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Aged Mice Lacking Fatty Acid Amide Hydrolase Show Reduced Neuroinflammation, Senescence, and Cognitive Impairment

THESIS

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ABSTRACT OF THE THESIS

Aged Mice Lacking Fatty Acid Amide Hydrolase Show Reduced Neuroinflammation, Senescence, and Cognitive Impairment

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The relationship between lipid-derived messengers and inflammation is a promising avenue for therapeutics in a number of diseases. The enzyme, Fatty Acid Amide Hydrolase (FAAH), has been implicated as an effective treatment point for afflictions such as pain, however, its involvement in the regulation of endogenous lipids involved in the inflammatory response in the central nervous system (CNS) has not yet been explored in great depth. Here, I demonstrate that aged mice lacking FAAH show not only a significantly reduced inflammatory profile (Il-1ß, Il-6, TNF-α, iNOS), within the CNS but also reduced incidence of glial fibrosis (GFAP), cellular senescence (p21, p53), decreased ceramide levels (d18:1/24:1) and improved learning and memory in the Object Location Memory Paradigm. The results suggest that inhibition of FAAH in aged mice is able to reverse the effects of neuroinflammation and perhaps lead to more 'successful' aging in these animals.

Chapter 1

Introduction: Inflammation and Lipids

The relationship between chronic inflammation and disease has outgrown its criticism as a correlational artifact and has begun to plant itself as a reliable contributor to the development of several pathologies. Inflammation has been implicated in many chronic diseases including: cancer, atherosclerosis, diabetes, and obesity (Freund et al., 2010) and also been shown to play a role in diseases of the Central Nervous System (CNS), which have contributed to massive increases in health care costs in the United States. In 2012 alone, $200 billion was spent on health care-related services for Alzheimer's Disease (AD) and related dementias, with a projected increase to $1.1 trillion by 2050 (Alzheimer's Association, 2014). While there is a vast literature on the relationship between AD and inflammation (Heneka & O'Banion, 2007; Mrak & Griffin 2001) the true pathological scope of inflammation inciting harm on the CNS is much wider. Diseases ranging from depression (Dantzer et al., 2008; Gimeno et al., 2009; Kuo et al., 2005), Parkinson's disease (Hirsch & Hunot, 2009; Long-Smith et al., 2009), schizophrenia (Kirkpatrick & Miller, 2013) and anxiety (O'Donovan et al., 2010) all have a place under the umbrella of neurodegenerative disease and each have a unique relationship with inflammation.

Inflammation as an Adaptive Response

The question then arises as to why inflammation has such an association with brain pathology. In order to explore this, we must first undertake the task of understanding what inflammation is. It should be noted that the process of inflammation is not a degenerative state that is to be avoided, but a normal and essential physiological response. This idea was stated clearly by the 18th century Scottish Physician, John Hunter, "inflammation in

itself is not to be considered as a disease, but as a salutary operation consequent to some violence or some disease" (Majno, 1975). As such, inflammation is not a maladaptive state but rather, an adaptive response to tissue injury or infection. The main goal of an animal's immune system in response to such an insult is to bring the organism back to homeostasis (Medzhitov, 2008). As we will see, a dysregulation of homeostasis can be the product of a variety of stimuli which, as an end result, affect the viability of tissues and the health of the organism.

Inflammation consists of four principal components: induction, prompted by exogenous inducers (pathogens, irritants) or endogenous signals triggered by tissue damage and the desequestration of cells and molecules; sensors, such as the toll-like receptor (TLR) family which will recognize and bind to products of induction such as lipopolysaccharides (LPS); mediators, such as cytokines, proteolytic enzymes, chemokines, eicosanoids and other lipid messengers that regulate the recruitment of immune cells and the physiological responses to inflammation; and finally, effectors such as the tissues and cells that are affected by the immune cascade (Medzhitov, 2008). Once the insult or infection is abrogated, a carefully coordinated repair phase involving a shift in lipid (Serhan, 2007; Serhan & Savil, 2005) and protein (Franceschi et al., 2007) based signaling brings the tissue back to homeostasis. Viewing inflammation in this way is advantageous as it allows it to be operationally defined in a manner consistent with its purpose and conceptualized as a process as opposed to a static state. It would seem that of the four parts, treatments that aim to curb inflammation should act on the mediators responsible for the signaling in the immune response, as inducers, sensors, and effectors offer impractical routes of treatment.

Para-Inflammation

Both endogenous and exogenous inducers can affect the basal state of cells, which will then rouse an effort to reinstate homeostasis (Medzhitov, 2008). Traditional examples often involve exogenous inducers (e.g., bacteria), which can elicit a rather extreme version of the immune response — the recruitment of plasma proteins and leukocytes (Freund et al., 2010) by macrophage and mast cell signaling (mediators) which alter the physiological state of the organism and induce the traditional descriptors of inflammation highlighting its presence in terms of clinical symptoms: redness, edema, pain, and heat (Saladin et al., 2007). However, endogenous inducers, such as stress (Buchanan et al., 2008), the accumulation of reactive oxygen species (ROS; Glass et al., 2010), apoptosis (Medzhitov, 2008), or the degradation of extracellular components (Sorokin, 2010) can occur in a graded manner, which does not necessarily elicit the same degree of inflammation. This state, which is more adequately described as 'para-inflammatory' (Medzhitov, 2008), displays levels of plasma cytokines and other factors that are elevated, but below a threshold that would make the inflammation clinically relevant (Freund et al., 2010). Para-inflammatory states are a common vestige in the aging process (Goto, 2009), which represents a formidable challenge because the accumulation of inflammation inducers – such as abnormal protein aggregation (Wyss-Coray & Mucke 2002) cellular senescence (Campisi, & d'Adda di Fagagna, 2007), toxic microenvironments (Campisi, 2005) and the accumulation of ROS (Glass et al., 2010) – are an inevitable consequence of aging.

Chronic Inflammation and Treatment

If the immune system is unable to resolve the issue, regardless of the inflammatory state being sub or para-threshold, a state of chronic inflammation can ensue (Lawrence & Gilroy, 2007). This is characterized by increased cytokine levels in circulation, consistent mobilization of lymphocytes and macrophages to the inflamed tissue, fibrosis, apoptosis and necrosis (Freund et al, 2010). Both necrosis and apoptosis can incite macrophage action (Medzhitov, 2008), which can further contribute to an inflammatory state and thus repeat the cycle. It is this chronic inflammatory state that is thought to play an essential role in the development of the aforementioned pathologies (Freund et al., 2010) and where typical routes of treatment have been aimed. Why certain mediators fail to bring the immune system into a resolved state is difficult to answer as the process involves several players. Despite this complexity, attempts have been made to alter several mediators of the inflammatory process in the treatment of disease.

Inhibition of the IL-1ß receptor, IL-1R, has been shown to reduce edema, injury, and glial activation in rodents (Allan & Rothwell, 2001). IL-1R antagonists have also been tested in normal volunteers with sepsis (Fisher et al., 1994) and rheumatoid arthritis with few side effects and good efficacy (Bresnihan, 2001). However, the extent of its treatment in other inflammatory conditions, especially in the CNS, has questionable efficacy. Other target mediators are those derived from lipid membranes (Lawrence et al., 2002), the most famous being the cyclooxygenase (COX) products, which are inhibited by aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs; Ferreira, 1972). These drugs work by preventing COX from converting arachidonic acid into the prostaglandins PGG_2 and PGH_2, which play key roles in pain and the inflammatory response (Seibert et al., 1995). Attempts

to administer NSAIDs in the treatment of neurodegenerative diseases have been met only with modest success (McGeer & McGeer, 2004). Additionally, this class of drugs causes severe gastrointestinal side effects (Buttgereit et al., 2001) and the inhibited COX derived prostaglandins have been shown to be involved in the resolution of inflammation (Gilroy et al., 1999), demonstrating NSAIDs as unattractive candidates for long-term treatment. However, there are several other classes of lipid mediators that have shown promise in ameliorating inflammation with minimal adverse effects.

Endocannabinoids and Inflammation

Other lipid mediators include the eicosanoids, lipoxins, and resolvins, which have shown themselves as essential players in immune regulation (Serhan, 2007; Serhan & Savil, 2005). Notably, attention has been paid to endogenous cannabinoid substances such as anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), which are also important modulators of the inflammatory response (Klein, 2005) and have shown promise in ameliorating inflammation and pain (Piomelli & Sasso, 2014). As mediators, endocannabinoids have been documented to be chemotactic agents for leukocytes (Klein, 2005; Oka et al., 2004) affecting the balance of Th1/Th2 cells (Yuan et al., 2002) and affecting other populations such as NK-cells and neutrophils (Pandey et al., 2009). While the peripheral effects of endocannabinoid action within the immune system are well documented (Berdyshev, 2000; Klein et al., 1998), endocannabinoids also have been shown to modulate inflammation and the immune response within the CNS. They modulate the actions of various cytokine cascades within the CNS (Correa et al., 2009; Correa et al., 2010; Klein et al., 1998) and have also been shown to directly inhibit IL-1ß and TNF-α (Nagayama et al., 1999). Endocannabinoids also affect the resident immune cells of the CNS; astrocytes

stimulated by LPS show attenuated iNOS release when given CB_1 cannabinoid receptor agonists (Molina-Holgado et al., 2002), LPS stimulated microglia given endogenous cannabinoids show attenuated transcription of various cytokines (Puffenbarger et al., 2000), and CB_1 agonists in glia co-cultures increase endogenous IL-1ra attenuating the effects of IL-1ß (Molina-Holgado et al., 2003).

Outside of cell culture, these same agonists have also demonstrated success in animal models of inflammation (Allan & Rothwell, 2001). Administration of cannabinoid agonists has been shown to be neuroprotective in various pathologies and injuries, in which inflammation is suspected. In models of multiple sclerosis, cannabinoid agonists have been shown to be neuroprotective by ameliorating behavioral deficits and decreasing cytokine signaling and immune activation within the CNS (Arévalo-Martín et al., 2003; Croxford & Miller, 2003). In mouse models of AD, treatment with the synthetic cannabinoid agonist WIN55,212-2 decreased the incidence of microglia activation, prevented cognitive impairment in a spatial navigation task, and lowered neurotoxicity (Ramírez et al., 2005). In other AD models, APP transgenic mice lacking the CB_1 receptor perform significantly worse in the Morris Water Maze than do wild-type controls (Stumm et al., 2013). Additionally, cannabinoids have also been shown to be effective in providing neuroprotection in animals with 6-OHDA lesions (Lastres-Becker et al., 2005) as well as in models of amyotrophic lateral sclerosis (Bilsland et al., 2006). In cases of trauma, cannabinoids administered after closed-head injury are able to decrease edema and improve clinical outcome, an effect that was countered by CB_1 antagonists (Panikashvili et al., 2001). Although not conclusive, the above offers sufficient evidence that the

endocannabinoid system can profoundly affect mediators of the immune response resulting in demonstrable successes in a variety of illnesses.

Other Fatty Acid Ethanolamides

In addition to endocannabinoids, another class of fatty acid ethanolamides, which includes oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), have been shown to play a role in the control of inflammation (Piomelli & Sasso, 2014). These lipids were once thought to exert their anti-inflammatory effects via the CB_2 receptor, but were later shown to selectively bind to the PPAR receptor family, specifically, PPAR-α, to exert their effects (Fu et al., 2003; Lo Verme et al., 2005). PPAR's are located ubiquitously throughout the nervous system (Moreno et al., 2004) and activation of PPAR's have been shown to be involved in the inhibition of transcription factors NF-κB and AP1, which can result in the transrepression of several other classes of inflammatory proteins such IL-1ß and TNF-α (Glass & Ogawa, 2006; Piomelli & Sasso, 2014). Additionally, PPAR agonists have been shown to be therapeutic in several pathologies, especially those with etiologies of chronic inflammation (Straus & Glass, 2007). *In vivo* and *In vitro* studies have shown administration of the PPAR-α endogenous lipid ligands OEA and PEA to have a multitude of anti-inflammatory effects which are likely mediated by this receptor. PEA is able to attenuate cytokine levels of TNF-α and IL-6 (Hesselink et al., 2013), and pharmacological inhibition of one of its degradative enzymes, N-acylethanolamine acid amidase (NAAA), can attenuate the inflammatory response in leukocytes and macrophages stimulated with the inflammatory polysaccharides carrageenan and LPS (Solorzano et al., 2009).

OEA can suppress the expression of iNOS (Fu et al., 2003; Colville-Nash et al., 1998) and has been shown to modulate the inflammatory response by decreasing COX-2 and

increasing the anti-inflammatory mediator Iκ-ßα (Sun et al., 2007). This same study also found OEA to be neuroprotective in cases of induced middle cerebral artery inclusion and this effect was mediated by the binding of PPAR-α (Sun et al., 2007). Clinically, OEA and PEA are found in significantly lower concentrations in the synovial fluid of rheumatoid arthritis and osteoarthritis patients relative to healthy controls (Richardson et al., 2008), and PEA has a history in treating sickness in several human trials for influenza and respiratory infections (Hesselink et al., 2013).

An intriguing idea for the role of OEA and PEA in inflammation is that PPAR-α binding can act as a 'no-go' signal to prevent the development of an immune response that would lead to inflammation (Piomelli & Sasso, 2014; Solorzano et al., 2009) and that events such as aging may throw this homeostatic set-point off which leads to increases in pro-inflammatory mediators (Erol, 2005). Interestingly, aged mice show significantly lower levels of OEA and PEA in the hippocampus (Maccarone et al., 2001) and PPAR-α activation was found to lower chronically active NF-κB in aged mice (Poynter & Daynes, 1998). Of interest, the basal levels of these fatty acid ethanolamides decreasing with age in the CNS (Maccarone et al., 2001) may alter the ability of these lipids and their receptors to put a brake on chronic inflammation.

Fatty Acid Amide Hydrolase

One of the gate keepers for the above lipids is the enzyme fatty acid amide hydrolase (FAAH). FAAH is a 63kDa membrane-bound 579 long amino-acid serine hydrolase serving as the main enzyme responsible for the degradation of AEA (Désarnaud et al., 1995; Bracey et al., 2002; Cravatt et al., 2001; Giang & Cravatt, 1997). While FAAH shows high selectivity for AEA, it has also been shown to degrade other fatty acid ethanolamides including OEA

and PEA (Désarnaud et al., 1995). FAAH converts AEA into arachidonic acid and ethanolamine, and PEA into palmitic acid and ethanolamine (Désarnaud et al., 1995; Natarajan et al., 1984). Rat and mouse FAAH share, respectively, 82% and 84% sequence homology with the human version of the enzyme as well as highly conserved hydrolysis rates of several substrates (Giang & Cravatt, 1997). This renders studies utilizing these models relevant to human variants of the enzyme.

FAAH is located throughout the body at differing levels, with a large presence in the brain and liver, and a minimal distribution in the heart, kidney, intestine, stomach, lung, spleen, and skeletal muscle (Désarnaud et al., 1995). Primarily, the distribution of FAAH in the brain targets it as an important enzyme in the regulation of the fatty acid ethanolamides as it is a primary regulator of these lipids in the brain (Cravatt et al., 2001; Kathuria et al., 2003). Within the brain, FAAH is expressed in many areas, most notably the large principal neurons of the hippocampus, neocortex, cerebellum and olfactory bulb (Egertova et al., 1998; Tsou et al., 1998). In tandem with the presence of FAAH, is the expression of CB_1 receptors in adjacent neurons (Egertova et al., 1998; Piomelli, 2003; Tsou et al., 1998), however, this co-localization is not ubiquitous and gives further evidence for the propensity of FAAH to cleave other substrates.

Inhibition of FAAH

Models to explore FAAH's potential as a treatment target have been developed and explored in a variety of pathologies – from anxiety (Kathuria et al., 2003) to pain (Jayamanne et al., 2006). A line of FAAH deficient mice has been developed (Cravatt et al., 2001) as well as FAAH inhibitors such as URB597 (Kathuria, et al., 2003) which have been shown to significantly increase the levels of AEA in the brain and have behavioral effects.

Both of these routes have shown promise in developing models to treat and understand the role of FAAH substrates in various disease states. Of relevance, FAAH knock-out mice show significant decreases in the hydrolysis of AEA and significant behavioral abnormalities when administered AEA (Cravatt et al., 2001). Additionally, FAAH KO mice were found to exhibit enhanced endogenous cannabinoid activity (up to 15x), significantly increased OEA and PEA activity in the brain (Cravatt et al., 2001), and demonstrate increased serotonergic signaling the brain (Cassano et al., 2011), which has also been shown to affect TNF-α-induced inflammation (Yu, et al., 2008).

FAAH in the Control of Inflammation

As described above, the propensity for the endocannabinoids as well as the PPAR-α agonists OEA and PEA to modulate inflammation is a promising avenue for therapeutics in chronic inflammation. In tapping the potential of drugs that selectively inhibit FAAH in both the periphery and the brain, scientists have been able to investigate the effects of raising endocannabinoids as well other fatty acids in the treatment of disease. One of the most demonstrable uses for FAAH inhibition is that for inflammatory pain (Piomelli & Sasso, 2014). Pharmacological inhibition of FAAH with URB597 has shown success in ameliorating hyperalgesia (Jayamanne et al., 2006) in several models with evidence for both PPAR-α and CB_1 as downstream receptors for its action (Sagar et al., 2008; Schlosburg, 2009). In the brain, inhibition of FAAH by URB597 has been shown to attenuate microglia activation after modeling traumatic brain injury in rodents (Katz et al., 2014) and FAAH deletion reduced ALS symptoms in mice with an SOD1 mutation (Bilsland et al., 2006). FAAH inhibition shows promising therapeutic potential in ameliorating the symptoms of inflammatory based maladies.

FAAH Knock-Out Mice as a Model

It has been conclusively shown that levels of AEA, OEA and PEA are significantly increased *in vivo* as a result of genetic ablation of FAAH (Cravatt et al., 2001). Knowing that AEA, OEA, and PEA can have powerful anti-inflammatory effects and therapeutic potential, the question arises as to whether long-term FAAH inhibition might alter common mediators of immune activation in aged mice, which are prone to states of chronic inflammation, and what effect this may have on their phenotype and behavior. To answer this question, a line of FAAH knock-out mice was bred and allowed to reach an old age (18-19 months). The aged animals were tested in a behavioral model of cognition and sacrificed. The following sections examine the effects of long-term FAAH inhibition on inflammatory mediators in these aged animals, and the effects that they may have on other vestiges of aging such as cellular senescence and cognitive impairment.

Chapter 2

'Inflamm-aging' in FAAH Knock-Out Mice

Throughout the lifespan, the culmination of exogenous and endogenous stressors (radiation, oxygen radicals, infection etc.) leads to the induction of several adaptive responses that then operate to maintain tissue homeostasis. As aging occurs, the accumulation of these stressors and the corresponding push to restore homeostasis, especially in immunity, can eventually lead to several physiological alterations which can result in a distinct phenotype colloquially characterized as 'inflamm-aging' (Franceschi et al., 2000). This phenotype is best represented with the occurrence of a para-inflammatory state (Medzhitov, 2008) that is, an elevated but clinically negligible level of cytokines and chemokines (Freund et al., 2010). Individual differences exist in the immune response which leads to a spectrum of varied health outcomes in aged subjects (Franceschi et al., 2000), and along these lines, individuals that experience healthy and 'successful' aging are found with overall lower levels of cytokines and other inflammatory mediators (Franchesi et al., 2007) and a decreased incidence of chronic disease in old age (Cevenini et al., 2013).

Here, the emergence of 'successful' aging and the absence of disease can be characterized by organisms that have an efficient and well-tempered immune response, which globally reduces the occurrence of inflammation and thus disease (Franceschi et al., 2000). Evidence suggests that aging can prompt a decrease in both the levels fatty acid ethanolamides (Maccarone et al., 2001) and CB_1 expression throughout the brain (Berrendero et al., 1998; Bilkei-Gorzo, 2012). Knowing the role that lipids play in immunity, one can postulate that tempering certain aspects of lipid metabolism throughout life can alter the immune response and thus increase the probability of 'successful' aging.

In this chapter, the link between 'inflamm-aging' and disease will be further established and a proposition for treatments that focus on reducing para-inflammation via the alteration of lipids in the CNS will be examined. Utilizing mice as a model, it will be argued that a global reduction in FAAH will decrease the incidence of an 'inflamm-aging' phenotype to render a more 'successful' aging process in these animals.

Neuroinflammation: An Exposé

There are over 36 cytokines expressed in the body, however, only a subset of these proteins and their receptors are expressed in the CNS and are considered "pro-inflammatory". The best studied are those from the class of Interleukins and Tumor Necrosis Factors: IL-1ß, IL-6 and TNF-α. Affecting both neurons and glia (Allan & Rothwell, 2001), each have receptors in the CNS with a high density of IL-1ß within the dentate gyrus of the hippocampus (Ban et al., 1991; Takao et al., 1990) and TNF within homogenates of the brainstem, basal ganglia, thalamus and cortex (Kinouchi et al., 1991). The mechanism of action for cytokines in the CNS is complex, consisting more of a synergy and interaction between these proteins and their receptors than a claim for the individual constituents themselves (Ownby, 2010). For example, TNF-alpha and IL-1ß can induce the expression of IL-6 (Benveniste et al., 1990) and IL-1ß can directly influence the expression of TNF-α (Bethea et al., 1992).

Cytokines can also serve as pyrogens, which influence the release of several eicosanoids (Rothwell & Hopkins, 1995) and other inflammatory mediators such as COX-2 (Serou et al., 1999), iNOS (Bonmann et al., 1997) and c-reactive protein (Singh-Manoux et al., 2014). This multi-faceted influence translates to the selective binding of cytokines to their receptors at which point they are able to alter vasculature (Giulian et al., 1988), the

permeability of the blood-brain barrier (Quagliarello et al., 1991), the induction of vasogenic edema (Holmin & Mathiesen, 2000) and the alteration of several other physiological parameters such as blood flow and temperature (Allan & Rothwell, 2001).

Despite these robust physiological alterations, cytokines also exert more subtle effects in the CNS, which do not necessarily result in an immune response. For example, IL-1ß has been shown to inhibit calcium release in the hippocampus (Plata-Salaman & Ffrench-Mullen, 1992) glutamate release in hippocampal synaptosomes (Murray et al., 1997), and long-term potentiation (LTP) in various hippocampal subregions (Bellinger et al., 1993; Cunningham et al., 1996; Katsuki et al., 1990). Along with IL-1ß, TNF-α has also been shown to inhibit LTP during administration of LPS on hippocampal slices (Cunningham et al., 1996). IL-1ß can also significantly interfere with striatal CB_1 receptor GABA activity by interfering with cholesterol metabolism (Rossi et al., 2012) and is able to interfere with BDNF signaling in the brain, a growth factor important for the modulation of LTP and cognitive function (Tong et al., 2008). These acute changes in LTP and other parameters within the hippocampus give clues to some of the ways inflammatory mediators may contribute to cognitive dysfunction in old age.

Glial Cells and the Modulation of Neurodegeneration

While the effect of cytokines on neurons is no doubt an important contributor to neurodegeneration, these same signaling pathways also affect glial cells which have been shown to be paramount to pathogenesis in the aging CNS (Cunningham, 2013; Giulian et al., 1993). Cytokines differentially affect glia cells and thus have a variety of effects on the surrounding microenvironment which can contribute to CNS pathology. Co-culture experiments suggest that isolated microglia and astrocytes separated by filtered chambers,

affect adjacent neuronal cultures differently. In response to cytokine signaling, astrocytes release growth factors such as NGF (Čarman-Kržan et al., 1991), however, microglia, the resident macrophages of the CNS, release various cytotoxic substances after cytokine stimulation, which can cause substantial neuronal loss (Giulian et al., 1993). While cytokine administration to astrocytes can cause neuronal proliferation, the stimulation of microglia can cause up to 50% of neuron loss over 48 hours (Giulian et al., 1993).

Microglia respond to various insults such as brain damage and infection in a manner analogous to peripheral monocytes and macrophages (Kreutzberg, 1996) by releasing highly reactive oxygen- and nitrogen-derived radicals (Hirsch & Hunot, 2009) and causing damage to surrounding tissue to further exacerbate CNS inflammation. However, astrocytes can also prove deleterious if cytokine signaling is aberrant, a real possibility in aging populations. Astrocytes have been shown to be active players in the CNS immune response (Farina et al., 2007) and can prompt increases in neurodegeneration (up to 90% loss of cells in hippocampal dentate gyrus) as a result of the overexpression of IL-6 (Campbell et al., 1993).

Evidence also suggests the make-up of our resident glia cells change as we age which may alter the surrounding CNS microenvironment and be conducive to increased inflammation and neurodegeneration (Barrientos et al., 2010). A phenotypic shift in microglia from a 'quiescent' state to a 'primed' state occurs with age and skews these cells to exaggerate immune responses when given both exogenous and endogenous inducers (Barrientos et al., 2010). Studies have shown significant up-regulation of MHCII in the aged brains of humans and rodents (Perry et al., 1993), as well as significant increases in CD11b and IFN-ϒ mRNA and decreases IL-10 and CD200 mRNA (Frank et al., 2006). Astroglia

fibrosis also occurs as a result of aging as is evidenced by significant increases in glial fibrillary acid protein (GFAP) in the CNS (Morgan et al., 1997; Yoshida et al., 1996).

As with cytokines, it is likely that the long-term activation and alterations of these resident immune cells can further lead to the effects of neurodegeneration and cognitive impairment seen in aging. With that said, it is important to remember that these players still lie in the service of the immune system and their existence within the CNS is in an effort to bring the surrounding tissue back to homeostasis. But as we have seen, the phenotypic changes in aged populations can result in a myriad of physiological changes (Allan & Rothwell, 2001), alterations in neurotransmitter release (Plata-Salaman & Ffrench-Millen, 1992) and modulations of LTP (Bellinger et al., 1993; Cunningham et al., 1996; Katsuki et al., 1990), which may result in changes in neuron morphology (Richwine et al., 2008) and eventually behavior (Chapman et al., 2012; Jurgens et al., 2012; McLinden et al., 2012).

'Inflamm-aging' in Animal Models

While the cellular effects of aging on inflammation present a compelling narrative, it is the pathological effects *in vivo* which are often the most telling. In rodents, the basal expression of several genes for cytokines, MHC class II, along with 33 other genes involved in inflammation such as C1q and CD68 are increased with age (Godbout et al., 2005) which corresponds to cognitive impairment as well (Gallagher & Pelleymounter, 1988). However, more strikingly, it is the administration of exogenous stressors in aged animals which have the ability to profoundly exacerbate inflammation, alter morphology within the brain, and generate notable cognitive dysfunction. This spill over from para-inflammation to the emergence of pathology after stress is the one of defining differences between young and

aged rodents as well as in humans (Allan & Rothwell, 2001) and this may be caused in part by the phenotypic changes seen within glial cells of the CNS (Barrientos et al., 2010).

Aged mice challenged with intra-peritoneal administration of LPS show increased expression of MHC class II microglia, IL-1ß, TNF-α, IL-6, with reduced BDNF and NGF relative to their younger counterparts (Richwine et al., 2008). These same mice also demonstrate significant architectural abnormalities in the branching of CA1 pyramidal neurons (Richwine et al., 2008) and show an exacerbated physiological response with prolonged aberrant body temperatures relative to young animals (Barrientos et al., 2009). Other studies utilizing different infection-inducing agents such as Poly I:C, direct inoculation with influenza or *E. coli,* or with over-expression of IL-1ß (Moore et al., 2009) have shown similar increases of inflammatory mediators in the hippocampus and cortex (Chapman et al., 2012; Jurgens et al., 2012; McLinden et al., 2012; Moore et al., 2009), phenotypic changes in glia (McLinden et al., 2012; Moore et al., 2009) in addition to significant cognitive impairment. Hippocampal-dependent behavioral changes in burrowing (McLinden et al., 2012), performance on fear conditioning (Chapman et al., 2012) and performance in the Morris water maze (Jurgens et al., 2012; Moore et al., 2009) show a significant decrement compared to young animals. These behavioral changes directly corresponded to increased Iba-1 activity in the hippocampus (Jurgens et al., 2012), lower BDNF levels (Chapman et al., 2012), increased levels of GFAP (Moore et al., 2009) and altered morphology in the dentate gyrus (Jurgens et al., 2012). Still other studies which exclude the use of exogenous immune activators are also able to show increased inflammatory mediators and cognitive impairment following mild stress in aged animals (Buchanan et al., 2008). The same telling facts are also applicable to human models despite

some criticisms in the utilization of mice as a relevant model in inflammation research (Leist & Hartung, 2013; Seok et al., 2013). The parallels of the inflammatory response between humans and rodents are clear as will be seen in the next section.

'Inflamm-aging' in the Human CNS

In humans, as in mice, the average serum levels of several pro-inflammatory cytokines and chemokines increase with age. Humans show 2-4 times the levels of cytokines and chemokines in individuals older than 50 (Freund et al., 2010). This increase of various inflammatory mediators has been found to be associated with a number of diseases in the CNS. Alzheimer's patients show significant increases of TNF-α in serum as well as IL-1ß and IL-6 (Licastro et al., 2000). These increased basal levels of cytokines also occur in aged Parkinson's disease patients (McGeer & McGeer, 2004) and aged subjects with mild cognitive impairment (Magaki et al., 2007). As demonstrated earlier, inflammatory mediators often interact with one another in a wide spread 'network' to contribute to CNS dysfunction (Ownby, 2010). In humans, C-reactive protein, is significantly increased in aged subjects and has been linked to distinct changes in mental abilities (Kuo et al., 2005). This protein is modulated by the cytokine IL-6, which in itself is predictive of cognitive decline in old age (Singh-Manoux et al., 2014) and also shows a significant increase throughout aging (Maggio et al., 2006; Wei et al., 1992). In addition, the levels of GFAP are significantly increased in aged humans as in rodents (Nichols et al., 1993) along with morphological and phenotypic changes in microglia (Mrak & Griffin, 2005) which may contribute to an inflammatory microenvironment in the brain.

While cytokine expression can be linked to existing pathologies within the human CNS, it can also correlate directly with clinical outcomes and can exacerbate or lead to the

genesis of cognitive dysfunction in aged populations (Allan & Rothwell, 2001) due to the presence of altered glial phenotypes (Barrientos et al., 2010). Studies indicate that acute insults such as ischemia or stroke (Allan & Rothwell, 2001), depression (Wolkowitz et al., 2010), and surgery (Kálmán et al., 2006), can lead to cognitive dysfunction while increasing the levels of pro-inflammatory cytokines. A notable example is that of postoperative cognitive dysfunction (Rasmussen, 2006), where following surgery, aged patients experience symptoms of cognitive decline that occur in conjunction with sharp increases in IL-6 (Kálmán et al., 2006). The obvious parallels of inflammation on cognitive dysfunction in aged humans and animals allows for the development and use of murine models to explore effective treatments.

Modulation of FAAH for 'Successful' Aging

As described earlier, the effects of inflammation can be potentially mitigated with the administration of various pharmaceutical agents acting on the CB_1 receptor. This includes their ability to directly modulate cytokines (Arévalo-Martín et al., 2003; Croxford & Miller, 2003;) affect glia activation (Molina-Holgado et al., 2002), and decrease inflammation in animal models (Panikashvili et al., 2001; Piomelli & Sasso, 2014; Ramírez et al., 2005). These effects also apply to the ligands which bind to the PPAR-α subfamily (Glass & Ogawa, 2006; Hesselink et al., 2013; Straus & Glass, 2007) which may serve as a fundamental control point in the inflammatory response (Erol, 2005) and also have a history of affecting health outcomes in animal models (Sun et al., 2007) and in clinical use as well (Hesselink et al., 2013).

Individual differences in the immune response lead to a spectrum of varied health outcomes in aged subjects (Franceschi et al., 2000). In this view, 'successful' agers are

those whom have a more well-tempered response to various stressors, and of relevance here, those corresponding to immunity (Franchesi et al., 2007). Perhaps an altered lipid metabolism plays a role in the probability of an organism to age 'successfully'. Evidence for this lies in the fact that aging can promote a decrease in the levels of OEA and PEA (Maccarone et al., 2001) as well as reports of age-related decreases of CB_1 receptor binding and mRNA levels in the CNS (Berrendero et al., 1998; Bilkei-Gorzo, 2012), which can be inconsistent across studies. Perhaps by genetically altering the expression of the degradative enzyme FAAH, we may be able to instigate a tonic increase in the ligands that bind to both the CB_1 and PPAR-α receptors, and develop a phenotype that is represented by decreased inflammation leading to 'successful' aging over the life-span. I hypothesize that a long-term increase in AEA, OEA and PEA, obtained by knocking-out FAAH, will down-regulate the expression of common inflammatory mediators found in the brain: IL-1ß, IL-6, TNF-α (Ban et al., 1991; Kinouchi et al., 1991; Takao et al., 1990) and other inflammatory markers such as iNOS and those for glia fibrosis (GFAP). To test this hypothesis, a cohort of aged transgenic FAAH knock-out mice were utilized to explore various markers of inflammation in both the hippocampus and cortex.

Methods

A colony of male FAAH knock-out mice were crossbred with wild-type C57BL6/J mice (*n*= 8 WT; *n* = 10 FAAH -/-). The mice were bred and maintained in the animal facilities at the University of California, Irvine (Irvine, CA) under standard conditions (12:12 light-dark cycle, with lights on at 7:30; temperature at 20 Cº, and *ad libitum* access to food and water). The mice were housed in groups of 3-4 and allowed to age for 18 months before the experiments took place. Mice were euthanized with isofluorane and

sacrificed by decapitation. After sacrifice, the brains were removed and the hippocampus and cortex were dissected out on a glass cover slip over ice. The hemispheres of each region were counterbalanced between groups. The brains were flash frozen in liquid nitrogen and stored at -80^{o}C until analysis.

RNA analysis

RNA was extracted from frozen tissues utilizing TRIzol reagent (Invitrogen, Carlsbad, CA) and molecular biological grade chloroform, 2-propanol, and ethanol in the method of a traditional phenol-chloroform extraction. Briefly, tissues were homogenized using a sterile 27.5 gauge needle in approximately 1mL of TRIzol reagent. An equal volume (1:1) of molecular biological grade chloroform was then added and the samples were centrifuged at 10,000G for 15 minutes. The supernatant was taken, mixed with 400µL of 100% 2-propanol and then centrifuged again for 30 minutes at 10,000G. The 2-propanol was then discarded and 70% EtOH was added to further precipitate the RNA. After centrifuging for another 15 minutes at 10,000G the remaining EtOH was discarded and 30mL of RNAse free water was added. Reverse transcription of total RNA (2 µg) was then conducted using oligo(dT)12–18 primers for 50 min at 42°C. mRNA levels were measured by quantitative real-time polymerase chain reaction (RT-PCR) with a Mx 3000P system (Stratagene, La Jolla, CA). The following primers and fluorogenic probes were purchased from Applied Biosystems (TaqMan Gene Expression Assays, Foster City, CA): Interleukin-1ß (Rn00580432_m1), Interleukin-6 (Rn01410330_m1), TNF-α (Rn99999017_m1) iNOS (Mm00440502_m1), and GFAP (Mm01253033_m1). mRNA levels were normalized using GAPDH.

Data Analysis

Relative RNA levels were analyzed with a two-tailed unpaired students *t*-test where statistical significance was determined if $p < .05$. Results are expressed as $M \pm S.E.M.$ Analyses were performed using GraphPad Prism software (version 5.04).

Results

First, in order to investigate the general trend of inflammatory mediators in the CNS, the cortex was dissected and utilized to examine the basal levels of various cytokines (IL-6, IL-1ß, TNF-α) and other mediators of inflammation (iNOS) between the aged FAAH -/- and wild-type animals. mRNA analysis revealed a significant attenuation of the major pro-inflammatory cytokines within the cortex: IL-6 (0.062 ± 0.008 vs. 0.037 ± 0.004; $p< .05$) and TNF-α (0.209 ± 0.048 vs. 0.103 ± 0.011; $p< .05$) were significantly lower in FAAH $^{-/-}$ mice compared to age-matched wild-type controls (**Fig. 2a & 2b**). IL-1ß showed a decreasing trend in the cortex (0.156 ± 0.035 vs. 0.094 ± 0.008; $p = 0.09$), however, this was not significant. In concert with these findings, there was a notable but non-significant decrease in the production of iNOS in the cortex (0.233 ± 0.031 vs 0.172 ± 0.016; $p = 0.08$).

Next, these same mediators were investigated in the hippocampus. There was a significant decrease in the cytokines IL-6 (0.078 ± 0.011 vs. 0.043 ± 0.003; $p < .01$), IL-1ß (0.16 ± 0.03 vs. 0.09 ± 0.02; $p < .05$), and TNF-α (0.34 ± 0.056 vs. 0.21 ± 0.022; $p < .05$), in aged FAAH$^{-/-}$ mice compared to their age-matched controls (**Fig. 2c, d, & e**). There was also a significant decrease in iNOS (0.144 ± 0.015 vs. 0.103 ± 0.012; $p < .05$) in the FAAH -/- cohort (**Fig. 2f)**. Finally, in an effort to indirectly test for the presence of glial fibrosis within the hippocampus, mRNA analysis of the astrocytic marker GFAP was run for this region. FAAH $^{-/-}$ mice displayed a marginal, but significant decrease in total GFAP RNA

expression (190.3 ± 8.1 vs. 167 ± 6.9; $p < .05$) which is indicative of decreased fibrosis in astroglia (**Fig. 2g)**.

Discussion

Aging produces a robust para-inflammatory phenotype (Franceschi et al., 2000; Freund et al., 2010; Goto, 2009), which can be demonstrably attenuated by pharmacologically blocking the receptors of inflammatory mediators (e.g., IL-1ß; Allan & Rothwell, 2001) or indirectly, for example, by altering lipid modulators involved in immune regulation (Klein, 2005). Of interest, endocannabinoids along with other fatty acid ethanoloamides can directly alter the cytokine levels in the CNS (Correa et al., 2009; Correa et al., 2010; Glass & Ogawa, 2006; Klein et al., 1998) and perhaps reduce chronic inflammation (Straus & Glass, 2007). While sufficient evidence has shown the endocannabinoids and other fatty acid ethanolamides to be successful in reducing inflammation in other models (Piomelli & Sasso, 2014), here we demonstrate this principle in a model of aging.

Various inflammatory mediators increase with normal aging (Freund et al., 2010), however, aged animals with a life-time reduction in FAAH show significantly reduced mediators of inflammation in both the hippocampus and cortex relative to age matched controls. This is significant as studies have found lower relative levels of inflammatory mediators to be associated with a reduced incidence of disease (Cevenini et al., 2013) and reduced cognitive impairments (Kuo et al., 2005; Magaki et al., 2007). In addition, lower levels of GFAP also demonstrate a phenotypic shift within glia cells which has been shown to be a factor in various CNS diseases and correlated with behavioral impairments in animals (Barrientos et al., 2010; Moore et al., 2009). Thus, mice with life-time reductions of

FAAH demonstrate a degree of 'successful' aging relative to their age-matched counter-parts.

These results are intriguing considering that aged animals show significantly reduced levels of both OEA and PEA in the hippocampus (Maccarone et al., 2001) and reduced CB_1 expression in the CNS (Berrendero et al., 1998; Bilkei-Gorzo, 2012). This may collectively reduce the ability of these ligands to bind to PPAR-α and CB_1 thus making animals more susceptible to increasing levels of inflammation (Erol, 2005). Therefore, it is interesting that a lifetime increase of these lipid levels has the effect of reducing the mRNA levels of several inflammatory cytokines in the CNS (IL-1ß, TNF-α, IL-6) and therefore affecting other parameters such as iNOS levels and the occurrence of fibrosis in glia (Morgan et al., 1997; Yoshida et al., 1996). The mechanism of action behind this can be rather direct as in the case of AEA directly affecting cytokine levels (Correa et al., 2009; Correa et al., 2010; Klein et al., 1998) or indirectly at the level of transrepression of cytokines via PPAR binding and the subsequent inhibition of transcription factors such as NF-κB and AP1 (Glass & Ogawa, 2006). While not shown, preliminary data from these animals suggests that the mRNA of the p65 subunit of NF-κB was not altered, however more experiments would be necessary to conclude that a reduction in the binding of NF-κB was responsible for these drops.

While exciting, there are still several limitations with these data. The total levels of various endocannabinoids were not examined in this study, despite sufficient evidence suggesting that the deletion of FAAH has the overall effect of raising levels of AEA, OEA, and PEA (Cravatt et al., 2001). Also, additional controls including a young cohort of FAAH -/- animals and the examination of lipids and CB_1 expression within these animals are critical

to fully demonstrate the hypothesis that alterations in lipid metabolism can alter the probability of 'successful' aging. In addition, future experiments should also look at chronic administration of various NAAA inhibitors to further tease out the effects that alterations in OEA, PEA and AEA may have upon the organism. At this point, it can be suggested that a lifetime reduction in FAAH reduces inflammation in aged mice, however more work is needed to conclude how this takes place.

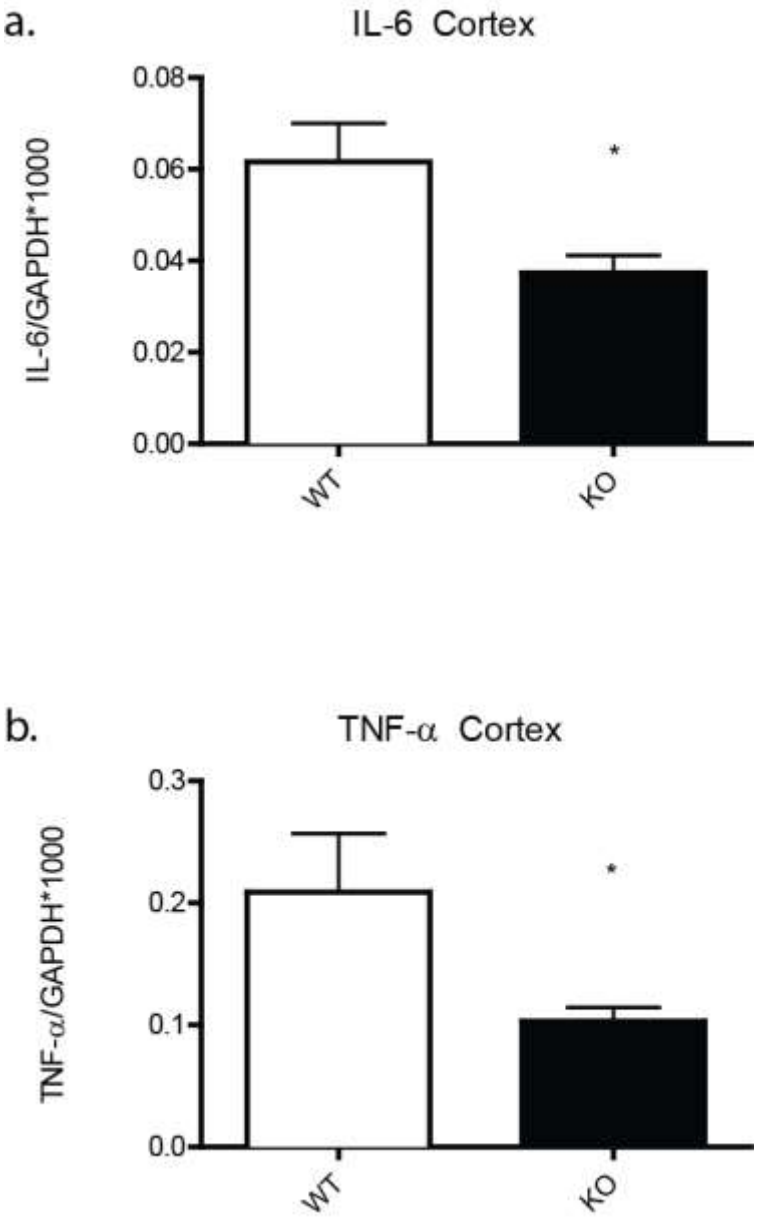

Figure 2a & b: The levels of IL-6 (2a) and TNF-α (2b) within the cortex of aged (18-19 month) mice. KO represents the FAAH -/- line and WT signifies Wild-Type. There was a significant decrease in the level of transcription for both IL-6 and TNF-α (*p*>.05). mRNA values are normalized to GAPDH.

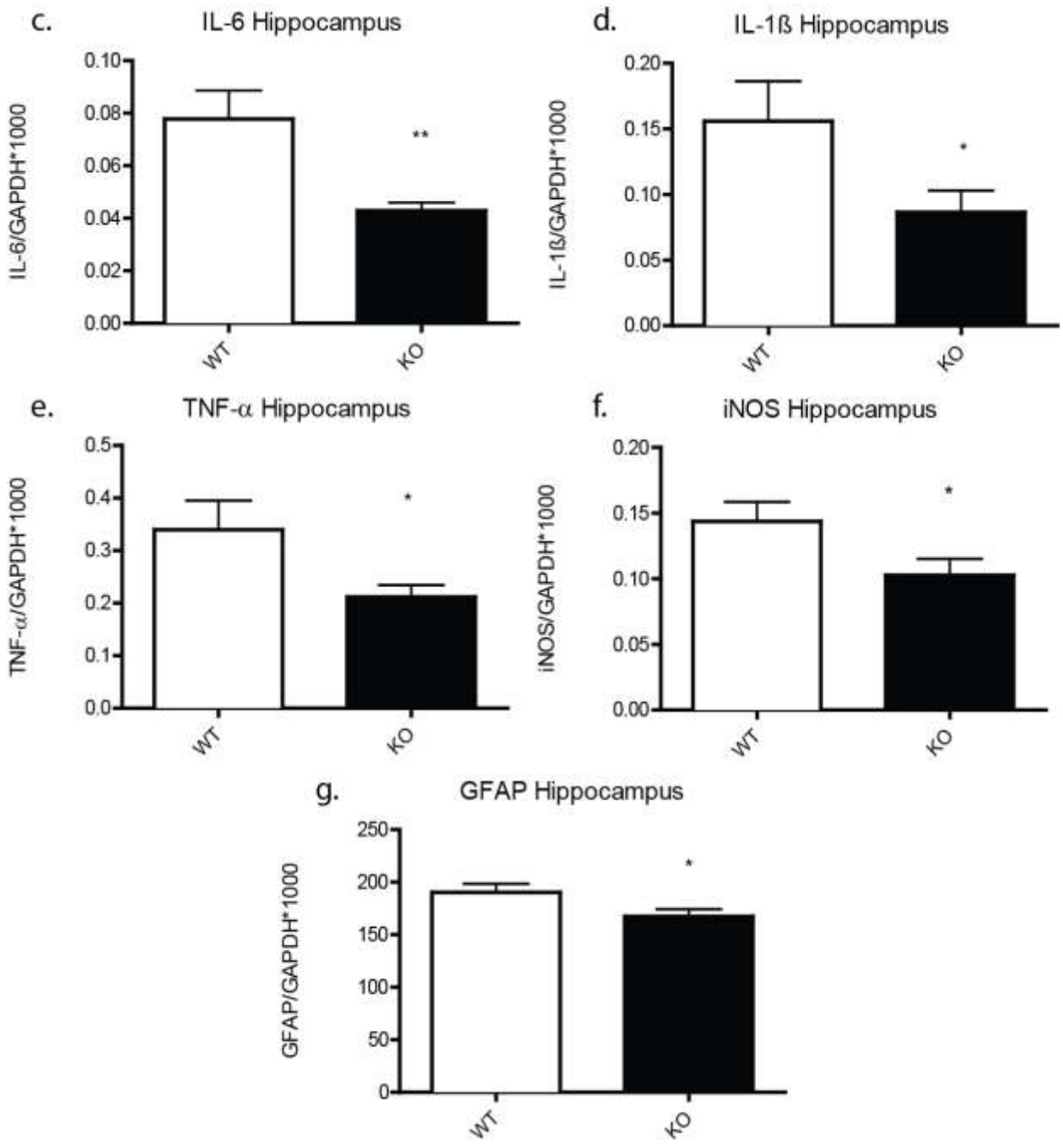

Figure 2c-g: mRNA levels of IL-6 (2c), IL-1ß (2d), TNF-α (2e), iNOS (2f) & GFAP (2g) in the hippocampus of aged (18-19 month) mice. There was a significant decrease in the level of transcription for IL-6 (*p*<.01) and IL-1ß (*p*<.05), TNF-α (p<.05), iNOS (p<.05) and the astrocytic marker GFAP (*p*<.05) in the KO mice compared to age match controls. mRNA values are normalized to GAPDH.

Chapter 3

Cellular Senescence and FAAH

Inflammation and aging are deeply connected to a third biological process: cellular senescence. This process was first formally described by Hayflick and colleagues in the 1960's, who showed that after many replications, cells in culture gradually diminish and eventually lose the ability to divide despite ideal conditions (Hayflick, 1965). Many of the normal processes of aging such as the shortening of telomeres (Harley et al., 1992) and the accumulation of ROS (Glass et al., 2010) are known factors which contribute to cellular senescence, however there was initial skepticism that this process was linked to aging and disease in animals (Campisi & d'Adda di Fagagna, 2007). Despite this, senescence has been found in the vicinity of areas subject to age-related pathologies such as atherosclerosis (Erusalimsky & Kurz, 2005) and osteoarthritis (Price et al., 2002) and has been shown to accumulate with age *in vivo* (Erusalimsky & Kurz, 2005; Jeyapalan et al., 2007).

While senescence may be an underlying byproduct of aging that can have deleterious effects on the health of various tissues, the process of senescence is thought to be primarily an anti-tumor mechanism (Sager, 1991), where under certain conditions, cells express various cell cycle inhibitors to suppress the growth of tumors and cause the cell to enter the senescent state (Campisi, 2013). This is analogous to inflammation, which over the life span can actually reduce the fitness of an organism if left unchecked. Formally, this process is known as antagonistic plieotropy, that is, a mechanism which provides high levels of fitness early in life may become deleterious later in life due to little selective pressure for it to be eradicated from the genome (Campisi & d'Adda di Fagagna, 2007).

Inflammation and cellular senescence share many parallels as a result, and as we will see, treatments which involve reducing inflammation may also help curb cellular senescence.

The Senescence-Associated Secretory Phenotype

As a cell enters replicative senescence, there are several phenotypic changes that can promote these cells to disrupt tissue homeostasis and make for a maladaptive microenvironment (Campisi & d'Adda di Fagagna, 2007). Several alterations in gene expression can lead to the expression of at least 40 different factors including growth factors, cytokines, chemokines and proteases (Coppé et al., 2010). The expression of these factors occurs in a graded manner in a phenotype known as the senescence-associated secretory phenotype (SASP). The SASP contributes many factors that are well known to be pro-inflammatory (e.g. IL-6, IL-1ß, IGF-1) and are among the most robustly secreted (Freund et al., 2010). Additionally, senescent cells resist common mechanisms of apoptosis induction (Campisi & d'Adda di Fagagna, 2007) and remain metabolically active while driving the SASP.

The SASP can contribute factors that are able to degrade the surrounding basement membrane and the extra-cellular matrix (Campisi, 2005; Hopkin, 2001), which when degraded, can trigger the response of the immune system (Medzhitov, 2008), induce the production of ROS species (Glass et al., 2010) and allow for the removal of diseased tissue via cytokine signaling (Xue et al., 2007). These increases in ROS and inflammation can further exacerbate the occurrence of cellular senescence in an organism (Bitto et al., 2010; Yu et al., 2012) and contribute to a particular microenvironment that is conducive to disease. With this said, it is conceivable that the accumulation of senescent cells within a specific area, such as the CNS, can result in increased neuroninflammation due to the SASP

phenotype altering the microenvironment (Chinta et al., 2014). While the evidence clearly argues that cellular senescence can significantly impact peripheral tissues and cells (Erusalimsky & Kurz, 2005; Jeyapalan et al., 2007), the impact of cellular senescence in the CNS is speculative as the most identifiable cell type, neurons, do not undergo any replicative processes. However, evidence suggests that glia, the most common cell type within the CNS, do replicate and exhibit degrees of cellular senescence (Chinta et al., 2014) and this has the potential to affect the viability of neurons within the CNS.

Senescence in the Brain

There is evidence that both microglia and astrocytes can undergo cellular senescence (Chinta et al., 2014). Microglia in particular, have a high rate of cell turn over, which utilizes either the mitosis of parenchymal microglia or the migration of bone-marrow derived progenitors to replenish the population during injury or infection (Streit, 2006). Of special interest, the proliferation of microglia occurs more rapidly in older animals (Conde & Streit, 2006), which has the downstream effect of shortening telomeres and has been demonstrated in rat microglia *in vitro* (Flanary & Streit, 2004) and *in vivo (*Flanary et al., 2007). This has also been demonstrated in human AD patients whom have significantly shortened telomeres in their microglia, compared to controls *(*Flanary et al., 2007). Additionally, post-mortem studies in aged humans show the presence of 'dystrophic' microglia, which demonstrate morphological changes and other aberrant cytoplasmic processes that are not consistent with an activated or quiescent state (Streit et al., 2004).

The other main cell type in the CNS, astrocytes, exhibit several characteristics that are suggestive of senescence. Cultured astrocytes treated with hydrogen peroxide

demonstrate growth arrest, increased expression of p21 and p16, and the presence of the senescence marker beta-galactosidase (Bitto et al., 2010). Similar changes occur in cultured human astrocytes when subjugated to ionizing radiation (Zou et al., 2012) and also can be found in astrocytes extracted from the aged brains of rats, which also demonstrate a reduced ability to ensure the survival of co-cultured neurons (Pertusa et al., 2007). Considering the above, it can be suggested that cellular senescence does occur in the CNS, and that these changes in glia have the potential to affect the microenvironment of the brain via the SASP. In turn, this may alter the viability of other cell types (i.e., neurons) and lead to several of the neurodegenerative symptoms associated with aging (Chinta et al., 2014). While senescence along with chronic inflammation may be unavoidable perils of the aging process, understanding the signaling pathways and molecular regulation of cellular senescence may allow for interventions that can have implications of treating age-based pathologies.

The Induction of Cellular Senescence.

Much like inflammation, cellular senescence can be initiated by a wide range of environmental stimuli including mitogenic signals, DNA damage, and oxidative stress (Campisi, 2005). Downstream from these effectors are several important regulators of cellular senescence, including the response element p53 and the cyclin-dependent kinase inhibitors (CDKI's) such as p21 (Xiong et al., 1993), which are induced well before the SASP or other morphological changes that characterize senescent cells. While these elements are not the only modulators important in senescence (Medcalf et al., 1996) they have been shown to be critical in the induction of senescence and can be triggered by DNA damage or telomere shortening (Campisi & d'Adda di Fagagna 2007), both which are a phenomenon

representative of aging cells. Early studies demonstrated that aged fibroblasts show 10-20 times higher expression of p21 than younger cells (Noda et al., 1994), and other studies demonstrated significant increases in DNA binding of p53 in aged cells as well (Atadja et al., 1995) of which p21 is a crucial transcriptional target (el-Deiry et al.,1998).

Concurrent with the effect that senescence has on inflammation, mice with overexpression of p53 show much more robust signs of aging and have shortened life spans (Maier et al., 2004; Tyner et al., 2002). Additionally, overexpression of p21 has been found to upregulate several genes, including those that code for APP (precursor to ß-amyloid) and SAA, an inflammatory protein involved in atherosclerosis (Chang et al., 2000). Conversely, reductions in p53 and p21 extend the lifespan of human fibroblasts (Itahana et al., 2001), reduce ischemic damage in rodents (Crumrine et al., 1994) and prevent telomere or DNA damage induced senescence (Beauséjour et al., 2003; Brown et al., 1997). This evidence suggests that the induction of p53 and p21 can induce senescence and modulate both inflammation and general health in animals.

Ceramide and Cellular Senescence

In addition to the response elements p53 and p21, the sphingolipid ceramide, which is a critical component of cell membranes (Ben-David & Futerman, 2010) and an essential lipid for neuronal development (Imgrund et al., 2009; Zhao et al., 2011), has been directly implicated in the initiation of cellular senescence (Mouton & Venable, 2000; Venable et al., 1995). Ceramide can also influence inflammation as it serves as a crucial immunomodulator that is able to alter the binding capacity of NF-kB thus altering the expression of other cytokines (Ballou et al., 1996). On the other end, inflammatory

cytokines such as TNF-α and IL-1ß can lead to the hydrolysis of the precursor sphingomyelin into ceramide (Kolesnick & Golde, 1994) thus perpetuating the cycle.

While beyond the scope of this work, ceramides differ in the lengths of their acyl chains and several species of ceramide require a variety of enzymes which have a differential distribution in various tissues (Grösch et al., 2012). These different species may also be able to elicit a variety of different physiological effects (Ben-David & Futerman, 2010) and show differential increases as a result of aging and disease (Cutler et al., 2004; Filippov et al., 2012). Considering ceramide's relationship with senescence and inflammation, it is not surprising then that specific ceramide species, specifically those with longer chain fatty acids, have been shown to increase over the lifespan (Cutler et al., 2004). They are also significantly elevated in the brains of AD patients (Filippov et al., 2012) and are correlated with several pathologies as a result of impaired sphingolipid metabolism (Haughey et al., 2010) such as diabetes (Haus et al., 2009) atherosclerosis (Bismuth et al., 2008) and neurodegenerative disorders (Filippov et al., 2012).

Inhibition of FAAH on Cellular Senescence

Much like inflammation, reducing cellular senescence in organisms has the potential to significantly affect the health outcomes of these animals in old age. In accordance with this, the removal of senescent cells has the ability to reverse several age-related physiological changes in aged animals such as cataracts, sarcopenia, and the loss of subcutaneous fat (Baker et al., 2011). Furthermore, dietary restriction reduces the expression of senescent cells in the liver of aged animals (Wang et al., 2010) and is well known to increase the longevity of several animal species (Heilbronn & Ravussin, 2003).

With this in mind, it is an interesting question as to how other more acute treatments, such as a reduction in FAAH, may affect cellular senescence and the health of aged animals.

Research has shown that endocannabinoids along with other fatty acid ethanolamides increase the synthesis of ceramide by stimulating sphingomyelin hydrolysis and *de novo* ceramide synthesis (Hansen, 2010; Velasco et al., 2005). This ultimately initiates apoptosis of nearby cell bodies (Hannun, 1995) and can have significant effects on reducing the size of tumors in mice (Velasco et al., 2005). From this evidence, one can propose that the lifetime elimination of FAAH would increase the levels of AEA, OEA, and PEA and thus drive an increase in ceramide levels which could increase inflammation (Ballou et al., 1996) and senescence (Mouton & Venable, 2000; Venable et al., 1995). However, the previous data suggest a more complex relationship between FAAH and inflammation that go beyond these views arising primarily from cancer research.

In aging research, there are many parallels between age-related increases in inflammation (Franceschi et al., 2000), senescence (Erusalimsky & Kurz, 2005; Jeyapalan et al., 2007), ceramide (Cutler et al., 2004) and disease, which suggest that there is another potential relationship between them. The fact that the genetic ablation of FAAH results in a significant decrease in neuroinflammation, hints that its substrates may be affecting other upstream processes responsible for it. The modulation of ceramide is one such process that shows an inverse relationship with levels of TNF-α and IL-1ß (Kolesnick & Golde, 1994) and is known the modulate inflammation (Ballou et al., 1996). The other is senescence, which also shares a similar inverse relationship with inflammation (Bitto et al., 2010; Yu et al., 2012) and can act as a crucial immunomodulator through the SASP (Campisi & d'Adda di Fagagna, 2007).

How a reduction in FAAH affects these other processes remains an unanswered question. In considering the evidence that AEA can increase ceramide levels (Hansen, 2010; Velasco et al., 2005) one could predict a significant increase in senescent cells in aged animals after the genetic ablation of FAAH. However, the anti-inflammatory effects we have already seen would argue for a decrease in cellular senescence and perhaps ceramide. To explore this, the senescent markers p53 and p21, in addition to brain levels of various ceramides were examined in both aged FAAH and WT strains.

Methods

A colony of male FAAH knock-out mice were crossbred with wild-type C57BL6/J mice (*n*= 8 WT; *n* = 10 FAAH -/-). The mice were bred and maintained in the animal facilities at the University of California, Irvine (Irvine, CA) under standard conditions (12:12 light-dark cycle, with lights on at 7:30; temperature at 20 Cº, and *ad libitum* access to food and water). The mice were housed in groups of 3-4 and allowed to age for 18 months before the experiments took place. Mice were euthanized with isofluorane and sacrificed by decapitation. After sacrifice, the brains were removed and the hippocampus and cortex were dissected out on a glass cover slip over ice. The hemispheres of each region were counterbalanced between groups. The brains were flash frozen in liquid nitrogen and stored at -80^{o}C until analysis.

RNA Extractions

RNA was extracted from frozen tissues utilizing TRIzol reagent (Invitrogen, Carlsbad, CA) and molecular biological grade chloroform, 2-propanol, and ethanol in the method of a traditional phenol-chloroform extraction. Briefly, tissues were homogenized using a sterile 27.5 gauge needle in approximately 1mL of TRIzol reagent. An equal volume

(1:1) of molecular biological grade chloroform was then added and the samples were centrifuged at 10,000G for 15 minutes. The supernatant was taken, mixed with 400μL of 100% 2-propanol and then centrifuged again for 30 minutes at 10,000G. The 2-propanol was then discarded and 70% EtOH was added to further precipitate the RNA. After centrifuging for another 15 minutes at 10,000G the remaining EtOH was discarded and 30mL of RNAse free water was added. Reverse transcription of total RNA (2 μg) was then conducted using oligo(dT)12–18 primers for 50 min at 42°C. mRNA levels were measured by quantitative real-time polymerase chain reaction (RT-PCR) with a Mx 3000P system (Stratagene, La Jolla, CA).

The RNA fluorogenic probes purchased from Applied Biosystems (TaqMan Gene Expression Assays, Foster City, CA) were: p21 (Mm04205640_g1) & p53 (Mm017131290_g1). mRNA levels were normalized using GAPDH.

Lipid extractions

Frozen tissue samples were weighed and homogenized in cold methanol containing appropriate internal standards. Lipids were extracted by adding chloroform and water (2/1, vol/vol) and fractionated through open-bed silica gel columns by progressive elution with chloroform/methanol mixtures. Fractions eluted from the columns were dried under nitrogen, reconstituted in chloroform/methanol (1:4, vol/vol; 0.1 ml) and subjected to LC/MS analyses. Protein concentration was measured using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA).

LC/MS analyses

Sphingolipids were analyzed by LC/MS, using an Agilent 1100 LC system coupled to an ESI-ion-trap XCT mass detector. Ceramide species were separated on a Poroshell 300 SB

C18 column (2.1 x 75 mm i.d., 5 μm; Agilent Technologies) maintained at 30°C. A linear gradient of methanol in water containing 5 mM ammonium acetate and 0.25% acetic acid (from 80% to 100% of methanol in 3 min) was applied at a flow rate of .89 ml-min^{-1}. Detection was in the positive mode, capillary voltage was 4.5 kV, skim1 -40 V, and capillary exit -151 V. Nitrogen was used as drying gas at a flow rate of 12 l-min^{-1}, temperature of 350 °C and nebulizer pressure of 80 psi. Helium was used as collision gas. Ceramide species were identified by comparison with authentic standards (Avanti Polar Lipids, Alabaster, AL). Extracted ion chromatograms were used to quantify the following ceramides: (d18:1/16:0) $[M+H]^+$ (*m/z* = 538.5>520.5>264.3), (d18:1/16:0) $[M+H]^+$ (*m/z* = 540.5>522.5), (d18:1/18:0) $[M+H]^+$ (*m/z* = 566.5>548.5>264.3), (d18:1/18:0) $[M+H]^+$ (*m/z* = 568.5>550.5), (d18:1/24:0) $[M+H]^+$ (*m/z* = 650.6>632.8>264.3), (d18:1/24:1) $[M+H]^+$ (*m/z* = 650.6>632.8), using (d18:1/12:0) $[M+H]^+$ (m/z = 482.5>464.5>264.3) as internal standard.

Data Analysis

Relative RNA and ceramide levels were analyzed with a two-tailed unpaired students *t*-test where statistical significance was determined if $p < .05$. Results are expressed as *M* ± *S.E.M.* A Pearson correlation was also performed and is expressed as an r^2 value. Analyses were completed using GraphPad Prism software (version 5.04).

Results

In an effort to investigate the overall incidence of senescence in the CNS, relative RNA levels of the response element p53 and the cyclin-dependent kinase inhibitor p21 were examined in the cortex. mRNA analysis revealed significant decreases of p21 (14.09 ± 1.94 vs. 7.83 ± 0.658; $p< .01$) and p53 (4.23 ± 0.270 vs. 3.62 ± 0.084; $p< .05$) in the cortex of

FAAH -/- mice compared to age-matched controls (**Fig. 3a & b)** . These same analyses in the hippocampus revealed that p21 was significantly lower in the FAAH -/- relative to age matched controls (9.70 ± 1.72 vs. 5.40 ± 0.411; $p < .05$). However, p53 (2.56 ± .294 vs. 2.03 ± 0.169; *ns*) was not significantly different (**Fig. 3c & d)**.

Next, the effects that deleting FAAH has on ceramide levels were examined. Among several species of ceramide, (14:0, 16:0, 18:0, 24:0, 24:1 and 26:0) there was a significant decrease in the levels of ceramide (d18:1/24:1) in the cortex of FAAH -/- mice (0.185 ± 0.031 vs. 0.110 ± 0.013; $p < .05$; **Fig 3e**). There were neither significant changes for the other ceramide species nor any differences in the hippocampus. To determine whether these changes in ceramide (d18:1/24:1) bore any relationship to cellular senescence, a regression analysis was performed to determine if a correlation existed between ceramide (d18:1/24:1) and p21. Pooling data from both the WT and FAAH -/- strains, there was a significant correlation between p21 and ceramide (d18:1/24:1) content in the cortex ($r^2 = .48$; **Fig 3f**). Similar correlations were carried out between other species of ceramide and with the response element p53, but there were no significant relationships. This suggests that animals with lower levels of ceramide (d18:1/24:1) in the CNS may also have lower incidence of cellular senescence (p21) in the same area.

Discussion

Cellular senescence, much like chronic inflammation, is an adaptive process that fits within the framework of antagonistic pleiotropy and progressively decreases the fitness of an organism throughout the aging process (Campisi & d'Adda di Fagagna, 2007). In accordance with this, cellular senescence has been linked to several age related pathologies (Campisi & d'Adda di Fagagna, 2007; Erusalimsky & Kurz, 2005; Jeyapalan et al., 2007),

which also occur in relation to increases in ceramide (Bismuth et al., 2008; Filippov et al., 2012; Haughey et al., 2010; Haus et al., 2009) as well as inflammation (Freund et al., 2010). Since evidence suggests that cellular senescence occurs in the CNS and may play a role in neurodegenerative diseases (Chinta et al., 2014) the incidence of crucial proteins (p21, p53) and lipids (ceramide) responsible for the initiation of senescence were examined to see if the life-time elimination of FAAH was able to alter these levels in a way that mirrored its effects on inflammation.

Here, it is demonstrated that long-term inhibition of FAAH reduces the levels of two crucial proteins involved in cellular senescence: p21 and p53. While the decrease of p53 was marginal in the cortex of the aged animals, the decreases in p21 transcription were robust in both cortex and hippocampus, indicating a very real effect that the inhibition of FAAH has on these promoters of cellular senescence. In addition, the levels of ceramide (d18:1/24:1) were decreased in the FAAH -/- and the levels of this lipid were significantly correlated with the expression of p21 across both groups.

An increase of senescence in glia and other support cells can cause significant phenotypic changes that result in the release of several inflammatory factors (Coppé et al., 2010) including IL-6, IL-1ß, IGF-1, which are among the most robustly secreted (Freund et al., 2010). This phenotypic change, known as the senescence-associated secretory phenotype (SASP), allows pro-inflammatory factors to accumulate in the tissues as senescent cells resist apoptosis while simultaneously driving the SASP (Campisi & d'Adda di Fagagna, 2007). The release of these factors causes a change in the microenvironment, which has several effects that can further trigger an immune response (Medzhitov, 2008). It is possible that with age, an increase of senescent cells within the CNS renders a

population of glia with an active SASP. This SASP would drive neuroinflammation and could eventually lead to several of the neurodegenerative effects of aging as a result of inflammation, and perhaps not coincidentally, senescence. Therefore, by decreasing senescent cells one could decrease inflammation and the resulting physiological changes. Decreasing FAAH significantly lowers both the markers of senescence and neuroinflammation and is in line with this hypothesis. However, more work is needed to clarify that there is a causal relationship between these two as a result of FAAH inhibition. Despite this, the parallels and therapeutic potential for this are exciting.

The decreases in ceramide (d18:1/24:1) and it's directional relationship with p21 become problematic as the literature suggests that the substrates of FAAH can increase the levels of ceramide in animals (Hansen, 2010; Velasco et al., 2005), which could eventually increase the incidence of cellular senescence (Mouton & Venable, 2000; Venable et al., 1995). The correlation between ceramide (d18:1/24:1) and the CDKI p21 support this fact, however, the net changes in several ceramides were negligible in this study. This may demonstrate a more complicated relationship between ceramide and senescence as there are several groups of ceramides each with their own physiological effects (Ben-David & Futerman, 2010) and differential distribution in the body (Grösch et al., 2012). Additionally, ceramide synthesis is also affected by the expression of inflammatory cytokines which we know to be lower in FAAH knock-outs (Kolesnick & Golde, 1994). Regardless, these data suggest that a decrease in FAAH leads to an increase in several substrates (AEA, OEA, PEA), which decrease ceramide (d18:1/24:1) levels and reduce known markers for cellular senescence. This reduction may then decrease the incidence of the SASP within the CNS, which would eventually decrease neuroinflammation and lead to

a more ‘successful’ aging process. However, much more evidence is needed to make any definitive statements on this claim.

With that said, each of these factors has a significant relationship with cognitive function in old age. Considering that these reductions in neuroinflammation and cellular senescence were shown to occur in the hippocampus as well as the cortex, it is reasonable to believe that a decrease in FAAH could also potentially affect learning and memory in aged animals. The next set of experiments set out to explore this idea.

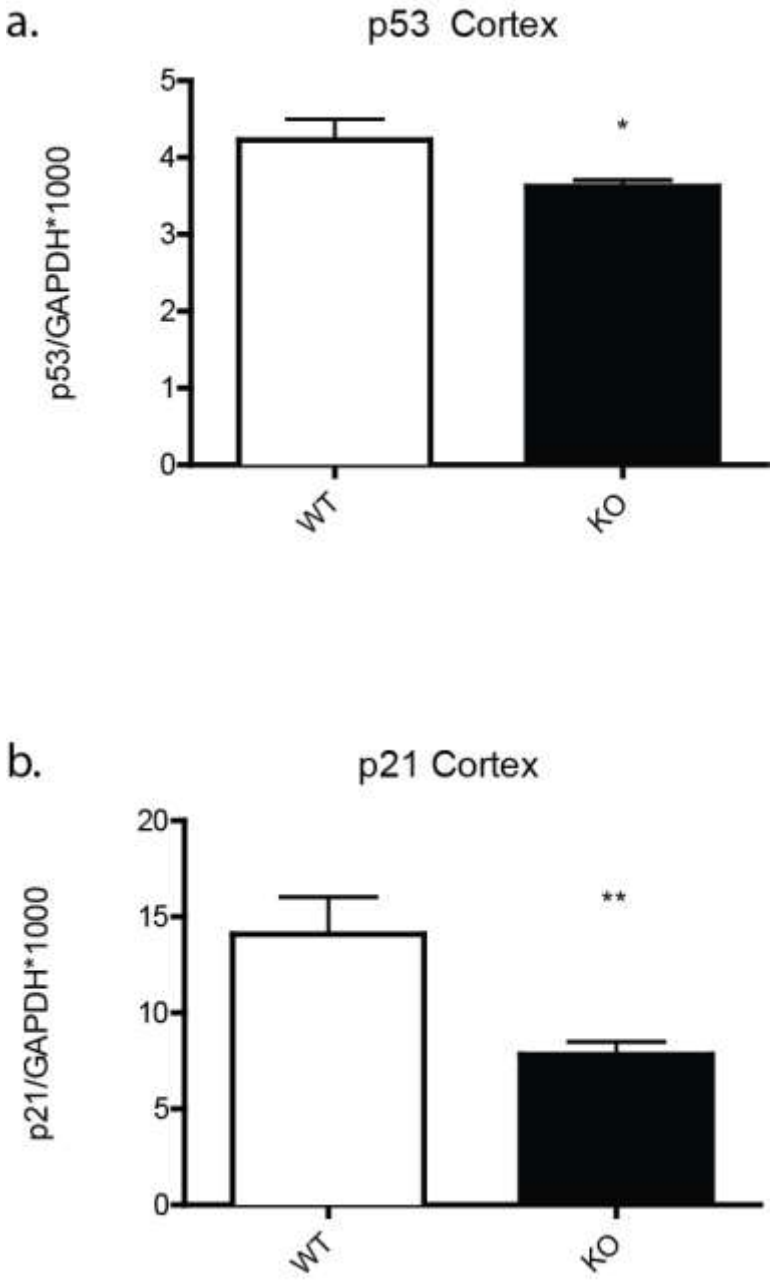

Figure 3a & b: The levels of p53 (3a) and p21(3b) within the cortex of aged (18-19 month) mice. KO represents the FAAH -/- line and WT signifies Wild Type. There was a significant decrease in the level of transcription for both p53 and p21 in the KO mice compared to age match controls (p<.05 for both analyses).

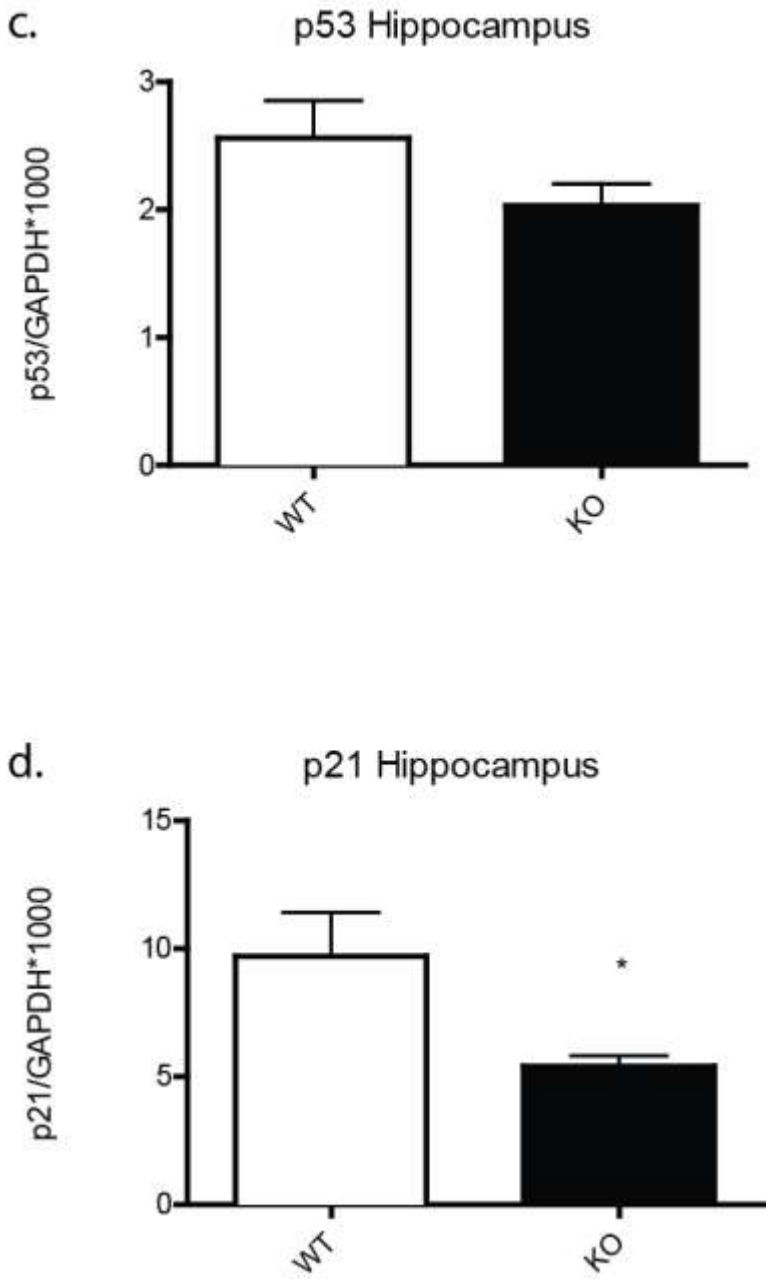

Figure 3c & d: The levels of p53 (3c) and p21 (3d) within the hippocampus of aged (18-19 month) mice. KO represents the FAAH -/- line and WT signifies Wild Type. There was a significant decrease in the level of transcription for p21 in the KO mice compared to age match controls (p<.05), however p53, while lower, was not significant.

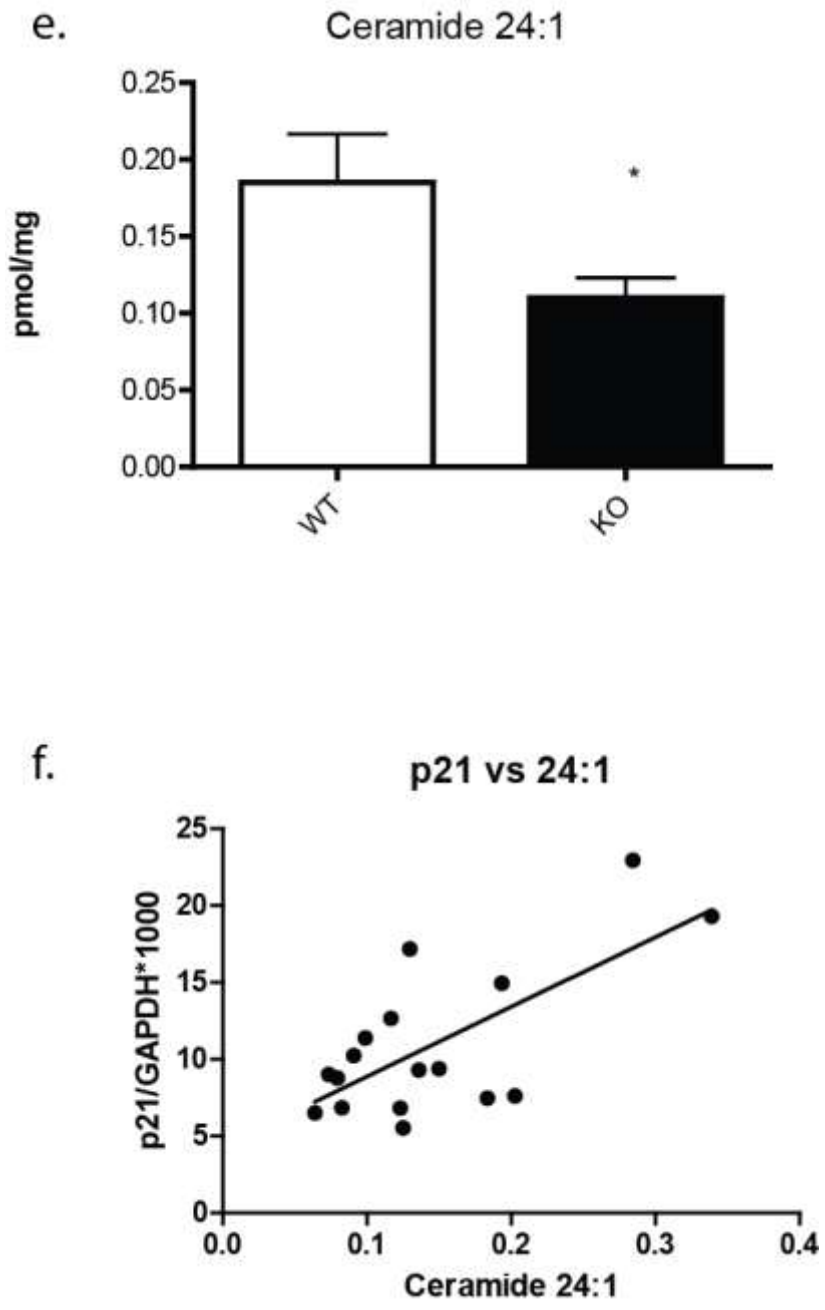

Figure 3e & f: The levels of Ceramide (d18:1/24:1;3e) within the cortex of aged (18-19 month) mice. KO represents the FAAH -/- line and WT signifies Wild Type. There was a significant decrease in the levels of Ceramide (d18:1/24:1) in the cortex of KO mice compared to age match controls (p<.05) Also, there was notable correlation (3f) between the levels of Ceramide (d18:1/24:1) and p21 (r^2 = .48), both in cortex.

Chapter 4

Learning and Memory in the Aged FAAH Knock-Out

As individuals age several crucial cognitive processes decline including: processing speed, verbal recall, and most importantly, memory (Laursen, 1997; Yankner et al., 2008). While various types of memory have been found to exist in mammalian species (Squire & Knowlton, 1993) research focused on the loss of declarative or place memory, which is modulated by the hippocampus (Packard & McGaugh, 1996), demonstrates the most robust alteration throughout aging (Gallagher & Pelleymounter, 1988). This loss is highly conserved across several species (Yankner et al., 2008) and several rodent models have demonstrated significant learning and memory loss as a result of normal aging (Gallagher & Pelleymounter, 1988). The aging brain differs on both the molecular and system level, which may contribute to some of deficits seen above (Yankner et al., 2008). In the hippocampus, differences in spine density (Geinisman et al., 1986), a decrease in immediate early genes such as *arc* in the dentate gyrus (Small et al., 2004) a loss of volume in the dentate gyrus and adjacent hippocampal subregions (Small et al., 2002), and decreased LTP (Landfield et al., 1978) are all apparent in aging subjects and have been associated with impairments in learning and memory (Yankner et al., 2008). In an effort to combat this, various treatments and interventions from histone deacetylase inhibitors (Intlekofer et al., 2013), aerobic exercise (Cotman et al., 2007), environmental enrichment (Jankowsky et al., 2005) and various nootropics (Lynch, 2004) have demonstrated themselves as effective strategies in improving memory and reversing impairment. Here, another strategy, namely the elimination of FAAH, will be examined for its propensity to ameliorate learning and memory deficits in aged animals as well.

Inflammation and Memory

The immune system plays a crucial role in modulating learning and memory in both a positive and negative way (Yirmiya & Goshen, 2011). To one extent, inflammatory cytokines such as IL-1ß have been shown to improve memory at low doses (Gibertini, 1998) and other studies have shown that complete inhibition of inflammatory mediators can have a net negative effect on LTP (Schmid et al., 2009). On the other end, the chronic dysregulation of homeostasis in the immune response coupled with systemic infections in old age (Cunningham et al., 2009), high doses of cytokines due to disease or infection (Yirmiya & Goshen, 2011), and age-related microglia sensitization (Barrientos et al., 2010) can have a significant contribution to memory loss. Sustained levels of chronic inflammation brought about by aging can precipitate memory loss in aged subjects (Yirmiya & Goshen, 2011) and these effects are reflected differentially in aged versus young animals (Chen et al., 2008). Underlying these effects are perhaps an aggregation of senescent cells (Jeyapalan et al., 2007), dysregulated lipid metabolism (Maccarone et al., 2001; Cutler et al., 2004), and increased basal levels of pro-inflammatory mediators (Freund et al., 2010) which have demonstrated effects on the viability of LTP and other processes related to learning and memory (Bellinger et al., 1993; Murray et al., 1996; Plata-Salaman & Ffrench-Mullen, 1992; Tong et al., 2008).

To combat this, treatments aimed at chronic inhibition of inflammatory cytokines have been shown to attenuate learning and memory deficits in aged animals. Long term inhibition of IL-ß in the hippocampus of aged animals demonstrated improved learning and memory performance on fear conditioning (Gemma et al., 2005). Similar results were seen utilizing inhibitors for IL-6, which was shown to prolong LTP and improve learning and

memory (Spooren et al., 2011). This is in addition to the literature on exercise, which has shown reduced inflammation concurrent with learning and memory benefits in several AD models (Cotman et al., 2007).

Previously, it was shown that the genetic ablation of FAAH works to decrease markers of inflammation and senescence in aged mice. Attributable to this is the tonic increase of several fatty acid ethanolamides, which are known to produce profound anti-inflammatory effects. A controlled inflammatory response is one way that age-related learning and memory deficits may be rescued as studies have suggested (Barrientos et al., 2010; Cotman et al., 2007; Gemma et al., 2005). Therefore, it is reasonable to hypothesize that the reduced inflammation in the hippocampus of aged FAAH-/- animals will also have effects on learning and memory. In order to test this, a separate cohort of aged FAAH -/- and WT mice was tested for differences in performance in the object location memory (OLM) paradigm. This task is of interest, especially in aged animals, because it operates independently of any external stress (e.g. shock, water) that could potentially modulate memory processes (Vogel-Ciernia & Wood, 2014).

Methods

A separate cohort of 16 male FAAH knockout mice and age matched wild type controls were utilized for the behavior studies (*n*= 8 WT; *n*= 8 FAAH -/-). The mice were bred and maintained in the animal facilities at the University of California, Irvine (Irvine, CA) under standard conditions (12:12 light-dark cycle, with lights on at 7:30; temperature at 20 Cº, and *ad libitum* access to food and water). The mice were housed in groups of 3-4 and allowed to age for 18 months before the experiments took place. All behavior testing took place between 18:00-19:00. The following behavioral procedures for the object

location memory (OLM) paradigm were adopted from Vogel-Ciernia & Wood, 2014. Performance on the task is dependent upon an animals' ability to remember the location of a stationary object twenty-four hours after its misplacement.

Object Location Memory (OLM) Procedure

Before introduction to the OLM apparatus, the mice were handled for two minutes per day for six consecutive days in their holding rooms. On the sixth day, the mice were transported to the behavior room, where they allowed thirty minutes of rest to acclimate to their environment. After thirty minutes, the animals were introduced to the OLM apparatus which consisted of four open topped white, opaque, acrylic boxes (30cm x 23cm x 21.5cm) with the chamber floors coated 1cm deep with their home-cage bedding. A hanging LED light above the apparatus measured at roughly 45 LUX during the experiments.

At the start of the habituation phase, which lasted five consecutive days after handling, the animals were introduced to the OLM apparatus in groups of four and were allowed to explore for five minutes. After five minutes, the animals were taken out and returned to their home cages. During this time, animal feces were removed, the bedding within the chambers was shuffled around, and the walls were cleaned with H_2O and 70% EtOH to minimize odor. On the sixth day, the animals were introduced into the chamber; however, two glass beakers were placed in the center of the chamber. The glass beakers sat roughly 7cm apart from each other and the animals were then given ten minutes to explore the beakers. Twenty-four hours later, the animals were re-introduced into the apparatus; however, one of the beakers was placed in a novel location, roughly 11cm

across from the initial location of the unmoved beaker. The animals were allowed to explore for two minutes and their responses were recorded.

Behavioral analysis

The total time an animal spent 'exploring' an object was defined as the amount of time the animals' nose was within 1cm of the object and pointing directly at the object. Other behaviors such as climbing, looking over the object or digging were not counted as exploration. Three independent graders blind to the conditions of the experiment evaluated these measures. Their results followed similar trends and analogous statistical significance. A discrimination index (DI) was calculated to measure an animals' preference for a particular location. The discrimination index is calculated as follows: $DI = (Time\ Exploring\ Novel\ Location - Time\ Exploring\ Stationary\ Location) \div (Time\ Exploring\ Novel\ Location + Time\ Exploring\ sSationary\ Location)\ x\ 100$

A discrimination index of zero would indicate no preference while a discrimination index ranging from 25-45 is indicative of memory consolidation (Vogel-Ciernia & Wood, 2014). In addition to the time spent exploring each individual beaker, the total exploration time was also recorded to control for overall activity. The data were analyzed using two-tailed unpaired students *t*-test where statistical significance was determined if $p < .05$. Results are expressed as $M \pm S.E.M.$ Analyses were performed using GraphPad Prism software (version 5.04).

Results

First, to control for individual differences in exploration, the animals total exploration time was calculated. The total time animals spent exploring the apparatus did not differ across groups (5.36± 1.74 vs. 5.57 ± 1.04; *p = n.s;* **Fig 4a**). However, when the DI

was calculated for the preference of a novel location, the aged FAAH-/- had a significantly higher discrimination index to their age matched counter parts (-4.316 ± 7.65 vs. 26.66 ± 8.13; $p < .05$) (**Figure 4b.)** This is indicative that these animals had more intact learning and memory processes in comparison to their aged-matched controls.

Discussion

Sustained levels of chronic inflammation brought about by aging can precipitate memory loss in subjects. Underlying these effects are the aggregation of senescent cells (Jeyapalan et al., 2007), dysregulated lipid metabolism (Maccarone et al., 2001; Cutler et al., 2004), and increased basal levels of pro-inflammatory mediators (Freund et al., 2010) which affect processes related to learning and memory (Bellinger et al., 1993; Murray et al., 1997; Plata-Salaman & Ffrench-Mullen, 1992; Tong et al., 2008). Studies demonstrate a critical regulatory role for cytokines on these processes (Gibertini, 1998; Schmid et al., 2009; Yirmiya & Goshen, 2011), as aging animals ubiquitously demonstrate significant impairment as a result of sudden increases in inflammatory cytokines (Barrientos et al., 2010; Cunningham et al., 2009). Treatments that aim at reducing inflammation have had success in rescuing learning and memory in aged animals (Cotman et al., 2007; Gemma et al., 2005), which led to the investigation of how a FAAH -/- phenotype may influence these processes in old age.

The following results demonstrate that knocking out FAAH allows aged mice to perform significantly better in a task that measures hippocampal based memory. While there are many studies showing memory impairments as a result of the administration of CB_1 agonists (Riedel & Davies, 2005), these results match the predictions that decreasing inflammation in the brain via the elimination of FAAH has notable benefits on learning and

memory, as has been seen in previous experiments (Cotman et al., 2007; Gemma et al., 2005; Ramírez et al., 2005). However, at this point the effects on learning and memory are only correlative to the reduction in inflammation and can still be explained with other interpretations of the results. For example, using the FAAH inhibitor URB597, investigators were able to directly enhance learning and memory in young rodents, and this was modulated via PPAR-α activation (Mazzola et al., 2010). Additional studies have also shown that strains of FAAH -/- mice have improved rates of extinction and acquisition on a fixed platform water-maze task (Varvel et al., 2007). Therefore, it is possible that the effects seen are independent of neuroinflammation. To parse this out, essential controls such OLM performance on a younger cohort of mice and the administration of an acute FAAH inhibitor such as URB597 in aged animals, are necessary to show that these effects are related to reductions in inflammation and not the acute effects of FAAH inhibitors on learning and memory. Regardless, these findings, in conjuncture with the data suggesting reduced neuroinflammation and senescence in the CNS of aged FAAH knockouts, merit more investigation and they may serve as a possible route of therapy in several age related neurodegenerative diseases.

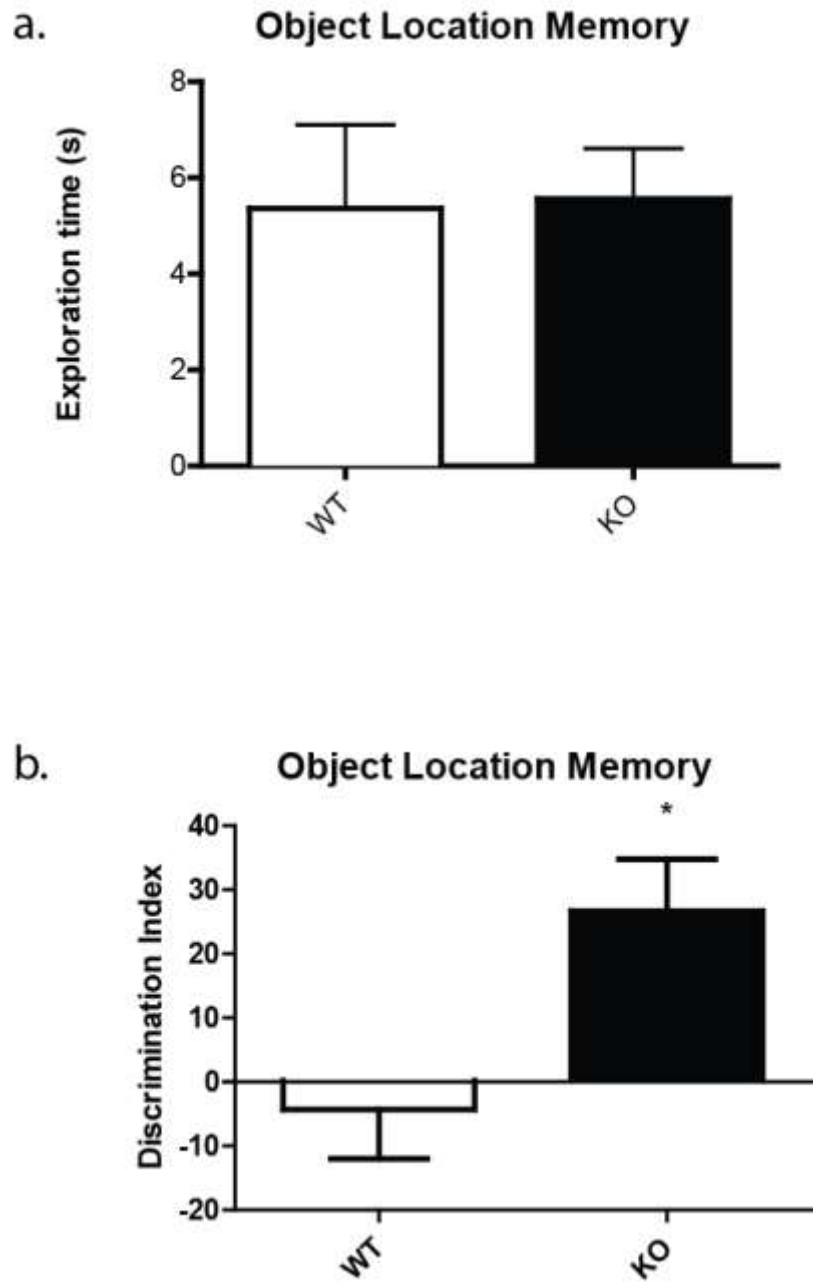

Figure 4a & b: The behavior of aged mice (18-19 month) within the OLM paradigm. KO represents the FAAH -/- line and WT signifies Wild Type. There was no significant in the total exploration time (4a), however FAAH -/- mice explored the novel location (4b) significantly more than aged matched controls (p<.05). This trend was consistent across three independent graders blind to the conditions.

Chapter 5

Conclusions and Summary

Neurodegenerative diseases constitute one of the biggest issues facing society in the next few decades. The tremendous costs, projected upwards of $1.1 trillion for Alzheimer's disease alone (Alzheimer's Association, 2014), demonstrate an essential need for more research into methods that aid in the treatment of these diseases. The role of inflammation in these pathologies has been demonstrated almost unequivocally: several lines of evidence suggest that common neurodegenerative diseases such as Alzheimer's disease (Heneka & O'Banion, 2007; Mrak & Griffin 2001), Parkinson's disease (Hirsch & Hunot, 2009; Long-Smith et al., 2009) and depression (Dantzer et al., 2008; Gimeno et al., 2009; Kuo et al., 2005) are associated with significant increases in inflammatory cytokines and chemokines (Freund et al., 2010). The importance of lipid signaling in controlling these levels and its therapeutic potential in several models from anxiety (Kathuria et al., 2003) to pain (Jayamanne et al., 2006) render endogenous lipid messengers as attractive candidates for the treatment of such maladies. Utilizing a cohort of aged FAAH -/- mice, several experiments were carried out to investigate the phenotypes of these animals in relation to age-related increases in inflammation, senescence, and cognitive impairment. The data herein suggest that:

A.) A lifetime reduction of FAAH in the aged mouse has the ability to curb inflammation in the CNS, as evidenced by a significant decrease in several pro-inflammatory markers (IL-1ß, IL-6, TNF-α, iNOS,). These decreases were associated with a lower expression of GFAP in the CNS, which may indicate a reduction of glial-fibrosis.

B.) FAAH knock-out mice demonstrate a significant decrease in key protein regultors of cellular senescence (p21, p53), and such a decrease is concurrent with a reduction in ceramide (d18:1/24:1), a modulator of both inflammation and senescence.

C.) As predicted by the results outlined above, the same animals show improved learning and memory on a hippocampal-based memory task.

While several studies have suggested that increasing endocannabinoid and other fatty acid ethanolamides have the ability to reduce inflammation (Correa et al., 2009; Correa et al., 2010; Glass & Ogawa, 2006; Klein et al., 1998) this study replicates this effect on aged mice (18-19 month) in a transgenic FAAH knockout strain. The proposed decreases in OEA, PEA (Maccarone et al., 2001) and CB_1 expression in aged animals (Berrendero et al., 1998; Bilkei-Gorzo, 2012) create the possibility that a lifetime decrease in FAAH allows these animals to avoid the inflammatory effects of reduced endocannabinoid signaling by having a higher circulation of the various substrates of FAAH. More exciting however, is the significant reduction in the senescent modulators p21 and p53, as cellular senescence is known to play a critical role in the modulation of inflammation. In accordance with this, the incidence of cellular senescence in the aged animal has the capability to alter baseline levels of several inflammatory mediators (Campisi & d'Adda di Fagagna, 2007; Coppé et al., 2010), which could be influenced by a decrease in senescence. One effect that a reduction in FAAH may have is preventing the onset of cellular senescence in the CNS, which would result in a net reduction in the SASP and therefore a reduction of inflammatory mediators that can result in improved learning and memory.

However, more comprehensive experimentation needs to be done to conclusively suggest the directional relationship of reduced inflammation and senescence leading to improvements in learning and memory. Primarily, a young cohort of FAAH knockouts is needed to conclude that these effects are limited to an aging phenotype and not subject to the more acute effects that elevations in endocannabinoids have on inflammation. A more detailed lipidomic approach is also needed to measure the levels of OEA, PEA, AEA and other lipids, despite the evidence that FAAH knock-out mice exhibit elevated levels in previous literature (Cravatt et al., 2001). Additionally, measures of ß-galactosidase staining for the actual presence of senescent cells are necessary to conclude that the changes in p21 and p53 mRNA expression result in an actual modulation in senescence. Finally, protein analysis of many of the inflammatory mediators is important to ensure that the changes in mRNA result in the translation of these inflammatory mediators. Despite this, these results are promising for future research to investigate the role that modulation of the endocannabinoid system has on age-related pathologies.

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