BIOBEHAVIORAL RESPONSES TO IMMUNE SKEW AND FREE FATTY ACIDS

BY

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DISSERTATION

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ABSTRACT

A well-described set of biobehaviors and cognitive dysfunction emerge after stimulation of the immune system with pathogen-associated or danger-associated molecular patterns, and the endogenous cytokines elaborated in the immunologic response are key effectors (Dantzer et al., 2008). Just as an organism’s response to a pathogen is defined by the host immune system status, so too is the basal cytokine environment altered by the endogenous immune skew (Gordon and Martinez, 2010). Shifting of cytokine skew has the capability to affect not only the immune and behavioral responses to exogenous stimuli, but to potentially alter biobehaviors observed in the naïve state (Moon et al., 2011).

To test our hypothesis that skewing the immune system can alter biobehaviors exhibited in mice, we utilized two distinct models. In Chapter 2, the project of endogenous immune skew, interleukin-4 (IL-4) knock-out (KO) mice were behaviorally phenotyped. We have found that IL-4 KO animals display differences in burrowing, social exploration, elevated zero maze and the open field test, without any depressive-like behavior (forced swim test (FST), saccharin preference) or cognitive dysfunction (novel object recognition, novel object location, Morris water maze). In Chapter 3, the second project utilized a novel exogenous stimulus, palmitic acid (PA). We observed a dose-dependent decrease in home cage locomotion was noted two hours after PA treatment. This locomotor deficit was not dependent on canonical proinflammatory signaling pathways (Toll-like receptor 4, Myeloid differentiation primary response gene 88, interleukin-1 receptor 1, interleukin-6, Tumor necrosis factor α) and was not PA specific. After resolution of acute effects, PA treated animals displayed anxiety-like behavior (elevated zero maze, novel object investigation) but not depressive-like behavior (FST) or cognitive deficit (Y
maze). Alterations to neurotransmitter balance in PA-treated mice were demonstrated in the amygdala and hippocampus.

In conclusion, we demonstrated unique behavioral phenotypes in our endogenous immune skew and exogenous stimulus models. While these two approaches differed in some parameter responses, both models resulted in anxiety-like biobehaviors in mice. Taken together, these results suggest that a spectrum of effectors can lead to a common behavior. Further investigation is warranted to determine if and when common pathways for the observed behavior emerge, which could potentially lead to novel pharmaceutical treatment targets for anxiety and related psychopathologies.
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# TABLE OF CONTENTS

CHAPTER 1: LITERATURE REVIEW .......................................................................................... 1

CHAPTER 2: IL-4 KNOCK-OUT MICE DISPLAY ANXIETY-LIKE BEHAVIOR
WITHOUT COGNITIVE DEFICIT .................................................................................. 20

CHAPTER 3: THE SATURATED FATTY ACID, PALMITIC ACID, INDUCES
ANXIETY-LIKE BEHAVIOR IN MICE ................................................................. 33

CHAPTER 4: CONCLUSION .............................................................................................. 50

FIGURES AND TABLES .................................................................................................. 54

REFERENCES ................................................................................................................. 68
CHAPTER 1: LITERATURE REVIEW¹

Summary

The macrophage (MΦ) is an essential cellular first responder in the innate immune system, sensing, alerting, removing and destroying intrinsic and extrinsic pathogens. While congenital aplasia of granulocytes, T or B lymphocytes leads to serious disease, lack of MΦs is incompatible with life. The MΦ, however, is not a monomorphic entity. These constructers, repairers and defenders of the body are diverse in form and function. What controls MΦ phenotype is beginning to be understood and involves a complex interplay of origination, location and microenvironment. Common to all MΦ developmental pathways are pro-inflammatory and anti-inflammatory cytokines. MΦs respond to these bioactives in distinct ways developing recently recognized activation phenotypes that canonically support bacterial clearance (classical activation), parasite defense/tissue repair (alternative activation) and anti-inflammation (de-activation). Critically, the same cytokines which orchestrate immune defense and homeostasis dramatically impact sense of wellbeing and cognition by eliciting sickness symptoms. Such behaviors are the manifestation of pro/anti-inflammatory cytokine action in the brain and are a direct consequence of MΦ function. This review describes the “new” archetypal MΦ activation states, delineates microglia phenotypic plasticity and explores the importance of these macrophage activation states to sickness behavior.

**Introduction**

Clinical manifestations of infection and inflammation have been recognized since antiquity — pain, swelling, redness, loss of function and fever that are known to be accompanied by behavioral manifestations — malaise, lethargy and anorexia. These collective sickness behaviors are driven by cytokines, and are an integral part of the acute phase response initiated by innate immune effectors, especially phagocytic cells (Johnson, 2002; Kelley et al., 2003). Sickness behavior comprises an intrinsic motivational state responsive to infectious agents (Hart, 1988; Dantzer et al., 2008). It is probable that as part of the acute phase response to infection, sickness behavior evolved as a mechanism for reducing pathogen load or minimizing pathogen damage (Read et al., 2008; Ra˚berg et al., 2009). While these immune-associated biobehavioral manifestations (immunobehaviors) may confer a survival advantage to the host, those that linger unresolved after elimination of infection may be maladaptive and contribute adversely to conditions such as ageing, major depressive disorder and obesity.

The immune system defends the body against pathogenic insults arising from both the external and internal environment. Immune responses are divided into innate and adaptive, with an intricate interchange between the two systems. While adaptive immunity utilizes somatic recombination and mutation to generate antigen-specific antibodies and T-cell receptors (TCRs), innate immunity relies on pattern recognition receptors (PRRs) encoded in the germline to recognize conserved motifs commonly displayed by pathogens or dangerously dysfunctional or transformed cells. PRRs are somewhat promiscuous in comparison with the specificity of immunoglobulins and TCRs, but they are immediately available within and on the surface of cells, especially phagocytic dendritic cells, neutrophils and macrophages (MΦ) (Flannagan et al.,
2009; Spits and Di Santo, 2011) that function in barrier maintenance and immune surveillance. These phagocytes are critical to both health and disease, ensuring recognition and removal of pathogens (Flannagan et al., 2009) and cellular debris (Mosser and Edwards, 2008). PRR ligand interactions stimulate the production of cytokines, which modulate function of both innate and adaptive immune cells. It is cytokine-mediated communication within the neuroimmune system that is key to optimal immunological progression from innate to adaptive when pathogens challenge a host.

While the host cytokine response to a pathogen challenge amplifies immunologic function and fosters immune system coordination, it also generates sickness behaviors (Dantzer and Kelley, 2007). As key cytokine producers, MΦs play a role in the balance of sickness and health through influencing immunobehaviors. Therefore, the degree and type of response displayed by MΦs has the potential to alter physical, emotive and cognitive function. Here we provide an overview of MΦs, MΦ activation states, and the interplay between MΦs and other immune cells. We also discuss MΦ cytokine profiles, the requirement of said cytokines for sickness behavior, future research and translational approaches to capitalize on MΦ plasticity.

**Background**

Originating from hematopoietic-derived stem cell progenitors in the bone marrow, committed progenitor cells develop into separate subpopulations of granulocyte-MΦ precursors or MΦ/dendritic cell (DC) progenitors (MDPs) (Geissmann et al., 2010), which then differentiate into monocytes or common DC precursors. Monocytes emigrate from the bone marrow and circulate in the blood for 1—3 days. Circulating monocytes may be attracted to an area of
inflammation by chemokines, then utilize surface molecules such as integrins to adhere and migrate into the tissue (Imhof and Aurrand-Lions, 2004), where interferon (IFN)-γ, interleukin (IL)-6 and macrophage colony-stimulating factor promote differentiation into MΦs (Delneste et al., 2003). A fraction of circulating monocytes are available to become resident, tissue-specific MΦs (Gordon and Taylor, 2005), such as those of the lung and liver. Brain-resident MΦs, called microglia, are a unique subset of these resident cells. Whereas other tissue-resident MΦs can be renewed by circulating monocytes, hematopoietic progenitors do not significantly add to the microglial population after birth (Ginhoux et al., 2010). Rather, myeloid progenitors are believed to establish the adult microglial population during early peri-natal development and proliferate locally, although the exact nature of this process is still unclear.

Circulating monocytes and tissue MΦs survey the body and are the first cellular responders of the innate immune system, capable of a variety of actions that depend on the nature of the threat. Resident tissue MΦs can recognize and phagocytose invading organisms, encapsulating the invader in an intracellular vesicle known as a phagosome. Such engulfed materials elicit responses designed to neutralize the pathogen (Flannagan et al., 2009). Recognition of pathogen-associated molecular patterns (PAMPs) on the surface of the invaders, as well as endogenous danger-associated molecular patterns (DAMPs) produced in response to insults, is the role of extracellular and intracellular PRRs. The PAMPs that PRRs can identify are conserved microbial molecular patterns that seldom grossly mutate, allowing for generalized binding to a number of pathogens without prior exposure (Martinon et al., 2009). Signaling through these receptors triggers a number of bodily defense coordinators, including cytokines, chemokines, adhesion molecules and enzymes essential to host maintenance and protection
(Rock and Kono, 2008; Fukata et al., 2009). MΦs also process phagosomal contents and present the resulting antigens on their surface thus activating cells of the adaptive immune system (Gordon, 2007). Phagocytic capability of the MΦ is critical to both health and disease, ensuring recognition and removal of pathogens (Flannagan et al., 2009) and cellular debris (Mosser and Edwards, 2008). MΦ cytokine production is also key to the pathogen response, impacting bodily processes both immune and non-immune, and it is this chemical communication that allows for coordination between disparate cells and distant sites. It has recently become clear that MΦs are not monochromatic in their effector response, but demonstrate a spectrum of novel activation profiles that alter the message they communicate. This concept of phenotypic alteration of immune cells was noted first in the Th cell where cytokines and other signals in the local environment interacted to drive Th subset differentiation defined by specialized cytokine profiles designed to execute specific immune tasks (Mosmann and Sad, 1996). Since this discovery, it is now increasingly evident that MΦs exhibit a similar responsiveness to signals received. Cytokines from multiple immunologic sources and other stimuli, including PAMPs on infectious and gut commensal microorganisms, synergize to alter the gene expression profiles of MΦs (Fig. 1.1), resulting in varied responses to immune activation (Gordon and Martinez, 2010). This gradient or ‘skewing’ of phenotypes is clarifying how MΦs carry out their manifold functions within the innate immune system. It is still not clearly defined, however, how this MΦ skewing relates to immunobehaviors.

**Classical versus non-classical MΦ activation**

Macrophages were recently shown to exhibit heterogeneous responses to stimulation, and classification of MΦ activation subsets somewhat parallels Th1 and Th2 polarization (Mills et
The first distinctly defined pathway of MΦ activation was classical activation (M1) (Gordon and Martinez, 2010). The M1 phenotype is co-induced from monocytes/resting macrophages by PAMPs and IFN-γ. Natural killer (NK) cells produce a transient burst of IFN-γ that primes MΦs, and Th1 cells contribute additional IFN-γ responsible for phenotype maintenance (Mosser and Edwards, 2008). M1 cells use arginine as a substrate for inducible nitric oxide synthase (iNOS, aka NOS2) resulting in high-output production of microbicidal nitric oxide (NO) (MacMicking et al., 1997). In contrast to classical activation, the alternative activation (M2) pathway is, canonically, a response to parasitic infestation or tissue damage. The M2 phenotype is supported by IL-4 and IL-13 elaboration from Th2 cells, which promote a humoral response against extracellular pathogens. M2 cells use arginine as a substrate for arginase 1 (Arg1), degrading arginine to ornithine, which is a precursor for polyamines, proline, and collagen needed in wound healing (Gordon and Martinez, 2010). In addition to classification by cytokine-induced activation and arginine metabolism, M2 cells were also marked by high expression of the MΦ mannose receptor (MR) which is important for cell—cell recognition, serum glycoprotein turnover, and neutralization of pathogens through recognition of complex carbohydrates on glycoproteins (Lee et al., 2002; Colton, 2009).

As immunologic characterization has progressed, a polarized dichotomy is no longer sufficient to describe all types of Th cells. New T helper subsets have been identified, including Th17 and regulatory T cells (Treg) which are necessary to the elimination of extracellular pathogens and modulation of immune function, respectively. In addition, other possible T cell subtypes may exist such as Th3 and Th9 (Mosmann and Sad, 1996; Zhu et al., 2010), although the importance of these Th phenotypes has yet to be fully elucidated. Similarly, the functional
categorization of MΦs covers a complex landscape involving resident cells and elicited cells in homeostatic offense, immune defense, and disease-related dysfunctions. Recently, it has been recognized than many immune cells contribute to the wide range of MΦ activation states (Fig. 1.1), including NK cells, neutrophils and other macrophages (Tsuda et al., 2004). When Mills et al. proposed the M1—M2 dichotomy they recognized the definitions could represent ends of a continuum of MΦ activation phenotypes. Further study of the diversity of MΦ responses to activating stimuli have peppered that continuum with M1 and M2 subsets characterized by differences in activating cytokines, receptor expression, and chemokine response (Martinez et al., 2009). Most notable of these divisions is the deactivated MΦ (dMΦ). Previously considered M2 or an M2 variant, the dMΦ exhibits a unique phenotype (Table 1.1). This skew is induced in large part by TGF-β and IL-10 from Treg cells, a tolerogenic Th cell generated through exposure to parasites and harmless microorganisms as described by the hygiene hypothesis (Rook and Lowry, 2008; Rook et al., 2011). Additional MΦ deactivating molecules exist and are produced from multiple sources immune and non-immune. These bioactives include glucocorticoids and prostaglandins (Fig. 1.1). dMΦs promote anti-inflammation including enhanced IL-10 and decreased IL-12 release (Mosser and Edwards, 2008). MΦs associated with cancer are often deactivated in the tumor microenvironment contributing to neoplastic growth (Jaiswal et al., 2010). IL-10 released after immune stimulation may also aid in resolution of inflammation after immune challenge (Liu et al., 2006; Pils et al., 2010).

Non-classical activation phenotypes alter the responses of the MΦ to various stimuli when compared to M1. MΦs primed with IL-4, IFN-γ or IL-10 exhibit distinct basal gene profiles. Treatment of M1s with lipopolysaccharide (LPS, also known as endotoxin), an
immunogenic component of Gram-negative bacteria cell walls, induces secretion of tumor necrosis factor (TNF)-α and IL-1β. Upon LPS stimulation, dMΦs produce less TNF-α and IL-1β (Dagvadorj et al., 2008; Schaljo et al., 2009; Chusid et al., 2010) and IL-1β signaling is decreased (Strle et al., 2008). While M2s treated with LPS still produce pro-inflammatory cytokines with similar time kinetics to M1s, the quantity released is lower (El Chartouni and Rehli, 2010; Gratchev et al., 2006). However, this phenotype of reduced inflammatory mediator production can be reversed. Changing the cytokine environment alters established MF phenotypes (Stout et al., 2005) suggesting that an immediate response to an inflammatory stimulus may be drift to non-classical MΦ phenotypes relative to M1. Additionally, a prolonged exposure to a new cytokine milieu can further skew or re-skew the MΦ activation profile.

While much of the work involving MΦ activation is focused on the periphery, microglia, the resident MΦs of the central nervous system (CNS), also exhibit a number of activation states. As with peripheral MΦs, microglia respond to the prevailing cytokine milieu and display markers of M1, M2 or deactivation (Colton, 2009). This is important because microglia complete immune-to-brain communication by releasing a variety of pro-inflammatory bioactives in response to pro-inflammatory peripheral signals (Dantzer et al., 2008). Thus, MΦ activation states of both the peripheral and neuroimmune systems should drastically alter the behavioral response to inflammation.

Microglia activation can be skewed in a manner similar to that of peripheral MΦs, and microglial phenotypic skew may potentially influence central response to an immune challenge. Microglia and peripheral MΦs treated with IL-4 and IL-13 display similar gene profiles (Colton
et al., 2006), indicating that Th2 cytokines can drive microglial alternative activation. Similar to peripheral MΦs, pretreatment of microglia in culture with IL-4 mitigates the induction of TNF-α and iNOS after LPS stimulation (Kitamura et al., 2000). Resident microglia also express the M2 markers Ym1 and Arg1, and lack NO production in an IL-4 dependent manner (Ponomarev et al., 2007). In addition to its direct action on microglia, IL-4 increases expression on neurons of CD200, a membrane glycoprotein that inhibits glial activation (Lyons et al., 2007). The exogenous signals affecting microglia, however, are much different than those of MΦs. Protected by the blood-brain barrier, microglia are not directly exposed to circulating bioactives such as cytokines or PAMPs. Instead, crosstalk between the CNS and peripheral signals occurs in several areas, most notably the subarachnoid space, ventricle walls and choroid plexus (Derecki et al., 2010), and signals from the periphery can induce endogenous CNS mediators by signaling through endothelial cells or reverse neurotransmission via the vagus nerve (Dantzer et al., 2008). Endogenous microglial inflammatory stimuli include neurotransmitter alterations (i.e. glutamate) and ATP. Microglia also respond to loss of neural input becoming repressed in function through tonic stimulation of CX3CR1 (Ransohoff and Perry, 2009). Despite the differences between inflammatory activators of MΦs and microglia, distinct activation states remain a consistent theme in the cellular responses.

**Classical MΦs and sickness behavior**

Animals and people display a set of immune driven manifestations collectively known as ‘‘sickness behavior’’ in response to pathogenic infection. In animals these immunobehaviors include anorexia, anhedonia, decreased locomotion, truncated social exploratory behavior, reduced grooming, diminished burrowing and loss of cognitive ability, all of which can be
induced experimentally by injection of LPS and/or pro-inflammatory cytokines (Dantzer et al., 1998). For many years sickness behaviors were considered maladaptive, but a review by Hart re-imagined these behaviors suggesting that they evolved to cope with disease (Hart, 1988). Hart proposed that increased sleep combined with reduced grooming, feeding and locomotion conserved energy needed to combat infection. Piloerection helped maintain increased body temperature for inhibiting pathogen growth. These ideas were expanded upon, and a detailed review of sickness behavior (Dantzer and Kelley, 2007) summarizes the history of sickness research beginning in the mid-1950s to current theories, with some work emphasizing sickness behavior as a non-specific response, an expression of a motivational state or as part of a pain defense system.

While the evolutionary development of sickness behavior is likely to remain in debate, the mechanisms behind these immunobehaviors have drawn increased interest. Initially, the observation that pro-inflammatory cytokines increased after infection or LPS administration sparked the idea that these bioactives were responsible for the sickness symptoms seen. IL-1β and TNF-α are among such cytokines up-regulated in the brain (Gatti and Bartfai, 1993) and periphery (Zuckerman et al., 1989). Peripheral or central administration of IL-1β and/or TNF-α induce immunobehaviors that mimic those seen in infection or endotoxin administration (Dantzer et al., 1998). How these peripheral signals communicate with the brain to produce behavioral change is still unresolved, although several redundant mechanisms have been proposed. These mechanisms include: (1) cytokine stimulation of afferent nerves (e.g. vagal); (2) cytokine access to the brain at the circumventricular organs (CVO) due to lack of a robust blood-brain barrier; (3) cytokine transport to the brain from the blood via cytokine transporters;
and (4) cytokine stimulation of brain prostaglandins by brain-based endothelial cells and perivascular MΦs (Dantzer et al., 2008). One of these pathways may be principally responsible for the central response to a peripheral infection or they may all work in concert. Regardless of the specific mechanism, MΦs play an important role in mediating the sickness response due to their ability to recognize microbial components and produce pro-inflammatory cytokines that invariably trigger the innate immune system in the brain.

**Non-classical MΦs and sickness behavior**

Since pro-inflammatory cytokine elaboration by M1s induce immunobehaviors, it would seem likely that bioactives released by M2s or dMΦs could potentially modify sickness behavior. Exogenous IL-10 given centrally abrogates both CNS and peripheral LPS-induced loss of locomotor activity and social exploration (Nava et al., 1997; Bluthe´ et al., 1999). The researchers hypothesized that this effect was in part due to the ability of IL-10 to deactivate MΦs and inhibit the activity and production of pro-inflammatory cytokines. Indeed, IL-10 knock out (KO) mice challenged peripherally with LPS exhibit a prolonged and exaggerated proinflammatory cytokine induction in plasma and brain when compared to wild-type controls (Krzyszton et al., 2008; Richwine et al., 2009), and this increased inflammatory state is associated with deficits of cognitive and motor function.

As with dMΦs, there is a paucity of research specifically investigating behavioral effects of M2 activation. Currently, МΦ phenotype research focuses on immunological response over biobehavioral change. A review of the literature finds few studies directly concerned with alterations in behavioral responses due to МΦ skew. Based on previous studies demonstrating a
link between adaptive immunity and cognition, Derecki et al. (2010) investigated the mechanism for this phenomenon finding it regulated by T cell-derived IL-4. Through the use of T cell deficient mice and adoptive immune cell transfer, they determined that IL-4-producing Th cells are recruited to the subarachnoid spaces, ventricle walls, and choroid plexus after a cognitive training task. This aforementioned T cell response was shown to promote brain-derived neurotrophic factor (BDNF) production by astrocytes as well as regulate the inflammatory phenotype of meningeal myeloid cells. Interestingly, these studies demonstrated that M2s appear to act in a T cell-independent manner to improve cognitive function in T cell deficient mice (Derecki et al., 2011).

In addition to the work by Derecki et al., we have investigated diet-induced immune alterations in endotoxin mediated sickness. This research showed that feeding mice a diet supplemented with the soluble fiber pectin protects mice from LPS-induced sickness behavior (Sherry et al., 2010). The pectin fed mice displayed basal up-regulation of IL-1 receptor antagonist (IL-1ra) and IL-4, a Th2 skew, and peritoneal MΦs expressing M2 markers that, when stimulated ex vivo with LPS, demonstrated dampened pro-inflammatory cytokine production. When the soluble fiber diet was fed to IL-4 KO mice, the effect of pectin on Th cell skew and sickness behavior was reduced, suggesting that IL-4 was key.

While the Derecki et al. and Sherry et al. studies may be unique in their aims to directly investigate the impact of M2 activation on behavior, they are not alone in revealing a MΦ activation state effect on immune-mediated sickness. Other researchers have essentially studied the skewing mechanism without measuring standard MΦ activation state markers. One such area
of investigation has examined the roles of IL-4 and IL-13 through exogenous administration of these bioactives as well as exploring their function in KO mice. IL-4 KO mice administered intraperitoneal LPS showed a greater reduction in exploratory behavior than wild-type controls, and this was associated with attenuated proinflammatory cytokine production by glia (Lyons et al., 2009). Intracerebroventricular administration of IL-4 12 h prior to intraperitoneal LPS diminished the endotoxin-induced decrease in social exploration in rats (Bluthe´ et al., 2002). However, intracerebroventricular administration of IL-13 prior to intraperitoneal LPS had no effect. When LPS was administered concurrently with IL-13, behavioral changes potentiated (Bluthe´ et al., 2001). The above findings raise the possibility that IL-13 is not as effective as IL-4 in inducing the M2 phenotype. Additionally, the work by Bluthe´ et al. found that co-administration of intracerebroventricular IL-4 or IL-13 with intraperitoneal LPS served to enhance LPS-induced sickness behavior, and research from a different group found that intracerebroventricular IL-4 during high fat diet feeding increased hypothalamic pro-inflammatory cytokine gene expression (Oh-I et al., 2010). These results indicate that whereas a pre-existing cytokine-induced M2 phenotype may reduce the classical response, IL-4 or IL-13 may serve to enhance the inflammatory functions of MΦs when provided concurrently with an immune stimulus.

Another wide-ranging line of research involving immuno-behaviors concerns those elicited by IL-1. While not directly examining M1 versus M2 skew in sickness, extensive work has been performed detailing MΦ generated IL-1. The IL-1 system is very important to immunobehaviors. Similar to LPS, IL-1β reduces rodent activity in an open field and decreases 24 h food intake and body weight (Wieczorek et al., 2005). In opposition to IL-1β and the
lesser-studied IL-1α, is the endogenous IL-1 receptor antagonist, IL-1ra, and the decoy receptor IL-1r2. These bioactives modulate the IL-1 system and appear to be critical to inflammation down-regulation and/or resolution. Treatment of human monocytes with IL-13 enhanced IL-1r2 and IL-1ra expression and decreased the activity of caspase-1, the enzyme responsible for production of mature IL-1β (Scotton et al., 2005). Studies in diabetic mouse models, which exhibit IL-4 resistance, showed that such mice fail to appropriately up-regulate IL-1ra and IL-1r2 in response to immune challenge (O’Connor et al., 2005). Therefore, M2s may not only reduce their production of IL-1β but also increase production of IL-1 antagonists.

Peripheral IL-1ra is effective at blocking the decrease in social and food motivated behavior after peripheral IL-1β administration. Furthermore, centrally administered IL-1ra has been repeatedly shown to block the decrease in social exploration induced by both central and peripherally injected IL-1β (Kent et al., 1992; Bluthe´ et al., 1995, 1997). Neutralizing peripherally administered IL-1β appears sufficient to ablate certain sickness behaviors. Furthermore, IL-1 signaling in the brain appears essential for IL-1β-induced immunobehaviors, as centrally administered IL-1ra inhibits the effects of both intraperitoneal and intracerebroventricular delivered IL-1β. Decrease in sweetened milk intake after LPS or influenza infection is attenuated by intraperitoneal IL-1ra (Swiergiel et al., 1997), and peripheral LPS-induced depression in food intake is partially reversed. Up-regulation of pro-inflammatory cytokine expression in the hypothalamus is blocked by intracerebroventricular IL-1ra (Laye´ et al., 2000), suggesting that IL-1 is important to but not solely responsible for LPS- and infection-related sickness behavior. Obesity induced by a high fat diet (HFD) augments serum IL-1ra, and obese mice recover (after a hypoxia challenge) social exploratory activity five times faster than
mice fed a normal low fat diet (LFD) (Sherry et al., 2009). This effect of a HFD was attenuated by neutralization of IL-1ra with administration of an anti-IL-1ra antibody. Interestingly, the source of this endogenous IL-1ra is visceral fat M\(\Phi\)s but the activation state of these fat-based M\(\Phi\)s is still unclear (Lumeng et al., 2007; Zeyda et al., 2007). In fat from HFD-fed mice, M2 markers are substantially increased as is the “dendritic cell marker” CD11c leading some to term these M\(\Phi\)s “non-inflammatory M1-like M\(\Phi\)s” (Li et al., 2010; Shaul et al., 2010). These unique fat-based M\(\Phi\)s appear tied to insulin resistance and may be the source of IL-1ra in HFD-fed mice.

Exogenous cytokine administration has shed some light on M\(\Phi\) skew in sickness behavior. However, further investigation of specific M\(\Phi\) phenotypes is necessary, and an animal model with natural M\(\Phi\) skewing would be valuable to understanding the role of M\(\Phi\) activation states in immunobehaviors. Different strains of mice show disparate responses to immune stimuli, and an important strain comparison for this review is between C57BL/6 and Balb/c mice. Treatment of lymphocytes isolated from spleen with the mitogen Concanavalin A elicits cytokine production. Stimulated splenocytes from C57BL/6 mice secrete high IFN-\(\gamma\) and low IL-4 indicating a Th1 skew, whereas an opposite cytokine ratio is observed in splenocytes from Balb/c mice (Mills et al., 2000). As mentioned earlier, cytokines produced by Th cells influence the activation of M\(\Phi\)s, so it would be expected that the M\(\Phi\) populations of C57BL/6 and Balb/c mice would exhibit different skews. Mills et al. has shown that in response to ex vivo stimulation with IFN-\(\gamma\) or LPS, C57BL/6 M\(\Phi\)s demonstrate an increase in iNOS and decrease in arginase activity, while Balb/c M\(\Phi\)s show only an increase in arginase. A comparison of C57BL/6 and Balb/c mice could, therefore, be an important bridge between M\(\Phi\) activation state and
immunobehaviors. Unfortunately, research comparing the behavior of these two mice strains has been limited, largely confined to anxiety, stress and memory. Cognitive deficit induced by stress was seen in Balb/c but not in C57BL/6 mice (Palumbo et al., 2010), and Balb/c mice showed higher home cage but lower open field activity (Tang et al., 2002). These results suggest basal and stimulated behavioral differences between mice strains, thus, use of naturally skewed mice could add significantly to the knowledge base regarding MΦ-mediated behavioral responses.

Another mouse model with potentially disparate MΦ activation states is that of young versus aged mice. Aged mice exhibit an increase in pro-inflammatory cytokine production and glial reactivity markers in the basal state (Sparkman and Johnson, 2008). Additionally, microglia in aged animals show the primed response (Godbout et al., 2005) characterized by exaggerated cytokine elaboration to innate immune challenge similar to what is observed in mouse models of chronic neurodegeneration (Combrinck et al., 2002; Cunningham et al., 2005). Aged animals challenged with LPS display prolonged sickness, higher cytokine induction and more severe cognitive deficits when compared to young animals (Godbout et al., 2008; Huang et al., 2008; Wynne et al., 2010). Depressive-like symptoms are also exacerbated in LPS treated aged mice compared to young mice evidenced by increased immobility in the forced swim and tail suspension tests (Godbout et al., 2008). Down-regulation of IL-1 signaling, either through IL-1ra administration or dietary resveratrol, an antioxidant that reduces LPS-generated IL-1, partially resolves the enhanced sickness behaviors seen in aged animals (Abraham and Johnson, 2009a,b). Aged mice also exhibit a longer diminution in locomotor activity compared to young mice in response to exogenous IL-1β (Sparkman and Johnson, 2008). Therefore, given a basal pro-inflammatory cytokine skew, enhanced microglial sensitivity to LPS and up-regulation in the
IL-1 signaling system, aged mice appear skewed M1. Whether this is due to loss of M2s/ dMΦs or due to a unique aged MΦ phenotype is not yet known but further research regarding MΦ phenotypes in ageing is important due to the increased incidence of neuroinflammation-associated disorders seen in elderly and diabese human populations.

**Concluding remarks**

MΦs appear critical to sickness behavior as evidenced by MΦ depletion studies causing an ablation of several LPS-induced immunobehaviors (Groeneveld et al., 1988), but MΦs as the sole cause of sickness behavior has yet to be proved definitively. While it is known that MΦ stimulation elicits cytokines and that these cytokines induce immunobehaviors, the connection between MΦs and sickness requires further study. Important questions still to be answered are whether MΦ-derived cytokines are exclusively responsible for immunobehaviors and what the roles are of various cytokines and cytokine sources in initiation, propagation, and termination of sickness. Through the use of radiation depletion, it was demonstrated that TLR4 on non-hematopoietic cells is essential for endotoxin-induced CNS inflammation, and this response is independent of systemic cytokines (Chakravarty and Herkenham, 2005). While this study showed a role for non-blood borne TLR4 in CNS inflammation, the specific cells in the CNS critical to actuating immunobehaviors remains unclear. Experiments critical to future research in sickness would benefit from the development of tools to knock-out MΦs, MΦ PRRs and MΦ cytokine production in an inducible and tissue/cell-specific manner.

In addition to examining the importance of MΦs and their skew to sickness behavior, further investigation is needed to unlock the secrets of human MΦs and translate activation states
observed in animal models to the clinical setting. Some markers of MΦ activation, such as nitric oxide production, may not be as important in humans as it is in mice. However, should it be found that human MΦ phenotypes reflect those observed in animal models, this would be very important to human health. Classic sickness behavior, dysregulated inflammation and cytokines are associated with depression in humans (Dantzer et al., 2008). Nearly 45% of malignant melanoma patients receiving interferon-α experience major depression (Musselman et al., 2001). Rates of depression are also higher in patients suffering from chronic inflammatory disorders, such as Alzheimer’s disease (Colton et al., 2006). Since MΦs and their precursors are relatively easy to isolate and culture from the human body (Davies and Gordon, 2004), these cells make therapeutic ex vivo bioengineering possible, where MΦs can be manipulated outside the body and then returned to the patient (Leor et al., 2006).

Whether sickness behavior is an adaptive trait that isolates the ill from the non-ill to curb the spread of infectious disease or is part of a reparative/regenerative process that is necessary to efficiently combat illness is open to debate. What is not in dispute is that cytokines such as those derived by MΦs are required for immunobehaviors. MΦs integrate a vast array of dynamic microenvironmental signals then adapt to coordinate the immunologic response to a changed physiologic state or microbial invasion. Given the different milieus in which MΦs reside, their phenotypes likely span an enormous spectrum controlled by things as pedestrian as pH and as exotic as strategic metals. To deconstruct this complexity it is reasonable to understand MΦ activation states as a trinity of archetypal phenotypes (M1, M2, and deactivated). However, this simplified scheme cannot predict why influenza can cause mild flu-like symptoms in some and potentially fatal cytokine storm in others. Neither can it answer other key questions including:
Do macrophage phenotypes impact mood or behavior? Or is the macrophage altering condition of the overweight/obese state manifest as a biobehavioral sickness. Thus, more research is needed to understand how and why macrophages make me sick and if these phenotypes can be manipulated in a therapeutic fashion to make me feel better.
CHAPTER 2: IL-4 KO MICE DISPLAY ANXIETY-LIKE BEHAVIOR WITHOUT COGNITIVE DEFICIT

Abstract

Endogenous immune skew has the potential to impact biobehaviors by altering naïve cytokine concentrations. As previous research has demonstrated cognitive deficits in interleukin (IL)-4 deficient mice, we sought to determine the complete biobehavioral phenotype in these animals. IL-4 knock-out (KO) animals were compared to wild-type (WT) C57BL/6 control mice in a behavior phenotype battery. Burrowing and social exploration were assessed to determine general behavior differences, depressive-like behavior was tested using the forced swim test (FST) and saccharin preference, the Morris water maze (MWM), novel object (NO) and novel location (NL) tests were performed for cognitive function, and the elevated zero maze (EZM) and open field test (OFT) were used to probe anxiety-like behavior. IL-4 KO mice showed altered generalized behaviors, with decreased burrowing and increased social exploration. No differences were seen in depressive-like behaviors or cognitive function between KOs and WT animals as assessed by FST, saccharin preference, MWM, NO and NL. In the EZM, IL-4 KO mice spent less time in the open arm, whereas KO animals spend more time in the center of the OFT without a difference in total locomotion versus WT controls. In conclusion, IL-4 KO mice display a unique biobehavioral phenotype when compared to WT controls, characterized by paradigm-dependent anxiety-like behavior, without differences in depressive-like behavior or

cognition. These data suggest that endogenous immune skew impacts unstimulated biobehaviors in mice.

**Introduction**

The interaction between the peripheral immune system and central nervous system (CNS) is a relatively young area of rapidly expanding focus (Dantzer and Kelley, 2007; Kelley and McCusker, 2014), with links found between inflammation and neurological and psychiatric diseases (Debnath and Venkatasubramanian, 2013; Haroon et al., 2012; Hou and Baldwin, 2012; Irwin and Rothermundt, ; Slavich and Irwin, 2014). Cytokines are thought to be a mechanism of this crosstalk (Dantzer et al., 2008) by altering neuronal functioning directly and indirectly (Felger and Lotrich, 2013; Yirmiya and Goshen, 2011) Specific cytokines have been implicated in biobehavioral complications, most notably the proinflammatory interleukin (IL)-1β and tumor necrosis factor (TNF)-α (Gosselin and Rivest, 2007; McAfoose and Baune, 2009; Wang and Shuaib, 2002). While the role of proinflammatory cytokines in biobehavioral alterations has been thoroughly studied, less is known about anti-inflammatory cytokines in immune-mediated behaviors.

Cytokines such as IL-4 and IL-10 play a critical role in balancing responses of the immune system. Whereas IL-1β and TNFα promote immune activation and proinflammation (Opal and DePalo, 2000), IL-4 and IL-10 are involved in alternative immune responses and resolution of inflammatory events (Dinarello, 2000). In animal models using lipopolysaccharide to stimulate a proinflammatory response with subsequent cytokine-induced behavior changes, administration of exogenous IL-10 or IL-4 have been shown to mitigate the biobehavioral effect
of the stimulus (Bluthé et al., 1999; Bluthé et al., 2002). As the overall balance of immune function and responsiveness is mediated by the endogenous cytokine environment of the organism (Mosser amd Edwards, 2008), a dysfunction in anti-inflammatory cytokine production likely influences naïve biobehaviors similar to their proinflammatory relatives (Moon et al., 2011). Indeed, mice lacking IL-10 display depressive-like and anxiety-like behavior (Mesquita et al., 2008), while IL-4 knock-out (KO) animals reportedly demonstrate cognitive dysfunction (Derecki et al., 2010).

In this study, we expanded upon previous research in IL-4 KO mice. While animals lacking IL-4 have been found to be cognitively impaired, and could be rescued by adoptive transfer of wild-type (WT) T cells (Derecki et al., 2010) or alternatively activated macrophages (Derecki et al., 2011), the full behavioral profile in this strain has not yet been defined. Recent studies in IL-1 receptor KO mice have shown the benefit of complete behavioral phenotyping, as one study found these animals to have hippocampal memory deficits (Avital et al., 2003) while the other demonstrated hyperactivity and decreased anxiety, but without impaired hippocampal-dependent memory (Murray et al., 2013). As behavior testing can be confounded by a number of factors (York et al., 2012), we sought to determine the whole biobehavioral picture of IL-4 KO mice.

**Methods**

*Materials-* All reagents were purchased from Sigma-Aldrich (St Louis, MO).
Animals- Animal use was performed according to protocols approved by the Institutional Animal Care and Use Committee at the University of Illinois. Wild-type C57BL/6J (WT) and interleukin (IL)-4 knock out (KO) mice (on a C57BL background) were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in-house. Mice were provided food and water ad libitum in standard shoebox cages. Animals were group housed (up to 8 per cage), then moved to individual housing the day prior to testing unless noted otherwise. Housing temperature (72 °F) and humidity (45–55%) were controlled, and a 12/12h reversed dark-light cycle (2100–0900 h) was maintained. Animal behavior was video recorded using a Sony HDR-XR500V Night Shot capable video camera (Tokyo, Japan). Mice used were between 8 and 20 weeks of age. All behavior testing was completed under red lights in the dark cycle unless otherwise indicated. A total of 146 mice were used.

Burrowing- As previously described (Kaczmarczyk et al., 2013), mice were individually housed overnight in the presence of a burrow (20 cm long polyvinyl chloride (PVC) piping capped at one and raised 1.3 cm at the open end). Testing was initiated by filling the burrow with approximately 200 g of food pellets. Food was only available from the burrow, but water was provided ad libitum. Mice were allowed to interact with the filled burrow for 10 h, at which point burrowing amount was determined from combined weight of burrow and food before and after testing.

Social Exploration- Social exploratory behavior was performed as we have described (Sherry et al., 2010). Briefly, a novel male 3- to 4-week-old conspecific mouse (challenge mouse) was confined to a 7.62 × 7.62 cm wire mesh enclosure placed in the home cage of the
test mouse. The test mouse was exposed to the challenge mouse and video recorded for 5 min. Investigation time (nose contact) of the challenge mouse by the test mouse was quantified by a blinded observer.

*Forced swim test*- A clean white cylindrical PVC container (diameter 16 cm; height 31 cm) was filled with 20 cm of water maintained at 25 ± 1 °C for each mouse. Mice were moved from their home cage to the test container and video recorded for 5 min. Immobility was evaluated from the video as we have described (Park et al., 2011).

*Saccharin Preference*- Saccharin preference testing was performed as previously described (Lavin et al., 2011). Briefly, mice were singly housed in standard cages with two bottles of water three days prior to testing for acclimation. Testing was started at the beginning of the dark cycle by replacing one of the waters (randomized to right verses left) with a 0.4% sodium saccharin solution (Sigma-Aldrich). Bottles were weighed before and after 24 h of access and total consumption calculated. Results presented as % saccharin preference (saccharin volume divided by total fluid volume consumed).

*Morris Water Maze*- Mice were tested as previously described (Sparkman et al., 2006). Briefly, in a 100 cm diameter by 30 cm deep circular tank filled with water (21-23°C), a 10 cm diameter transparent round platform was placed 0.5-1cm below the water surface. Animals were trained for 4 days (acquisition phase) at three trials per day. Mice were placed in one of three quadrants other than the one containing the hidden platform and allowed to swim for 60 s or until reaching the platform. If mice failed to locate the platform, they were placed on the platform for
30 s. After three consecutive trials mice were returned to their home cage and placed under a heat lamp for 10 min. On day 5 the platform was removed and each mouse was tested (probe trial) for 60 s. On day 6 the platform was placed in the quadrant opposite the original learned quadrant (reversal training) and mice were trained for three trials. On day 7 mice were tested (reversal test). All phases of training and testing were video recorded by a camera mounted above the maze and analyzed using Noldus Information Technology EthoVision XT 7. All testing was performed during the dark phase, with spatial clues illuminated by dim white light.

Novel Location Recognition- Mice were individually housed in a large rat-sized cage (26 cm x 48 cm x 21 cm) with a spatial cue marked on one side. During the training phase, two identical objects were presented to the animal on one side of the cage. After overnight training, the objects were removed from the testing arena and sanitized with ethanol. Testing was initiated after 1 hr. During testing, one of the objects was replaced in the same location as during training (familiar location). The second object was placed on the opposite end of the arena (novel location). Investigation was video recorded for 5 min and evaluated by a blinded observer. Novel and familiar location % investigation was calculated by dividing the time spent examining each location by the total investigation time.

Novel Object Recognition- Novel object recognition was examined as previously described (Chiu et al., 2012; York et al., 2012). Mice were individually housed overnight in a rat cage-sized arena (26 cm × 48 cm × 21 cm) containing two identical objects (training). After overnight training, objects were removed and cleaned and sanitized with ethanol. Testing was initiated after 1 h. During testing, two objects were replaced in the cage, one seen during
training (familiar object) and one unfamiliar object (novel object). Object investigation was video recorded for 5 min and evaluated by a blinded observer. Novel and familiar object % investigation was calculated by dividing the time spent examining each object by the total object investigation time.

Zero maze- As previously described (Kaczmarczyk et al., 2013), a zero maze, with 4 quadrants, 2 of which had high walls (14 cm), was under white light illumination. Mice were placed in the high wall section to begin testing and video recorded for 5 min. Time spent in the non-high wall area (open arm) was quantified by a blinded observer.

Open Field Test- As we have described (York et al., 2012), mice were placed in a novel open field arena (66 cm length x 45.7 cm width x 22.9 cm height) with lighting positioned to generate a 9 cm shadow from each side wall. Mice were allowed to explore the novel arena and video recorded for 5 min. Noldus Information Technology EthoVision XT 7 was used to track the mouse, determining mouse center point movement and time spent in the shadowed and non-shadowed areas of the arena. Results are presented as raw time (s) and distance moved (cm).

Statistics- Data are expressed as mean ± SEM. Analysis was conducted using Sigma Plot 11.2 (Systat Software, Chicago, IL). One-way ANOVA was run for all experiments except Morris Water Maze, which required two-way repeated measures ANOVA for Task Acquisition. Post-hoc comparison used Tukey’s test. Statistical significance was determined as p<0.05.
Results

*IL-4 KO mice display burrowing and social exploration differences*- Figs. 2.1A and 2.1B show IL-4 KO mice display decreased food pellet burrowing (WT vs IL-4 KO, 156.0 ± 18.0 vs 79.8 ± 23.2, p=0.017) and increased social exploration of a novel juvenile (WT vs IL-4 KO, 130.0 ± 5.0 vs 148.4 ± 6.9, p=0.039) when compared to WT mice.

*IL-4 KO mice do not exhibit depressive-like behavior*- Fig. 2.2A shows that WT and IL-4 KO mice do not differ in immobility in the forced swim test (WT vs IL-4 KO, 127.0 ± 6.0 vs 121.0 ± 9.3, p=0.580). Fig. 2.2B demonstrates WT and IL-4 KO mice have the same preference for a saccharin-sweetened solution over water (WT vs IL-4 KO, 93.7 ± 0.5 vs 92.9 ± 0.8, p=0.398).

*Spatial and non-spatial memory is not impaired in IL-4 KO mice*- During the acquisition phase of Morris water maze (Fig. 2.3A), WT and IL-4 KO mice both decrease in latency to find the hidden platform without a strain interaction (Strain effect p=0.897, Acquisition Day effect p<0.001, Strain x Acquisition Day effect p=0.878; Day 1 vs Day 2, 49.5 ± 2.5 vs 40.1 ± 3.7, p=0.336; Day 1 vs Day 3, 49.5 ± 2.5 vs 29.3 ± 5.3, p=0.004; Day 1 vs Day 4, 49.5 ± 2.5 vs 25.1 ± 4.1, p<0.001; Day 2 vs Day 3, 40.1 ± 3.7 vs 29.3 ± 5.3, p=0.213; Day 2 vs Day 4, 40.1 ± 3.7 vs 25.1 ± 4.1, p=0.043; Day 3 vs Day 4, 29.3 ± 5.3 vs 25.1 ± 4.1, p=0.870). In the probe trial (Figs. 2.3B-2.3D), WT and IL-4 KO mice do not differ in % time spent in platform quadrant (WT vs IL-4 KO, 46.8 ± 6.4 vs 47.1 ± 6.1, p=0.972), latency to platform (WT vs IL-4 KO, 34.9 ± 6.9 vs 29.6 ± 7.2, p=0.603) or number of platform crossings (WT vs IL-4 KO, 3.1 ± 0.9 vs 2.3 ± 0.7, p=0.454). Fig. 2.3E demonstrates that WT and IL-4 KO mice both spend significantly more %
time investigating an object in a novel location (WT Novel vs Familiar Location, 57.6 ± 1.9 vs 42.4 ± 1.9, p<0.001; IL-4 KO Novel vs Familiar Location, 60.4 ± 4.0 vs 39.6 ± 4.0, p=0.003).

Fig. 2.3F shows that WT and IL-4 KO mice both investigate a novel object preferentially over a familiar object (WT Novel vs Familiar Object, 70.2 ± 2.5 vs 29.8 ± 2.5, p<0.001; IL-4 KO Novel vs Familiar Object, 66.7 ± 3.4 vs 33.3 ± 3.4, p<0.001).

Anxiety-like behaviors differ in IL-4 KO versus WT mice- Fig. 2.4A shows IL-4 KO mice spend significantly less time in the open arms of the elevated zero maze than WT controls (WT vs IL-4 KO, 62.8 ± 10.0 vs 24.4 ± 6.5, p=0.003). Figs. 2.4B and 2.4C demonstrate that while WT and IL-4 KOs move the same distance in the open field test (WT vs IL-4 KO, 3491.1 ± 156.0 vs 3275.1 ± 143.9, p=0.338), the IL-4 KO mice spend significantly more time in the center (non-shadowed) area than WT animals (WT vs IL-4 KO, 47.9 ± 9.2 vs 76.2 ± 8.0, p=0.048).

Discussion

Burrowing food and other material is an innate behavior in rodents that is easily performed in a laboratory setting (Deacon, 2006), and used as a measure of generalized alterations (Deacon, 2009; Jirkof et al., 2013) suggestive of potential disturbances in pain (Jirkof et al., 2010) and depressive-like (Lavin et al., 2011) and anxiety-like behaviors (Bailey and Crawley, 2009). We found that IL-4 KO mice burrow significantly less food from a burrow placed in their home cage than WT controls (Fig. 2.1A). Additionally, adult mice will explore a novel conspecific juvenile mouse introduced into their home cage, and perturbances in this parameter are seen in biobehaviors such as sickness (Fu et al., 2010) or anxiety (Bailey and Crawley, 2009). Fig. 2.1B demonstrates IL-4 KO mice have significantly higher exploration of a
novel juvenile than WT mice. These differences in burrowing and social exploration indicate that there is a behavioral phenotype difference between WT and IL-4 KO animals that requires behavior-specific tests to identify.

Assessment of depressive-like behavior in mice can be conducted using the sucrose/saccharin preference test and forced swim test (York et al., 2012). In the forced swim test, behavioral despair is defined as reduced drive to escape and is quantified by increased immobility (Hales et al., 2014). IL-4 KO mice do not differ from WT control animals in immobility in the forced swim test (Fig. 2.2A). The sucrose/saccharin preference test evaluates loss-of-interest (anhedonia) by preference for a solution sweetened with sucrose or saccharin versus water (Hales et al., 2014). No difference was observed between WT and IL-4 KO animals in the saccharin preference test (Fig. 2.2B). As no anhedonia or behavioral despair is evident when comparing strains, IL-4 KO mice do not exhibit a depressive-like phenotype.

The Morris water maze is a test used frequently in rodent models to assess spatial learning and memory where animals are trained and tested to use distal visual cues to locate a hidden, submerged escape platform (Vorhees and Williams, 2006). In the acquisition phase, mice are trained in multiple trials per day starting from different positions for each trial, and upon completion of each trial animals are allowed to remain on the platform to orient themselves to the spatial cues. Latency to platform is assessed for each trial, and impaired spatial learning is defined as a higher time searching for the platform over the course of the acquisition phase (York et al., 2012). Fig 2.3A demonstrates that WT and IL-4 KO mice do not differ in latency to platform during the acquisition phase, indicating no deficit in spatial learning. After completion
of acquisition, animals are tested for memory in a probe trial, where the platform is removed from the maze and the time spent in the platform quadrant, latency to the location previously occupied by the platform, and number of platform crossings are assessed (York et al., 2012). IL-4 KOs do not differ from WT in time in quadrant (Fig. 2.3B), latency to platform (Fig. 2.3C) or number of platform crossings (Fig. 2.3D), demonstrating no difference between strains in spatial memory. As previous research has suggested IL-4 KO mice display decreased cognitive function (Derecki et al., 2010), we sought to confirm our results with further memory testing. Capitalizing on the natural preference of mice for novelty, the novel object and novel location tests probe cognition by determining if a mouse can distinguish an unfamiliar object (non-spatial memory) or object placement (spatial memory) from a familiar one, respectively (Weible et al., 2009). A mouse should spend significantly more time exploring the novel versus the familiar object or location, and a deficit is defined as when exploration between the novel and familiar are not statistically different (York et al., 2012). We found that WT and IL-4 KO mice both spend significantly more time investigating the novel versus familiar location (Fig. 2.3E) and object (Fig. 2.3F), suggesting no deficits in spatial or non-spatial memory and confirming previous results from the Morris water maze (Figs. 2.3A-D).

A number of paradigms are available to test anxiety in rodents, utilizing aversive events or approach-avoidance conflict (Bailey and Crawley, 2009). First we used the elevated zero maze (EZM), which has been shown to be a powerful model in which to study anxiety (Singh et al., 2007). In the EZM, mice displaying anxiety-like behavior will spend less time in the open, brightly-lit versus the closed, dark arms of the maze (York et al., 2012). We found that IL-4 KO mice explore the open arms of the EZM less than the WT controls (Fig. 2.4A), suggestive of
anxiety-like behavior of IL-4 KOs in the EZM. To expand upon this finding, we then sought to determine the nature of the anxiety-like phenotype in the IL-4 KO mice. Anxiety can be thought of as situation-dependent (“state” anxiety) or situation-independent (“trait” anxiety) (Bailey and Crawley, 2009), and may be differentiated by comparing performance on multiple anxiety tests. The open field test (OFT) is another common paradigm for testing anxiety (Gould et al., 2009), utilizing bright lights and social isolation in a novel environment. In the OFT, in addition to quantifying anxiety as a reduction in time spent in the center of the testing arena, total locomotion can be assessed (York et al., 2012). IL-4 KO mice do not differ from WT controls in total locomotion (Fig. 2.4B). However, IL-4 KOs spend significantly more time in the center versus WT controls (Fig. 2.4C), which is not indicative of an anxiety like-phenotype in the OFT. Taken together, our results from testing anxiety-like behaviors in different paradigms suggest an anxiety-like phenotype in conditions specific to the EZM. These data are in line with current descriptions of “state” anxiety, where animals display anxiety-like behavior only in certain paradigms and an anxiolytic phenotype in others (Avgustinovich et al., 2000; O’Leary et al., 2013; Steimer, 2011).

In summary, we found that IL-4 KO mice display anxiety-like behavior in the EZM (Fig. 2.4A), but not the OFT (Fig. 2.4C), without concomitant depressive-like behavior (Figs. 2.2A-B) or cognitive deficits (Figs. 2.3A-F). The results of our cognitive testing did not replicate previous work in IL-4 KO animals that demonstrated deficits in learning and memory (Derecki et al., 2010). As we have demonstrated here that IL-4 KO animals display an anxiety-like phenotype that appears dependent upon the paradigm used, this may contribute to the differences seen between our results and those previously published. Anxiety has been shown to affect
performance in cognitive tests such as the MWM (Harrison et al., 2009), and our protocol differed from that used in previous testing of IL-4 KOs. Specifically, we trained and tested animals during their active phase (dark cycle), whereas Derecki et al. tested mice during their inactive phase (light cycle) (Derecki et al., 2010). These results are not surprising in that similar results have been demonstrated, where a cognitive deficit was found by one group in IL-1 receptor KO mice (Avital et al., 2003), then later an anxious phenotype without cognitive dysfunction was demonstrated by another group in the same strain (Murray et al., 2013). In addition to reinforcing the importance of considering the complete biobehavioral profile of an animal and testing conditions when interpreting behavior results, our data importantly suggest a link between endogenous immune skew and behavior phenotype, specifically anxiety-like behavior. Future research investigating the neurochemical basis for and reversibility of immune-mediated anxiety are warranted and could lead to novel therapeutic interventions for neuropsychiatric complications.
CHAPTER 3: THE SATURATED FATTY ACID, PALMITIC ACID, INDUCES ANXIETY-LIKE BEHAVIOR IN MICE

Abstract

Excess fat in the diet can impact neuropsychiatric functions by negatively affecting cognition, mood and anxiety. We sought to show that the free fatty acid (FFA), palmitic acid, can cause adverse biobehaviors in mice that lasts beyond an acute elevation in plasma FFAs. Mice were administered palmitic acid or vehicle as a single intraperitoneal (IP) injection. Biobehaviors were profiled 2 and 24 hrs after palmitic acid treatment. Quantification of dopamine (DA), norepinephrine (NE), serotonin (5-HT) and their major metabolites was performed in cortex, hippocampus and amygdala. FFA concentration was determined in plasma. Relative fold change in mRNA expression of unfolded protein response (UPR)-associated genes was determined in brain regions. In a dose-dependent fashion, palmitic acid rapidly reduced mouse locomotor activity by a mechanism that did not rely on TLR4, MyD88, IL-1, IL-6 or TNFα but was dependent on fatty acid chain length. Twenty-four hrs after palmitic acid administration mice exhibited anxiety-like behavior without impairment in locomotion, food intake, depressive-like behavior or spatial memory. Additionally, the serotonin metabolite 5-HIAA was increased by 33% in the amygdala 24 hrs after palmitic acid treatment. Palmitic acid induces anxiety-like behavior in mice while increasing amygdala-based serotonin metabolism. These effects occur at a time point when plasma FFA levels are no longer elevated.

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Introduction

Overweight/obesity is associated with a variety of organic comorbidities including cardiovascular disease, stroke, type 2 diabetes (T2D) and cancer (Dixon, 2010; Li et al., 2013). Recently, neuropsychiatric complications such as depression, cognitive impairment and anxiety are seen as serious adverse sequelae in overweight/obese individuals (Lopresti et al., 2013; Burkhalter and Hillman, 2011; Gariepy et al., 2010). Over-nutrition due to an excess intake of dietary fat is causally linked to the overweight/obese state (Astrup, 1999; Lissner and Heitmann, 1995), and more recently to metaflammation-linked neurodegenerative disease (de la Monte, 2008) and adult obstructive airway disease-associated, cognitive impairment, depression and anxiety (Gariepy et al., 2010; Kanoski and Davidson, 2011; Preiss et al., 2013). More recently, high-fat diet (HFD)-induced over-nutrition is tied to neuropsychiatric morbidities prior to the onset of inflammation, hyperglycemia and weight gain (Davidson et al., 2013; Kaczmarczyk et al., 2013) indicating that excess fat in the diet is, itself, harmful to psychological health.

One consequence of a HFD is an increase in circulating free fatty acids (FFAs) (Boden, 2011). Canonically, elevated plasma FFAs are associated with insulin resistance, non-alcoholic fatty liver disease (NAFLD), pre-diabetic neuropathy, decreased aortic distensibility, and ischemic stroke (Brands et al., 2011; Capurso and Capurso, 2012; Leamy et al., 2013; Lupachyk et al, 2012; Rider et al., 2012; Yaemsiri et al., 2013). Much less, however, is known about the impact of FFAs on the brain and behavior outside of an impact on food intake (Obici et al., 2002). We recently demonstrated that a short-term HFD in mice impairs object-based memory and causes anxiety-like behavior after 1 wk of feeding, suggesting that a HFD can negatively impact amygdala-related processes in a relatively rapid fashion (Kaczmarczyk et al., 2013).
Since the brain is rich in receptors that can recognize FFAs including Toll-like receptors (TLRs) (Crack and Bray, 2007) and free fatty acid receptors (FFARs) (Ma et al, 2008; Nakamoto et al, 2012), FFAs have a real potential to modulate higher brain function.

Anxiety disorders, including generalized anxiety disorder (GAD), obsessive-compulsive disorder (OCD), panic disorder and post-traumatic stress disorder (PTSD) are among the most commonly reported neuropsychiatric conditions with a lifetime prevalence of nearly 29% (Kessler and Wang, 2008). Organically, how anxiety develops is poorly understood but metabolic interventions (exercise (Stathopoulou et al., 2006), weight loss (Petry et al., 2008) and diet modification (removal of caffeine (Smith, 1988) and alcohol (Kushner et al., 2000)) are often suggested as ways to reduce and/or stave off GAD or panic disorder. Given that palmitic acid is the most abundant saturated fatty acid in most diets (Kien et al., 2013) and its elevation in plasma is associated with poor clinical outcomes and disease progression for both the metabolic syndrome and obesity (Vessby, 2003; Mayneris-Perxachs et al., 2013), the question addressed here is whether FFAs can negatively impact biobehaviors in mice.

**Methods**

**Materials-** All reagents and chemicals were purchased from Sigma-Aldrich. All primers were purchased from Applied Biosystems.

**Animals-** Animal use was conducted in accordance with Institutional Animal Care and Use Committee approved protocols at the University of Illinois. Wild-type C57BL/6J (WT), TLR4 knock out (KO), Myeloid differentiation primary response gene 88 (Myd88) KO,
interleukin (IL)-1 receptor-1 (IL-1R1) KO, IL-6 (IL-6) KO, tumor necrosis factor-α (TNF-α) KO mice (all on a C57BL background) were originally purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were group housed (8 per cage) in standard shoebox cages and provided water and food ad libitum, then moved to individual housing in standard shoebox cages the day prior to treatment unless noted otherwise. Housing temperature (72 °F) and humidity (45–55%) were controlled as was a 12/12 h reversed dark-light cycle (2100–0900 h). Video recording of animal behavior was performed under red light using a Sony HDR-XR500V Night Shot capable video camera (Tokyo, Japan). All treatments at each time point represent separate cohorts of mice, except locomotor activity which was a repeated measure. Mice used were between 8 and 16 weeks of age. All behavior testing was completed in the dark cycle under red light illumination unless otherwise indicated. A total of 382 mice were used.

Injectables- FFAs (palmitic acid, palmitoleic acid, myristic acid, decanoic acid, octanoic acid and valeric acid) were administered IP in a vehicle of castor oil at a volume of 50 µl/mouse. Palmitic acid was administered for the dose response at 0.3, 3, and 30 µmol/mouse, after which all free fatty acids (FFAs) were tested at 30 µmol/mouse. All FFA injections occurred at the onset of the dark cycle.

Spontaneous locomotion- Locomotion was measured by videography in conjunction with automated video tracking software (Noldus Information Technology EthoVision XT 7 (Leesburg, VA)), as we have described (York et al., 2013; York et al., 2012). At times indicated after treatment, mice were video recorded in their home cage for 5 min. Distance moved (cm) was determined, and results are presented as percent vehicle control.
Object investigation- As we have described (York et al., 2012), the subject mouse was placed in a novel home cage-sized arena with two objects 10 cm apart at one end. Mice were video recorded for 10 min and investigative behavior of the objects was evaluated using EthoVision XT 7. Results were expressed as raw investigation time (s).

Zero maze- As previously described (Kaczmarczyk et al., 2013), mice were transferred from their home cage to the high wall section of a Zero maze, which was divided into 4 quadrants, 2 of which had high walls (14 cm). Testing was completed under white light illumination and video recorded for 5 min. Time spent in the non-high wall area (open arm) was quantified as we have described (York et al., 2012).

Food intake- As we have described (York et al., 2012), prior to treatments and, at each time point indicated, food was weighed. Food intake was calculated as the difference in weight of food removed from the feed bowl.

Forced swim test- The test mouse was transferred from its home cage to a clean white cylindrical PVC container (diameter 16 cm; height 31 cm) containing 20 cm of water maintained at 25 ± 1 °C. Total swim duration was 5 min, and immobility was evaluated from the video record as we have described (York et al., 2012; Park et al., 2011).

Y-maze- Testing was performed as previously described (Kaczmarczyk et al., 2013; York et al., 2012). The subject mouse was placed into one of the 3 maze arms. The Y-maze used was
symmetrical, clear and Plexiglas (arms = 40 cm in length × 9 cm in width × 16 cm in wall height). Maze side walls were decorated with black triangles, black circles, or black diagonal lines. Movement was video recorded for 5 min and scored by hand by a blinded observer. Arm entry required all 4 legs of the subject mouse to enter an arm. Perfect alternations were defined as exploration of all three arms sequentially given 3 opportunities independent of a right or left arm choice at initiation. Imperfect alternations were defined as entry into all three arms in 4 opportunities. Results were calculated as the number of perfect or imperfect alternations divided by total opportunities.

**Plasma FFAs-** Mice were euthanized and blood collected via cardiac puncture in BD Microtainer Tubes with Lithium Heparin (BD Diagnostics, Franklin Lakes, NJ) on ice. Blood was centrifuged at 2,000 x g for 10 min at 4°C and plasma collected. Plasma FFAs were measured on aAU680 Chemistry System (Beckman Coulter, Brea, CA) using a non-esterified fatty acid module (Randox Laboratories Ltd., UK).

**Brain region collection-** As we have described (Chiu et al., 2012), mice were euthanized and immediately perfused through the left ventricle with 30 mL of ice-cold PBS. Brains were removed and regions dissected.

**HPLC-** Mouse brain regions were solubilized as previously described (O’Connor et al., 2009) in 0.1 N HClO4 and 25 μM ascorbate using an ultrasonic tissue disruptor (Sonic’s & Materials, Inc., Newtown, CT). Homogenates were centrifuged at 12,000 x g for 10 min at 4 °C. Supernatants were diluted in 0.02 N HClO4 and analyzed by HPLC for norepinephrine (NE),
normetanephrine (NME), 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA),
dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) using
an ESA Coulochem III detector with a 5041 Enhanced Analytical cell containing a glassy carbon
electrode (+320 mV) (Thermo Scientific, Sunnyvale, CA). Mobile phase (pH = 3.0) consisted of
75 mM NaH2PO4, 25 μM EDTA (disodium salt), 0.45 mM octanesulfonic acid and
triethylamine in acetonitrile:water (5:95, v:v) and was pumped at 0.2 μL/min through a 2 mm x
150 mm ODS Hypersil column (Thermo Scientific, Sunnyvale, CA). Protein concentrations in
tissue pellets were determined using the DC Protein Assay (BioRad, Hercules, CA) and read on
ELx800 Absorbance Microplate Reader (BioTek, Winooski, VT) after being solubilized in RIPA
buffer (150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton and 10 mM Tris, pH 7.4) using a 60
Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA). Results were quantified as ng
analyte/mg brain region protein, and expressed as raw values and as ratios.

*Monoamine oxidase (MAO) activity*- MAO activity was determined as previously
described (Kaczmarczyk et al., 2013). Briefly, brain regions were freeze fractured and
homogenized in reaction buffer containing 50 mM NaCl (Fisher Scientific, Fair Lawn, NJ), 10%
glycerol, 1 mM EDTA, 50 mM HEPES, pH 7.4 (USB Corporation, Cleveland, OH). Lysates
were clarified at 16,000 × g for 15 min at 4 °C and the supernatant protein concentrations
determined using the DC Protein Assay (Bio-Rad, Hercules, CA) as above. Sample protein
concentrations were normalized within regions with reaction buffer. MAO activity was tested
using the MAO-Glo Assay System (Promega, Madison, WI) and quantified with an ImageQuant
LAS-4000 CCD camera quantitative imager (GE Healthcare, Piscataway, NJ) coupled to Multi
Gauge Software (GE Healthcare, Piscataway, NJ).
*qPCR*- RNA was extracted, cDNA generated, and gene expression was determined as previously described (Kaczymarczyk et al., 2013). In brief, mRNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). TaqMan Gene Expression primer for X-box binding protein 1 (Xbp1) (Mm00457357_m1), ER degradation enhancer mannose alpha-like 1 (Edem1) (Mm00551797_m1), heat shock protein 90 beta member 1 (Hsp90b1) (Mm00441926_m1) and activating transcription factor 4 (Atf4) (Mm00515325_g1) were used in real-time PCR performed on a 7900 HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan Universal PCR Master Mix. Parallel amplification of GAPDH (Mm00484668_m1) was performed to normalize gene expression. Relative fold change of target genes was performed by comparing ΔCts.

**Statistics**- Data are expressed as mean ± SEM. Data analysis was conducted using Sigma Plot 11.2 (Systat Software, Chicago, IL). One-way or two-way ANOVA were run with or without repeated measurements where needed. Tukey’s test was used for post-hoc comparison procedures. All statistical analysis included testing for treatment x time interactions. Statistical significance was determined as p<0.05.

**Results**

*Palmitic acid decreases spontaneous locomotion*- Fig. 3.1 demonstrates that 30 and 3 µmol, but not 0.3 µmol, of IP-administered palmitic acid induces a decrease in locomotion 2 h after injection compared to vehicle-treated controls (Treatment effect p<0.001; 30 µmol vs. vehicle, 26.5% ± 5.1 vs 100% ± 5.8, p<0.001; 3 µmol vs. vehicle, 58.1% ± 3.73 vs. 100% ± 5.8,
p=0.002; 0.3 µmol vs. vehicle, 105.7% ± 11.5 vs. 100% ± 5.8, p=0.947). The effect of palmitic acid on locomotion is dependent on dose (30 vs. 3 µmol, p=0.019).

**Palmitic acid-induced loss of locomotion is not dependent on TLR4, MyD88, IL-1, IL-6 or TNFα**- Fig. 3.2 shows that palmitic acid decreased locomotion 2 h after IP administration in WT, TLR4, MyD88, IL-1R1, IL-6 and TNFα KO mice (TLR4 KO vehicle vs. palmitic, 100.0 ± 7.9 vs. 45.7 ± 5.5, p<0.001; MyD88 KO vehicle vs. palmitic, 100.0 ± 8.5 vs. 41.5 ± 4.2, p<0.001; IL-1R1 KO vehicle vs. palmitic, 100.0 ± 8.7 vs. 52.9 ± 8.9, p=0.002; IL-6 KO vehicle vs. palmitic, 100.0 ± 8.7 vs. 38.3 ± 6.5, p<0.001; TNFα KO vehicle vs. palmitic, 100.0 ± 7.1 vs. 36.1 ± 10.0, p<0.001). The decrease observed in KO animals was not different than WT (data not shown; WT vs. TLR4 KO strain x treatment p=0.804; WT vs. MyD88 KO strain x treatment p=0.890; WT vs. IL-1R1 KO strain x treatment p=0.089; IL-6 KO strain x treatment p=0.696; WT vs. TNFα KO strain x treatment p=0.337).

**Chain length is important to FFA-dependent loss of spontaneous locomotion**- Fig. 3.3 shows that IP-administered palmitic acid (16:0), palmitoleic acid (16:1), myristic acid (14:0) and decanoic acid (10:0) reduced locomotion at 2 h (vehicle vs. palmitic, 100.0 ± 7.8 vs. 31.4 ± 2.4, p<0.001; vehicle vs. palmitoleic, 100.0 ± 7.8 vs. 29.0 ± 4.5, p<0.001; vehicle vs. myristic, 100.0 ± 7.8 vs. 27.4 ± 4.8, p<0.001; vehicle vs. decanoic, 100.0 ± 7.8 vs. 53.9 ± 4.5, p<0.001). Loss of locomotion caused by palmitoleic, myristic and decanoic acids were not different from palmitic acid (palmitic vs. palmitoleic, 31.4 ± 2.4 vs. 29.0 ± 4.5, p=1.000; palmitic vs. myristic, 31.4 ± 2.4 vs. 27.4 ± 4.8, p=1.000; palmitic vs. decanoic, 31.4 ± 2.4 vs. 53.9 ± 4.5, p=0.064). Locomotion after administration of octanoic acid (8:0) and valeric acid (5:0) was not different from vehicle
(vehicle vs. octanoic, 100 ± 7.8 vs. 87.3 ± 6.8, p=0.631; vehicle vs. valeric, 100.0 ± 7.8 vs. 78.1 ± 5.0, p=0.078) and was significantly greater than that seen for the other FFAs administered
(octanoic vs. palmitic, 87.3 ± 6.8 vs. 31.4 ± 2.4, p<0.001; octanoic vs. palmitoleic, 87.3 ± 6.8 vs.
29.0 ± 4.5, p<0.001; octanoic vs myristic, 87.3 ± 6.8 vs. 27.4 ± 4.8, p<0.001; octanoic vs.
decanoic, 87.3 ± 6.8 vs. 53.9 ± 4.5, p=0.001; valeric vs. palmitic, 78.1 ± 5.0 vs. 31.4 ± 2.4,
p<0.001; valeric vs. palmitoleic, 78.1 ± 5.0 vs. 29.0 ± 4.5, p<0.001; valeric vs. myristic, 78.1 ±
5.0 vs. 27.4 ± 4.8, p<0.001; valeric vs. decanoic, 78.1 ± 5.0 vs. 53.9 ± 4.5, p=0.036).

**Mice exhibit anxiety-like behavior 24 hrs after palmitic acid administration** - Figs. 3.4A and 3.4B show that palmitic acid induces a decrease in time spent investigating an unfamiliar object (vehicle vs palmitic, 41.7% ± 4.8 vs 23.6% ± 4.8, p=0.013) and a decrease in time spent in the open arm of a Zero maze (vehicle vs palmitic, 54.4 sec ± 6.1 vs 35.4 sec ± 3.2, p=0.015) at 24 h after treatment, which resolves by 48 h (vehicle vs palmitic, 54.6 sec ± 7.0 vs 56.0 sec ± 6.3, p=0.886). Fig. 3.4C demonstrates that there is a decrease in locomotion 2 h after palmitic acid administration, and that locomotion in palmitic acid-treated mice is not different from controls at 24 h post treatment (treatment x time effect, p<0.001; 2 h vehicle vs 2 h palmitic,
100% ± 2.9 vs 37.2% ± 9.0, p<0.001; 24 h vehicle vs 24 h palmitic, 100% ± 9.1 vs 80.2% ± 9.0,
p=0.102; 2 h palmitic vs 24 h palmitic, 37.2% ± 9.0 vs 80.2% ± 9.0, p<0.001). Fig. 3.4D illustrates that food intake decreases in palmitic acid-treated animals in the 24 h interval after administration, and increases to levels seen in controls from 24 to 48 h after treatment (treatment x time effect, p<0.001; 0 to 24 h vehicle vs 0 to 24 h palmitic, 3.1 g ± 0.17 vs 0.9 g ± 0.4,
p<0.001; 24 to 48 h vehicle vs 24 to 48 h palmitic, 3.2 g ± 0.1 vs 2.5 g ± 0.2, p=0.061, 0 to 24 h palmitic vs 24 to 48 h palmitic, 0.9 g ± 0.4 vs 2.5 g ± 0.2, p<0.001). Figs. 3.4E and 3.4F show
that at 24 h after administration, palmitic acid-treated mice do not display differential immobility in the forced swim test (vehicle vs palmitic, 107.0 sec ± 8.1 vs 113.3 sec ± 15.7, p=0.731) or Y-maze perfect alternations (vehicle vs palmitic, 61.7% ± 1.9 vs 62.3% ± 4.0, p=0.894).

Palmitic acid increases the serotonin metabolite 5-HIAA in the amygdala- Table 3.1 shows that 24 hrs after palmitic acid administration mice had a 33% increase in 5-HIAA in the amygdala. Table 3.2 demonstrates that 24 hrs after palmitic acid administration there was a 25% decrease in the 5-HT:5-HIAA ratio in the amygdala and a 42% increase in the DA:DOPC ratio in the hippocampus. Table 3.3 shows no difference in brain MAO-A or MAO-B activity 24 hrs after palmitic acid administration. Fig. 3.5 shows that 24 hrs after palmitic acid administration plasma FFAs were no longer elevated (treatment x time effect p=0.038; 2 h vehicle vs 2 h palmitic, 0.88 mmol/l ± 0.17 vs 1.63 mmol/l ± 0.10, p<0.001; 4 h vehicle vs 4 h palmitic, 0.76 mmol/l ± 0.09 vs 1.76 mmol/l ± 0.09, p<0.001; 8 h vehicle vs 8 h palmitic, 0.94 mmol/l ± 0.17 vs 1.45 mmol/l ± 0.21, p=0.018; 12 h vehicle vs 12 h palmitic, 1.00 mmol/l ± 0.09 vs 1.45 mmol/l ± 0.13, p=0.026; 24 h vehicle vs 24 h palmitic, 0.70 mmol/l ± 0.17 vs 0.83 mmol/l ± 0.13, p=0.511).

Genes associated with the un-folded protein response (UPR) are not up-regulated by palmitic acid in the brain- Table 3.4 demonstrates gene expression of Xbp1, Edem1, Hsp90b1 and Atf4 24 hrs after palmitic acid administration, in the cortex, hippocampus and amygdala.
Discussion

Over-nutrition predisposes individuals to a variety of adverse outcomes including neuropsychiatric sequelae (Dixon, 2010; Li et al., 2013; Lopresti and Drummond, 2013; Burkhalter and Hillman, 2011; Gariepy, 2010). Furthermore, especially in obesity, elevated plasma FFAs correlate with certain psychological alterations (Kien, 2009; Tsuboi et al., 2013). While circulating FFAs are implicated in the development of morbidities associated with a western-style HFD, the mechanisms underlying the complications of an obesogenic diet and the role of FFAs is still unclear (Kaczymarczyk et al., 2013; Lupachyk et al., 2012; Wen et al., 2011; Eguchi et al., 2012). Inflammation is a well-described consequence of over-nutrition and is a potential mechanism by which HFDs can trigger insulin resistance, diabetes and diabesity-related co-morbid conditions (Gregor and Hotamisligil, 2011). Acute inflammation, through pro-inflammatory cytokines, causes sickness behaviors such as reduced locomotion, anorexia, memory impairment and anxiety (Dantzer et al., 2008). Therefore, given that FFAs can drive a pro-inflammatory phenotype in astrocytes, microglia and neuronal stem cells (Gupta et al., 2012; Tracy et al., 2013; Yuan et al., 2013), pro-inflammatory cytokines would seem a likely antecedent to adverse behaviors induced by FFAs. Here we show that TLR4 KO, MyD88 KO, IL-1R1 KO, IL-6 KO and TNFα KO mice all developed an FFA-dependent reduction in spontaneous locomotion. These finding indicate that the canonical pathway of FFAs binding to TLR4 (Lee et al., 2001; Yin et al., 2013) inducing classical macrophage activation (Moon et al., 2011) with elaboration of IL-1, IL-6 and TNF-α is not responsible for the behaviors observed.

While these findings do not exclude inflammation as a mechanism by which FFAs can adversely impact the brain, they do indicate that FFAs serving as a LPS lipid A analog in the stimulation of TLRs, especially TLR4, is not a likely mechanism by which FFAs promotes anxiety. Further
evidence that canonical inflammatory pathways may not be directly involved in the biobehavioral impact of administered FFAs is that both saturated palmitic acid, and monounsaturated palmitoleic acid induced loss of locomotion, as did the long chain FFA, myristic acid, and the longer medium chain FFA, decanoic acid. Work in vitro comparing unsaturated FFAs with saturated FFAs demonstrate that the unsaturated FFAs are ineffective at generating a classical inflammatory response in macrophage-derived cells when TNFα generation is examined (Lee et al., 2001). Interestingly, the short chain FFA, valeric acid, and the shorter medium chain FFA, octanoic acid, did not affect locomotion at the times measured suggesting that fatty acid chain length and not saturation are important to the biobehaviors observed.

Since TLRs did not appear responsible for the behaviors observed and FFA chain length impacted efficacy, a potential mechanism by which certain FFAs could trigger the effects seen is via FFAR1. FFAR1-3 are a closely related family of receptors that were previously identified as the orphan G-protein receptor (GPR)s: GPR40, GPR43 and GPR41, respectively (Stoddart et al., 2008). In 2003, a variety of groups identified free fatty acids as the ligands for these GPRs (Stoddart et al., 2008). Medium and long chain fatty acids were the agonists for FFAR1 while short chain fatty acids, with a chain length of less than 6 carbons, were the agonists for FFAR2 and FFAR3 (Stoddart et al., 2008). Briscoe et al. characterized the ability of 42 different long-chain FFAs to activate FFAR1 in FFAR1 transfected HEK293 cells via measurement of intracellular calcium ([Ca2+]) release because FFAR1 after binding fatty acids activates phospholipase C (PLC) raising [Ca2+]. In support, palmitic acid was one of the most potent
activators of FFAR1 with an EC50 of 10 μM. Tissue distribution of FFAR1 is relatively ubiquitous with the highest mRNA expression in the brain (Briscoe et al., 2003).

While longstanding overnutrition is linked to alterations in brain function and neuropsychiatric complications (de la Monte and Wands, 2008; Bruce-Keller et al., 2009), short-term overnutrition, in both rodents and humans, can also generate adverse biobehaviors. These neuropsychiatries include cognitive dysfunction, attention disorders and anxiety-like behaviors. Short-term HFD feedings appear particularly detrimental to attention, spatial memory and anxiety and tends to manifest prior to the clear onset of metabolic dysfunction (Kaczymarczyk et al., 2013; Edwards et al., 2011; Murray et al., 2009; Valladolid-Acebes et al., 2011). To investigate the possibility that a single exposure to FFAs could precipitate such biobehavioral changes, we utilized the forced swim test, Y-maze, object investigation and elevated Zero maze to probe FFA-treated mice for depressive-like behavior, spatial memory defects, and anxiety-like behavior, respectively. We found that palmitic acid induced anxiety-like behavior that could be measured 24 hrs post administration which was a time point at which palmitic acid-dependent impairments in locomotion and food intake had resolved. This time frame fits well with human fear/anxiety paradigms because these emotions frequently manifest/persist well beyond the putative eliciting event and are commonly unassociated with memory impairment and depression (Milanak et al., 2013).

An important reason why we were able to identify the FFA-dependent behaviors observed was likely due to how we administered the FFAs. Since Lee et al. first demonstrated that saturated fatty acids activate TLR4 (Lee et al., 2001), investigators in the field have
generally prepared saturated free fatty acids as 10:1 saturated free fatty acid: albumin complexes in ethanol because saturated FFAs are insoluble in most physiologically relevant aqueous solutions. Albumin has a high affinity for saturated FFAs ($10^7$ for palmitic acid, as example) (Spector, 1975) and this high-affinity can hinder FFA bioavailability and FFA-dependent activation of FFAR1, as shown by Stoddart et al. (Stoddart et al., 2007). Therefore, instead of using the method of Lee et al. in our preparations of palmitic acid, we used a novel oil-in-oil delivery system with castor oil as the carrier. This approach potentially enabled improved FFA bioavailability. In addition, we did not have to deliver ethanol which confounds behavioral testing and has anxiolytic properties (Gilles et al., 2006). Importantly, we administered a relatively low dose of palmitic acid that caused only a doubling of plasma FFAs. This level of FFA is often seen after an ingestion of a high-fat meal or a short-term fast (Dole, 1956).

Dysfunctional neurotransmitter availability and signaling is associated with many neuropsychiatric sequelae, including memory loss, depression, delirium and anxiety (Blier and El Mansari, 2013; Gressier et al., 2013; Johansen et al., 2011). Recently, we demonstrated that mice fed a HFD for 1 wk exhibit memory impairment and anxiety like behaviors that were connected to disrupted brain-based dopamine metabolism (Kaczymarczyk et al., 2013). Here we found that 24 hrs after palmitic acid administration the hippocampal DA:DOPAC ratio was elevated in the hippocampus and that the 5-HT:5-HIAA ratio was elevated in the amygdala. This dopamine ratio supports the contention that FFAs may be responsible for HFD-mediated dysregulated dopamine metabolism in the brain and that the addictive nature of HFDs may be related to perturbed brain levels of dopamine (Vucetic and Reyes, 2010). Previously, in our HFD feeding work, we did not look at the amygdala or at serotonin/serotonin metabolism. In that the amygdala is critical to
emotive learning (Hamann et al., 1999) and anxiotal states (Davis, 1992) and defects in serotonergic signaling in the amygdala are associated with anxiety (Matsuda, 2013), our current findings suggest that palmitic acid-dependent increase in 5-HIAA may be responsible for the anxiety seen. Correlatively, increased 5-HIAA in the amygdala is associated with anxiety-like behavior in mice (Neufeld-Cohen et al., 2010). Recently it has been noted that palmitic acid can be lipotoxic to cells resulting in an accumulation of reactive oxygen species (ROS) (Egnatchik et al., 2014) that fosters endoplasmic reticulum (ER) stress (Havashi et al., 2014) and subsequent apoptosis (Shen et al., 2014). Additionally, ER stress has been implicated in the FFA-induced complications of obesity (Boden, 2009; Melo et al., 2014). To determine if ER stress is involved in the observed neurotransmitter alterations and anxiety-like behavior, we quantified changes in expression of genes downstream of the ER stress response (van Galen et al., 2014). No upregulation of genes associated with ER stress signaling were found, suggesting that ER stress and lipotoxicity in the brain are not responsible for the anxiety-like behaviors demonstrated in our model.

Our data demonstrate an acute lasting behavioral response to the FFA, palmitic acid, which appears distinct from overnutrition-associated comorbidities driven by canonical inflammatory pathways. These data are the first to show that palmitic acid administration is anxiogenic and suggest that modest serum elevations in long-chain FFAs may underlie neuropsychiatric complications observed in metabolic diseases like obesity and T2D. Further exploration into the metabolic origins of anxiety is warranted with serum long-chain FFAs an intriguing new bioactive. Therefore, elucidating metabolic mechanisms that contribute to mental
illness will likely yield promising biomarkers and drug targets to diagnosis and treat a variety of psychiatric illnesses.
CHAPTER 4: CONCLUSION

Summary

We hypothesized that an imbalance in immune mediators would result in an altered biobehavioral phenotype in mice (Moon et al, 2011). Through the IL-4 KO model of endogenous immune skew, we demonstrated an altered behavioral profile in naïve animals, characterized by exhibition of anxiety-like behavior in a context-dependent manner (Moon et al., In Process). Further, we demonstrated exogenous administration of a ubiquitous compound, palmitic acid, could also induce anxiety-like behavior in mice (Moon et al., 2014). These data are collectively the first to show 1) the biobehavioral phenotype of IL-4 KO mice and 2) the biobehavioral phenotype resulting from acute administration of palmitic acid. Importantly, these results indicate that anxiety can be precipitated by diverse stimuli, suggesting a common pathway to eliciting anxiety-like behaviors.

Pitfalls and Future Directions

While novel pathways inducing anxiety-like behaviour have been suggested based on this work, we did not definitively identify the pathways involved. A critical next step is to determine the mechanisms behind the biobehavioral phenotypes we have described in our models.

In the biobehavioral phenotype of IL-4 KO animals, a neurological correlate has not been identified. While we found context-specific anxiety-like behavior in IL-4 KO mice, we did not identify changes in brain structure, cellular function, or neurotransmitter levels in these animals. Further research remains to investigate the mechanisms behind the behaviour alterations and if
these behaviors are chemically reversible or due to permanent functional changes. Additionally, we did not investigate the effect on circadian rhythm of IL-4 depletion, as this could influence the behaviors noted in the animals due to shifts in activity levels. Also, while we identified a role for IL-4 in development of biobehavioral disturbances, we did not localize the effect to a specific cell type. Generating animals without IL-4 receptor (IL-4R) on specific cell types using Cre-Lox recombination could identify cell types involved in the unique behaviour phenotype associated with lack of IL-4. Irrespective of the cell type involved, ameliorating the anxiety-like behaviour in the IL-4 KO animals was not achieved. Previous research using IL-4 KO mice has found resolution of behavior changes through adoptive transfer of immune cells from WT animals (Derecki et al., 2010). Repletion of IL-4 by adoptive transfer of WT immune cells or administration of exogenous IL-4 in IL-4 KO animals would be beneficial to understanding the role of this cytokine in biobehavior.

In the palmitic acid administration paradigm, a signalling location and receptor remains to be defined. Some potential sites of sensing the exogenous palmitic acid that were not investigated could be the vagus nerve or hypothalamus. Vagal sensing of peritoneal contents is a common pathway for other immune-mediated biobehavioral responses (Dantzer et al., 2008) and could be probed using animal with a sub-diaphragmatic vagotomy. Should vagotomised mice still respond to palmitic acid administration, it is likely that the signal is relayed directly to the brain. The hypothalamus is a likely candidate, as it is rich in FFAR1, a receptor that can bind palmitic acid (Nakamoto et al., 2012). However, whether these signals are propagated by FFARs, as we proposed based on the FFA panel (Fig. 3.3), or other mechanisms also remains unanswered. An inhibitor of FFAR1 was not available at the initiation of these studies.
However, the FFAR1 antagonist GW1100 has recently become commercially available and would be useful to determine the role of FFAR1 in this paradigm. Studies using intraperitoneal versus intracerebroventricular injection of the antagonist would not only determine if FFAR1 plays a role in palmitic acid-induced anxiety, but would also be an important indicator of peripheral versus central FFA signalling. Additionally, generation of FFAR1 KO animals, especially utilizing Cre-Lox recombination technology to generate cell-specific KOs, would be an important next step in identifying the cellular response in palmitic acid treatment. Lastly, direct treatment of the responsive cells with palmitic acid in culture would conclude this course of study by delineating the signalling cascade in response to FFAs.

**Clinical Implications**

Psychological pathologies are complex states that contribute significantly to suffering, yet the neurological basis of these disorders are often not well understood, hindering efforts at pharmacologic intervention. Of the neuropsychiatric conditions, anxiety disorders affect nearly one third of the population (Kessler and Wang, 2008). Research currently suggests a potential role for most of the neurotransmitter systems in the pathogenesis of anxiety (Bukalo et al., 2014), with serotonin signalling/metabolism (Matsuda 2013; Neufeld-Cohen et al., 2010) being the most well-studied candidate. Approved pharmacologic treatment of anxiety currently includes benzodiazepines, monoamine oxidase inhibitors, tricyclic antidepressants, and selective serotonin and norepinephrine reuptake inhibitors (Bukalo et al., 2014). However, given the diverse mechanisms of action of these treatments, anxiety pharmacotherapy is not well defined. Our research demonstrates that anxiety-like behaviour can develop in two unrelated animal models, one of endogenous immunologic skew and the other of free fatty acid administration.
Further research delineating the mechanisms behind the anxiety-like behaviors we observed would advance the understanding of the pathogenesis of anxiety and could potentially yield novel therapeutic interventions.
Fig.1.1 *Macrophage (MΦ) phenotype skew is a product of multiple influences*. T helper (Th) and other immune cells produce cytokines that influence macrophage activation states. Pathogen-associated molecular patterns (PAMPs) and IFNγ from Th 1 and natural killer (NK) cells synergize to generate M1 MΦs. Neutrophil and Th2 cell IL-4 and IL-13 prime M2 MΦs. Glucocorticoids, prostaglandins and regulatory T cell (Treg) TGF-β and IL-10 deactivate MΦs, and dMΦ-produced IL-10 feeds-back to enhance the deactivated phenotype.
<table>
<thead>
<tr>
<th>Macrophage</th>
<th>Marker</th>
<th>Associated functions</th>
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<tbody>
<tr>
<td>M1</td>
<td>iNOS</td>
<td>Produces microbicidal NO from arginine</td>
</tr>
<tr>
<td></td>
<td>↑ IL-1β, TNF</td>
<td>↑ Inflammatory activity</td>
</tr>
<tr>
<td>M2</td>
<td>↓ IL-1β, TNF</td>
<td>↓ Inflammatory activity</td>
</tr>
<tr>
<td></td>
<td>IL-1ra</td>
<td>Antagonizes IL-1 signaling</td>
</tr>
<tr>
<td></td>
<td>Insulin-like growth factor 1</td>
<td>Tissue proliferation/repair</td>
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<tr>
<td></td>
<td>Arginase 1</td>
<td>Produces wound-healing compounds from arginine</td>
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<tr>
<td></td>
<td>Mannose receptor</td>
<td>Uptake of mannosylated antigens</td>
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<tr>
<td></td>
<td></td>
<td>Pathogen recognition</td>
</tr>
<tr>
<td>dMΦ</td>
<td>IL-10</td>
<td>Inhibit proinflammatory cytokine production/action</td>
</tr>
<tr>
<td></td>
<td>↓ IL-12</td>
<td>↓ cytotoxic lymphocyte generation/activity</td>
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<tr>
<td></td>
<td></td>
<td>↓ IFN-γ production</td>
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**Table 1.1 Characteristic markers of the 3 main MΦ phenotypes.** M1 MΦs express high levels of iNOS and proinflammatory cytokines to fight invading microorganisms. M2 MΦs exhibit a gene profile conducive to wound healing and produce anti-inflammatory IL-1ra. A high IL-10/IL-12 ratio in dMΦs promotes resolution of inflammation through decreasing cytotoxic cell generation and proinflammatory cytokine actions.
Fig. 2.1 *IL-4 KO mice display burrowing and social exploration differences.* (A) C57BL/6J mice (WT) and IL-4 KO mice were allowed free access to a food pellet-filled burrow and contents were assessed 10 h later. Results are expressed as means ± s.e.m.; n=14-16, *p<0.05. (B) Novel conspecific juvenile mice were introduced into the home cage of WT and IL-4 KO mice for 5 min and investigation of the juvenile by test mouse was quantified. Results are expressed as means ± s.e.m.; n=18, *p<0.05.
Fig. 2.2 *IL-4 KO mice do not exhibit depressive-like behavior.* (A) WT and IL-4 KO mice were subjected to the forced swim test for 5 min and total immobility was quantified. Results are expressed as means ± s.e.m.; n=7-9, *p<0.05. (B) WT and IL-4 KO mice were allowed free access to water and a 0.4% saccharin solution for 24 h. Quantities of each fluid were recorded and saccharin consumption expressed as the percent of total fluid consumption. Results expressed as means ± s.e.m.; n=17-18, *p<0.05.
Fig. 2.3 *Spatial and non-spatial memory is not impaired in IL-4 KO mice.* (A) WT and IL-4 KO mice were trained in the Morris Water Maze at 3 trials per day for 4 days. Latency to platform was quantified for each trial, and results are the average of 3 trials for each day. Results are expressed as means ± s.e.m.; n=8, *p<0.05. (B-D) On the 5th day of testing in the Morris Water Maze, the hidden platform was removed and mice were tested in 3 trials of 60 seconds. Time spent in platform quadrant (B), latency to platform (C) and number of platform crossings
**Fig. 2.3 (cont.)** (D) were quantified for each trial, and results are the average of 3 trials. Results are expressed as means ± s.e.m.; n=8, *p<0.05. (E) WT and IL-4 KO mice were subjected to Novel Location test for 5 min. Exploration of objects was quantified and investigation of each location expressed as percent of total investigation time. Results are expressed as means ± s.e.m.; n=7-9, *p<0.05. (F) WT and IL-4 KO mice were subjected to Novel Object test for 5 min. Exploration of objects was quantified and investigation of each object expressed as percent of total investigation time. Results are expressed as means ± s.e.m.; n=16, *p<0.05.
Fig. 2.4 Anxiety-like behaviors differ in IL-4 KO versus WT mice. (A) WT and IL-4 KO mice were tested in the Elevated Zero Maze for 5 minutes and time spent in open arms of the maze was quantified. Results are expressed as means ± s.e.m; n=15, *p<0.05. (B-C) WT and IL-4 KO mice were allowed to explore an open field arena for 5 minutes. Total locomotor activity and time spent in center of arena were quantified. Results are expressed as means ± s.e.m; n=5, *p<0.05.
**Fig. 3.1** Palmitic acid decreases spontaneous locomotor activity. Mice were injected intraperitoneal (IP) with vehicle or palmitic acid (0.3, 3 or 30 µmol) and home-cage locomotion was measured 2 h later. Results are expressed as means ± s.e.m.; n=8-9. Bars without a common superscript are different (p<0.05).
Fig. 3.2 Palmitic acid-induced loss of locomotion is not dependent on TLR4, MyD88, IL-1, IL-6 or TNFα. C57BL/6J mice (WT) and mice with a genetic knock-out (KO) of TLR4, MyD88, IL-1r1, IL-6, and TNFα were injected IP with vehicle or 30 µmol palmitic acid and home-cage locomotion was measured 2 h later. Results are expressed as means ± s.e.m.; n = 7-12. (*p<0.05 vs vehicle treated control).
Fig. 3.3 Chain length is important to FFA-dependent loss of spontaneous locomotion. Wild type (WT) mice were injected IP with vehicle or 30 µmol of palmitic (C16:0), palmitoleic (C16:1), myristic (C14:0), decanoic (C10:0), octanoic (C8:0) or valeric (C5:0) acid and home-cage locomotion was measured 2 h post-injection. Results are expressed as means ± s.e.m.; n = 8. Bars without a common superscript are different (p< 0.05).
Fig. 3.4 Mice exhibit anxiety-like behavior 24 hrs after palmitic acid administration.
Fig. 3.4 (cont.) (A) Mice were injected IP with vehicle or 30 µmol palmitic acid and time spent investigating novel objects in a test arena was quantified. Results are expressed as means ± s.e.m.; n = 7. (*p < 0.05 versus vehicle treated control). (B) Mice were injected IP with vehicle or 30 µmol palmitic acid and time spent in the open arm of the elevated Zero maze was quantified 24 and 48 hrs post-treatment. Results are expressed as means ± s.e.m.; n = 8. (*p<0.05 versus vehicle treated control per time). (C) Mice were injected IP with vehicle or 30 µmol palmitic acid and home-cage locomotion was measured 2 and 24 h later. Results are expressed as means ± s.e.m.; n = 11-14. Bars without a common superscript are different (p< 0.05). (D) Mice were injected IP with vehicle or 30 µmol palmitic acid and food intake was measured from injection to 24 h and from 24 h to 48 h. Results are expressed as means ± s.e.m.; n = 9-10. Bars without a common superscript are different (p< 0.05). (E) Mice were injected IP with vehicle or 30 µmol palmitic acid and FST immobility was scored after 24 h. Results are expressed as means ± s.e.m.; n = 4. (*p < 0.05 versus vehicle treated control). (F) Mice were injected IP with vehicle or 30 µmol palmitic acid and Y-maze performance was scored at % perfect alternations after 24 h. Results are expressed as means ± s.e.m.; n = 8. (*p<0.05 versus vehicle treated control).
**Fig. 3.5** Plasma FFAs levels after palmitic acid administration. Mice were injected IP with vehicle or 30 µmol palmitic acid and plasma FFAs were measured at 2, 4, 8, 12 or 24 h later. Results are expressed as means ± s.e.m.; n = 6. Bars without a common superscript are different (p<0.05).
Table 3.1 - NE, NME, DA, DOPAC, HVA, 5-HT and 5-HIAA concentrations in the brain 24 h after palmitic acid administration

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Palmitic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex NE</td>
<td>2.34 ± 0.08</td>
<td>2.36 ± 0.13</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>2.28 ± 0.07</td>
<td>2.44 ± 0.12</td>
</tr>
<tr>
<td>Amygdala</td>
<td>3.28 ± 0.17</td>
<td>3.43 ± 0.41</td>
</tr>
<tr>
<td>Cortex NME</td>
<td>0.42 ± 0.05</td>
<td>0.33 ± 0.05</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.45 ± 0.05</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>Amygdala</td>
<td>0.29 ± 0.06</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>Cortex DA</td>
<td>8.49 ± 1.12</td>
<td>9.52 ± 0.95</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.07 ± 0.01</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Amygdala</td>
<td>1.12 ± 0.13</td>
<td>1.12 ± 0.13</td>
</tr>
<tr>
<td>Cortex DOPAC</td>
<td>1.27 ± 0.08</td>
<td>1.15 ± 0.11</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Amygdala</td>
<td>0.29 ± 0.04</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>Cortex HVA</td>
<td>1.59 ± 0.13</td>
<td>1.79 ± 0.16</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.28 ± 0.02</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>Amygdala</td>
<td>0.43 ± 0.07</td>
<td>0.47 ± 0.07</td>
</tr>
<tr>
<td>Cortex 5-HT</td>
<td>1.69 ± 0.06</td>
<td>1.70 ± 0.08</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>1.70 ± 0.24</td>
<td>1.91 ± 0.23</td>
</tr>
<tr>
<td>Amygdala</td>
<td>5.96 ± 0.37</td>
<td>5.89 ± 0.53</td>
</tr>
<tr>
<td>Cortex 5-HIAA</td>
<td>1.01 ± 0.05</td>
<td>1.07 ± 0.04</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>2.75 ± 0.15</td>
<td>3.01 ± 0.21</td>
</tr>
<tr>
<td>Amygdala</td>
<td>2.01 ± 0.16</td>
<td>2.67 ± 0.24*</td>
</tr>
</tbody>
</table>

Results are expressed as means; n=5-6. Significant difference at *p<0.05 vs vehicle.

Table 3.2 – NE:NME, DA:DOPAC, DA:HVA and 5-HT:5-HIAA concentrations in the brain 24 h after palmitic acid administration

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Palmitic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex NE:NME</td>
<td>6.05 ± 0.73</td>
<td>7.90 ± 0.96</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>5.34 ± 0.54</td>
<td>6.82 ± 0.83</td>
</tr>
<tr>
<td>Amygdala</td>
<td>12.85 ± 1.87</td>
<td>9.79 ± 1.44</td>
</tr>
<tr>
<td>Cortex DA:DOPAC</td>
<td>6.56 ± 0.58</td>
<td>8.73 ± 1.40</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.93 ± 0.06</td>
<td>1.32 ± 0.14*</td>
</tr>
<tr>
<td>Amygdala</td>
<td>4.15 ± 0.59</td>
<td>4.28 ± 0.70</td>
</tr>
<tr>
<td>Cortex DA:HVA</td>
<td>5.27 ± 0.39</td>
<td>5.28 ± 0.17</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.25 ± 0.25</td>
<td>0.35 ± 0.07</td>
</tr>
<tr>
<td>Amygdala</td>
<td>2.88 ± 0.45</td>
<td>2.61 ± 0.49</td>
</tr>
<tr>
<td>Cortex 5-HT:5-HIAA</td>
<td>1.69 ± 0.08</td>
<td>1.60 ± 0.08</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.63 ± 0.10</td>
<td>0.66 ± 0.11</td>
</tr>
<tr>
<td>Amygdala</td>
<td>3.01 ± 0.16</td>
<td>2.25 ± 0.21*</td>
</tr>
</tbody>
</table>

Results are expressed as means; n=5-6. Significant difference at *p<0.05 versus vehicle.

Table 3.3 – MAO-A and -B activity in the brain 24 h after palmitic acid administration

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Palmitic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex MAO-A</td>
<td>4871 ± 112</td>
<td>5203 ± 223</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>11429 ± 455</td>
<td>11753 ± 547</td>
</tr>
<tr>
<td>Amygdala</td>
<td>9938 ± 481</td>
<td>10317 ± 354</td>
</tr>
<tr>
<td>Cortex MAO-B</td>
<td>1572 ± 117</td>
<td>1889 ± 180</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>3540 ± 94</td>
<td>4602 ± 778</td>
</tr>
<tr>
<td>Amygdala</td>
<td>2728 ± 227</td>
<td>2662 ± 167</td>
</tr>
</tbody>
</table>

Results are expressed as means; n=6.

Table 3.4 – Brain expression of the UPR genes Xbp1, Edem1, Hsp90b1 and Atf4 24 hrs after palmitic acid administration

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Palmitic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex Xbp1</td>
<td>1.00 ± 0.08</td>
<td>0.95 ± 0.05</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>1.00 ± 0.06</td>
<td>1.12 ± 0.06</td>
</tr>
<tr>
<td>Amygdala</td>
<td>1.00 ± 0.64</td>
<td>0.97 ± 0.07</td>
</tr>
<tr>
<td>Cortex Edem1</td>
<td>1.00 ± 0.07</td>
<td>0.95 ± 0.03</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>1.00 ± 0.10</td>
<td>1.12 ± 0.05</td>
</tr>
<tr>
<td>Amygdala</td>
<td>1.00 ± 0.07</td>
<td>0.97 ± 0.05</td>
</tr>
<tr>
<td>Cortex Hsp90b1</td>
<td>1.00 ± 0.05</td>
<td>0.98 ± 0.04</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>1.00 ± 0.10</td>
<td>1.06 ± 0.05</td>
</tr>
<tr>
<td>Amygdala</td>
<td>1.00 ± 0.07</td>
<td>0.97 ± 0.05</td>
</tr>
<tr>
<td>Cortex Atf4</td>
<td>1.00 ± 0.05</td>
<td>0.81 ± 0.03*</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>1.00 ± 0.10</td>
<td>1.04 ± 0.05</td>
</tr>
<tr>
<td>Amygdala</td>
<td>1.00 ± 0.07</td>
<td>0.89 ± 0.05</td>
</tr>
</tbody>
</table>

Results are expressed as means; n=5-8. Significant difference at *p<0.05 versus vehicle.
REFERENCES


Gratchev, A., Kzhyskowska, J., Köthe, K., Muller-Molinet, I., Kannookadan, S., Utikal, J., Goerdt, S., 2006. MΦ1 and MΦ2 can be re-polarized by Th2 or Th1 cytokines, respectively, and respond to exogenous danger signals. Immunobiology. 211: 473-486.


receptor GPR40 in the neurogenic niche of adult monkey hippocampus. Hippocampus 18: 326-333.


