics. This response is an important aspect of current sickness behavior frameworks\textsuperscript{102}, providing a mechanistic connection for how individuals experiencing illness show strong tendencies to decrease movement and feeding behaviors.

In summary, we describe a previously unappreciated role of the pattern recognition receptor Tlr2 in regulating active state consolidation throughout the circadian day. As mentioned in the introduction, understanding mechanisms controlling active/inactive
state transitions are highly relevant to understanding sleep/wake cycle regulation. Our data suggest that interventions to block Tlr2 signaling may be novel and promising avenues for treatment of insomnia and related sleep disturbances. Further research to identify (1) how different pattern recognition receptors influence the orexinergic/MCH neuronal “switch”, (2) how signaling from these molecules ultimately affects neuronal architecture and (3) how pattern recognition receptors influence neuronal function are critical to close current knowledge deficits.
References


Chapter 4: Mutation of MHC I H2-Kb Leads to adult onset progressive obesity due to reduced orexin neuron activity and increased sedentary behavior

Introduction

Age Related Deficits in Energy Balance and Metabolism

Aging has a dichotomist effect on weight and metabolic status, where some individuals trend towards obesity, yet others towards frailty. Grossly, age-related changes in body composition have been described in both genders and consist of the loss of lean body mass and an increase in fat deposition\textsuperscript{1-3}. However, it is clearly evident that not all elderly individuals gain weight, and only a portion of those that do, gain a significant enough amount of weight to be deemed obese. Another fraction of the elderly population loses weight, and a small percentage of those individuals can even reach a state of frailty. Frailty is a geriatric condition characterized by unintentional weight loss, reduced capacity for physical activity, low muscle strength, and slower gait speeds\textsuperscript{4}. Importantly, an individual on either end of this spectrum, either obese or frail, have considerably increased risk for downstream health complications.

Globally, over 1.4 billion adults are overweight (BMI > 25) and more than half a billion are considered obese (BMI > 30). By 2030 these numbers could rise considerably to 2.16 billion overweight (38\% of population) and 1.12 billion obese (25\% of population) individuals if the current rate of change continues\textsuperscript{5, 6}. Obesity leads to increased risk of comorbid conditions including heart disease, stroke, type II diabetes, and certain forms of cancer culminating in over 2.8 million obesity related deaths per year\textsuperscript{7}. The prevalence of frailty varies greatly depending on whether it is defined as a phenotype or as an accumulation of deficits\textsuperscript{8}. The prevalence of frailty is 14\% when defined as a phenotype, where 3 of the 5 following criteria are met: 1) weight loss, 2)
fatigue and exhaustion, 3) weakness, 4) low physical activity and slowness, and 5) mobility impairment. This prevalence increases to 24% when frailty is viewed as a collection of symptoms, diseases, conditions, and disability and based on risk accumulation theory. A summary analysis of survival rates for frail older adults compared to non-frail older adults average roughly 50% for frail patients when using the phenotypic description and 15% when using the accumulation of deficits model. Additionally, using pooled prevalence data, the estimated increased mortality risk of frailty was concluded to be between 3-5%. A 2017 study by Kiu, et al., used latent class analysis of data collected from the I-Lan Longitudinal Aging Study (ILAS) and subdivided patients with frailty into three groups, those with mobility impairments (slowness and weakness), those without mobility impairments (weight loss and exhaustion), and those with low physical activity. Analysis of healthcare complications in each group demonstrated that those with mobility impairments have the worst outcomes characterized by poorer body composition, worse bone health, reduced cognitive ability, lower survival rates, and worse overall health outcomes compared to robust controls. Individuals with non-mobility based frailty still showed significant health concerns with higher risk of metabolic serum abnormalities and poorer bone health.

There is much debate within the literature on how to clinically define frailty and obesity, but definitions and classifications will only be truly relevant for patients if factors contributing to the development and pathophysiology of these diseases are understood to a point where therapeutic options become available. Therefore, it is critical to identify risk factors that underlie the development of obesity and frailty, and more generally regulate metabolic and energy balance changes in aging. The etiology of obesity is multifactorial, but twin studies suggest between 40-70% of obesity cases arise due to an underlying genetic cause. Currently, only 15% of obesity phenotypes have been linked
to specific genes, including MC4R and leptin mutations\textsuperscript{15-17}. Therefore, a large percentage of obesity phenotypes likely have genetic contributions that are yet to be described. The etiology of frailty is just as complicated, as it requires deficits in multiple functional areas for diagnosis and each of these deficits can arise from a multitude of pathologies. One well-described precursor to frailty development is behavioral adaptation to declining physiologic reserves\textsuperscript{4}. The etiology of these changes is multifactorial, encompassing environmental and age-related physiologic changes. While there is much focus on peripheral mechanisms of obesity and frailty development such as diet and exercise, CNS contributions, particularly hypothalamic function, likely play a significant role in the development of these conditions but are poorly understood.

\textit{Functions of the Hypothalamus in Energy Balance}

Specific hypothalamic nuclei function to link nutrient sensing to energy balance regulation through control of feeding, drinking, and arousal behaviors. In general, energy balance can be considered the equilibrium of caloric intake and expenditure. The hypothalamus can be split into three gross anatomical regions, the anterior hypothalamus, posterior hypothalamus, and tuberal hypothalamus. Each of these regions is made up of several small nuclei that fall either medially or laterally, and contain distinct populations of cells with specific functions in the regulation of energy balance through orexinergic or anorexigenic signaling. Three critical hypothalamic areas for the studies presented here are the arcuate nucleus, paraventricular nucleus, and lateral hypothalamus.

The arcuate nucleus (ARC) is a collection of neurons located in the mediobasal hypothalamus adjacent to the third ventricle and superior to the median eminence. Through specific cell types and receptor expression the arcuate nucleus integrates peripheral signaling on nutrient status to the CNS. Activation of proopiomelanocortin
(POMC) neurons by leptin, glucose, insulin, or other signaling molecules that indicate a nutrient high state, leads to the release of α-melanocyte stimulating hormone (α-MSH) at POMC axon terminals. α-MSH acts on melanocortin receptors to activate an anorexigenic path, thereby suppressing food intake. Contrarily, neuropeptide Y/agouti related peptide (NPY/AgRP) cells within the ARC provide orexigenic signaling through the action of NPY on NPY receptors (NPYR). Each of these paths also possess reciprocal inhibition to one another where cocaine- and amphetamine-regulated transcript (CART) functions as an antagonist to NPY receptors, and AgRP as an antagonist to melanocortin receptors. Activation of NPY/AgRP neurons by grehlin leads to release of AgRP from axon terminals, providing antagonistic activity to α-MSH at melanocortin receptors and strongly inhibits POMC perikarya within the arcuate nucleus through NPY and GABA^{18-27}. Axons from the ARC project primarily rostral from the arcuate to innervate the dorsomedial hypothalamic nucleus (DMH), paraventricular nucleus (PVN), and the lateral hypothalamus in succession during postnatal development^{28}.

The paraventricular nucleus of the hypothalamus lies laterally to the superior portion of the third ventricle and receives input from multiple brain regions, including, but not limited to, the arcuate nucleus, lateral hypothalamus, preoptic area, pons, and medulla^{29,30}. Neurons within the PVN are commonly classified into two groups, magnocellular (large) and parvocellular (small). Parvocellular autonomic neurons project to the nucleus tractus solitarius, interomediolateral column of the spinal cord, and dorsal motor nucleus of vagus, which are all key regions for the regulation of sympathetic functions^{31-34}. Through downstream projections and synapses from these PVN targets, it is connected to multiple organ systems with critical autonomic functions. PVN connects to the liver, pancreas, white and brown adipose tissues to regulate hepatic glucose flux, adipose
deposition, thermogenesis, peripheral glucose uptake, and pancreatic secretion\textsuperscript{35-39}. In addition to autonomic projections, the PVN functions as a critical regulator of neuroendocrine function. Parvocellular neuroendocrine cells release signaling peptides that regulate the production of pituitary hormones through projections to the median eminence and access to the pituitary portal system\textsuperscript{31}. Magnocellular neuroendocrine neurons project to the posterior pituitary, where they release vasopressin and oxytocin to the systemic circulation. Both GABAergic and glutamatergic interneurons also exist within the PVN and function to integrate input and organize output in response to energy status\textsuperscript{31, 40, 41}. POMC neuronal protections from the ARC synapse on both magnocellular and parvocellular neurons expressing receptors of \(\alpha\)-MSH, where release leads to satiety as previously described\textsuperscript{42}. Furthermore, NPY/AgRP neurons from the ARC synapse with parvocellular neurons in the PVN, where NPY activity leads to increased feeding\textsuperscript{43, 44}. The formation of PVN inputs from the ARC is developmentally regulated process that occurs in the postnatal period in mice, and is critically tied to leptin signaling. Data suggests a postnatal leptin surge that occurs in mice is important for the development of ARC projections, as well as dendritogenesis, neurogenesis, and synaptogenesis\textsuperscript{45-48}. Leptin deficiency leads to impaired development of ARC projections to the PVN. This can be rescued by exogenous leptin therapy, but only during the postnatal period as adult treatment with leptin has no effect in restoring this pathway\textsuperscript{28}. Impaired development of POMC and NPY/AgRP connections to the PVN leads to impairments and energy balance and may be involved in the development of obesity phenotypes.

The lateral hypothalamus (LH) is also a critical region for the maintenance of energy balance. The LH is less well defined and partitioned into discrete cell populations or nuclei compared to other regions of the hypothalamus. Within the lateral hypothalamus
there are scattered populations of neurons that express both excitatory and inhibitory neurotransmitters, as well as several neuropeptides. *In situ* hybridization and immunohistochemistry studies have identified populations of neurons positive for excitatory vesicular glutamate-2, inhibitory GABA neurons, orexin/hypocretin (Orx), melanin concentrating (MCH), neurotensin (Nts), and galanin (GAL). Each of these cell populations have important roles in the regulation of energy balance, but in the context of this project we will focus on the orexinsergic neuron system of the lateral hypothalamus.

Orexins, also known as hypocretins, were identified concurrently by two independent groups and were found to be endogenous ligands for two orphan G-protein coupled receptors. Receptors for orexin A and B are found throughout the brain and participate in a multitude of pathways, but are primarily studied for their role in the regulation of arousal and feeding behaviors. Within the hypothalamus receptors for orexin A are enriched in the ventromedial hypothalamic nucleus and orexin B in the paraventricular nucleus. Outside of the hypothalamus orexin A receptors are present in tecta, the hippocampal formation, dorsal raphe, and locus coeruleus, and orexin B receptors in the cortex, nucleus accumbens, subthalamic and paraventricular thalamic nuclei, and anterior pretectal nucleus. In humans, orexin neurons project to locus coeruleus, dorsal raphe nuclei, amygdala, suprachiasmatic nucleus, basal forebrain, brainstem, and spinal cord. Functionally, orexin neurons have been shown to be glutamatergic and therefore excitatory to their target neurons in multiple brain regions. Orexin neurons receive predominantly excitatory Vglut2 synaptic input and electrophysiology studies show that orexin neuron resting membrane voltages are intrinsically depolarized, making them particularly sensitive to changes in excitatory synaptic input. Recent data indicate that the orexin system is plastic, where synaptic
Changes occur in response to both daily and long term fluctuations in neuronal activity. Orexin levels in the CSF are low during resting phases and high during periods of activity, which correlates with electrical activity patterns recorded in orexin neurons during these states. However, environmental changes can impact this process. C57BL/6 mice fasted for 12 hours show significant potentiation of orexin neurons, evident by increased frequency of mini excitatory post-synaptic currents and increased excitatory synapses on the orexin containing cell bodies. Tuning of synaptic strength can also occur in response to changes in nutritional status through leptin signaling in an activity dependent manner. Additionally, maternal obesity, early malnutrition, and gestational caloric restriction have all been shown to alter the development of hypothalamic projections and increase the risk for long-term deficits in energy balance. Despite a clear impact for environmental factors in regulating synaptic plasticity and function, clear mechanisms underlying these changes are poorly understood.

Within multiple regions of the brain immune proteins have been shown to play important roles in synaptic development, pruning, plasticity, and maintenance but their roles within the hypothalamus have yet to be elucidated. Our previous research has implicated immune proteins and pattern recognition receptors with regulation of aging phenotypes including changes in energy balance. However, the impacts of specific immune molecules in regulating energy balance phenotypes are currently unknown.

Expression of Immune Proteins in the Aging Hypothalamus

In an aging-mouse model that shows progressive weight loss and characteristics of frailty, immune proteins are increased in the aging hypothalamus (General Introduction: Figure 1 and 2). The hypothalamus is a critical brain region for the regulation of energy balance including regulation of activity, feeding, drinking, and sleep. Despite clear roles for immune proteins in the regulation of synapses during development and in
neurodegenerative disease, there is little known on the role of immune proteins in regulating hypothalamic function and downstream behaviors. Therefore, we set out to assess the role of a particular class of immune proteins, class I major histocompatibility complex proteins (MHC I). MHC I proteins were selected as several members of family were increased in the aged hypothalamus and they have been shown to function in synaptic regulation in other regions of the brain.

**Major Histocompatibility Complex Proteins**

Class I major histocompatibility complex proteins (MHC I), also known as human leukocyte antigens (HLA) are highly polymorphic transmembrane proteins with over 50 known gene sequences corresponding to the protein class. MHC I proteins are expressed on nearly all nucleated cells and function to present peptide fragments 8-10 amino acids in size generated from endogenous and exogenous protein. Classical MHC I proteins (MHC Ia) are highly polymorphic and function in adaptive immunity, while non-classical proteins (MHC Ib) are less polymorphic, more tissue specific, and function in both innate and acquired immunity. MHC I proteins are encoded by a ~3.6-Mb cluster of genes within the mammalian genome. Classical MHC Ia molecules in mice are encoded by the K and D regions and homologous to human HLA-A, HLA-B, and HLA-C. Non-classical MHC Ib molecules are encoded by the M, Q, and T regions and homologous to human HLA-E, HLA-F, HLA-G, and HLA-H. MHC I proteins are composed of a membrane spanning alpha-chain forming the α1, α2, and α3 domains. The heavy (α) chain non-covalently interacts with soluble β2-microglobulin to form a heterodimer and a peptide binding groove in the α1α2 or α1β1 domains. MHC I peptides are loaded by the peptide-loading complex (PLC) composed primarily of heterodimeric transporter associated with antigen processing and supporting proteins. Following loading, MHC I proteins are trafficked via selective recruitment into cargo
vesicles to the plasma membrane to present peptides to recipient cells\textsuperscript{86}. MHC I genes are both constitutively expressed and modulated by several pathways targeting cis-acting regulatory promoter elements containing binding sites for nuclear transcription factor kappa B (NF-κB) and interferon-regulatory factor (IRF) family members\textsuperscript{87-89}. Both NF-κB and interferon pathways have well documented roles in inflammatory signaling, and NF-κB signaling is critical for proper nervous system function\textsuperscript{90}. Additionally, in the CNS MHC I expression can be regulated by neuronal activity through a calcium dependent mechanism involving CREB signaling\textsuperscript{91-93}.

**Major Histocompatibility Complex Proteins in the CNS**

A growing body of work has shown a significant role for MHC I proteins in select areas of the CNS. Within the normal developing nervous system MHC I proteins are expressed on cortical, hippocampal, and cerebellar neurons with higher expression earlier in development\textsuperscript{94-96}. MHC I proteins are expressed at the synapse where they colocalize with both pre and post synaptic proteins\textsuperscript{97}. Expression of MHC I in the nervous system is regulated by neuronal activity through a calcium dependent mechanism involving CREB signaling\textsuperscript{91-93}.

Functionally, MHC I proteins have been shown to be important for neuronal development and synaptic function. Loss of MHC I surface expression using TAP1/β2-microglobulin double knockout mice (β2M\textsuperscript{-/-}TAP\textsuperscript{-/-}) results in increased hippocampal synaptic density and increased action potential frequency\textsuperscript{97}. Additionally, β2M\textsuperscript{-/-}TAP\textsuperscript{-/-} mice show several changes in excitatory synaptic pathways within hippocampal circuits. The AMPA/NMDA ratio is significantly lower at β2M\textsuperscript{-/-}TAP\textsuperscript{-/-} synapses compared to WT in the Schaffer collateral/CA1 synapses, which reflects an increase in NMDAR-mediated currents\textsuperscript{98}. These changes likely underlie enhanced NMDAR dependent LTP and the loss of LTD in β2M\textsuperscript{-/-}TAP\textsuperscript{-/-} mice\textsuperscript{99}. MHC I was shown to regulate NMDAR-induced AMPA
receptor trafficking and tonically inhibit NMDAR function\textsuperscript{98}. Behaviorally, MHC I deficient β2M\textsuperscript{-/-}TAP\textsuperscript{-/-} mice have deficits in hippocampal dependent memory including object recognition, social recognition, and contextual fear memory\textsuperscript{100}. β2M\textsuperscript{-/-}TAP\textsuperscript{-/-} mice also exhibit sex-specific increased stress responses to saline injection, measured by quantification of activity and exploratory behaviors in open-field and elevated plus maze paradigms. Despite well documented roles for MHC I proteins in select regions of the CNS, functions and expression of MHC I proteins in the hypothalamus are poorly understood. Additionally, the roles of specific MHC I isoforms are poorly described.

MHC I isoform H2-Kb (also called H2-K1) expression is increased in the aged hypothalamus in a mouse model of energy balance dysregulation. MHC I proteins have defined functions in synaptic development, and given the plastic nature of hypothalamic connections, we deemed MHC I H2-Kb a priority target. As mechanisms underlying the regulation of energy balance on the neuronal level are poorly understood, we set out to determine if MHC I proteins are important for this process. As a first step, we chose to characterize MHC I expression in the hypothalamus from postnatal development to early adult. Additionally, we designed and carried out a longitudinal trial to assess homecage behavior, basal and active state metabolism, and body composition at 2-3 months, 5-6 months, and 11-12 months in cohorts of male C57BL/6J (WT) and mice carrying a mutation in H2-Kb. B6.C-H2-K\textsuperscript{bmf}/ByJ (H2-K\textsuperscript{bmf}) carry mutations in 7 nucleotides leading to three amino acid substitutions including Glu152Ala, Arg155Tyr, and Leu156Tyr\textsuperscript{101,102}. These three amino acid substitutions result in large changes in the size and polarity of amino acid side chains, thereby altering the crystal structure of H2-Kb. Changes in crystal structure to lead to the impaired antigen presentation, 18-fold reduced thermostability, and inability to elicit a T cell response from known H2-Kb presented viral peptides including those from SEV, OVA, or VSV observed in H2-K\textsuperscript{bmf} mutants\textsuperscript{101-103}.\textsuperscript{101,102}
Based on this data, the H2-Kb antigen presentation function in H2-K^{bm1} mice is significantly impaired and therefore can serve as a model to test the potential function of H2-Kb in the hypothalamus. As we noted marked changes in energy balance regulation as early as 2-3 months in H2-K^{bm1} mice, we performed follow up studies to determine underlying causes for the observed progressive obesity phenotype.

**Methods and Materials**

**Mice and Animal Husbandry**

B6.C-H2-K^{bm1}/ByJ male mice (H2-K^{bm1}, Jackson Labs #001060) and wild-type C57BL/6J mice (Jackson Labs #000664) were used for all studies. The bm1 mutation in H2-K^{bm1} mice consists of 7 nucleotide differences resulting in three amino acid substitutions (Glu152Ala, Arg155Tyr, and Leu156Tyr) that occur in the crest of the α2α helix^{101-103}. H2-Kb in these mice has impaired antigen presentation of classic H2-Kb viral peptides, reduced thermostability, and inability to elicit a CTL response with viral peptide binding, and therefore serves as a model of impaired H2-Kb function^{101-103}. Upon arrival at UNMC, mice were singly-housed in micro isolator cages in rooms with a 12:12 lighting cycle (lights on 0600 CST), provided with chow (Envigo Teklad #7012) and water *ad libitum*. Vivarium temperatures ranged between 20-23 °C. Mice were given two weeks to recover from transportation stress and acclimate to our facility before any additional testing was performed. Breeder triads (2 ♀ and 1 ♂) of H2-K^{bm1} and C57BL/6J mice were bred to produce homozygous mutant and wild type mice for developmental studies. Mice were separated by sex at weaning. All studies were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

**Behavioral Analysis**
8 mice of each genotype (2-3 months) were chosen for control and experimental groups. Mice were singly-housed in the home cage monitoring (HCM) chambers and allowed to habituate for 5 days; we then collected data for 16 consecutive days to ensure at least 14 days of data for each animal. Mice within the system have access *ad libitum* to water and powdered chow (PicoLab Mouse Diet 20, #5058). Behavioral analysis was repeated on the same cohorts of mice at 5-6 months of age to assess longitudinal changes in behavior. For detailed description of home cage monitoring system see Chapter 2 Methods and Goulding et al., 2008.

**Active and Resting State Temperature Analysis**

Temperatures were assessed in WT and H2-K^bm1^ mice at 2-3 months, 5-6 months, and 11-12 months of age during both the circadian light and dark cycles, corresponding to resting and active states, respectively. Mouse temperatures were taken using a mouse rectal temperature probe and thermometer (Thermoworks Microtherma 2) accurate to 0.1 °C between the hours of 2:00 - 3:00 PM for resting state (light cycle) and 11:00 PM – 12:00 AM (dark cycle) for active state. Temperature data was analyzed by two-tailed Student’s t-test in Microsoft Excel.

**DEXA Scanning and indirect calorimetry**

Following each round of home cage testing, we performed indirect calorimetry (Oxymax, Columbus Instruments) and dual-emission x-ray absorptiometry (DEXA; Piximus I, GE-Lunar) studies to assess mouse resting and activity associated metabolic status and body composition, respectively. For full details on system parameters see Chapter 2 Methods for Metabolic Analysis.

**Glucose Tolerance Testing**
Glucose tolerance tests (GTT) were performed on cohorts of WT and H2-K<sup>bm1</sup> mice at 6-8, 14-16, 32-34, and 45-47 weeks of age. Mice were fasted for 10 hours prior to testing. Mice were restrained using restraining tube and the tails were cut just distal to the bone (~1-2 mm) to initiate blood flow. Baseline fasting blood glucose levels were measured at this time. Mice then received an intra-peritoneal injection of D-glucose (20% solution, 2g/kg body weight) and blood glucose was measured at 15, 30, 45, 60, 90, and 120 minutes post injection. All measurements were made using Bayer Contour blood glucose meter and test strips. Data was then analyzed by two-way ANOVA using SPSS controlling for genotype and time. If a significant genotype by time interactions was present, comparisons at individual time points were made by two-tailed Student’s t-tests.

**Insulin ELISA**

Whole blood samples collected from 10 hour fasted WT and H2-K<sup>bm1</sup> mice were allowed to clot at room temperature and serum was prepared by centrifugation at 4000G for 15 minutes at 4° C. Serum samples were then removed and stored at -80° C until further processing. An Ultra-Sensitive Mouse Insulin ELISA kit (Crystal Chem #90080) was used with mouse insulin standards to analyze fasting insulin levels at 4-6 weeks, 14-16 weeks, and 32-34 weeks, and 45-47 weeks of age. The assay was carried out using the manufacturer’s protocol for the low range assay (0.1 ng/mL – 6.4 ng/mL). The plate was read using a SynergyMx plate reader (BioTek) loaded with Gen5 software (v1.10). Insulin levels at 4-6 and 14-16 weeks were either undetectable or at the bottom of the standard curve and therefore unreliable. Comparisons of fasting serum insulin levels in 32-34 and 45-47 week old mice was performed using two-tailed Student’s t-tests between WT and H2-K<sup>bm1</sup> groups at each age in Microsoft excel. A total of 8 biological replicates were used for WT mice and 6 for H2-K<sup>bm1</sup> mice at each age.
**Tissue Isolation for Protein Studies**

To collect tissues for protein isolation, H2-K\textsuperscript{bmt} and wildtype mice were euthanized with CO\textsubscript{2}, followed by rapid decapitation. Postnatal C57BL6/J mice were rapidly decapitated without CO\textsubscript{2} euthanasia. The cranium was opened and the brain was removed. Under a dissecting microscope, individual brain regions were isolated, placed in 1.5 mL conical tubes and immediately frozen in a dry ice ethanol slurry. Tissues were stored at -80°C until further processing.

**Western Blotting**

RIPA buffer (1% Triton X-100, 0.1% SDS, 50mM Tris (pH 7.4), 107 mM NaCl, Halt Phosphatase Inhibitor (ThermoFisher #78420, 1:100), Protease Inhibitor (Sigma #P8340, 1:100), 5 mM EGTA, and 5 mM EDTA) was added to thawed tissue and sonicated for 3 cycles of 2 seconds each. Lysate was then centrifuged at 14,000G for 30 min at 4° C. Protein concentration was quantified by BCA assay. Protein samples were run on 4-15% SDS gels and transferred to PVDF membranes. Membranes were blocked in 5% non-fat dry milk in tris-buffered saline with 0.1% tween for all non-phospho specific antibodies for 1 hour at room temperature. For antibodies to detect phosphorylated proteins, membranes were blocked in 5% bovine serum albumin for 1 hour at room temperature. Membranes were probed with primary antibodies (Table 1) overnight at 4° C and incubated with species specific secondary HRP antibodies for 1 hour at room temperature.

**Quantitative RT-PCR and Analysis**

Developmental gene expression of postnatal mouse tissue was measured using quantitative real time PCR (Eppendorf realplex\textsuperscript{2} Master Cycler). Total RNA was extracted from RNAlater-preserved hypothalamic tissue taken from C57BL/6 mice (P1,
P5, P15, P28; n = 4 per time point) using a Tissuelyser II system (Qiagen) according to manufacture protocol. RNA was purified using an RNeasy Mini Kit (Qiagen #74104) with on-column DNase digestion (Qiagen #79254).

RNA quality was assessed by Agilent RINapico 6000 chips (Agilent # G2938-90046) on a bioanalyzer (Agilent 2100). RNA purity was assessed using a spectrophotometry (NanoDrop II, Thermo, Waltham MA) to measure 260/230 and 260/280 ratios. RNA degradation was assessed by micro gel electrophoresis (BioAnalyzer 2100, Agilent Technologies). Only RNA samples meeting minimum purity and quality were used for downstream analysis. Specifically, any samples with A260/A280 ratios less than 1.8 were deemed unsuitable for analysis secondary to RNA contamination. Additionally, any samples with RNA integrity numbers (RINs) < 7.0 were deemed unsuitable for analysis secondary to RNA degradation. One-Step RT-PCR was performed using Verso SYBR Green 1-Step kits (Thermo Scientific Catalog #AB4105) with all samples run in triplicate.

All reactions were processed with no enzyme and no template controls. Quantitect primers (Qiagen) were used for all reactions (Table 2). RT-PCR data was analyzed for relative expression using the $2^{(-\Delta \Delta CT)}$ method in Microsoft Excel. A geometric mean housekeeping gene model was constructed using three common reference genes found to be stable during development (Tfrc, Gapdh, and Gusb). For review of this method please see Vandesompele et al., 2002. Gene stability was assessed using RefFinder which aggregates results from BestKeeper, Normfinder, and Gennorm.

**Tissue Processing for Immunohistochemistry and immunofluorescence**

Mice were deeply anesthetized with Avertin and transcardial perfused with 4% PFA in PBS. Briefly, following anesthesia, mice were sprayed with ethanol and the chest cavity was opened. The diaphragm was dissected away to expose the apex of the heart.
### Table 1. List of antibodies used for works presented in Chapter 4.

<table>
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<th>Target Protein</th>
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<th>Dilution</th>
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### Table 2. List of primers used for works presented in Chapter 4.

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A butterfly needle connected to a transcardial perfusion pump was placed into the left ventricle near the apex of the heart. Immediately the pump was started and the right atrium was opened with a dissection scissors. Mice were perfused with PBS for 3 minutes to clear blood, followed by 5 minutes with 4% PFA/PBS. Following perfusion, organs including liver, heart, muscle, kidneys, brain, eyes, and ependymal fat were collected, weighed, photographed, and post fixed in 4% PFA/PBS for 24 hours at 4°C. Tissues were then cyroprotected with 30% sucrose at 4 °C until the tissues sank. Tissues were embedded in Tissue Tec OCT, and flash frozen in a beaker of isopentane in a dry ice and ethanol slurry. Tissue blocks were stored at -80 °C until further processing.

**Tissue Sectioning**

OCT tissue blocks were allowed to equilibrate to the cryostat temperature of (-22°C to -17°C, depending on tissue type) for 30 minutes. Tissue sections were collected and directly mounted on glass slides. Liver tissue was sectioned at 8 µm and brain tissue at 16 µm. After collection, slides were stored at -80°C until further processing.

**Oil Red O Staining and Quantification**

Liver sections were washed 2 times in distilled water, followed by equilibration in 100% ethylene glycol for 2 minutes. Ethylene glycol was removed and 0.25% Oil Red O (Sigma-Aldrich #O1516) was added to the slide for 10 minutes. Slides were rinsed in 3 washes of 85% ethylene glycol to differentiate, followed by 2 washes of distilled water. Slides were cover slipped with an aqueous mounting media and imaged via light microscopy at 10x magnification. 40x magnification images were taken as representative inset images. Quantification of integrate density was performed using ImageJ and genotype comparisons were made by Student's t-test in Microsoft Excel.
**Fluorescent Immunohistochemistry**

Tissue sections were allowed to warm to room temperature for 30 minutes prior to washing. Slides were washed 3 times in PBS to remove excess OTC, followed by blocking in 5% bovine serum albumin in PBS. For synaptic analysis and cell signaling, 0.2% triton-X 100 was added to blocking, primary, and secondary antibody solutions. To enrich for surface expression, triton was not added for developmental MHC I staining. To assess MHC I and PSD95 colocalization, tissue sections were blocked and incubated with MHC I antibody overnight at 4° C in the absence of permeabilization agents. This was followed by another round of blocking, permeabilization with triton, and staining with PSD95 overnight at 4° C. Primary and secondary antibodies (Table 1) were diluted in 0.5% BSA in PBS with or without triton. Slides were incubated in primary antibody solution overnight at 4° C and in secondary for 2 hours at room temperature. Tissue was washed, counterstained with DAPI and cover-slipped with prolong diamond anti-fade reagent.

**Confocal Microscopy**

All confocal images were collected using a Zeiss LSM 700 laser scanning confocal microscope with either Plan-Apochromat 40x/1.3 Oil DICIII M27 (0.26 um resolution) or EC Plan-Neofluar 20x/0.50 M27 (0.67 um resolution) objectives.

**MHC I and synaptic protein colocalization**

Z stack confocal images (X: 26.59 um, Y: 26.59 um, Z: 11.99 um) were collected from the arcuate nucleus, paraventricular nucleus, and the lateral hypothalamus using at 40X with 6X digital zoom on tissue sections stained for surface MHC I and synaptic proteins as described above. Colocalization analysis was performed using both object based and intensity based methods with IMARIS software (Bitplane). First, object based
colocalization was assessed using the detect spots function to identify all MHC I, VGlut2, and PSD95 puncta. We then quantified all colocalized puncta centers within 400nm of each other. Additionally, intensity based colocalization was performed using the colocalization software within IMARIS. Briefly, an identical mask setting was applied for each channel in all samples, followed by automatic threshold calculation. A colocalization channel was then built and colocalization statistics were generated (see Costes, et al., 2004 for more information). The Mander’s coefficient, Pearson’s coefficient, and the % colocalized volume were calculated.

Quantification of Vglut1, Vglut2, GAD65, PSD95 positive synaptic puncta

To assess excitatory and inhibitory synapse densities in the hypothalamus, z-stack confocal images were collected from tissue sections isolated from adult 14-15 month WT and H2-K^bm1 mice. Images were acquired at 40x with 2x zoom (X: 80.0 µm, Y: 80.0 µm, Z: 8.11 µm) with a 0.353 µm z-stack step distance and 1000 x 1000 pixel resolution. Excitatory (Vglut1, Vglut2) and inhibitory (GAD65) synapses were imaged in the lateral hypothalamus, paraventricular nucleus and arcuate nucleus. Images were loaded into IMARIS software and puncta densities were quantified using the detect spots function controlling for puncta size and quality. Differences between genotypes were determined by two-tailed Student’s t-tests using the average puncta counts for each sample.

Quantification of NPY positive axonal puncta

The distribution and density of NPY projections was quantified in the anterior (near bregma -1.9) and posterior (near bregma -2.4) arcuate nucleus, dorsal and ventral aspects of the lateral hypothalamus, paraventricular nucleus, and dorsomedial nucleus of the hypothalamus. Images were acquired by confocal microscopy at 40x with 4x zoom to generate z-stacks (X: 40 µm, Y: 40 µm, Z: 6.0 µm) at 500 x 500 resolution with a 0.468 µm z-stack step distance. Images were quantified in IMARIS software using the
detect spots function controlling for puncta size and quality. Differences between genotypes were determined by two-tailed Student’s t-tests using the average puncta counts for each sample.

**Quantification of synaptic input onto orexin positive neurons**

Confocal z-stack images of orexin positive neurons in the lateral hypothalamus were acquired at 40x with 2x zoom (X: 80 µm, Y: 80 µm, Z: 15.3 µm) at 1000 x 1000 resolution with a 0.450 µm z step distance. Images were loaded into IMARIS to detect synaptic puncta and generate surfaces for orexin neurons. To generate surfaces for orexin positive neurons, individual regions of interest were created around orexin positive cells. Using the create surfaces function 3D surfaces were generated for individual orexin neurons. An intensity threshold was created for each channel and maintained for all samples. The threshold for surface generation was optimized for each individual cell to extend the surface just distal to the border of orexin staining. This method allowed us to reliably create surfaces that captured the soma and some proximal dendritic processes of orexin neurons. Using the detect spots function, all Vglut2 and NPY puncta were identified within the image frame controlling for quality and size. Finally, using the split into surface objects function all puncta in contact with each of the individual orexin neurons were identified. The total number of synaptic puncta in contact with each cell was then divided by the total cell surface area to generate individual cell synaptic densities. The primary outputs generated from this analysis included total Vglut2 and NPY puncta counts, average cell surface area, and the number of Vglut2 and NPY puncta per 100 µm² of orexin surface area. We analyzed 55 WT and 57 H2-Kbm1 orexin positive neurons across 4 biological replicates per genotype. Data for surface area and synapses by surface area were first analyzed by two-way ANOVA to assess the effect of genotype on each of the perimeters above using IBM SPSS Statistics 22.
Secondly, we performed boot-strap analysis for 1000 permutations of the data using Matlab software. Data for Vglut2 puncta per field, and NPY puncta per field were analyzed by two-tailed Student’s t-tests of the average counts per sample.

**cFOS induction via fasting**

To assess the gross activity level of orexinergetic neurons, we employed a fasting cFOS induction assay. 3-month old C57BL/6 and H2-K\textsuperscript{bm1} mice were split into fed (n = 3 per genotype) and fasted (n = 4 per genotype) groups. Animals were fasted for 24 hours followed by perfusion and tissue processing as described above. Brain sections were then immunostained as described previously for cFOS, Orexin, and DAPI (Table 1). All samples were masked and blinded prior to image collection and quantification. To maximize the field of view, confocal z-stack images were collected at 20x with 0.8x zoom (X: 400 µm, Y: 400 µm, Z: 12.3 µm) at 1924 x 1924 with a 2.46 µm z-step. Maximum projection images were generated from confocal z-stacks and analyzed using ImageJ. A minimum of 5 images were collected per sample with each image containing a variable number of orexin neurons. The number of cFOS positive cells, Orexin positive cells, and cFOS/Orexin double positive cells were quantified using the cell-counter plugin in ImageJ. For each image a weighted percentage of double positive cells was generated using the formula: % double cFOS/orexin positive cells in image x (# of orexin cells in image / total # orexin neurons imaged for sample). One animal from each the WT fed and H2-K\textsuperscript{bm1} fed group was removed as they were significant outliers using Dixon’s test. Importantly, the removal of these points does not affect the primary comparison of this study between WT fasted and H2-K\textsuperscript{bm1} fasted mice. The resulting weighted percentages were analyzed by One-way ANOVA with Bonferroni post-hoc analysis using IBM SPSS statistical software.

_Fasting Activity Assay_
WT and H2-K\textsuperscript{bm1} mice were placed into the homecage monitoring system and allowed to habituate for 4 days to establish baseline behavior. This acclimation period allows mice to explore the cage environment, find the food and water sources, establish a niche, and build a nest. Following this acclimation period mice were fasted under two paradigms to assess activity patterns in response to fasting. In the first paradigm, food was removed 15 minutes prior to the initiation of the light cycle in efforts to place mice in a 12-hour fasted state at the onset of the dark cycle. In the second paradigm, food was removed 15 minutes prior to the onset of the dark cycle to place mice in a 12-hour fasted state at the onset of the light cycle. In both paradigms movement and feeding behaviors were analyzed using the homecage system. Data collected from 2 fed days was used to create a model of activity including movement (cm) and feeding attempts (number and duration) for the fed state to serve as control data for overall analysis and analysis of how fasting affects each mouse. Activity data was generated by aligning 6 minute bin data for each day and aggregating that data to 60 minute bins. For the light cycle fasting analysis, this amounted to a 20 hour period from 11:30 AM to 7:30 PM. For dark cycle fasting a 12 hour period from 6:30 PM to 6:30 AM was analyzed. Overall movement between WT and H2-K\textsuperscript{bm1} mice in the fasted versus fed states was compared by two-tailed Student’s t-tests Bonferroni corrected for 2 comparisons on the sum of movement for WT (n= 7-8) and H2-K\textsuperscript{bm1} (n = 7) mice for both fasting paradigms. A two-way ANOVA was used to assess the effect of genotype on activity at individual timepoints with fasting. If a significant interaction of genotype*time (p<0.05) was noted, individual two-tailed Student’s t-tests Bonferroni corrected for 2 comparison were performed between fed and fasted groups of each genotype. Differences in movement ratios between fed and fasted states of WT and H2-K\textsuperscript{bm1} mice was assessed by two-tailed Student’s t-test. Two primary measures of feeding behavior were quantified during fasting paradigms. First, we used the number of photo beam breaks to signify the number of feeding
attempts mice made in fed and fasted conditions. Second, we quantified the duration of time the photo beam was broken in seconds. The aggregate counts and duration were then analyzed between WT and H2-K\textsuperscript{bni} mice in the fed and fasted states. Differences between groups were calculated by two-tailed Student’s t-tests with Bonferroni correction for four comparisons. We compared the effect of fasting within each genotype, and the effect across genotype for the same condition.

Results

Class I MHC molecules are dynamically expressed during hypothalamic development

MHC class I proteins have previously been shown to be expressed within the hippocampus, cortex, and cerebellum, primarily during neurodevelopment\textsuperscript{97, 99, 110}. MHC I proteins colocalize with both excitatory pre and post-synaptic proteins, but appear to be enriched at the post-synaptic density (Goddard et al., 2007). The majority of research on MHC I proteins in the CNS has targeted hippocampal neurons, due the observation that they play a role in synaptic plasticity and therefore learning and memory. Many of these studies focus on developmental timelines with little focus on later life time points. Our data, and data from several other studies have demonstrated increased expression of MHC I related proteins with aging, suggesting a reactivation of pathways employed during neuro development\textsuperscript{111-114}. Interestingly, we noted increased expression of MHC I proteins in the aged hypothalamus of a mouse with deficits in energy balance regulation. There are exceptionally few studies interrogating the functions of MHC I proteins in the hypothalamus, and therefore we set out to characterize MHC I expression in the hypothalamus in efforts to better understand roles that MHC I proteins may play in hypothalamic processes necessary for regulating metabolic function.
To determine if MHC Class I proteins are expressed during hypothalamic development, we employed a multifold approach using western blotting and confocal microscopy. Western blot analysis demonstrated that hypothalamic MHC class I proteins are dynamically expressed through postnatal development, corresponding inversely with the expression of excitatory (Vglut2 and PSD95) and inhibitory (Gephyrin) synaptic proteins. Western blot analysis of MHC I protein shows a reduction in expression from postnatal day 1 through adult (3 months) (P1 1.0 ± 0.17, P3 0.89 ± 0.08, P5 0.90 ± 0.16, P9 0.70 ± 0.08, Adult 0.44 ± 0.08; p < 0.01 for comparisons between P1, P3, P5 and Adult by one-way ANOVA) (Figure 1). During this period excitatory synaptic proteins Vglut2 and PSD95, as well as inhibitory protein gephyrin, increase suggesting maturation of synaptic networks. One pitfall of Western blot analysis is the inability to localize protein expression to distinct hypothalamic subregions. Therefore, to determine if the reduction of MHC I protein was localized to distinct hypothalamic nuclei we preformed immunohistochemistry on non-permeabilized tissue sections to enrich for MHC I surface expression. We assessed expression by confocal microscopy of the arcuate nucleus, paraventricular nucleus, and lateral hypothalamus. Our data demonstrates a significant reduction in immunofluorescent intensity between P5 and P15 in the arcuate nucleus (Arc: P5 46.78 ± 7.39 a.u., P15 33.42 ± 4.47 a.u., p < 0.05) and lateral hypothalamus (LH: P5 35.09 ± 7.19 a.u., P15 22.29 ± 3.44 a.u., p < 0.05) (Figure 2A,B,D,E). Analysis of the paraventricular nucleus demonstrated a trending reduction in MHC I staining intensity that failed to reach significance (PVN: P5 22.20 ± 4.24 a.u., P15 19.02 ± 0.96) (Figure 2C,F). Also of note, within each region the pattern of immunofluorescence appears to change from diffuse staining, to a more localized pattern on individual cells. This may indicate that subsets of neurons retain higher levels of somatic MHC I expression than others within the hypothalamus, but further analysis of single cell expression is necessary to confirm this finding.
Figure 1. **MHC I protein expression in the hypothalamus decreases during postnatal development and coincides with increases in synaptic proteins.** To determine the pattern of MHC I protein expression in the hypothalamus we probed for hypothalamic lysates with an antibody recognizing multiple MHC I isoforms and with antibodies for synaptic proteins. (A) Representative Western blot for hypothalamic lysates from P1 to adult (3 months) probed for MHC I, Vglut2, PSD95, Gephyrin, and GAPDH. (B) Quantification of MHC I band intensity demonstrates a reduction in MHC I protein expression during postnatal development. The reduction in MHC I expression appears to coincide with the increased expression of excitatory (Vglut2 and PSD95) and inhibitory (Gephyrin) synaptic proteins. Data represents 3 biological replicates per time point. Significance of fold change was determined by one-way ANOVA with Bonferroni post-hoc analysis. Significance is indicated by a = p < 0.01 compared to P1, b = p < 0.01 compared to P3, c = p < 0.01 compared to P5. Error bars represent standard deviation from the mean.
Figure 2. MHC I expression decreases with postnatal age in the developing hypothalamus. Developmental hypothalamic tissue was stained for MHC I in the absence of detergent to enrich for MHC I proteins expressed on the surface of cells. (A-C) Representative images of MHC I expression in the arcuate nucleus (Arc), lateral hypothalamus (LH), and paraventricular nucleus (PVN) at P5 and P15. Visually a reduction in florescent intensity and change in distribution are noted in all three regions. (D-F) Quantification of mean fluorescent intensity revealed a significant reduction in the Arc (Arc: P5 46.78 ± 7.39 a.u., P15 33.42 ± 4.47 a.u., p < 0.05) and LH (LH: P5 35.09 ± 7.19 a.u., P15 22.29 ± 3.44 a.u., p < 0.05), but only a trending reduction in the PVN (PVN: P5 22.20 ± 4.24 a.u., P15 19.02 ± 0.96). Data is representative of 4 independent samples at P5 and 3 at P15. Statistical differences determined by two-tailed student t-test. * indicates p < 0.05. Error bars represent standard deviation of the mean. a.u. indicates arbitrary units for florescent intensity.
Class I MHC molecules colocalize with synaptic proteins during hypothalamic development

MHC class I protein expression in hippocampal and cortical neuron populations colocalizes with both pre and postsynaptic proteins of excitatory synapses (Goddard et al., 2007). To determine if MHC class I proteins in the developing hypothalamus colocalize with synaptic proteins, we performed confocal microscopy on tissues stained with an MHC I antibody and markers of excitatory synaptic proteins including vesicular glutamate transporter 2 (Vglut2) and postsynaptic density protein 95 (PSD95). To enrich for surface MHC I expression, tissues were blocked and incubated overnight with MHC I antibody in detergent free conditions followed by a second round of blocking and incubation with primaries for synaptic proteins. Using confocal microscopy and IMARIS software we performed both object based and intensity based colocalization of synaptic and MHC I proteins. MHC I proteins colocalize with both pre and postsynaptic proteins dynamically during postnatal hypothalamic development. MHC I proteins show a greater percentage of colocalization with postsynaptic protein PSD95 compared to presynaptic Vglut2, and the percentage of colocalization with both synaptic markers is reduced with developmental age in multiple hypothalamic regions (Figure 3). Using intensity based colocalization, we note a similar significant decrease in both the Mander’s and Pearson’s Coefficient of colocalization, and the percent of ROI colocalized from P5 to P15 in multiple hypothalamic nuclei (data not shown). This data clearly demonstrates that similar to the hippocampus, MHC I proteins colocalize with excitatory synaptic proteins in early postnatal hypothalamic tissue, but colocalize at higher levels with postsynaptic proteins. MHC I proteins have been shown to decrease during development in other regions and in response to neuronal activity in vitro. Our data demonstrates developmental differences in MHC I expression and colocalization within a less
Figure 3. MHC I colocalizes with pre- and post-synaptic proteins early during neurodevelopment. Tissue sections from P5, P10, and P15 WT mice were immunostained for MHC I under detergent free conditions followed by staining for synaptic proteins Vglut2 and PSD95. (A) Representative images of P5 and P15 hypothalamic tissue from within the arcuate nucleus. Far right panels in A show colocalized puncta (within 400 nm) quantified using IMARIS software. Images from paraventricular nucleus and lateral hypothalamus show similar staining patterns. Scale bar represents 2 mm. (B) The percent of PSD95 and Vglut2 puncta that colocalize with MHC I puncta were quantified in the arcuate nucleus, paraventricular nucleus, and lateral hypothalamus. MHC I colocalizes more with post-synaptic PSD95 than pre-synaptic Vglut2 in in all three regions early in development. With increasing age there is a decrease in MHC I colocalization with both excitatory pre and post-synaptic proteins in the arcuate nucleus and lateral hypothalamus. Within the paraventricular nucleus, MHC I colocalization with Vglut2 decreases with development, but there was no significant difference in colocalization with PSD95. For P5 and P10, data is representative of 4 biological replicates. For P15, data represents 3 biological replicates. Differences in colocalization percentages were determined using one-way ANOVA for each brain region and synaptic marker. For comparisons, a indicates a significant difference (p < 0.05) from P5, b indicates a difference from P10. Error bars represent standard error of the mean.
characterized region of the CNS. This data makes an argument for synaptic roles for MHC I proteins in the hypothalamus, potentially analogous to those in other regions of the CNS.

**Specific Class I MHC isoforms and β2-microglobulin are differentially expressed during hypothalamic development**

The commercially available MHC I antibodies, including the antibody used for the studies above, recognize multiple MHC class I isoforms. Due to this lack of specificity for individual MHC Class I isoforms, it is challenging to determine how specific isoforms change during hypothalamic development. To begin to answer this question, we performed RT-PCR analysis for several isoforms of MHC I including H2-Kb, H2-Db, H2-Q1, H2-Q10, and the major histocompatibility complex light chain beta-2 microglobulin on P1, P5, P15, and P28 C57BL/6 mice. Developmentally, gene expression is very dynamic and many housekeeping genes (HKGs) used for RT-PCR normalization are not spared from developmental differences as neuronal ad glial populations migrate, proliferate, and differentiate. Therefore, we employed a multiple HKG model to best normalize expression data. We used a geometric averaging method to establish a model of HKG expression for each sample\(^\text{105}\). The three genes selected (GusB, GAPDH, and TfrC) were chosen as they had the highest stability when assessed using several methods (geNorm, Best Keeper, Normfinder). Upon RT-PCR analysis we identified expression differences between MHC I isoforms. Transcript expression for H2-Kb increases from P1 to P28 (P1 1.02 ± 0.25, P28 2.13 ± 0.56, p < 0.01), whereas expression of H2-Db and H2-Q1 slightly decrease or remain stable through postnatal hypothalamic development (**Figure 4**). H2-Q10 was not detected in the hypothalamus at any age, suggesting expression is either too low to detect in whole tissue RNA extract or completely absent within this brain region. Interestingly, H2-Kb expression was the only
Figure 4. MHC I isoforms are differentially expressed in the postnatal hypothalamus. Specific antibodies for MHC I isoforms are poorly developed making it challenging to characterize expression at the protein level. In efforts to characterize the expression patterns of specific MHC I isoforms in the developing hypothalamus, qRT-PCR was performed for H2-Kb, H2-Db, H2-Q1, H2-Q10, and β2-microglobulin (β2M). Expression data was normalized to a geometric average of three stable housekeeping genes (Gusb, Gapdh, and Tfrc) and then normalized to P1 levels to generate a fold change from P1. H2-Q10 expression was not detected in hypothalamic RNA and is therefore not represented above. Expression levels for H2-Db and H2-Q1 either remained stable or decreased during postnatal development. Expression of H2-Kb increased later in hypothalamic development, suggesting a potential continued role in adulthood. β2M, levels drastically increased from P1 to P28 in the hypothalamus. All comparisons were made by one-way ANOVA with Bonferroni post-hoc testing. For comparisons, a indicates p < 0.05 compared to P1, b indicates p < 0.05 compared to P5, c indicates p < 0.05 compared to P15. Data represents 4 biological replicates per time point. Error bars represent standard deviation of the mean.
isoform to increase and maintain relatively high levels throughout development. As MHC I proteins have been implicated in synaptic plasticity, and the hypothalamus maintains the ability to tune and rewire itself, we hypothesized that H2-Kb may have a continuing role in regulating hypothalamic synapses. Additionally, H2-Kb transcript is significantly elevated in the hypothalamus in a mouse model of age-associated energy balance deficits, suggesting that H2-Kb may play a role in these processes. To assess if H2-Kb functions in regulating hypothalamic behaviors, we organized a longitudinal study of mice carrying a mutated from of H2-Kb, and age-matched C57BL/6 controls using our state-of-the-art home cage behavioral system.

*Mutation of Class I MHC H2-Kb leads to an obesity phenotype characterized by increased body mass and increased adiposity, despite marked hypophagia*

As H2-Kb is expressed in the developing hypothalamus, persists into adulthood, and is over expressed in an aging model of metabolic energy balance deficits, we chose to evaluate energy balance and hypothalamic mediated behaviors in mice carrying a mutation in H2-Kb (H2-Kb<sup>bm1</sup>). H2-Kb<sup>bm1</sup> mice demonstrate no significant differences in body weight during postnatal development through roughly 1.5 months of age (Figure 5A). However, early observations of H2-Kb<sup>bm1</sup> mice at 2-3 months of age demonstrated a small, but significant increase in body weight (WT 23.32 ± 0.51 g, H2-Kb<sup>bm1</sup> 26.92 ± 0.52 g, adjusted p < 0.01) and adiposity (2-3 months: WT 11.45 ± 0.19%, H2-Kb<sup>bm1</sup> 15.34 ± 0.82%, adjusted p < 0.01) as measured by DEXA scan (Figure 5B,D). H2-Kb<sup>bm1</sup> mice continue to gain significantly more weight and store more body fat compared to WT controls as they aged. At 5-6 months of age H2-Kb<sup>bm1</sup> mice were more than 30% heavier (WT 27.53 ± 0.63 g, H2-Kb<sup>bm1</sup> 36.26 ± 0.73 g, adjusted p < 0.0001) and had roughly double the adiposity as WT controls (WT 14.56 ± 0.51%, H2-Kb<sup>bm1</sup> 27.80 ± 1.77%, adjusted p < 0.0001). This weight gain and fat deposition occurred until animals were
Figure 5. *H2-K<sup>bm1</sup>* mice exhibit a progressive obesity phenotype with onset in early adulthood. (A) Assessment of WT and H2-K<sup>bm1</sup> mice from birth to early adulthood demonstrate no significant differences in body weight. (B) Longitudinal analysis of cohorts of WT and H2-K<sup>bm1</sup> mice demonstrates progressive weight gain starting at 2-3 months of age and progressing as they age. H2-K<sup>bm1</sup> and WT mice gain weight with age, but H2-K<sup>bm1</sup> mice remain significantly heavier at 5-6 and 11-12 months. (C) Representative pictures of WT and H2-K<sup>bm1</sup> mice at 11-12 months of age demonstrate increase in central body mass. (D) DEXA scan analysis revealed significant increases in adiposity with age in both WT and H2-K<sup>bm1</sup> mice. However, H2-K<sup>bm1</sup> mice have a significantly higher percentage of body fat than controls at all ages assessed. For panel A, P0/1 n = 17 per genotype; P7 WT n = 5, H2-K<sup>bm1</sup> n = 17; 1 month WT n = 5, H2-K<sup>bm1</sup> n = 4; 1.5 month WT n = 8, H2-K<sup>bm1</sup> n = 8. For panel B and C, n = 8 mice per cohort for 2-3 months and 5-6 months, n = 4 for 11-12 months, p < 0.05 when denoted by # for WT vs H2-K<sup>bm1</sup> at each age; by $ within genotypes compared to 2-3 months of age; by F within genotypes compared to 5-6 months. All comparisons made by two tailed student t-test with Bonferroni correction for 9 comparisons. Error bars represent SEM.
processed for tissue studies at 11-12 months of age (Weight: WT 30.70 ± 1.55 g, H2-K^{bm1} 43.51 ± 0.78 g, adjusted p < 0.01; Adiposity: WT 16.71 ± 2.01%, H2-K^{bm1} 33.34 ± 1.22%) *(Figure 5B,C,D)*. Much of the fat deposition was in central white adipose tissue, similar to fat deposition seen in obese adults with metabolic syndrome. Remarkably, increased weight and adiposity in H2-K^{bm1} mice occurs despite significant hypophagia *(Figure 6)*, indicating that obesity is in H2-K^{bm1} is not a result of increased caloric intake.

**H2-K^{bm1} mice have progressively impaired glucose clearance and increased fasting insulin levels**

Obesity is accompanied by changes in endocrine function that can increase an individual’s risk for development of downstream complications like diabetes mellitus type II. To determine if H2-K^{bm1} mice showed signs of endocrine dysfunction we performed glucose tolerance testing (GTT) and measured fasting insulin levels at 4 time points during the development of obesity. We noted no differences in GTT results at 4-6 or 14-16 weeks of age *(Figure 7A,B)*. Fasting insulin levels in mice of both genotypes at these ages were either undetected or at the bottom of the standard curve and therefore unreliable. However, at 32-34 weeks of age H2-K^{bm1} mice showed a trending genotype by time interaction and elevated blood glucose level at the 90 minute time point in GTT (WT 190.5 ± 11.12 mg/dL, H2-K^{bm1} 229.8 ± 9.20 mg/dL, p < 0.05) *(Figure 7C)*. 32-34 week old H2-K^{bm1} mice also demonstrated a significant increase in fasting insulin levels compared to controls (WT 0.120 ± 0.011 ng/mL, H2-K^{bm1} 0.448 ± 0.037 ng/mL, p < 0.00001) *(Figure 7E)*. At later stages of obesity development H2-K^{bm1} mice have a reduced peak glucose level, but significantly (p<0.0001, two-way ANOVA genotype*time) impaired clearance upon GTT assessment at the 90 and 120 minute time points (90 min: WT 223.4 ± 19.0 mg/dL, H2-K^{bm1} 275.3 ± 23.1 mg/dL, p < 0.05; 120 minutes: WT 185.9 ± 10.5 mg/dL, H2-K^{bm1} 248.3 ± 23.1 mg/dL, p < 0.01) *(Figure 7D)*.
**Figure 6.** *H2-K*<sup>bml</sup> *mice are hypophagic in the homecage monitoring system compared to WT controls.* The amount of daily food consumption is calculated while mice are in the homecage monitor system and normalized to body weight. At 2-3 months there is no genotypic difference in food intake. However, *H2-K*<sup>bml</sup> mice at 5-6 months and 11-12 months are significantly hypophagic compared to WT controls. Interestingly, increased consumption is not observed with increasing weight gain in *H2-K*<sup>bml</sup> mice, as normalized intake is significantly reduced when comparing 2-3 month and both older ages. This suggests that hyperphagia is not a driving factor of the obesity phenotype in *H2-K*<sup>bml</sup> mice. n = 8 mice per cohort for 2-3 months and 5-6 months, n = 4 for 11-12 months, p < 0.05 when denoted by # for WT vs *H2-K*<sup>bml</sup> at each age; by $ within genotypes compared to 2-3 months of age. WT vs *H2-K*<sup>bml</sup> at 11-12 months, p = 0.077. All comparisons made by two tailed student t-test with Bonferroni correction for 9 comparisons. Error bars represent SEM.
Figure 7. H2-K<sup>bm1</sup> mice show age-associated reduced clearance of glucose upon glucose challenge and increased fasting serum insulin levels. Glucose tolerance tests were performed on WT and H2-K<sup>bm1</sup> mice at 4-6, 14-16, 32-34, and 45-47 weeks of age as they progressively gained body weight and increased in adiposity. (A, B, and C) At early time points there are no discernable differences between WT and H2-K<sup>bm1</sup> mice, aside from subtle trends. However, at 32-34 and 45-47 weeks of age clear differences are demonstrated. (D) H2-K<sup>bm1</sup> mice at 45-47 weeks of age show a significant (p < 0.0001) genotype*time interaction when assessed by two-way ANOVA. This difference is carried by a statistically significant reduction in maximum glucose levels and demonstration of higher glucose levels at the final time point. (E) H2-K<sup>bm1</sup> mice have increased fasting serum levels of insulin at 32-34 weeks (p < 0.001) and 45-47 weeks (p < 0.01) of age compared to WT controls. Measurements of fasting insulin levels at younger ages were lower than the detectable range, and therefore were not reported. For A-D, all time points were analyzed by Two-way repeated measures ANOVA. To determine statistical differences at specific time points two-tailed t-tests were used. 4-6 weeks WT n = 7, H2-K<sup>bm1</sup> n = 7; 14-16 weeks WT n = 8, H2-K<sup>bm1</sup> n = 7; 32-34 and 45-47 weeks WT n = 8, H2-K<sup>bm1</sup> n = 6. For E, statistical differences were assessed by two-tailed student t-tests. For both time points WT n = 8, H2-K<sup>bm1</sup> n = 6. For all panels * indicates p < 0.05, ** p <0.01, *** p < 0.001. Error bars represent standard error of the mean.
H2-K\textsuperscript{bmi1} mice also display a significant increase in fasting insulin levels compared to controls, suggesting the onset of insulin resistance (WT 0.253 ± 0.024 ng/mL, H2-K\textsuperscript{bmi1} 0.970 ± 0.243 ng/mL, p < 0.01) (Figure 7E). This data demonstrates additional complications of progressive obesity in H2-K\textsuperscript{bmi1} mice, which begin to mimic characteristics of metabolic syndrome.

\textit{Adult H2-K\textsuperscript{bmi1} mice have hepatomegaly, hepatic steatosis and increased pericardial fat deposition}

As we noted a progressive obesity phenotype in H2-K\textsuperscript{bmi1} mice, we performed necropsy and tissue processing of 12-13 month old mice to further characterize the obesity phenotype. Upon necropsy, it was evident that H2-K\textsuperscript{bmi1} mice had increased liver size and pallor, as well as increased pericardial adipose deposition (Figure 8A). Animals were perfused for immunohistochemistry, following perfusion the liver was removed and weighed. H2-K\textsuperscript{bmi1} mouse livers were visibly larger and had an increased mass compared to wild-type controls (WT 1.54 ± 0.18 g; H2-K\textsuperscript{bmi1} 2.41 ± 0.31 g, p< 0.05) (Figure 8B). Further analysis to quantify fat deposition in the liver revealed marked hepatic steatosis, evident by a significant increase in Oil Red O staining intensity in H2-K\textsuperscript{bmi1} mice (WT 1.0 ± 0.25 a.u., H2-K\textsuperscript{bmi1} 2.35 ± 0.39 a.u., p = 0.027, normalized to WT) (Figure 8C,D). These data demonstrate that H2-K\textsuperscript{bmi1} mice acquire downstream pathological consequences of obesity including what may be signs of nonalcoholic fatty liver disease (the hepatic manifestation of metabolic syndrome\textsuperscript{115}).

\textit{Metabolic Assessment}

We performed indirect calorimetry on 2-3 and 5-6 months old H2-K\textsuperscript{bmi1} mice and WT controls to evaluate measures of basal and activity associated metabolism. Significant differences were noted between genotypes at both ages in several measures of metabolic status. However, after taking adiposity differences into account through
Figure 8. *H2-K*<sup>bmi</sup> mice have significant hepatomegaly and steatosis compared to WT controls. (A) Representative images of WT and *H2-K*<sup>bmi</sup> mice during terminal perfusion showing increased liver size and pallor (asterisk), and deposition of pericardial adipose tissue (arrow). (B) Representative images of fixed liver samples and quantification of liver mass revealed significant hepatomegaly in *H2-K*<sup>bmi</sup> mice at 11-12 months of age (WT 1.54 ± 0.18g, *H2-K*<sup>bmi</sup> 2.41 ± 0.31g, p = 0.048). Further analysis demonstrated increased fat deposition within *H2-K*<sup>bmi</sup> liver tissue. (C) Representative images at 10X and 40X of Oil Red O stained liver sections from 11-12 month old WT and *H2-K*<sup>bmi</sup> mice. (D) Quantification of Oil Red O staining intensity normalized to WT liver shows a significant increase in fat deposition in *H2-K*<sup>bmi</sup> liver tissue (WT 1.0 ± 0.25 a.u., *H2-K*<sup>bmi</sup> 2.35 ± 0.39 a.u., p = 0.027). n = 4 per group, a.u. represents arbitrary units for normalized intensity. All comparisons made by two-tailed student t-tests.
analysis of covariance testing these differences were negligible. This demonstrates that changes in metabolic status in H2-K\textsuperscript{bm1} mice are a result of increased adiposity and not specifically an effect of genotype alone. For completeness of presenting metabolic data, 2-3 month and 5-6 month indirect calorimetry data is presented in Figure 9 and Figure 10, respectively. Overall, this analysis allows us to conclude that obesity in H2-K\textsuperscript{bm1} is not a result on an underlying metabolic difference in basal or activity-associated metabolic function.

**Obesity in H2-K\textsuperscript{bm1} mice results from increased sedentary states, despite marked hypophagia**

In effort to determine if underlying behavioral differences trigger the development of obesity in H2-K\textsuperscript{bm1} mice, we assessed homecage behavior in cohorts of WT and H2-K\textsuperscript{bm1} mice at 2-3 months and 5-6 months of age. In the overall analysis we noted significant differences in overall 24-hour time budgets and active states temporal organization. First, H2-K\textsuperscript{bm1} are significantly less active than WT controls. At 2-3 months of age H2-K\textsuperscript{bm1} mice spend an increased percentage of their day in inactive states (H2-K\textsuperscript{bm1} 63.00 ± 3.89\%, WT 54.48 ± 5.3\%, p = 0.0093, critical p = 0.01). A trending reduction in percentage of time spent feeding (H2-K\textsuperscript{bm1} 6.47 ± 0.97\%, WT 8.72 ± 2.42\%, p = 0.029, critical p = 0.01) and other non-forward locomotor activity (H2-K\textsuperscript{bm1} 26.07 ± 3.14\%, WT 30.66 ± 4.11, p = 0.025, critical p = 0.01) were observed, but failed to reach significance when adjusted for multiple comparisons (Figure 11A). When mice were reassessed at 5-6 months of age these differences became even more striking. H2-K\textsuperscript{bm1} mice spend roughly 10\% more time (2.4 hours/day) inactive compared to controls (H2-K\textsuperscript{bm1} 70.85 ± 6.61\%, WT 61.07 ± 6.01\%, p = 0.0079, critical p = 0.01). Additionally, H2-K\textsuperscript{bm1} at 5-6 months spend significantly less time feeding (H2-K\textsuperscript{bm1} 6.40 ± 1.50\%, WT 11.27 ± 4.21\%, p = 0.0082, critical p = 0.01). (Figure 11B). Time budget data confirms reduced feeding
Figure 9. Indirect calorimetry measures of basal and activity associated metabolic function in 2-3 month old WT and H2-K^bmi. (A) Overall basal metabolic function in H2-K^bmi mice is unchanged from WT mice when normalized to adiposity for all measures. (B) Overall activity associated metabolic function in H2-K^bmi mice is unchanged from WT mice when normalized to adiposity for all measures. Data is representative of 8 mice per genotype. Data analyzed by analysis of covariance (ANCOVA).
Figure 10. **Indirect calorimetry measures of basal and activity associated metabolic function in 5-6 month old WT and H2-K^{bm1} mice.** (A) Overall basal metabolic function in H2-K^{bm1} mice is unchanged from WT mice when normalized to adiposity for all measures. (B) Overall activity associated metabolic function in H2-K^{bm1} mice is unchanged from WT mice when normalized to adiposity for all measures. Data is representative of 8 mice per genotype. Data analyzed by analysis of covariance (ANCOVA).
in H2-K^{bmi} observed in daily chow intake measurements. It also clearly demonstrates that H2-K^{bmi} mice are significantly less active than WT controls. Further analysis of state probabilities revealed that H2-K^{bmi} mice have lower active state probabilities in the last 4 hours of the dark cycle. At 2-3 months, H2-K^{bmi} show a clear trend towards reduced active state probability in this time window (Figure 11C). At 5-6 months H2-K^{bmi} mice show a significant reduction in active state probability at several time points organized around the end of the dark cycle (Figure 11D). This data clearly demonstrate that the generation of obesity in H2-K^{bmi} mice is not due to hyperphagia, but a result of increased sedentary states, despite reduced food intake. To identify potential CNS mechanisms of reduced feeding and activity in H2-K^{bmi} mice, we performed molecular and cellular analysis to assess hypothalamic circuits underlying the regulation of feeding and activity.

**Mutation of Class I MHC H2-Kb leads to reduced NPY expression in the arcuate nucleus**

Our initial assessment began with measuring gross levels of synaptic and neuropeptide proteins in the hypothalamus. MHC I proteins have been shown to play a role in regulation of synaptic density in hippocampal and cortical networks. To determine if mutation of H2-Kb leads to synaptic alterations in the hypothalamus of adult mice, we applied a multi-fold approach looking at excitatory, inhibitory, and hypothalamic specific synaptic proteins across the entire hypothalamus and within in distinct hypothalamic subregions. To assess for changes in total synaptic protein levels in the hypothalamus, protein lysates from 9-10 month old wild-type and H2-K^{bmi} mice were probed with antibodies for specific synaptic proteins. We noted no significant alteration in gross protein expression for excitatory synaptic proteins (Vglut1, Vglut2, PSD95) or inhibitory synaptic proteins (gephyrin) by western blot in total protein lysate (data not shown). However, mutation of H2-Kb led to a trending reduction in NPY expression in total
Figure 11. *H2-K<sup>bm1</sup>* mice spend more time in inactive states and are less likely to be active compared to WT controls. 2-3 month and 5-6 month old WT and H2-K<sup>bm1</sup> mice were analyzed in the homecage monitoring system. (A) At 2-3 months H2-K<sup>bm1</sup> mice spend a significantly higher percentage of their day in an inactive state (black segment) compared to WT controls (H2-K<sup>bm1</sup> 63 ± 3.89%, WT 54.48 ± 5.3%, p = 0.0093, critical p = 0.01). A trending reduction in percentage of time spent feeding (H2-K<sup>bm1</sup> 6.47 ± 0.97%, WT 8.72 ± 2.42%, p = 0.029, critical p = 0.01) and other non-forward locomotor activity (H2-K<sup>bm1</sup> 26.07 ± 3.14%, WT 30.66 ± 4.11, p = 0.025, critical p = 0.01) were also observed. (B) 5-6 month old H2-K<sup>bm1</sup> mice spend roughly 10% more of the 24 hour day in an inactive state (H2-K<sup>bm1</sup> 70.85 ± 6.61%, WT 61.07 ± 6.01%, p = 0.0079, critical p = 0.0). Additionally, H2-K<sup>bm1</sup> mice spend less time feeding compared to WT controls (H2-K<sup>bm1</sup> 6.40 ± 1.50%, WT 11.27 ± 4.21%, p = 0.0082, critical p = 0.01). (C) H2-K<sup>bm1</sup> show a trending reduction in active state probability in the final hours of the dark cycle compared to controls. (D) At 5-6 months of age H2-K<sup>bm1</sup> mice are significantly less likely to be in an active state during the final two hours of the dark cycle and first hour of the light cycle compared to WT controls. All comparisons made by two-tailed t-tests corrected for multiple comparisons. * indicates p < 0.01. Error bars indicate standard error of the mean.
hypothalamic protein lysates. This change was not accompanied by a significant change in the protein level of POMC (Figure 12A). NPY/AgRP neurons predominately project to the paraventricular nucleus where they antagonize the anorexigenic action of alpha-MSH release by POMC neurons and promote feeding behavior through the orexigenic action of NPY onto NPY receptor populations. However, there exists an asymmetric inhibitory circuit within the arcuate nucleus where NPY/AgRP neurons provide tonic local inhibition of POMC neurons whenever NPY/AgRP neurons are active. While western blot analysis can be informative, it lacks the spatial resolution needed to detect where changes in NPY specifically occur. In efforts to identify the origin of reduced NPY in H2-Kbm1 mice and determine if there are other synaptic changes, we performed immunohistochemistry and confocal microscopy. Synaptic changes in specific regions of the hypothalamus were assessed in fixed tissue sections from 12-13 month old mice stained for excitatory (Vglut1, Vglut2), inhibitory (GAD65), and NPY positive synapses. Images were collected from the arcuate nucleus, paraventricular nucleus, and lateral hypothalamus. Using this method, not only did we confirm a reduction in NPY expression, but we identified a neuroanatomical location for this change. H2-Kbm1 mice have a ~30% reduction in NPY+ puncta within the arcuate nucleus (WT 442.1 ± 63.7, H2-Kbm1 328.1 ± 65.24 NPY puncta per field, p < 0.05), and this reduction is carried by the largest change occurring within the posterior arcuate (~bregma -2.4) (WT 415.1 ± 73.20, H2-Kbm1 266.6 ± 71.81 NPY puncta per field, p < 0.05) (Figure 12B,C). No changes were observed in other hypothalamic subregions assessed. It is possible that lower NPY levels in the arcuate nucleus would lead to reduced inhibition of anorexigenic POMC neurons, thereby driving hypophagia. This result can be viewed as an underlying potential mechanism for hypophagia observed in H2-Kbm1 mice. However, it is also possible that lower levels of NPY occur as a
Figure 12. *H2-K*<sup>bm1</sup> mice have reduced NPY axon puncta density in the arcuate nucleus. Immunohistochemistry and Western Blot were performed with an antibody targeting neuropeptides to assess for genotypic differences. (A) Western blot analysis of whole hypothalamic lysate revealed a trending reduction in NPY expression in *H2-K*<sup>bm1</sup> mice, but no change in POMC expression. (B) Representative images of anterior and posterior arcuate nucleus NPY distribution in WT and *H2-K*<sup>bm1</sup> mice at 20x and 160x. Anterior images were taken at roughly bregma -1.9 and posterior images were taken at bregma -2.4. A clear reduction in NPY staining is observed at bregma -2.4. Scale bar equals 50 mm in 20x image and 10 mm in 160x image. (C) Quantification of NPY positive puncta per field at 160x revealed a significant reduction in the arcuate nucleus (WT 442.1 ± 63.7, *H2-K*<sup>bm1</sup> 328.1 ± 65.24 NPY puncta per field, p < 0.05), driven by change in the posterior arcuate (WT 415.1 ± 73.20, *H2-K*<sup>bm1</sup> 266.6 ± 71.81 NPY puncta per field, p < 0.05). Data represents average puncta per field for 4 biological replicates per genotype. Comparisons were made by two-tailed student t-tests and * indicates significance p < 0.05. Error bars represent standard error of the mean.
compensatory mechanism in response to obesity, indicating the arcuate nucleus in H2-K<sup>bm1</sup> mice is functioning properly and responding to changes in metabolic status.

**Mutation of Class I MHC H2-Kb leads to alterations in synapse density in select regions of the hypothalamus**

Mutation of H2-Kb also leads to changes in excitatory synaptic density within specific nuclei of the hypothalamus. Vglut1<sup>+</sup> puncta are significantly increased within the arcuate nucleus (WT 1050.0 ± 256.1, H2-K<sup>bm1</sup> 1385.6 ± 90.7 puncta per field, p < 0.05), which is accompanied, by a trending increase in inhibitory GAD65<sup>+</sup> puncta (WT 2280.7 ± 242.7, H2-K<sup>bm1</sup> 2814.6 ± 460.5 puncta per field, p = 0.086) (*Figure 13A,C,D*). We did not note any differences in puncta density in the paraventricular hypothalamus, but did note large variability between samples (*Figure 13C,D,E*). Within the lateral hypothalamus there is a trending reduction in the number of Vglut2 positive synaptic puncta (WT 3207 ± 341.6, H2-K<sup>bm1</sup> 2696 ± 304.5 Vglut2 puncta per field, p = 0.067) (*Figure 13B,E*). One of the synaptic targets of Vglut2 synapse in the lateral hypothalamus are orexin neurons. Altered active states and arousal can be directly linked to the function and excitation of orexin neurons in the lateral hypothalamus. So, next we chose to assess if the trending reduction in Vglut2 puncta affected the input to orexin positive neurons in the lateral hypothalamus.

**Mutation of Class I MHC H2-Kb leads to reduced excitatory input onto orexin A positive lateral hypothalamic neurons**

Initiation of active states is driven by excitation of orexin positive neurons scattered throughout the lateral hypothalamus. Orexin neurons have a 10-fold increase in somatic mini-excitatory postsynaptic current frequency compared to mini-inhibitory post synaptic currents, driven by asymmetric excitatory glutamatergic innervation compared to inhibitory GABAergic innervation<sup>75, 116, 117</sup>. Additionally, glutamatergic antagonists have
Figure 13. *H2-K*<sup>bm1</sup> mice have altered hypothalamic excitatory synapse density. Excitatory and inhibitory synapse density was measured in the arcuate nucleus (ARC), paraventricular nucleus (PVN), and lateral hypothalamus (LH) by immunohistochemistry with antibodies targeting Vglut1, Vglut2, and GAD65. (A) Representative images from the arcuate nucleus demonstrating increased Vglut1 immunoreactive puncta in *H2-K*<sup>bm1</sup> mice compared to WT controls. A trending increase was also observed in the number of GAD65 puncta per field. (B) Representative images of Vglut2 immunoreactivity in the WT and *H2-K*<sup>bm1</sup> lateral hypothalamus. (C) Quantification of Vglut1 puncta demonstrated a significant increase in Vglut1 immunoreactive synapse density in the arcuate of *H2-K*<sup>bm1</sup> mice compared to controls (WT 1050.0 ± 256.1, *H2-K*<sup>bm1</sup> 1385.6 ± 90.7 puncta per field, p < 0.05). No differences were noted in the PVN or LH. (D) Quantification of GAD65 puncta demonstrated a trending increase in the arcuate nucleus of *H2-K*<sup>bm1</sup> mice (WT 2280.7 ± 242.7, *H2-K*<sup>bm1</sup> 2814.6 ± 460.5 puncta per field, p = 0.086). (E) Analysis of Vglut2 puncta density demonstrated a trending reduction in the lateral hypothalamus of *H2-K*<sup>bm1</sup> mice (WT 3207 ± 341.6, *H2-K*<sup>bm1</sup> 2696 ± 304.5 Vglut2 puncta per field, p = 0.067). Synapse counts are representative of 4 biological replicates per genotype. * indicates p < 0.05 by two-tailed student t-test. Scale bar in all images equals 20 mm.
been shown to greatly reduce action potential generation in orexin neurons; however inhibition of GABA\textsubscript{A} does not lead to an increase in spontaneous action potential firing. These results indicate that glutamatergic innervation is critical for proper orexin neuron function. Our data demonstrates that mutation of H2-Kb leads to a gross reduction in Vglut2 excitatory puncta in the lateral hypothalamus, without changes in additional excitatory synaptic (Vglut1) for inhibitory synaptic (GAD65) proteins. Considering H2-K\textsuperscript{bm1} mice show a remarkable reduction in daily activity and reduced Vglut2 excitatory synaptic puncta in the lateral hypothalamus, we hypothesized that orexin A positive neurons were receiving less excitatory input in H2K\textsuperscript{1bm1} mice compared to controls. To evaluate this, we performed confocal microscopy capturing z-stacks of hypothalamic sections stained with orexin A, Vglut2, and NPY. This was followed by 3D reconstruction of orexin A positive neurons and quantification of synaptic puncta that colocalized with the surface reconstruction of orexin positive neurons. Using this method, we found that H2-K\textsuperscript{bm1} mice show a significant reduction in Vglut2\textsuperscript{+} puncta / 100 µm\textsuperscript{2} of orexin A cell surface area (WT 5.81 ± 0.25, H2-K\textsuperscript{1bm1} 4.90 ± 0.20 Vglut2\textsuperscript{+} puncta per 100 µm\textsuperscript{2} orexin SA, p < 0.01), but no differences in NPY\textsuperscript{+} puncta (WT 0.99 ± 0.074, H2-K\textsuperscript{1bm1} 1.06 ± 0.10 NPY\textsuperscript{+} puncta per 100 µm\textsuperscript{2} orexin surface area) (Figure 14A,B,D). To further validate this finding, we performed bootstrapping analysis for 1000 permutations of the mean puncta density data for Vglut2\textsuperscript{+} and NPY\textsuperscript{+} synapses. Frequency distributions generated from this analysis clearly demonstrate differences between WT and H2-K\textsuperscript{1bm1} cells for Vglut2\textsuperscript{+} synapse density demonstrated by minimal overlap of the histograms (Figure 14C). Differences are not displayed in NPY\textsuperscript{+} density between genotypes, denoted by significant overlap of frequency distributions (Figure 14E). This data clearly demonstrates reductions in Vglut2 innervation of orexin neurons in the lateral hypothalamus. Functionally, orexin neurons have heightened sensitivity to excitatory changes, as they exist in an intrinsically depolarized state\cite{68-70}. Therefore, the small
Figure 14. **H2-K^bm1** orexin neurons have reduced Vglut2 excitatory input compared to WT controls. Orexin neurons in the lateral hypothalamus were identified by orexin A staining in tissue sections stained for Vglut2 and NPY to assess synaptic input to orexin neurons. (A) Representative imaging and data analysis sequence...Continued on next page.
Figure 14. Continued (A1) Original confocal z-stack images as displayed in IMARIS software are shown in the top row labeled raw. (A2) In the second row, images with spots and surfaces detected are displayed. (A3) Final output image displaying orexin neuron surface and synaptic puncta detected within the surface (Vglut2 in green, NPY in magenta). (B) Quantification of Vglut2 puncta per 100 $\mu m^2$ of orexin surface area (SA) demonstrated a significant genotype interaction and reduction in H2-K$^{bm1}$ mice when assessed by two-way ANOVA (WT 5.81 ± 0.25, H2-K$^{bm1}$ 4.90 ± 0.20 Vglut2 puncta per 100 $\mu m^2$ orexin SA, p < 0.01). (C) Bootstrap analysis for 1000 permutations of the data demonstrated minimal overlap in the frequency distributions for the mean Vglut2 puncta per 100 mm of orexin SA. (D) Quantification of NPY puncta per 100 mm of orexin surface area showed no difference between genotypes (WT 0.99 ± 0.074, H2-K$^{bm1}$ 1.06 ± 0.10 NPY puncta per 100 $\mu m^2$ orexin SA). (E) Bootstrap analysis of NPY data demonstrates major overlap in frequency distribution verifying no difference between genotypes. All data is representative of quantification of 55 cells for WT and 57 cells for H2-K$^{bm1}$ mice spread across 4 biological replicates per condition. Data was analyzed by two-way ANOVA. ** indicates p < 0.01. Error bars represent standard error of the mean.
changes observed may have large functional outcomes and investigating these outcomes may offer insight into the mechanism of obesity in H2-K\textsuperscript{bml} mice.

**Mutation of H2-Kb leads to reduced fasting induced cFOS induction in orexin neurons**

Fasting results in increased mini excitatory post synaptic potentials and excitatory synaptic density on orexin neurons within 12 hours\textsuperscript{75,118}. cFOS can serve as a marker for whether there are differences in orexin neuron response to fasting, as it is rapidly increased in neurons in response to firing\textsuperscript{119,120}. To determine if reduced excitatory drive onto orexin positive neurons results in a baseline reduction in orexin activation in response to an attenuation stimulus, we performed a fasting cFOS induction assay on 4-4.5 month old WT and H2-K\textsuperscript{bml} mice. At the time of testing, H2-K\textsuperscript{bml} mice were significantly heavier than WT controls (WT 24.50 ± 1.33 g, H2-K\textsuperscript{bml} 27.91 ± 2.40 g, p < 0.01), demonstrating the onset of the previously described progressive obesity phenotype. Mice were fasted for 24 hours (11:00 AM to 11:00 AM) and the brains were processed for immunohistochemistry. Confocal imaging for orexin A and cFOS was performed and the numbers of orexin, DAPI, cFOS positive neurons in addition to the number of cFOS/orexin double positive neurons were quantified. Remarkably, H2-K\textsuperscript{bml} mice fail to show cFOS induction upon fasting. Fasted H2-K\textsuperscript{bml} mice show a significant reduction in the percentage of orexin/cFOS double positive cells compared to fasted WT mice (WT Fasted 35.05 ± 4.77%, H2-K\textsuperscript{bml} Fasted 3.91 ± 2.33%, p < 0.01 by one-way ANOVA) (Figure 15). WT mice show a modest increase in the percent of orexin cells that are positive for cFOS staining; however this increase is not evident in H2-K\textsuperscript{bml} mice. Additionally, H2-K\textsuperscript{bml} mice show extremely low levels of cFOS positive orexin neurons in basal conditions. This data supports the hypothesis of reduced orexinergic activity in H2-K\textsuperscript{bml} mice, corresponding to the observed reduced excitatory synaptic density. In
Figure 15. *Orexin neurons in H2-Kbm1 mice fail to respond to fasting stimulus with increased activity.* WT and H2-Kbm1 mice were fasted for 24 hours and processed for immunohistochemistry to quantify orexin neurons activation in response to fasting. c-fos induction serves as a marker of neuronal activity. (A) Representative images of c-fos and orexin staining in WT and H2-Kbm1 mice in fed and fasted states. Yellow arrowheads indicate c-fos/Orexin double positive cells. White arrows indicate orexin positive c-fos negative cells. (B) Quantification of the percentage of c-fos positive orexin neurons revealed a significant reduction in H2-Kbm1 fasted mice compared to fasted controls. A clear increase was observed in WT mice with fasting and H2-Kbm1 mice appear to have low c-fos positive orexin neurons at baseline, but this was not analyzed for statistical difference due to the small sample size of mice in the fed states. Data is representative for n = 2 for WT and H2-Kbm1 fed and n = 4 for WT and H2-Kbm1 fasted groups. Comparison made by one-way ANOVA with Bonferroni post-hoc comparisons. ** indicates p < 0.01. Error bars represent standard deviation of the mean.
processing mice for the cFOS induction assay, we visually noted less activity in H2-K<sup>bm1</sup> mice compared to WT controls. WT mice were actively scurrying around the cage, whereas H2-K<sup>bm1</sup> mice appeared stationary in their bedding material. As orexin activation is a critical regulator of arousal, we chose to further analyze this finding in a quantitative manner.

**H2-K<sup>bm1</sup> mice fail to increase activity in response to dark cycle fasting**

Orexin administration results in elevated metabolic rates<sup>121</sup>, food intake<sup>56, 57</sup>, locomotor activity<sup>122</sup>, and wakefulness<sup>123</sup>. Similar behaviors are observed in fasting animals, where orexin plays a link between metabolic status and motivated behavior<sup>124-</sup><sup>127</sup>. As H2-K<sup>bm1</sup> mice appear to demonstrate orexin deficits, we wanted to determine if functional outcomes related to orexin activation are altered. As a first step towards this analysis, we quantified ambulatory activity measurements taken during indirect calorimetry evaluation. For indirect calorimetry, mice are fasted for roughly 12 hours prior to assessment. We noted significant periods of reduced activity counts in H2-K<sup>bm1</sup> mice. Activity differences are evident at early time points of analysis, where WT mice are the most active. Later in the assay, both genotypes show lower activity and the differences are less apparent (Figure 16A). At 2-3 months H2-K<sup>bm1</sup> mice showed a significant reduction in total ambulatory activity while in the indirect calorimetry system (WT 14099 ± 2413 ambulatory counts, H2-K<sup>bm1</sup> 6514 ± 1490 ambulatory counts, p < 0.05) (Figure 16B). At older ages, differences are still observed at specific time intervals (Figure 16C), but these differences become less dramatic with overall ambulatory activity just failing to reach significance (Figure 16D). To further determine if the orexinergic system fails to respond to fasting induced attenuation, we designed a 24 hour fasting paradigm within the homecage monitoring system where we can measure movement and feeding over the circadian day.
Figure 16. H2-K<sup>bm1</sup> mice do not increase activity in response to fasting and placement in a novel environment, suggesting deficits in orexin function. WT and H2-K<sup>bm1</sup> mouse activity data from indirect calorimetry analysis was assessed for ambulatory activity counts over 18 intervals of 17.5 minutes each at 2-3 months and 5-6 months of age. (A) At 2-3 months of age, H2-K<sup>bm1</sup> mice fail to respond to fasting by increasing activity counts when placed in a novel environment, especially early upon introduction to the cage. (B) H2-K<sup>bm1</sup> mice are significantly less active over the entire analysis period compared to WT controls (WT 14099 ± 2413 ambulatory counts, H2-K<sup>bm1</sup> 6514 ± 1490 ambulatory counts, p = 0.018). (C) At 5-6 months of age H2-K<sup>bm1</sup> mice still fail to respond to fasting with increased activity. (D) Overall ambulatory activity counts in 5-6 month H2-K<sup>bm1</sup> mice are lower than WT controls, but fail to reach statistical significance (WT 14776 ± 1278 ambulatory counts, H2-K<sup>bm1</sup> 10825 ± 1684 ambulatory counts, p = 0.083). Analysis for number of ambulatory counts over 18 cycles by two-way repeated measures ANOVA demonstrated a significant interval*genotype interaction (p < 0.001) for both ages. Within interval comparisons in A and C, and total ambulatory comparisons in B and D were made by two tailed student t-tests, * indicates p < 0.05, ** p < 0.01, *** p < 0.001. Error bars represent standard error of the mean. n = 8 mice per genotype at each age.
Mice were placed in the HCM system and allowed to acclimate for 4 days prior to fasting. For the first fasting paradigm, food was removed just prior (~15-30 minutes) to the onset of the light cycle. For the second paradigm, food was removed just prior (15-30 minutes) to the onset of the dark cycle. By performing both paradigms we were able to assess light and dark cycle behaviors corresponding to different levels of fasting. This is important as orexin signaling is temporally regulated with higher levels during the active (dark cycle). When food is removed at the onset of the dark cycle, fasted WT mice show a trending increase in overall movement over a 20 hour period following food removal, while fasted H2-K\textsuperscript{bm1} show no change compared to their baseline fed state (\textbf{Figure 17A}). Differences in movement in fasted WT mice appear to occur throughout most of the dark cycle, with minimal differences during the light cycle (\textbf{Figure 17B}). Analysis of 1 hour bin time data by repeated measures MANOVA only revealed a significant genotype effect, therefore no further analysis was performed this data. Using the fed baseline activity for the same duration of time for each mouse, the fold change in activity was assessed and averaged for each genotype. Overall there was a trending increase in movement ratio for fasted WT mice (\textbf{Figure 17C}). Looking at each mouse individually, 5 of 7 WT mice showed increased movement (>10% increase), 1 showed no change, and 1 had less (>10% decrease). Whereas, 3 of 7 H2-K\textsuperscript{bm1} mice had less movement, 2 showed no change, and 2 had increased movement (\textbf{Figure 17C}). When mice were fasted just prior to the dark cycle, this phenotype became even more prominent. Fasted WT mice again showed an overall trending increase in movement compared to their fed state over a 12 hour period following fasting (WT Fed 363.8 ± 34.8 m, WT Fast 447.9 ± 21.4 m, adjusted p = 0.12). Whereas, fasted H2-K\textsuperscript{bm1} showed a drastic reduction in activity compared to the fed state (H2-K\textsuperscript{bm1} fed 204.8 ± 19.9 m, H2-K\textsuperscript{bm1} fast 107.8 ± 6.1 m, adjusted p <
Figure 17. *H2-K*<sup>bm1</sup> fasted at the start of the light cycle do no show any increase in overall locomotor activity. Mice respond to fasting with increased orexin activity that drives increased movement and food seeking behaviors. To test if *H2-K*<sup>bm1</sup> demonstrate function aspects relating to deficits in orexin function, mice were fasted at the start of the light cycle and total movement was quantified for a 20 hour period of fasting. (A) WT mice show a trending increase in overall movement (WT Fed 444.75 ± 22.5 m, WT Fast 559.8 ± 59.8 m, adjusted p = 0.09), while *H2-K*<sup>bm1</sup> mice show no change (*H2-K*<sup>bm1</sup> Fed 245.0 ± 22.1 m, *H2-K*<sup>bm1</sup> Fast 234.6 ± 36.1 m, adjusted p = 0.81). Comparisons made by two-tailed Student’s t-test Bonferroni corrected for two comparisons. (B) Movement data organized by hourly bins during the dark and light cycle shows comparisons at individual time points. Comparison by repeated measures MANOVA demonstrated only a significant effect of genotype (p < 0.0001). (C) Total fasting movement for each mouse normalized to its own fed baseline total movement. Ratios greater than 1 signify greater movement under the fasting paradigm. Far left bars for WT and *H2-K*<sup>bm1</sup> represent aggregate ratios for all samples within a genotype (WT 1.25 ± 0.11, *H2-K*<sup>bm1</sup> 1.01 ± 0.19, p = 0.28). Bars on the right represent individual mice for WT (n = 7) and *H2-K*<sup>bm1</sup> (n = 7). Comparison of aggregate ratios by two-tailed Student’s t-test.
0.001) (Figure 18A). Analysis of individual time points throughout the dark cycle by repeated measures multivariate ANOVA revealed significant between subject effects for genotype and genotype by condition on movement distances. Increased movement in fasted WT mice occurs throughout the dark cycle, but with the largest changes towards the onset and offset times (Figure 18B). Comparing the average ratio of movement for fasted and fed WT and H2-K<sup>bm1</sup> mice, there was a clear increase in WT and decrease in H2-K<sup>bm1</sup> mice (WT 1.38 ± 0.24, H2-K<sup>bm1</sup> 0.56 ± 0.06, p < 0.01) (Figure 18C). On the level of individual mice 4 WT mice showed increased activity (>10%) and 4 showed relatively no change (<10% increase or decrease) from fed baseline. Whereas, all 7 H2-K<sup>bm1</sup> mice had less activity (>30% reduction) compared to fed baseline (Figure 18C).

Fasting induced orexin activity is known to drive feeding behaviors including food pursuit. Therefore, in addition to movement data we quantified the number of feeder photobeam breaks and total duration of time the beam was broken in both fasting paradigms. When mice were fasted at the start of the light cycle and followed for 20 hours, we observed a significant reduction in feeding attempts for H2-K<sup>bm1</sup> mice compared to their fed baseline (H2-K<sup>bm1</sup> fed 566 ± 73, fast 152 ± 35, p <0.001). Additionally, H2-K<sup>bm1</sup> fasted mice show a significant reduction compared to WT fasted mice (WT fast 442 ± 92, p < 0.05) (Figure 19A). Quantification of feeding attempt duration revealed a significant reduction in both WT fasted and H2-K<sup>bm1</sup> fasted mice compared to their fed baselines. Both fasted and fed H2-K<sup>bm1</sup> mice also had reduced feeding attempt durations compared to WT mice under the same conditions (Figure 19B). Analysis of feeding activity when fasted at the start of the dark cycle yielded roughly identical results with a reduction in feeding attempts in fasted H2-K<sup>bm1</sup> mice compared to fed baseline and fasted WT mice (Figure 19C) and reduced feeding attempt durations between conditions and genotypes (Figure 19D). WT mice when
Figure 18. H2-K^{bmi} fasted at the start of the light cycle do not show an increase in overall locomotor activity. Mice respond to fasting with increased orexin activity that drives increased movement and food seeking behaviors. To test if H2-K^{bmi} demonstrate function aspects relating to deficits in orexin function, mice were fasted at the start of the light cycle and total movement was quantified for a 20 hour period of fasting. (A) WT mice show a trending increase in overall movement (WT Fed 363.8 ± 34.8 m, WT Fast 447.9 ± 21.4 m, adjusted p = 0.12), while H2-K^{bmi} mice show a drastic reduction (H2-K^{bmi} Fed 204.8 ± 19.9 m, H2-K^{bmi} Fast 107.8 ± 6.1 m, adjusted p < 0.001). Comparisons made by two-tailed Student's t-test Bonferroni corrected for two comparisons. (B) Movement data organized by hourly bins during the dark and light cycle shows comparisons at individual time points. Comparison by repeated measures MANOVA demonstrates a significant between subject's effect of genotype (p < 0.00001) and genotype by condition (p < 0.01). (C) Total fasting movement for each mouse normalized to its own fed baseline total movement. Ratios greater than 1 signify greater movement under the fasting paradigm. Far left bars for WT and H2-K^{bmi} represent aggregate ratios for all samples within a genotype (WT 1.38 ± 0.24, H2-K^{bmi} 0.56 ± 0.0.06, p < 0.01). Bars on the right represent individual mice for WT (n = 7) and H2-K^{bmi} (n = 7). Comparison of aggregate ratios by two-tailed Student’s t-test.
Figure 19. H2-K\textsuperscript{bm1} and WT mice show altered feeding behaviors upon fasting.

Orexin neuron activity is known to drive feeding activity and food pursuit behaviors in mice upon fasting. To test if H2-K\textsuperscript{bm1} have deficits in feeding behaviors in response to fasting, we measured feeder photobeam breaks and duration of breaks in mice fasted at the start of the light cycle or start of the dark cycle. (A) H2-K\textsuperscript{bm1} mice have a reduced number of photobeam breaks when challenged by fasting at the start of the light cycle compared to fed controls and fasted WT mice. (B) The duration of time the photobeam was broken drastically decreased in both genotypes with fasting at the start of the light cycle. H2-K\textsuperscript{bm1} fed and fasted mice have reduced beam break duration compared to WT fed and fasted mice. (C) Similar to fasting at the start of the light cycle, when food is removed at the start of the dark cycle H2-K\textsuperscript{bm1} show a reduction in the number of beam breaks compared to fed baseline and fasted WT mice. (D) When fasted at the start of the dark cycle both H2-K\textsuperscript{bm1} and WT mice show reduced time spent breaking the feeder photobeam. H2-K\textsuperscript{bm1} fed and fasted mice show a significant decrease compared to WT condition matched controls. Data is representative of 20 hours of activity when fasted at the start of the light cycle (A,B) and for 12 hours of activity when fasted at the start of the dark cycle (B,C). Data is the mean of 7 mice per genotype for light cycle food removal (A,B) and 8 WT and 7 H2-K\textsuperscript{bm1} mice for dark cycle food removal (C,D). Error bars represent standard error of the mean. Comparisons made by two-tailed Student's t-tests Bonferroni corrected for 4 comparisons. $ denotes p < 0.05 when compared within genotype, # indicated p < 0.05 when compared to matched condition in opposite genotype.
fasted showed trending, but non-significant reductions in feeding attempts compared to their fed baseline. Overall, this data confirms reduced feeding in H2-K\textsuperscript{bm1} during the normal fed state and demonstrates that H2-K\textsuperscript{bm1} under fasting conditions may not maintain the same level of drive for feeding as fasted WT mice. This is supported by the data showing overall reductions in feeding duration for both genotypes, but a higher number of attempts at feeding in fasted WT compared to fasted H2-K\textsuperscript{bm1} mice.

**Adult H2-K\textsuperscript{bm1} mice are hypothermic during dark cycle activity**

In addition to sleep, arousal, feeding, and activity orexin neurons have also been linked to autonomic regulation including sympathetic activity, blood pressure, and thermoregulation among others\textsuperscript{128-131}. To determine if H2-K\textsuperscript{bm1} mice demonstrate differences in autonomic functions suggestive of further orexin dysfunction, we first chose to assess body temperature during resting (light cycle) and active (dark cycle) states at 2-3 months, 5-6 months, and 8-9 months of age. We observed no differences in resting body temperatures between genotypes at any age (Figure 20A,B,C). However, at 5-6 months of age H2-K\textsuperscript{bm1} mice are hypothermic compared to controls during the dark cycle (WT 36.48 ± 0.053 °C, H2-K\textsuperscript{bm1} 35.83 ± 0.125 °C, p < 0.001) (Figure 20B). This difference persists in 8-9 month old mice (WT 36.48 ± 0.09 °C, H2-K\textsuperscript{bm1} 36.175 ± 0.09 °C, p < 0.05) (Figure 20C). The difference between genotypes stems from a failure to increase body temperature, which may be a result of overall decreased activity in H2-K\textsuperscript{bm1} mice or due to direct deficits in orexin mediated regulation of autonomic function.

**Conclusions and Discussion**

Here, we present the first data clearly implicating the classical MHC I proteins in hypothalamic regulation of energy balance. MHC I proteins are dynamically expressed during hypothalamic development and colocalize with synaptic proteins. MHC I expression is reduced in the adult hypothalamus, but one isoform, H2-Kb increased in
Figure 20. H2-K^{bmt} mice are hypothermic during the active cycle at 5-6 and 8-9 months of age. Orexin neuron activity is known to function in autonomic regulation, including in thermogenesis. To determine if orexin deficits in H2-K^{bmt} mice extend to autonomic function, we measured body temperatures in WT and H2-K^{bmt} mice during the resting state (light cycle) and active state (dark cycle) at three different time points. (A) At 2-3 months of age there were no observed differences between WT and H2-K^{bmt} mice in resting or active state body temperatures. (B,C) At both 5-6 and 8-9 months of age H2-K^{bmt} mice are significantly hypothermic during the active state (5-6 months: WT 36.48 ± 0.05, H2-K^{bmt} 35.48 ± 0.13, p < 0.001; 8-9 months: WT 36.48 ± 0.09, H2-K^{bmt} 36.18, p < 0.05). No differences between genotypes were found during the resting cycle. 2-3 month data is representative of 4 WT and 8 H2-K^{bmt} mice. Data at 5-6 and 8-9 months is representative of 8 mice per genotype. Error bars signify standard error of the mean. * indicate p < 0.05, *** p < 0.0001 when compared by two-tailed Student’s t-test.
transcript expressing in adult mice suggesting a potential continued functional role. In efforts to identify potential functions of H2-Kb in the hypothalamus we performed behavioral phenotyping of mice with a mutation of the H2-Kb isoform. Mutation of H2-Kb leads to a significant early adult onset obesity phenotype with characteristics of metabolic syndrome. H2-Kb\textsuperscript{bm1} mice show progressive weight gain, central adipose deposition, glucose intolerance, insulin resistance, and hepatic steatosis. Interestingly, increased caloric intake does not underlie obesity in H2-Kb\textsuperscript{bm1} mice, as they are markedly hypophagic compared to WT controls. Our data reveals that obesity results from an underlying deficit in orexinergic neuron function, which leads to a drastic reduction in mouse activity and increased time spent in sedentary states. Functionally, orexin neurons in H2-Kb\textsuperscript{bm1} fail to respond to fasting stimulus, evident by significantly lower cFOS induction. H2-Kb\textsuperscript{bm1} mice also fail to increase activity when fasted and are significantly hypothermic during the active cycle, also indicating reduced orexin activity. Our data also shows reduced excitatory synaptic innervation of orexin neurons, providing a potential mechanism for orexin neuron deficits in H2-Kb\textsuperscript{bm1} mice. To our knowledge this is the first evidence for MHC I protein functions in the adult hypothalamus and orexinergic signaling.

Our studies focused on orexin A neurons in the lateral hypothalamus. Orexin A neurons signal through both orexin receptor 1 (OX1R) and orexin receptor 2 (OX2R) equally, whereas orexin B shows a 10-selectivity for OX2R\textsuperscript{132}. Functionally, OX1R primarily functions in feeding and reward\textsuperscript{133-135}, and OX2R is linked to the promotion of arousal\textsuperscript{136, 137}. As we noted functional differences in both behaviors, assessing orexin A activity was deemed a logical first assessment. However, as orexin B neuronal activity was not assessed we cannot conclude that mutation of H2-Kb leads affects orexin B function. Future analysis of this pathway will ultimately yield conceptually interesting
results, no matter the finding. If orexin B neurons are spared with the mutation of H2-Kb it would suggest a level of cell selectivity for MHC I protein regulation of synapses previously unappreciated in the hypothalamus. If orexin B neurons are affected, it implicates H2-Kb in overall regulation of orexin functions.

Orexin neuron activity is linked to behaviors primarily organized around the promotion of feeding, active states, and autonomic function. More recently, orexin signaling has implicated in regulation of motivated behaviors\textsuperscript{131}. H2-K\textsuperscript{bmm1} mice demonstrate significant impairments in each of these areas, which is highly indicative of orexinergetic dysfunction. Energy balance deficits in H2-K\textsuperscript{bmm1} mice can be summarized as obesity secondary to reduced activity, despite marked hypophagia. These changes clearly resemble those seen in models of orexin function antagonism through pharmacologic or genetic techniques. Administration of an orexin receptor antagonist or an antibody against orexin leads to a significant reduction in food intake, even when animals are in a fasted state\textsuperscript{135, 138-140}. Our data demonstrates reduced feeding in H2-K\textsuperscript{bmm1} mice when challenged with fasting, mimicking the results of orexin antagonism. We also observe lower daily activity in H2-K\textsuperscript{bmm1} mice at 2-3 and 6-7 months of age. Orexin deficient mice have also been described to have lower locomotor activity compared to WT mice\textsuperscript{141}. Administration of orexin centrally has been shown to increase locomotor activity and feeding\textsuperscript{142, 143}. Another clear behavioral change associated with orexin function is increased activity when challenged by fasting. Orexin deficient mice fail to increase activity in response to a fasting challenge\textsuperscript{127}. Similarly, H2-K\textsuperscript{bmm1} demonstrate reduced activity in the fasted state in two independent experimental paradigms presented in this study. Another role for the orexin system is in autonomic function, including the regulation of thermogenesis. Administration of orexin A by intracerebroventricular injection leads to increased body temperature in both fasted and
anesthetized rodents, demonstrating orexin regulation of body temperature independent of activity or feeding\textsuperscript{144, 145}. H2-K\textsuperscript{bm1} mice are significantly hypothermic during the active cycle, when orexin neurons have the highest level of activity and peak orexin levels in the CSF\textsuperscript{71-74, 146}.

Our data also suggests that H2-K\textsuperscript{bm1} mice may have deficits in motivated behavior. Administration of orexin leads to increased food pursuit behaviors when the food source is either highly palatable (altered diet) or when mice are placed under fasting\textsuperscript{147-149}. Our data assessing behavioral activity in the homecage system under fasting conditions revealed a reduction in the number of feeding attempts in fasted H2-K\textsuperscript{bm1} mice compared to fasted controls, suggesting that H2-K\textsuperscript{bm1} mice demonstrate reduced food pursuit. Fasting paradigms and conditioned feeding paradigms have been shown to lead to cFOS induction in orexin neurons\textsuperscript{75, 119, 120, 134, 150, 151}. Our data demonstrates no cFOS induction upon fasting, suggesting reduced orexin activation, which could impair food pursuit behaviors when food was removed from the original location of the food source. Even on a larger scale, in terms of obesity development, H2-K\textsuperscript{bm1} mice mimic deficits observed in mice with orexin deficiencies. Orexin deficient animal models often suffer from adult onset obesity, much like H2-Kb null mice\textsuperscript{141, 152-155}. Similarly, in humans, individuals with narcolepsy, a genetic condition characterized by reduced numbers of orexin neurons, have a two-fold increased risk for obesity development, despite often being hypophagic\textsuperscript{156}. However, there is a major difference between our data and others linking orexin deficiencies to obesity. The majority of the previously mentioned studies directly inhibited or ablated orexin neurons, whereas orexin deficits in our study appear secondary to mutation of MHC class I H2-Kb. The potential implications these results have in the generation of obesity phenotypes are not truly appreciated until mechanisms underlying MHC I expression and MHC I roles in other brain regions are detailed.
Further analysis of H2-K\textsuperscript{bm1} orexin neuron synapse density by electrophysiology and electron microscopy will yield a more detailed picture of synaptic density and dynamics of synapse function. Electron microscopy has demonstrated a higher density of asymmetric (assumed excitatory) synapses and smaller surface areas for orexin neurons than we detailed based on our approach, likely due to differences in resolution\textsuperscript{75}. Differences in cell surface area can be explained by the methods of calculation and our inclusion of proximal dendrites. Our reported low levels of NPY synaptic puncta in contact with surface reconstructions of orexin neurons is in accordance with low levels NPY innervation of orexin neurons\textsuperscript{75}. We did not assess GABAergic synapse density onto orexin neurons, since we noted no change in overall GABA immunoreactivity across the lateral hypothalamus. GABAergic synaptic contacts are a minor input to orexin neurons and occur at a rate of 2 per every 10 Vglut2 synapses\textsuperscript{75}. Performing electrophysiology on H2-K\textsuperscript{bm1} mice and matched controls in a fasting paradigm would offer potential explanation for the lack of cFOS induction observed in our studies. Fasting results in increased frequency of excitatory mEPSCs with no changes in mIPSCs, and is correlated with increased excitatory synapses in WT mice\textsuperscript{75}. Our data demonstrating absent cFOS induction implies this process is not functioning in H2-K\textsuperscript{bm1} mice. At the level of the synapse, MHC I proteins have been implicated in regulating both synapse density as well as function in hippocampal and cortical neurons\textsuperscript{93, 97, 98}. However, in contrast to our data, MHC I loss in these systems leads to increased excitatory synapse density and function\textsuperscript{97}. There is also evidence for roles of specific MHC I isoforms within distinct cell populations. Loss of H2-Kb and H2-Db in a double knockout model leads to deficits in formation of the laminar structure of the visual cortex and impaired synapse elimination, which can be rescued by restoring H2-Db, but not H2-Kb\textsuperscript{73}. We believe our data in context of the literature provides evidence for cell and brain region specific differences in MHC I protein function. Orexin
neuron synaptic connectivity is distinct from other neuron types, with high excitatory and low inhibitory tone, compared to a more equal distribution in interneurons, and high inhibitory tone on other types of long projecting neurons\textsuperscript{157}. This underlying difference may be a foundation for distinct roles of MHC I proteins. Expression patterns and levels of MHC I on the cell surface may also be critical in regulating neuronal properties.

MHC I genes are constitutively expressed, but influenced by several pathways targeting cis-acting regulatory promoter elements containing binding sites for nuclear transcription factor kappa B (NF-κB) and interferon-regulatory factor (IRF) family members\textsuperscript{87-89}. Both NF-κB and interferon signaling pathways have well documented roles in inflammatory signaling, and NF-κB signaling is critical for proper nervous system function\textsuperscript{90}. Additionally, in the CNS MHC I expression can be regulated by neuronal activity through a calcium dependent mechanism involving CREB signaling\textsuperscript{91-93}. The link between MHC I expression and inflammatory pathways may be of particular importance to understanding how MHC I proteins may impact obesity phenotypes. At the most basic level, viral infections can lead to either increased expression of MHC I proteins or reduced expression, often through interactions with the immune system. Select paramyxoviruses\textsuperscript{158}, retroviruses\textsuperscript{159}, and flaviviruses\textsuperscript{160} may increase MHC Class I expression. Conversely, adenoviruses\textsuperscript{161,162}, herpes viruses\textsuperscript{163-165}, pox viruses\textsuperscript{166,167}, and select retroviruses\textsuperscript{159,168-171} may reduce MHC Class I expression. The majority of data for infectious or inflammatory control of MHC Class I expression is centered within the immune system and little is known about how these mechanisms change neuronal expression of MHC I proteins. MHC I levels have been shown to increase in neurons in response to inflammation\textsuperscript{172}, CNS injury\textsuperscript{173}, seizures\textsuperscript{174}, and aging\textsuperscript{175}. Whether neuronal MHC I expression changes with specific infections, is less understood. If neuronal MHC I expression can be influenced in similar manners, it is not difficult to postulate on the
potential impact on metabolic functions. These impacts may be acute or possibly lead to alterations in hypothalamic signaling that may impact an individual's metabolic status chronically. In the short term, MHC I may play a role in regulating sickness behaviors (including increased sleepiness, anorexia, and lethargy), which have been directly tied to the suppression of orexin signaling. In the long term, dysregulated MHC I proteins may lead to variations in the connectivity and plasticity of hypothalamic networks, predisposing individuals to obesity development. While this theory has yet to be thoroughly tested, there is evidence that early life infections can result in obesity in animal models, but the underlying mechanisms are unclear.

The majority of our data detailed deficits in orexin system function as they likely drive the obesity phenotype observed in H2-Kbm1 mice. However, it is also important to note additional changes in synapses observed in H2-Kbm1 mice including increased Vglut1 excitatory and reduced NPY density in the arcuate nucleus. NPY neurons in the arcuate project to PVN and antagonize anorexigenic melanocortin neurons. NPY neurons also robustly inhibit POMC neurons within the arcuate through NPY and GABAergic projections, thereby providing tonic inhibition of POMC stimulation of melanocortin neurons when NPY neurons are active. Our data demonstrates a reduction in NPY density in the arcuate nucleus, which may indicate reduced inhibition onto POMC neurons. Reduced POMC inhibition would lead to increased anorexigenic activity and promote satiety. As H2-Kbm1 mice are hypophagic, it is possible that changes in arcuate function play a role in this finding. This neuronal pathway is also highly plastic and reduced NPY density may also be a response to obesity in efforts to curb weight gain. The predominant excitatory transmitter expressed in the majority of the hypothalamus, including the in the arcuate nucleus is Vglut2. Vglut1 expression is more limited within the hypothalamus and less is known about its functional roles and...
synaptic partners making our observation of increased Vglut1 density challenging to interpret. It is the hope that future work will better characterize this system and place our results into perspective.

The mechanisms underlying how mutation of H2-Kb results in altered excitatory synaptic density onto orexinergic neurons in the lateral hypothalamus is unknown, however we speculate that it is a result of altered binding and presentation of endogenous peptides or through reduced stability on the surface of neurons. H2-Kbm1 carries 7 nucleotide mutations, which lead to three amino acid substitutions\textsuperscript{101-103}. The resulting changes in amino acid side chains results in significant modification of the H2-Kb protein crystal structure and result in altered functional properties\textsuperscript{101-103}. H2-Kbm1 is 18-fold less thermostable on the surface of cells compared to wildtype H2-Kb. Interestingly, H2-K\textsuperscript{bm1} can still bind known H2-Kb antigens, but binding fails to elicit a CTL immune response, suggesting a lack of classical MHC I function\textsuperscript{101-103}. The mechanisms of involvement for MHC I proteins in synaptic development are still poorly understood, but our data demonstrating that mutation of H2-K\textsuperscript{bm1} leads to changes in synaptic density of orexin neurons suggests that proper binding and presentation of protein sequences is critical for the process. The question about what is bound in the clefts of MHC I proteins and the trans-synaptic partner recognizing MHC I proteins in the CNS is still poorly understood. We postulate that the MHC I presentation of self-proteins may serve as cell identifiers to other cell types in the CNS, where orexin neurons present small fragments of proteins that identify the neuron type potentially guiding synaptic targeting. Mutation of H2-Kb may then alter the presentation of these proteins and result in improper synaptic development. Another possibility is that mutated H2-Kb is presenting a new protein or set of proteins that alter its role in synaptic development. Future studies to isolate and identify what is presented on MHC I proteins in the CNS
from wildtype and mutated forms may begin to answer these questions. Additionally, future single cell sequencing experiments will offer more information on the expression patterns of MHC I proteins in the CNS including cell-to-cell variability within neuron types and across neuron types.

Our data demonstrates that classical MHC I isoform H2-Kb is expressed in the hypothalamus and colocalizes with synaptic proteins. Additionally, we show that mutation of H2-Kb leads to a profound progressive adult-onset obesity phenotype with characteristics of human metabolic syndrome. Obesity generation in H2-Kb<sup>bm1</sup> mice is likely due to deficits in the orexin system resulting from reduced excitatory drive onto orexin neurons in the lateral hypothalamus. The implication that immune proteins, particularly class I MHC proteins, may play a role in regulating acute and chronic synaptic architecture of the hypothalamus may open an entire new set of therapeutic targets for treating energy balance deficits including obesity.
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Overall Discussion

The works presented in this dissertation relate to a distinct theme that we believe underlie functional deficits associated with aging. In the most simplistic of terms, our data demonstrates that altered PRR activity in the brain leads to structural and functional changes in neurons resulting in behavioral deficits. The loss of Tlr2 and C3, and the mutation of MHC I H2-Kb all lead to downstream deficits in behaviors, albeit distinctly different behaviors. Furthermore, increased expression of these proteins is correlated with age-related functional deficits likely originating from the same brain regions with altered expression during aging. It is interesting to highlight the behavioral changes in context of the change in protein expression. To start, aging is associated with fragmentation of active states as described in Chapter 2 and increased hypothalamic expression of Tlr2. When Tlr2 is genetically deleted from mice, we observed consolidation of active states (i.e. reduced fragmentation). MHC I protein isoform H2-Kb is also elevated in the aged hypothalamus in mice with significant weight loss, coupled with altered energy balance regulation. When H2-Kb is mutated in mice, it results in a progressive obesity phenotype with multiple similarities to metabolic syndrome in humans. From these two models it appears that loss or mutation of immune proteins results in phenotypes opposite of those observed in our aged mouse models. However, loss of C3 or increased C3 expression in aging are both associated with synaptic phenotypes and locomotor impairments characterized by signs of gait ataxia. Therefore, modeling age related functional disorders is not a simplistic as the presence of absence of immune protein expression.

The effect of protein loss on functional behaviors may be dictated by different functional properties of immune proteins. MHC I and Tlr2 are transmembrane surface receptors with intracellular signaling capabilities, whereas C3 is a secreted protein that functions through activation of surface receptors and as an opsonin. Developmentally,
C3 functions in targeting synapses for pruning, as well as regulation of neuronal properties through binding of C3R\textsuperscript{1, 2}. In aging, the function of C3 in the brain is less clear. It is possible that C3 is increased in the aging brain in response to NF-κB activation by immune protein stimulation and works as a compensatory mechanism to remove dysfunctional synapses or cell debris. In support of this idea, data has shown that C3 expression is regulated by NF-κB activity. Our data demonstrates that C3 transcript expression is increased in response to Tlr2 ligation. Therefore, there may be different mechanisms underlying locomotor deficits in the aged brain and those in C3 deficient mice. The complex interplay of immune proteins and PRRs in the establishment of immune phenotypes has yet to be adequately detailed. However, it is intriguing to postulate that within proteins increased in aging, there are both those that function as drivers and brakes on CNS regulation.

It must also be appreciated that immune proteins appear to be regionally important in CNS regulation. Our data demonstrates that loss of Tlr2 and mutation of MHC I H2-Kb both affects hypothalamic functions, while C3 affects cerebellar function. Loss of Tlr2 and mutation of H2-Kb affect hypothalamic regulated behaviors tied to lateral hypothalamic function, but differ greatly in the phenotypic outcomes. Tlr2 deficient mice show consolidations of active and inactive states, suggesting increased stability of orexinergic signaling. Whereas, mutation of MHC I H2-Kb has the profound effect of drastically reducing orexinergic signaling, leading to progressive obesity and increased sedentary activity. Conversely, loss of C3 protein did not appear to alter hypothalamic function, as C3 mice overall did not demonstrate significant changes in metabolism, adiposity, or large changes in feeding behaviors in the HCM system. Loss of C3 did lead to significant impairments in locomotor functions including reduced gait speed and characteristics of gait ataxia. Our data is focused on hypothalamic and cerebellar
functions, but altered expression of immune proteins have also been linked to changes in other brain regions, including those involved in cognition\textsuperscript{3-7}.

The timing for when immune protein expression are altered (\textit{ie.} prenatal, postnatal, adult, aged) may also be critical to the development of behavioral deficits. Increases in immune protein expressing in the brain occurs gradually with aging, as does the expression of potential endogenous agonists. Interestingly, there is significant variability in the timeline of synaptic phenotypes present in immune protein knockout models. Complement 3 deficiency results in altered synapse density in the visual cortex as early as postnatal day 10\textsuperscript{1}; whereas, no changes are noted in the hippocampus until 4 months of age\textsuperscript{4}. Additionally, we noted no gross synaptic changes in the cerebellum at 1 month or 3 months of age. Similarly, mutation of MHC I proteins have been associated with postnatal synaptic changes in hippocampal neurons\textsuperscript{7}, as well as changes in 12 month old adult mice, but no apparent deficits at 6 months of age\textsuperscript{8}. These data demonstrate that immune proteins likely function in both the establishment and the maintenance of synaptic networks; therefore altering their expression at any point in life may have significant impact on neuronal functions. We propose that the gradual accumulation of extracellular agonists, endogenous or otherwise, drive dysregulation of cell signaling necessary for proper neuronal function through stimulation of immune receptors. Our analysis was centered around NF-\kappa B dysregulation based on previous data. However, other pathways are likely involved in addition to NF-\kappa B. Our protein array data suggests other potential target pathways including MAPK signaling, cell response to oxidative stress, and TGF-\beta signaling, among others.

Finally, it is important to recognize that ‘no cell is an island’, and environmental changes play crucial roles in regulating cell signaling. The classic functional perspective for all of the proteins we assessed is rooted in their role within the immune system and
response to infectious stimulus. While it was only mentioned briefly throughout the previous chapters, it must be stated that infectious states can have large effects on the expression levels of immune proteins. Expression levels of toll-like receptors, complement proteins, and MHC I can all change in response to infection\textsuperscript{9-16}. Interestingly, MHC I expression can be downregulated in response to specific viruses\textsuperscript{17-27}. Based on this evidence, and what we know about the function of immune proteins in synaptic regulation, it is possible that infectious states alter neuronal network functions both acutely and chronically. An acute effect of infection on neuronal function is supported by evidence demonstrating that sickness behaviors are linked to suppression of orexin signaling in the lateral hypothalamus\textsuperscript{28} and in part regulated by toll-like receptors\textsuperscript{29-32}. To date, much less known about the chronic effects of infections on neuronal structure and function. However, in support of chronic changes to hypothalamic function resulting from immune system insults, exposure to early life infection can lead to adult onset obesity in animal models\textsuperscript{33-36}. Hopefully, future studies will be able to determine if there is any correlation within pre- or postnatal infection and adult obesity in humans.

Overall, the study of immune protein roles in the maintenance of neuronal structure and function is in its relative infancy compared to what is known about the same proteins in the periphery. We believe that our data adds another level of appreciation to the complex functions immune proteins play in CNS regulated behaviors associated with daily living and a healthy lifestyle. It is our hope that future work in these areas will offer novel targets to combat functional declines in mobility and energy balance.
References


