Sex Differences of the Central Amygdala's Immune Response in EAE-Induced Chronic Pain

by

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Abstract

Multiple sclerosis (MS) is an autoimmune disease that is characterized by demyelination of central nervous system axons. Symptoms of MS are highly heterogenous, although some, such as motor deficits and chronic pain, are very common in those with the disease. Defined by the International Association for the Study of Pain as pain that persists or recurs for longer than 3 months, chronic pain represents a source of significant reductions in quality of life. To date, however, the underlying mechanisms facilitating chronic pain in MS remain largely unknown. One contributor to chronic pain is the immune system, which, although shown to act differently in males and females, displays converging mechanisms of facilitation of chronic pain. To date, the majority of this work has been performed in the spinal cord, but comparatively little is known about the role of brain-resident immune cells roles in chronic pain or how these cells may influence activity within pain processing regions of the brain. This project aims to characterize the sex differences in the immune response within the central amygdala, a major hub for incoming and outgoing pain signalling. Using a murine model of MS with stereotyped pain behaviours, Experimental Autoimmune Encephalomyelitis (EAE), I demonstrate that within the amygdala, EAE induced robust cytokine dysregulation. Additionally, using the Four Core Genotype murine model, I show that the observed inflammatory dysregulation was significantly greater in animals with XX sex chromosome complement and/or ovaries. To assess if microglia were the primary drivers for this dysregulation, I assessed microglial morphology, density, and colocalization with cytokines showing sex differences, *Cxcl1* and *Cxcl10,* and found minimal sex differences. However, astrocytes, another immunocompetent glial cell population within the brain, displayed significantly greater colocalization with tested cytokines. Furthermore, I observed a significant upregulation of *Gfap*+ astrocytes in the central amygdala of female EAE mice, but not males. Together, this study suggests that astrocytes may contribute to a greater extent than microglia to the sex differences in EAE-induced inflammation within the central amygdala.

Preface

This thesis is an original work by Adam C. Wass. This research project was approved by the Alberta Research Ethics Board and was conducted under the supervision of Dr. Anna Taylor, Dr. Bradley Kerr, and Dr. Jesse Jackson. All experiments involving laboratory animals were in compliance with the Canadian Council on Animal Care Guidelines and followed protocols approved by the University of Alberta Health Sciences Animal Care and Use Committee (AUP#00000274; AUP0002493). This research project received funding support from the Canadian Institutes of Health Research (Canadian Graduate Scholarship – Master's).

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I consider myself to be a very lucky person and much of this comes from the presence of many kind and loving people that have left a positive impact on my life.

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List of Abbreviations

ANOVA	Analysis of variance
ACC	Anterior cingulate cortex
BBB	Blood-brain barrier
BLA	Basolateral amygdala
CD4	Cluster of differentiation 4
CD45	Cluster of differentiation 45
CeA	Central nucleus of the amygdala
CFA	Complete Freund's Adjuvant
CNS	Central nervous system
DAPI	4'6-diamidino-2-phenylindole
DRG	Dorsal root ganglia
EAE	Experimental autoimmune encephalomyelitis
FCG	Four core genotypes
FISH	Fluorescent *in situ* Hybridization
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
IBA-1	Ionized calcium-binding adapter molecule 1
IC	Internal capsule
IHC	Immunohistochemistry
ITGAM	Integrin subunit alpha M
LPS	Lipopolysaccharide
MOG	Myelin oligodendrocyte glycoprotein
mRNA	Messenger ribonucleic acid
MS	Multiple Sclerosis
PFA	Paraformaldehyde
PKCδ	Protein kinase C delta
PRT	Paw response threshold
S1	Somatosensory cortex
SEM	Standard error of the mean
Som	Somatostatin
TLR	Toll-like receptor
XXF	XX sry-; Chromosome complement XX with ovaries
XXM	XX sry+; Chromosome complement XX with testes
XYF	XY sry-; Chromosome complement XY with ovaries
XYM	XY sry+; Chromosome complement XY with testes
WT	Wildtype

Chapter 1: General Introduction

1.1. Pain

Pain is a fundamentally beneficial bodily response, typically playing a critical role in the health and safety of the those who experience it. Although an unpleasant experience, pain promotes the cessation of harmful activities and encourages greater care to prevent further harm from occurring. Grasping a steaming hot kettle will not only elicit the reflexive removal of the hand from the kettle to prevent further tissue damage but will also result in a throbbing burning pain that serves as a potent negative reinforcer. This process involves a wide array of neural circuitry from the periphery to the brain that sums to both the sensory and emotional aspects of pain. Under normal circumstances, the experience of pain is transient, resolving alongside wound healing. However, in some cases, the tissue heals but the pain remains; in other instances, the generation of pain is entirely divorced from any kind of tissue damage, instead arising from dysfunction within the circuitry responsible for pain processing. In these cases, the experience of pain ceases to provide any benefit to the sufferer and becomes pathological in nature. The chronification of this pathological pain presents enormous social and economic issues. Afflicting 1 in 4 adults in Canada, chronic pain boasted an economic burden of $40 billion in 2019 (Canadian Pain Task Force, 2020). Globally, chronic pain conditions comprise 4 of the top 10 causes for years lived with disability (Rice et al., 2016). As chronic pain conditions are known to plague older populations more than younger, the need to find effective therapeutics to alleviate chronic pain states will only be exacerbated as the global population continues to increase in age (Fayaz et al., 2016; Zimmer et al., 2022). Additionally, as chronic pain disproportionally effects women more than men and the majority of research to date has been performed in male subjects, our development of pain therapeutics is hindered by the fact that we still lack an understanding of the potential sex differences that may exist in the generation and persistence of chronic pain (Osborne & Davis, 2022).

Pain is a multidimensional process with many different causes along a social, psychological, and biological spectrum (Cohen et al., 2021). The social environment is a unique mediator of pain as it does not require prospective harm to the self but is largely linked to the emotional experience of pain. In humans, it has been shown that simply witnessing the pain of a partner is sufficient to activate multiple regions of the brain that are typically activated in the presence of a noxious stimulus (Singer et al., 2004). Specifically, activation of regions important in the emotional processing of pain, such as the anterior insula

and anterior cingulate cortex (ACC), was found to be elicited via both the knowledge that one's partner was in pain as well as during the personal experience of pain. On the other hand, activation of the somatosensory cortex was specific to the experience of being in pain, indicating that knowing one's partner was in pain preferentially elicits the emotional aspects of pain but not the sensory. This social transfer of pain has also been demonstrated in mice, where mice housed within the same cage or even the same room as other mice experiencing pain will similarly develop a hyperalgesic phenotype (Smith et al., 2016). The same phenomenon has also been observed for the analgesic effects of morphine and has been shown to be mediated, at least in part, via ACC projections to the nucleus accumbens, suggesting that the activation of regions important in emotion and empathy is a major driver in the social transfer of pain in mice as well (Smith et al., 2021).

The degree to which a noxious stimulus is regarded as painful is dictated by how much attention is paid to the stimulus, the interpretation of the stimulus, the emotions that are currently being experienced, and many other psychological processes that can either enhance or diminish the degree to which pain is experienced (Linton et al., 2011). Many chronic pain diseases are comorbid with mood disorders such as depression, indicating a strong relationship between the two (Rodriguez-Lozano et al., 2022; Tölle et al., 2006). Depression has been reported to be as high as 56% in patients experiencing chronic pain (Bair et al., 2003) and in rodents the induction of chronic pain similarly induces depressive-like behaviours (Šutulović et al., 2023). While the psychological link to chronic pain is well established, the causal relationship remains undefined, underscoring the need to find neural correlates at a basic physiological level.

The sensory aspect of pain, or nociception, functions to communicate to the brain when potential or actual tissue damage is occurring. At a basic level, noxious pain signals are transmitted to the spinal cord via primary sensory neurons which synapse on secondary neurons. These signals are then propagated up the spinal cord to the brain for further processing. Once this information is communicated to the brain, a wide array of brain regions are recruited to integrate all of the available internal and external sensory information, ultimately dictating the perception of this pain at both a conscious and unconscious level (Bliss et al., 2016). This cortical pain network consists of structures such as the cingulate, frontal, and the somatosensory cortices that all act together to provide information about the

painful stimulus and generate a physiological response to the stimulus (Fenton et al., 2015). In chronic pain states, however, there is decreased emphasis on these cortical regions and a greater recruitment of subcortical ones, particularly the limbic system (Baliki & Apkarian, 2015). In a longitudinal study following 39 patients as they transitioned from subacute back pain to chronic back pain, Hashmi et al. (2013) used functional magnetic resonance imaging to demonstrate that brain activity in patients with chronic pain shifted from sensory regions to limbic regions such as the amygdala. Long known for its importance in emotion and memory, the amygdala is a major integration hub for painful information. Multiple subnuclei within the amygdala are involved in pain processing, but the central nucleus of the amygdala (CeA) in particular has attracted a considerable amount of attention in the last decade for its ability to modulate the experience of pain.

1.2. The Central Nucleus of the Amygdala in Pain

In line with the colloquial knowledge pertaining to the amygdala, the CeA was initially known as a locus for fear and anxiety (Davis M, 1992; Rosen & Schulkin, 1998). Early manipulations of the CeA were primarily performed via insertion of the rodent stress hormone, corticosterone, directly into the nucleus, facilitating an anxiogenic effect (Meerveld et al., 2001; Shepard et al., 2000). As time has progressed, however, the CeA has become more implicated in pain. The CeA receives information about pain via two distinct neuronal circuits (Neugebauer V, 2015). Processed pain information entering the CeA originates indirectly from the thalamus which provides inputs to the CeA via its projections to the basolateral amygdala. The second, more direct pathway consists of spinal projections to the parabrachial nucleus in the brainstem, which in turn projects directly into the lateral and capsular subdivisions of the CeA, after which the signal is sent out of the CeA or to the medial subdivision of the CeA for further processing before exit. The CeA serves as the primary output centre of the amygdala, sending signals to the brainstem, hypothalamus, and basal forebrain regions, modulating pain at both the sensory and cortical level (Neugebauer et al., 2020). Interestingly, the behavioural effect of these processes appears to be lateralized. The right CeA has been shown to be predominately pro-nociceptive, whereas the left CeA's function remains more contentious, having been found to be less pro-nociceptive than the right CeA, anti-nociceptive, or having no effect on pain at all (Allen et al., 2021).

In various pain models, the CeA has been shown to be active in response to noxious stimuli (Gonçalves & Dickenson, 2012; Ji et al., 2009; Wilson et al., 2019). With technological advancements, specific pain-related cell populations have been uncovered, allowing for a more thorough dissection of the neuronal cells that are involved in pain processing. Corticotropin releasing factor expressing neurons within the CeA have been implicated in nociceptive processing but seem to play a more significant role in the emotional components of pain (Mazzitelli et al., 2021; Mazzitelli et al., 2022; Regev et al., 2012). Recently, two other GABAergic neuronal cell populations within the CeA, neurons positive for either protein kinase C δ (PKCδ) or somatostatin (Som), have been shown to play a role in nociceptive processing and are dysregulated in neuropathic pain (Adke et al., 2021; Wilson et al., 2019). Chemogenetic activation of PKCδ+ neurons was able to induce mechanical hypersensitivity, while inhibition resulted in attenuation of nociceptive responses following sciatic nerve cuffing. Conversely, the chemogenetic inhibition of Som+ neurons resulted in a decrease in the mechanical nociceptive thresholds of naïve mice and a rise in the mechanical thresholds of mice experiencing neuropathic pain following chemogenetic activation. Together, the various neuronal populations of the CeA seem to work in concert to modify both the affective and sensory components of pain. However, how these neurons become dysregulated in chronic pain remains to be elucidated. Additionally, despite sex differences being noted in multiple pain-relevant pathways that involve the CeA (Cantu et al., 2022; Long et al., 2016; Presto & Neugebauer, 2022) many works continue to be performed in male subjects only, leaving gaps in the literature in how the CeA is modulated in chronic pain in both sexes.

While neurons have received the lion’s share of the attention regarding the changes in chronic pain within the brain and the CeA specifically, increasing emphasis is being placed on the role of the immune system in chronic pain. Microgliosis has been observed within the CeA in rodent models of chronic pain conditions (Dworsky-Fried et al., 2022; Fülöp et al., 2023; Nascimento et al., 2023; Yuan et al., 2020). Microglia have been shown to interact with CeA neurons in stress-related conditions via synaptic remodeling (Chen et al., 2024; Yuan et al., 2021). Synaptic pruning is, however, only one function of microglia and their other functions, such as cytokine production, have yet to be thoroughly explored in the CeA in chronic pain.

1.3. The Immune System's Role in Chronic Pain

For several decades it has been shown that immune cells and neurons communicate in chronic pain conditions to facilitate and perpetuate pain states. Peripheral immune cells can be divided into two broad classes: cells of the innate immune system and cells of the adaptive immune system. Innate immune cells mount rapid and unbiased attacks on molecules that could be deemed hazardous to the host and consist predominately of phagocytic cells, namely macrophages, neutrophils, eosinophils, and basophils. Adaptive immune cells, alternatively, are primarily composed of lymphocytes that respond specifically to pathogens to rid the host of it. In acute inflammation induced by, for example, a skin lesion, immune cells will localize to the injury site and release several inflammatory mediators that result in a stereotyped response including swelling, redness, and pain (Kandel et al., 2021).

In contrast to the periphery, the central nervous system (CNS) is an immune-privileged organ: the blood brain barrier (BBB) prevents most molecules and cells in the blood from permeating into the CNS parenchyma, protecting it from potentially damaging substances. As peripheral immune cells are restricted from entering the CNS under physiological conditions, the CNS requires a population of resident immune cells to keep the host healthy. The archetypal immune cell in the CNS is the microglia, but other cells, such as astrocytes, can also serve immune functions. In terms of morphology and immunoreactivity, microglia resemble peripheral macrophages although they have greater functional range (Perry & Teeling, 2013). Microglia are continuously surveying the brain parenchyma and responding to their surroundings to maintain homeostasis within the CNS. As CNS pathology is highly heterogenous and can result from a wide variety of factors such as stroke, injury, or neurodegeneration, the microglial response to insult must also be highly heterogenous. This adds a significant degree of complexity to the role of microglia in the facilitation of pain, for which most research has been done in the spinal cord. Additionally, injury to the CNS is not required for microglial reactivity in chronic pain conditions, as first shown in 1999 by Fu et al., who demonstrated that microglial immunoreactivity in the rat spinal dorsal horn increases following hind paw injection of formalin, adding further need to understand the function of microglia in pain.

Microglia have been shown to play a role in central sensitization in the spinal cord, the increased responsiveness of spinal nociceptive neurons to their normal or subthreshold afferent input (International

Association for the Study of Pain). Microglia have been shown to play a critical role in spinal nociceptor activation, releasing several pain-facilitating factors. In 2005, Coull et al. showed that microglial BDNF is released following peripheral nerve injury and that this BDNF binds to TrkB on neurons to facilitate increased excitability in lamina I neurons. Similarly, microglial IL-1β can bind to neuronal IL-1R to enhance glutamatergic signalling (Inoue & Tsuda, 2018). Following spared nerve injury, microglial TNFα is critical in the neuronal remodelling of the spinal cord (Liu et al., 2017). Liu et al. (2017) also showed that inhibition or ablation of microglia within the spinal cord was able to attenuate the pain in their spared nerve injury model, providing further support to the notion that microglia are incredibly important to the development and facilitation of pain. However, this study, like many others, was performed exclusively in male mice. The predominant use of male mice in this study and many others poses an issue in our understanding of the role of the immune system in pain, as an ever-growing body of literature suggests that, while pain phenotypes are often conserved between sexes, the mechanistic processes by which males and females generate pain may be different.

1.4. Sexual Dimorphisms in the Generation of Chronic Pain

It has been well established that chronic pain is more common in women than it is in men (Osborne & Davis, 2022). Despite this and increased mandates by funding agencies to incorporate both sexes in studies, there remains a male bias in most fields of research (Beery & Zucker, 2011). Immunology represents one of the few fields with a bias towards female subjects, in part due to an overwhelming majority of autoimmune diseases being more prevalent in women than men. However, the study of chronic pain, a sex-biased disease with known immune system involvement, has an extensive history of male-biased research. Mogil and Chanda (2005) reported that in the ten years leading up to 2005, 8 in every 10 studies published in *Pain* were performed in male subjects exclusively. This has contributed to a historical lack of understanding of the dissociable mechanisms by which males and females develop and maintain pain.

Within the past ten years there has been an increase of studies showcasing sexual dimorphisms in the neuroimmune contributions to pain. One of the early studies showcasing a stark contrast between the mechanisms facilitating pain in males and females demonstrated that spinal microglia mediated

mechanical hypersensitivity following spared nerve injury in male mice, but not in females (Sorge et al., 2015). Instead, female mechanical hypersensitivity was reported to be dependent on infiltrating T cells. This was particularly interesting considering that the presentation of pain was the same in both sexes. Since then, several studies have been performed demonstrating additional neuroimmune sexual dimorphisms within the spinal cord and dorsal root ganglia (DRG) in neuropathic pain models (Berta et al., 2016; Lopes et al., 2017; Luo et al., 2019; Luo et al., 2021). In contrast, only two studies have evaluated neuroimmune sex differences in pain-relevant regions of the brain of animals experiencing chronic pain, representing a major gap in pain literature. Two weeks following spared nerve injury, Michailidis et al. (2021) showed that microglial proliferation differed in the contralateral ventral ACC between females and males, although functional ramifications of this were not investigated. Using experimental autoimmune encephalomyelitis (EAE), a central neuropathic pain model, Dworsky-Fried et al., (2022) demonstrated that microglial activation within the CeA resulted in reduced morphine analgesic efficacy in female but not male mice. As microglia in these supraspinal pain-relevant regions have largely been unexplored in either sex, neuroimmune interactions have yet to be elucidated.

1.5. Sex Differences in MS and EAE

Multiple Sclerosis (MS) is a chronic, progressive disease more common in women that is characterized by demyelination of axons via an autoimmune attack within the CNS (Whitacre C., 2001). This disease is associated with a myriad of different symptoms including motor deficits, sensory aberrations, and cognitive impairment (McGinley et al., 2021). Among the many sensory aberrations, pain is a very common symptom in MS, afflicting more than half of all patients (Österberg et al., 2005). EAE is the most widely used murine model of MS and mimics many of the symptoms including progressive motor dysfunction and pain (Constantinescu et al., 2011). Motor dysfunction due to corticospinal axon demyelination and degradation manifests in an ascending order where the animal first experiences tail paralysis, commonly termed disease onset, before sequentially losing motor function of the hindlimbs and eventually the forelimbs. Prior to the presentation of these motor deficits, the animal will be hypersensitive to multiple sensory modalities, allowing for the of use of this model to study pain (Olechowski et al., 2009). As in the clinical population (Hirsh et al., 2009), there does not appear to be a sex difference in the

degree of pain experienced between males and females. However, despite similar behavioural phenotypes in the murine models of MS, the physiological processes that occur between male and female animals are different, particularly in regions that facilitate pain.

The DRG are the collection of cell bodies of sensory neurons that send both noxious and non-noxious information into the spinal cord from the periphery. Although EAE is considered to be predominately a disease of the CNS, the peripherally located DRG have also been shown to be dysregulated (Yousuf et al., 2019). Maguire et al. (2022) showed that sex differences exist in the degree of infiltrating immune cells of the DRG and that female and male DRG neurons respond differently to TNFα, a pleiotropic cytokine that is upregulated in EAE. Similarly, the degree of differentially dysregulated genes within the DRG in EAE has been shown to be greater in females (Friedman et al., 2019). This dysregulation in females was intimately associated with the immune system, with upregulations of several important immune genes, whereas in males the few dysregulated genes in EAE largely corresponded to transport, identifying that the immune system may display a greater degree of dysregulation in females in EAE. These studies suggest that not only do females display greater inflammatory responses in EAE than males, but that the responses of peripheral neurons to this inflammation also differs.

EAE is characterized by infiltration of immune cells into the CNS which subsequently mount an immune response against the myelin that surrounds the axons of neurons. At the level of the spinal cord, the activation of both infiltrating and resident immune cells, as well as the degree of demyelination that this autoimmunity results in, has been shown to differ between sexes. At EAE disease onset, when the mice lose motor function in their tails, male mice have greater astrogliosis and axonal damage than female mice do despite similar degrees of immune cell infiltration (Catuneanu et al., 2019). Conversely, at 20 days post-EAE induction, Wiedrick et al. (2021) found greater immune cell infiltration and demyelination in females compared to males. These authors also found that males displayed significantly greater chemokine expression in EAE than females, in contrast to what was found in the DRG by Friedman et al. (2019), although this may be due to the collection of tissue at a later timepoint (20 days vs. disease onset). Importantly, these studies demonstrate no sex differences in behavioural phenotype, indicating that, despite the differences in immune dysregulation between both sexes, there are convergent yet dissociable mechanisms facilitating EAE disease and pain.

While there is an increasing collection of studies showing sex differences in the immune response to EAE in peripheral and lower-order CNS structures like the DRG and spinal cord, the brain remains largely unexplored in terms of its role in pain in EAE. Given the increased prevalence of mood disorders concurrent with MS and the overlapping neurocircuitry between the emotion and pain circuitry, investigating the alterations within the brain that occur in MS is paramount to successful treatment of pain in patients with MS (Schubert & Foliart, 1993). The amygdala, and particularly the CeA, is well positioned to be involved in not only the nociceptive aspects of pain in MS, but also the affective. As a model of central neuropathic pain in MS, EAE has been shown to alter microglial and neuronal activity within the CeA in a sex dependent manner, though the immune mechanisms behind changes within this region in EAE have yet to be characterized.

1.6. Aims and Hypothesis

Given the current lack of effective treatment for chronic pain in MS, there is a significant need to understand the dissociable mechanisms by which men and women develop and maintain chronic pain states in this disease. Sex differences in the immune system have been shown in the facilitation of chronic pain, suggesting that treatments must address the sex-specific alterations that occur. By using EAE, an inflammatory autoimmune pain model with known sex differences, this project aims to increase our understanding of the immune dysregulation that occurs within a highly influential pain locus, the CeA. To do this, we had 3 goals:

Aim 1: Evaluate the sex differences in the inflammatory milieu of the CeA in EAE.

Aim 2: Evaluate the sex differences in microglial response to EAE in the CeA.

Aim 3: Assess other tissue-resident and infiltrating immune cells in the CeA as potential contributors to the inflammatory response.

I hypothesized that females would exhibit a greater inflammatory response within the central amygdala in EAE, and that this response would be primarily driven by microglia.

In anticipation of unveiling sex differences in the inflammatory milieu within the CeA, we utilized the Four Core Genotype (FCG) model to discern the origins of the differences. As such, we first validated

the use of FCG mice in EAE to study the presymptomatic characteristics of the disease as well as the immunological response at EAE onset.

Chapter 2: Four Core Genotype Mouse Model in EAE

2.1. Introduction

When sex is determined to be a relevant biological variable, sex hormones are often assumed to be the most likely culprit. However, due to the ability of some genes on the X chromosome to escape X-inactivation, sex differences can also originate from sex chromosome gene expression due to females having higher expression of these X-linked genes. Dissociating the influence of these factors is difficult and has historically been performed using gonadectomy, which neglects the influence of sex hormones on development. To attempt to better dissociate the influence of sex chromosomes and sex hormones, the FCG mouse model was made (Arnold & Chen, 2009). Briefly, the testes-determining, *Sry* gene was deleted from the Y chromosome and inserted onto chromosome 3, making an XY male where the *Sry* gene is independent of the Y chromosome. This male is then bred with a wildtype (WT) C57Bl/6J female, producing offspring with an XX or XY chromosome complement, wherein each genotype can possess either ovaries or testes due to the dissociation of the *Sry* gene from the Y chromosome. Consequently, the FCG model consists of the following four genotypes: XX*Sry*-, XX*Sry*+, XY*Sry*-, and XY*Sry*+ wherein the presence of the *Sry* gene determines whether the animal appears phenotypically female or male, independent of sex chromosome complement. In this model, if a difference is found between mice expressing XX and XY sex chromosomes, regardless of *Sry* expression, then the difference is considered to be due to sex chromosomes; if a difference is found between animals having ovaries and testes, regardless of sex chromosomes, the difference is considered to be due to sex hormones.

This mouse model has been used in dozens of publications in many different fields, including three studies that utilize the model in EAE. Published in 2008, the first of these three studies was also one of the earliest uses of the FCG model, demonstrating that the XX chromosome complement conferred greater EAE severity in SLJ mice and increased immune cell infiltration into the spinal cord, but not in the C57Bl/6J mouse line, suggesting an interplay of sex chromosome gene expression with strain-specific genes (Smith-Bouvier et al.). Elaborating on this work 11 years later, Itoh et al. (2019) found that two X-linked histone demethylases escaped X-inactivation in CD4+ T cells from XX FCG C57Bl/6J mice, resulting in genome-wide consequences on immune response in EAE. In the latest study to use the FCG model in EAE, Doss et al. (2021) used a C57Bl/6J FCG mouse line crossed with a non-obese diabetic strain to study chronic progressive EAE. The authors found that T helper cells transmit greater EAE

severity in FCG C57Bl/6J males due to sex-specific downregulation of the X-linked KDM5c gene in Th17 cells. These studies have largely focused on the disease course of EAE and exclusively used mice in the chronic disease stage of the EAE model. However, the ability to detect and quantify EAE-induced pain hypersensitivity is largely limited to the presymptomatic stage of the disease, prior to motor loss, which is currently mostly uncharacterized (Dworsky-Fried et al., 2022; Olechowski et al., 2009). As such, we aimed to establish that the early characteristics of EAE in FCG mice, including time to onset, weight loss, and nociceptive behaviours, were in line with that of WT C57Bl/6J males and females.

Recently, an X-Y gene translocation has been found in the FCG mice, leading some to question the validity of the model (Panten et al., 2023). The authors of this study demonstrate that this gene translocation is associated with alterations in the peripheral immune cells of the FCG XXF and XYM mice compared to WT C57Bl/6J females and males, respectively, raising concerns about the use of the FCG model in studies investigating sex differences in the immune system. Given that we sought to compare the behavioural responses of presymptomatic EAE FCG mice, we opted to characterize the central presymptomatic immune response as well in order to validate our immunological findings in the FCG model with WT C57Bl/6J mice. To investigate the immune response, we evaluated cytokine expression in EAE at both a protein and mRNA level in various pain-related regions. As EAE models a demyelinating disease, we also characterized the immune response within a major white matter tract, the internal capsule (IC). Given the previous findings using the FCG mice in EAE, we hypothesized that the FCG mice would display normal EAE disease course characteristic of female and male C57Bl/6J mice, and that the immune response would similarly be on par. Behaviourally, we find that FCG mice follow the expected EAE disease course, though we do present evidence that the gonadal females may differ from WT females. In terms of immune response, we find very few unexplainable strain differences, suggesting that there are minimal differences in immune response to EAE between FCG and WT C57Bl/6J mice within several pain-related regions of the brain.

2.2. Materials & Methods

2.2.1. Animals

Four Core Genotype Mice:

Male C57Bl/6J mice with the *Sry* gene (129-derived), responsible for testes development, translocated from the Y chromosome to chromosome 3 (provided by Dr. Arthur Arnold, UCLA) were bred with WT C57Bl/6J females to generate FCG mice. This permits the production of four groups of mice: gonadal females and gonadal males with either XX or XY sex chromosomes, allowing the dissociation of hormonal vs chromosomal sex effects. Mice were backcrossed onto a C57Bl/6J background for a minimum of eighteen generations. FCG mice ranged between 15-50 weeks in age.

Experimental Autoimmune Encephalomyelitis Induction:

To induce EAE, reagents provided by Hooke Laboratories (Cat. #: EK-2110) were used. Mice were subcutaneously administered myelin oligodendrocyte glycoprotein amino acids 35-55 (MOG_{35-55}; 50 µg/animal) emulsified in an inflammatory agent, Complete Freund's Adjuvant (CFA; 50 µL). Mice were given an intraperitoneal injection of pertussis toxin (100 ng) on the day of immunization and again 24 hours later. Control mice received subcutaneous injections of CFA and the subsequent intraperitoneal pertussis injections at the same times as the EAE mice.

Animal Ethics:

All animal procedures were conducted in compliance with the Canadian Council on Animal Care Guidelines and Policies, with approval from the University of Health Sciences Animal Care and Use Committee at the University of Alberta. Mice were housed in standard wire-top cages (1-5 per cage) in a temperature- and humidity-controlled environment with water and food was available *ad libitum*. Mice were kept on a 12-hour light/dark cycle and all experiments were performed during the light cycle.

2.2.2. Behaviour

EAE Behaviour:

EAE disease severity is classified on a five-point scale. A grade of 1 is considered disease onset where the animal first shows signs of motor deficits in the form of tail paralysis. A grade of 2 is characterized as having mild hindlimb weakness. A grade of 3 is severe hindlimb weakness in one or both

hindlimbs, where the animal struggles to support body weight on the hind limb. A grade of 4 is full paralysis of the hindlimbs. A grade of 5 is hindlimb and forelimb paralysis.

FCG mice (N=29 XXF, N=37 XXM, N=35 XYF, N=29 XYM) were weighed and monitored daily. Mechanical thresholds were assessed prior to disease induction and at disease onset (ie. tail paralysis), after which the animal was euthanized, and the brains were collected. Animals that did not show signs of disease onset by 20 days post-induction were assessed for mechanical thresholds and euthanized at that time. A genotype-matched CFA control animal was euthanized at the same time as each experimental animal on the day of onset. Mechanical hypersensitivity was assessed via von Frey filaments prior to EAE induction and at disease onset (grade 1). Thermal tail hypersensitivity was assessed on days 8, 9 or 13 post-induction via tail flick assay.

Von Frey Filaments:

Hind paw mechanical thresholds were assessed using the Up-Down method with von Frey filaments (Chaplan et al., 1994). Briefly, a 0.4 g filament was pressed into the hind paw and the reaction of the mouse was observed (no reaction or paw withdrawal). If the mouse did not respond to the mechanical stimulus, a higher force filament was applied until a pain response was observed, at which point the filament force was reduced. This process occurred four more times until five consecutive responses after the first positive response was recorded. These 5 responses were used to calculate the 50% positive response threshold for that hind paw – a measure of the mechanical nociceptive threshold of that mouse. Left and right hind paw thresholds were averaged for a single average for each animal.

Tail Flick Assay:

Mice were gently restrained in a soft conical tube and the distal inch of the tail was submerged in 49°C water. Time to withdraw the tail from the water was recorded. Reported tail withdrawal times for each mouse represent an average of three tail withdrawal tests, separated by 20 minutes each.

2.2.3. Post-Mortem

Luminex Multiplex Immunoassay

Following disease onset, a FCG EAE mouse and a genotype-matched CFA control were euthanized using pentobarbital, and brains were immediately removed following transcardial perfusion

with saline (0.9% NaCl). Brains were placed in a brain block and 2 mm punches corresponding to the amygdala and primary somatosensory cortex were collected and flash frozen on dry ice before being stored at -80°C. Punches were homogenized in tissue extraction reagent (Thermo Fisher Scientific, Burlington, ON, Canada) and the protein concentration of each sample was standardized to 2 mg/ml. Samples were sent to Eve Technologies (Calgary, AB, Canada) where a Luminex multiplex immunoassay was performed to observe the concentration of 45 different cytokines within each sample.

For WT C57Bl/6J Luminex analyses (N=9 females, N=18 males), some minor differences in protocol occurred. Following disease onset, the EAE mouse was euthanized, and brains were immediately removed following saline perfusion. CFA control mice were euthanized on day 20 post-induction. Brains were then flash frozen and stored at -80°C in Tissue Tek Optimal Cutting Temperature (O.C.T.™) compound (Fischer Scientific, Edmonton, AB, Canada). To obtain amygdalar samples, tissue was thawed and O.C.T.™ compound was removed prior to placement of the brain in a brain block for collection of 2 mm amygdala punches. Punches were homogenized in tissue extraction reagent (Thermo Fisher Scientific, Burlington, ON, Canada) and the protein concentration of each sample was standardized to 1 mg/ml. Samples were sent to Eve Technologies (Calgary, AB, Canada) where a Luminex multiplex immunoassay was performed to observe the concentrations of 45 different cytokines within each sample.

RNAscope Fluorescent in situ Hybridization (*FISH*):

Following euthanasia and transcardial perfusion with saline (0.9% NaCl), brains were immediately flash frozen on dry ice into cryomolds for the cryosectioning of 12 µm sections. Slides containing the slices were immersed in 4% paraformaldehyde (PFA) at 4°C for 15 minutes followed by 4 subsequent dehydrations in ethanol at room temperature for 5 minutes each (50%, 70%, 100%, 100%). Slides were allowed to air-dry for 5 minutes at room temperature and a hydrophobic barrier was drawn around the tissue using an Immedge® Hydrophobic Barrier PAP Pen. RNAscope Protease III (ACDbio) was added to each slide and the tissue was allowed to incubate for 30 minutes at room temperature. Slides were then washed in 1X phosphate-buffered saline twice. One hundred µL of an RNAscope probe (ACDbio) cocktail for targeted mRNA (Glial fibrillary acidic protein (*Gfap*) to label astrocytes, Integrin subunit alpha M (*Itgam*) to label microglia, *Cxcl1*, *Cxcl10*, *Il17*, *Il1β*, & *Tnfα*) was added to each slide and the slides were

left to incubate for two hours at 40°C. Slides were washed in 1X Wash Buffer (ACDbio) twice, then amplification reagents 1-4 were added sequentially with washes between each reagent incubation using 1X Wash Buffer. The slides were cover slipped using Prolong Gold with DAPI. Tissue was imaged using a Zeiss Axio Imager microscope with a 20X objective lens (Carl Zeiss, Oberkochen, Germany). Cells positive for fluorescent expression of cytokines were manually counted and colocalization with ITGAM and GFAP was assessed manually. Positive cells were determined by the presence of 3 or more puncta. Total cell counts of *Itgam*+ and *Gfap*+ cells were counted using QuPath (v0.4.4) (Bankhead et al., 2017).

No differences in protocol occurred when performing RNAscope FISH on FCG or WT tissue.

2.2.4. Statistics

Behaviour:

Statistical analyses of all behavioural assays were performed on GraphPad Prism (9.5.1). Survival curves were compared using Mantel-Cox log-rank test. Comparisons of 8 independent groups were performed using 3-way ANOVA. Comparisons of 4 independent groups over time were performed using repeated measures 2-way ANOVA. Post-hoc multiple comparisons tests were run using the Holm-Šídák test. Data are presented as means ± the standard error of the mean (SEM), and significance thresholds were set at $p < 0.05$.

Post-mortem:

Pairwise comparisons of cytokine protein concentrations used to generate volcano plots were performed using the rstatix package on R (4.2.2) by Julia Nickols. Differential cytokine expression between CFA controls and EAE animals was assessed using the Mann-Whitney U test with FDR correction for multiple comparisons. Significance thresholds were set at $q < 0.05$.

RNAscope FISH analyses comparing disease and strain differences were performed using GraphPad Prism (9.5.1). Comparisons of 4 independent groups were performed using 2-way ANOVAs, with follow-up Holm-Šídák post-hoc tests. Data are represented as means ± the SEM, and significance thresholds were set at $p < 0.05$.

2.3. Results

2.3.1. FCG mice demonstrate typical presymptomatic progression of EAE

As all our behavioural testing occurs at disease onset, we asked if there was a significant difference in our FCG mice in terms of time to disease onset. Gonadal female (XXF and XYF) mice onset significantly earlier than gonadal males (XYM and XXM), without a significant effect of chromosome complement (**Fig. 1B**; **Fig. S1**; effect of gonad: $F(1,69) = 5.055$, ***p = 0.0278***; effect of chromosome: $F(1, 69) = 0.0009132$, ***p = 0.9760***; interaction effect of gonad and chromosome: $F(1,69) = 0.1918$, ***p = 0.6628***). Prior to disease onset, we observed a marked decrease in weight of EAE animals that was greater in gonadal females than males, potentially due to lower rates of disease onset in gonadal males (**Fig. 1C,E**; effect of disease: $F(1,65) = 285.7$, ***p < 0.0001***; effect of genotype: $F(3,65) = 49.74$, ***p < 0.0001***; interaction effect between genotype and disease: $F(3,65) = 2.859$, ***p = 0.0436***) that was not observed in CFA animals (**Fig. 1C,D**; effect of disease: $F(1,53) = 0.6683$, ***p = 0.4173***; effect of genotype: $F(3,53) = 27.64$, ***p < 0.0001***; interaction effect between genotype and disease: $F(3,53) = 0.4153$, ***p = 0.7427***). EAE onset is characterized by mechanical allodynia but not thermal hyperalgesia (Olechowski et al., 2009). We therefore evaluated the mechanical thresholds of EAE mice at disease onset in comparison to the CFA controls and found that EAE mice had significantly lower nociceptive thresholds than CFA mice at EAE onset (**Fig. 1F**; effect of disease: $F(1,118) = 24.85$, ***p < 0.0001***). We also saw a significant interaction effect between EAE and gonads but not of chromosome (interaction effect between disease and gonads: $F(1,118)=4.311$, ***p = 0.0400***; interaction effect of disease and sex chromosome: $F(1,118) = 0.3620$, ***p = 0.5486***). Post-hoc analyses revealed that there was a significant difference between CFA and EAE nociceptive thresholds in XXM (***p = 0.0003***), XYF (***p = 0.0320***), and XYM (***p = 0.0089***) groups, but not the XXF group (***p = 0.6038***), indicating there may be genotype effects on nociceptive thresholds. Expanding on this, when comparing FCG gonadal females and gonadal males in CFA and EAE, there was not a significant difference between EAE gonadal females and males (***p = 0.4548***), but the CFA groups are trending towards significance (***p = 0.0640***) (**Fig. S2A**, effect of interaction: $F(1,122) = 4.430$, ***p = 0.0374***). However, when FCG XX and XY mice are compared, there is no effect of chromosome complement (**Fig. S2B**, effect of disease: $F(1,122) = 26.08$, ***p < 0.0001***; effect of chromosomes: $F(1,122) = 0.5812$, ***p = 0.4473***; interaction effect of disease: $F(1,122) = 0.1281$, ***p = 0.7211***). This is not a result of baseline differences in mechanical nociception (**Fig. S3**). Evaluating thermal nociceptive thresholds, we found no

effect of disease on tail withdrawal thresholds when tested across days 8, 9, or 13 (**Fig. 1G**; effect of disease: F(1,109) = 0.3050, ***p = 0.5819***), indicating that the FCG mice may not experience thermal hyperalgesia in EAE during the presymptomatic period. However, when displayed as a function of time to onset, we do see evidence to support a reduction in thermal thresholds 1 day prior to disease onset, albeit in a very small sample size (**Fig. S4).**

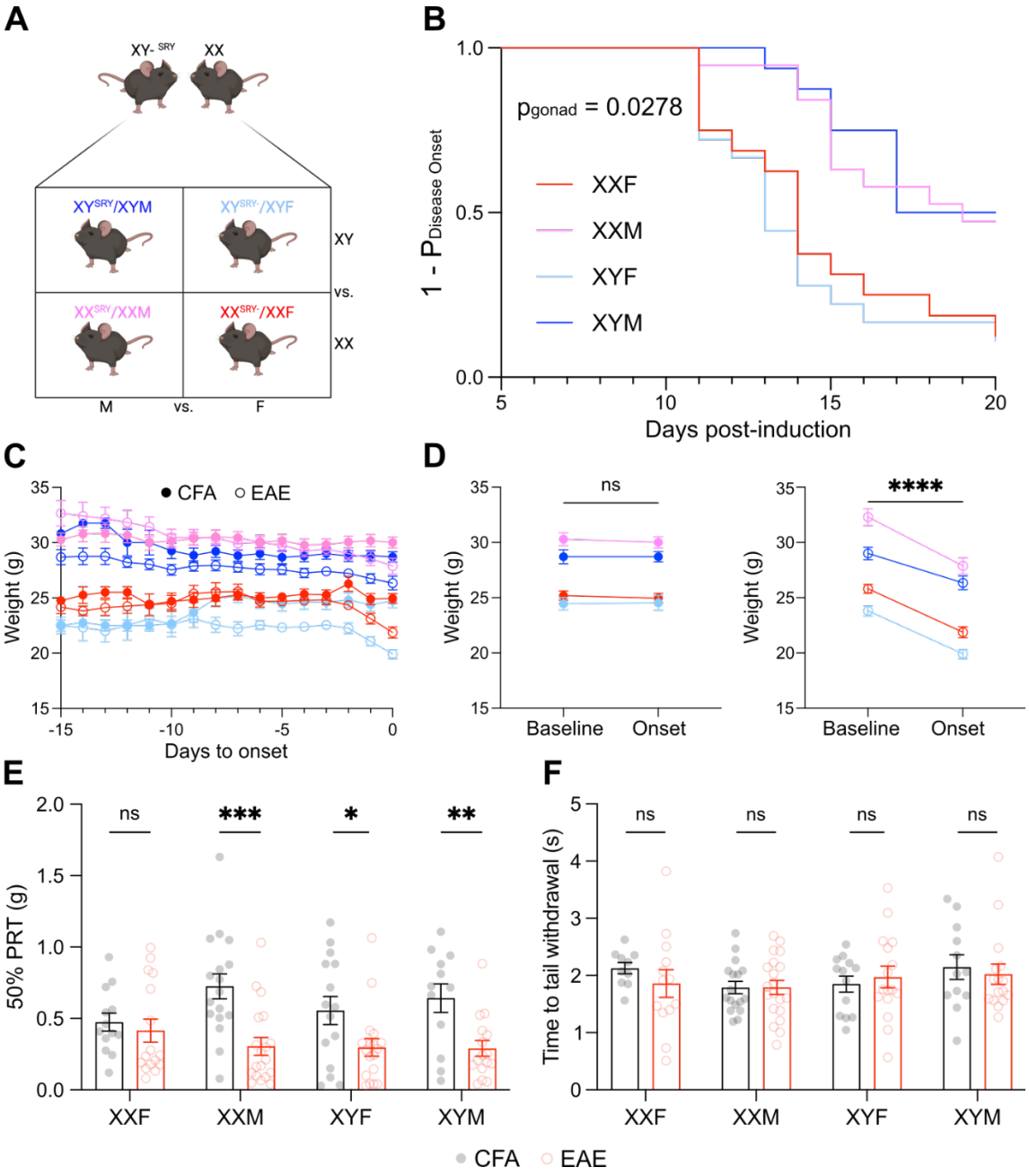

Fig. 1: FCG mice demonstrate typical presymptomatic progression of EAE. (A) Schematic depiction of the FCG mouse model. **(B)** Survival curve showing that animals with ovaries reach disease onset, characterized by tail paralysis, sooner than animals with testes, regardless of chromosome complement. **(C)** Weights of CFA and EAE FCG mice leading up to disease onset. **(D)** EAE mice experience significant weight loss at disease onset (right), whereas CFA animals do not (left). **(E)** FCG EAE mice display mechanical allodynia at disease onset, tested by von Frey. XXF mice do not display significant differences compared to controls, whereas the 3 other genotypes have significantly lower nociceptive thresholds in EAE than CFA controls. **(F)** Tail flick thermal thresholds are not changed in EAE compared to CFA in any genotype during the presymptomatic period, tested at days 8, 9, or 13. Data presented as mean ± SEM.

2.3.2. The inflammatory response between FCG and WT C57Bl/6J mice display minimal differences

To evaluate whether the inflammatory response within the amygdala in EAE differed between FCG and WT mice, we assessed the cytokine response in EAE via a Luminex assay in both mouse strains (**Table 1**; **Fig. S5**). We were able to compare 25 cytokines between the two strains and found that 8 cytokines were differentially dysregulated between the two models (CCL11, CCL17, CCL21, CX3CL1, CXCL2, IL-10, IL-13, and VEGF). CCL11 was upregulated in the FCG EAE mice but not in the WT mice. Two cytokines (CCL17 and CCL21) were upregulated in the WT mice but not in the FCG animals. The largest group of differentially regulated cytokines were cytokines that were downregulated in the FCG EAE mice but not dysregulated in the WT animals (CX3CL1, CXCL2, IL-10, IL-13, and VEGF).

Table 1: Effect of EAE on cytokine protein within the amygdala of FCG and WT C57Bl/6J mice.

	Regulation in EAE vs. CFA	
Cytokine	Four Core Genotype	WT C56Bl/6J
CCL11	Upregulated	No effect
CCL12	Upregulated	Upregulated
CCL17	No effect	Upregulated
CCL21	No effect	Upregulated
CCL22	Upregulated	Upregulated
CX3CL1	Downregulated	No effect
CXCL2	Downregulated	No effect
CXCL9	Upregulated	Upregulated
CXCL10	Upregulated	Upregulated
IL-10	Downregulated	No effect
IL-13	Downregulated	No effect
VEGF	Downregulated	No effect

Bolded cytokines are different between strains.

Cytokines omitted due to concentrations being too low for a comparison: CCL2, CCL4, CCL19, CXCL1, CXCL5, EPO, G-CSF, GM-CSF, IL-3, IL-5, IL-6, IL-7, IL-11, IL-12p40, IL-15, IL-20, LIF, LIX, TIMP-1, TNFα.

Cytokines omitted because EAE did not have an effect in either group: CCL3, CCL5, CCL20, CSF-1, IFNβ-1, IL-1α, IL-1β, IL-2, IL-4, IL-9, IL-12p70, IL-16, IL-17.

Sexes are pooled.

To further test whether the inflammatory response to EAE was different between FCG and WT mice in the CNS, we compared 5 cytokines of interest (*Cxcl1*, *Cxcl10*, *Il1β*, *Il17*, & *Tnfα*) using RNAscope

within 4 different regions (BLA, CeA, IC, and S1). *Cxcl1, Cxcl10,* and *Il17* were selected for further exploration based on the results of the cytokine Luminex, while *Il1β* and *Tnfα* were selected based on their association with microglial-driven inflammation. Of the 20 analyses, 3 had a significant effect of strain (*Cxcl1*, *Cxcl10* in the IC; *Il1β* in the S1) and 1 had a significant interaction effect of disease and strain (*Il1β* in the BLA) (**Table 2**; **Fig. S7**). Within the IC, WT C57Bl/6J animals expressed more *Cxcl1*+ and *Cxcl10*+ cells. In the S1, FCG animals express more *Il1β*+ cells than WT C57Bl/6J animals. Within the BLA, *Il1β*+ cells were significantly upregulated in WT EAE mice, but not in the FCG. *Il17*+ cells were not found in any region and therefore are not shown. Due to small sample size, sex was not evaluated.

Table 2: Differences in the effect of EAE on cytokine mRNA within various regions of the brain of FCG and WT C57Bl/6J mice.

Cytokine	Region	Effect
Il1β	BLA	Interaction (p = 0.0472)
Cxcl1	IC	Strain (p = 0.0169)
Cxcl10	IC	Strain (p = 0.0306)
Il1β	S1	Strain (p =0.0079)

All other analyses did not show a significant effect of strain or an interaction of disease and strain. Sexes are pooled.

2.4. Discussion

2.4.1. Four Core Genotype mice in a model of EAE

At the conclusion of this project, only a handful of papers have been published using FCG mice in EAE and none have focused on the early stages of the disease. As such we initially set out to establish that the FCG mice followed the traditional pattern of EAE onset as well as the expected nociceptive responses. While it has been previously reported that female SLJ mice induced with EAE have an earlier day of disease onset post-immunization compared to males (Papenfuss et al., 2004; Voskuhl et al., 1996), the same has not been observed in C57Bl/6J mice induced with EAE using pertussis (Catuneanu et al., 2019; Huntemann et al., 2022; Mifflin et al., 2019; Wiedrick et al., 2021). As FCG mice are backcrossed on the C57Bl/6J line and our model of EAE induction utilized the administration of pertussis, our finding that gonadal females had significantly earlier disease onset than gonadal males, regardless of sex chromosome complement, was unexpected. This may be due to residual effects of the initial breed of the *Sry* gene, which originated from the 129-mouse line, although this type of sex difference has not been assessed in this mouse line and this is unlikely to have overt effects on mouse behaviour to this degree. Alternatively, in light of our cytokine data, it is possible that the FCG gonadal females mount a greater immune response to EAE, facilitating more rapid corticospinal degradation and thereby decreasing the time to disease onset.

Our finding pertaining to a lack of influence of sex chromosomes on disease onset is consistent with the only other study that has analyzed EAE disease onset in C57Bl/6J FCG mice, although the design of that study intentionally prevented the assessment of sex hormones (Smith-Bouvier et al., 2008). Estrogens have, however, been shown to be largely neuroprotective in rodent models of EAE, in line with the clinical observation that MS severity in women is attenuated by pregnancy (Voskuhl & Palaszynksi, 2001). Testosterone levels between XXM and XYM mice are similar and consistent with WT males, but sex hormones may vary between the XXF and XYF genotypes, as evidenced by the early loss of estrous cycle in XYF mice compared to XXF mice (Arnold & Chen, 2009; Dhakal et al., 2023). In a study evaluating different characteristics of FCG mice, Corre et al., (2016) reported similar testosterone and estrogen levels between the XX and XY genotypes in gonadal males and females, respectively, but they also find age-dependent differences in luteinizing hormone and follicular stimulating hormone. Other pituitary hormones have not been compared over longer time points in the FCG mice, but it is possible

that there are also differences in these that could influence behaviour. As the mice used in our experiments had a significantly greater age range and erred towards being older, it is possible that the circulating hormone levels of our FCG mice varied by a significant amount, influencing the observed EAE phenotype. Further work should be done to characterize the hormones of FCG mice and their influence on EAE behaviours.

We found an influence of genotype on the weight loss at EAE onset, although this is likely mediated by the fact that some of the male animals did not onset prior to day 20, and therefore did not lose weight by the time of euthanasia. However, weight loss was still seen across all genotypes. This is largely in line with previous reports of EAE-induced weight loss by studies such as Huntemann et al. (2022), wherein they find no sex difference in weight loss due to EAE. This indicates that EAE FCG mice lose weight at disease onset in a similar fashion to that of a WT C57Bl/6J mice.

This is the first study assessing nociceptive responses in EAE of FCG animals during the presymptomatic stage of the disease. We found that EAE animals generally displayed significantly reduced nociceptive thresholds at onset, as has been shown in previous studies using WT C57Bl/6J mice (Catuneanu et al., 2019). Interestingly, we found that there was also a significant interaction effect between gonad and disease. This appears due to reduced nociceptive thresholds in the CFA group of gonadal females. This is a surprising finding considering that in previous EAE studies, CFA females do not display a significant reduction in nociceptive thresholds (Ding et al., 2022; Wang et al., 2017). It is possible that the gonadal females in FCG mice differ from WT females in their response to CFA, presumably with a heightened immune response, which may explain the decreased nociceptive thresholds in the XXF mice following CFA administration. As a heightened general immune response of the gonadal females in EAE could potentially explain both a sooner time to disease onset as well as greater reductions in mechanical sensitivity in our CFA controls, more work should be done to investigate the peripheral and central immune response of FCG mice to ensure consistency with previous works in WT C57Bl/6J mice.

Although some studies have shown thermal sensitivity in EAE (Aicher et al., 2004; Mirabelli et al., 2019), only Dworsky-Fried et al. (2022) has demonstrated its occurrence during the presymptomatic stage of the disease. Alternatively, Olechowski et al. (2009) were unable to find a change in thermal

thresholds at any point. Consistent with the latter, we found that the FCG mice did not develop thermal hyperalgesia at the time of testing during the presymptomatic stage of the disease. This may be due to the time of testing: Aicher et al. (2004) showed that thermal tail withdrawal latencies vary over the time course of the disease, although they also report marked hypoalgesia prior to onset of motor symptoms. As we tested the FCG mice at days 8, 9, and 13 post-induction, it is possible that the range of disease courses across all mice prevented a significant effect from being shown. Consistent with this, when revisiting our tail withdrawal data, we observed that EAE animals that onset the day following testing did experience a drop in thermal thresholds, although it is worth noting that our sample size is very small, and we were unable to assess effects of genotype (**Fig. S4**). This, alongside our other behavioural testing both prior to and at disease onset, suggest that the FCG mice largely follow the expected behavioural disease course of WT C57Bl/6J males and females.

2.4.2 The FCG inflammatory response to EAE within the brain is comparable to that of C57Bl/6J mice

The FCG mouse model has been used in dozens of studies since the model's inception, including in several inflammatory models. However, recent evidence has demonstrated that the FCG mouse line has a 3.2 MB X-Y chromosome translocation not observed in WT C57Bl/6J mice containing several genes that influence the immune system (Panten et al., 2023). We therefore evaluated EAE-induced inflammatory proteins in the amygdala in both FCG and WT mice (**Table 1**; **Fig. S5**). Further, we evaluated 5 cytokines via RNAscope within the basolateral amygdala (BLA), CeA, IC, and somatosensory cortex (S1) (**Table 2**; **Fig. S7**).

In our Luminex assay, we compared 25 cytokines and found that 8 cytokines were differentially dysregulated between FCG and WT mice in EAE. Six cytokines were found to be dysregulated in the FCG mice, but not in the WT animals. This is likely exaggerated by a lower sample size in the WT tissue (n = 4-8 for CFA, n = 6-19 for EAE) than FCG (n = 20-36 for CFA, n = 24-41 for EAE) which would result in fewer cytokines being determined as significant if the effect size is small. Additionally, we obtained lower total protein concentrations from WT as compared to FCG mice, further limiting our ability to detect smaller differences between groups. For example, in the FCG Luminex, IL-10 was found to be

downregulated in the FCG amygdala but not in the WT tissue. However, in a previous study by Acharjee et al. (2018) utilizing the same Luminex assay performed in our study, the authors found that C57Bl/6J female EAE mice had a significant reduction of IL-10 compared to CFA controls in the amygdala, suggesting that our failure to find downregulation of IL-10 in the amygdala of WT females in EAE may be due to our inability to detect the difference. Additionally, that study was performed exclusively in female WT animals whereas our assay was performed using only 2 female and 6 male CFA animals, so we may be masking some inflammatory effects due to a male-dominant control group. Lastly, only 4 WT EAE mice (3 males and 1 female) reached disease onset prior to euthanasia, which could also be exacerbating the differences between strains as the majority of FCG mice did reach disease onset. Of the 8 cytokines that we found to be different between our strains in the Luminex assay, 5 could potentially be explained by these reasons (CCL11, CXCL2, IL-10, IL-13, and VEGF) due to significance being found in the FCG mice, but not the WT animals. However, the 3 remaining cytokines found to be differentially dysregulated – CCL17, CCL21, and CX3CL1 – cannot be explained by these reasons.

CCL17 conventionally binds to CCR4 to act as a powerful chemokine for Th2 cells and monocytes, as well as dendritic cells, but not granulocytes, suggesting that it serves important roles in the pathogenesis of EAE (Ness et al., 2006). Unsurprisingly then, we found CCL17 was upregulated in our WT amygdala, in line with a previous study that found similar results in the spinal cord (Ruland et al., 2017). In contrast to that, our FCG mice displayed no dysregulation in CCL17. Within the CNS, CCL17 is produced by microglia (Scheu et al., 2017), although a 2018 study reported a small population of CA1 neurons that expressed CCL17 as well (Fülle et al.). Interestingly, CCL17 is found within the same gene cluster as CX3CL1 on the murine chromosome 8, another cytokine with contrasting dysregulation between strains (Ness et al., 2006).

Within the CNS, CX3CL1 is a prominent neuron-microglial communicator, wherein CX3CL1 is released by neurons and binds to CX3CR1 on microglia to induce functional changes (Pawelec et al., 2020). In patients with relapsing-remitting MS, CX3CL1 has been shown to be increased in the CSF compared to healthy controls (Blauth et al., 2015). In the CNS of C57Bl/6J mice, CX3CL1 is upregulated in the brain (Mills et al., 2012), spinal cord, and the DRG at EAE onset (Zhu et al., 2013). Our FCG results directly contradict this, finding that seemingly regardless of genotype, there is downregulation of CX3CL1

expression in the amygdala of our FCG EAE animals. This effect also seems to be consistent across different brain regions as our S1 punches saw similar reductions (**Fig. S6A**). However, CX3CL1 was also not dysregulated in the amygdala of our WT EAE animals, which is also in contrast to previous studies, although, as mentioned previously, this may be an effect of smaller sample sizes or lack of EAE disease onset in most of our WT animals. Still, as we found our FCG mice had an unprecedented downregulation of CX3CL1, neuronal-microglial communicators that are dysregulated in EAE may be different in the FCG mice compared to C57Bl/6J mice. Given the genetic link of CCL17 with CX3CL1 and the unexpected change in disease for both cytokines, it is possible that the differences in concentrations observed in these cytokines are due to alterations in gene transcription or translation, warranting further investigation.

In line with our assay in WT animals, CCL21 has been shown to be upregulated in the CNS in EAE (Bielecki et al., 2015; Columba-Cabezas et al., 2003). CCL21 canonically binds to CCR7 to facilitate migration of leukocytes such as dendritic cells, T cells, and B cells in homeostatic or pathological conditions (Hughes et al., 2018; López-Cotarelo et al., 2017). However, CCL21 can also be upregulated in neurons following peripheral nerve injury (Biber et al., 2011) and has also been found to be upregulated in the spinal cord of EAE onset animals (Schmitz et al., 2013). While CCR7 is the primary target for CCL21 in the periphery, CCL21 also binds to CXCR3 on microglia, representing another means for neuronal-microglial communication in the CNS (Biber et al., 2011; Rappert et al., 2002). However, we found that in our FCG mice, in opposition to our own and other's findings in WT C57Bl/6J animals, that CCL21 was not upregulated in the amygdala of EAE mice. This, in addition to our findings with CCL17 and CX3CL1, adds further interest to potentially strain-dependent dysregulation of neuronal-immune communication within the amygdala. Panten et al. (2023) investigated the effect of the previously unknown X-Y translocation in peripheral immune cells and hepatocytes but did not investigate central nervous immune cells such as microglia, nor did they look at neurons. It is possible that the genetic alterations in the FCG mice, particularly the reported translocation of the toll-like receptor (TLR) genes, *Tlr7* and *Tlr8,* has altered the immune response of neurons to pathological conditions. As Friedman et al. (2019) displayed sex-dependent changes in the expression of *Tlr7* and *Tlr8* in the DRG of EAE mice, it is possible that the alterations to these genes in FCG animals has significant implications to the immune response within the CNS neurons as well. Our findings demonstrate that, while in regard to total cytokine

release, the amygdalar inflammatory response is largely conserved between FCG and WT mice, further work is required to fully elucidate the immune differences between the strains. This is especially apparent in terms of neuronal cytokine release, where little is currently known.

The results of the RNAscope analyses were also largely consistent across the two models in pain-relevant regions, with only 4 of 20 analyses showing any effect of strain. The greatest differences were found in the IC, where both *Cxcl1* and *Cxcl10* were found to have greater expression in WT C57Bl/6J mice than FCG mice, suggesting that WT mice may have a greater chemotactic profile in white matter tracts than FCG mice in EAE. Given the demyelinating nature of EAE within the spinal cord, reduced chemoattraction of immune cells to white matter could have implications for the progression of the disease and axonal degradation, although we did not observe an unexpected time to disease onset in our FCG mice. Further work should be done to characterize the chemotactic profile within regions of concentrated white matter in FCG mice.

Within pain-relevant regions, the only significant difference between strains was found in *Il1β*+ cell expression. In the BLA, there was a significant upregulation of *Il1β*+ cells in EAE in WT animals but not FCG mice. As IL1β protein was not found to be upregulated in either of our Luminex experiments, nor was it upregulated in another study looking at EAE females, it is possible that the upregulation in the mRNA of IL1β does not translate to the protein level (Acharjee et al., 2018). In the S1, we found significantly more *Il1β*+ cells in our FCG mice than our WT animals, suggesting that the pro-inflammatory protein may be expressed at higher levels in the S1 of FCG mice than WT animals. As IL1β in the S1 can alter sensory transmission, this may influence pain signalling in FCG animals (Won et al., 1995). However, FCG animals display nociceptive thresholds that are typical of WT mice, so higher levels of IL1β likely plays a negligible role in the overt behaviours of the animals.

Taken together, we find few differences between FCG mice and WT mice in EAE. FCG mice display some sex differences during EAE onset uncharacteristic of typical C57Bl/6J mice that may be explained by an underlying exaggerated immune response in gonadal females. Similarly, we find FCG mice display typical EAE nociceptive thresholds, although we also find that the CFA control gonadal females have lower nociceptive thresholds following CFA and pertussis administration than expected that may, again, be explained by an exaggerated immune response in the gonadal females. Directly

comparing the immune response within the amygdala, we find very few unexplainable differences between the FCG and WT animals, although we do find 3 cytokines that may implicate alterations in the immune response of neurons to EAE which warrants further investigation. Lastly, our RNAscope data suggested that few differences exist in the immune response at the transcriptional level, although differing levels of chemotactic factors present in the white matter tracts of FCG animals may have ramifications for the overall disease presentation. Continued work should be done to ensure that findings in FCG mice are consistent with WT mice. However, as FCG mice largely follow the expected disease course of EAE, display typical nociceptive behaviour, and share largely overlapping immune responses to EAE with WT mice, we posit that our findings in the FCG mice are consistent with that of WT mice and consider it a valid model of EAE.

Connecting text: Chapter 2 and Chapter 3

Having determined that the behavioural phenotype of EAE in FCG mice is largely in line with WT C57Bl/6J mice and that the immune response to EAE within a collection of regions, as determined at a transcriptional and translational level, is consistent with the expected response of WT animals, we sought to perform an in-depth analysis of the sex differences in the CeA immune response to EAE using this model. Using the FCG mice, we measured protein concentrations of various cytokines in the amygdala in EAE in each independent genotype and find that the greatest dysregulation in cytokine expression was in XXF mice, and the least was in XYM mice. The other two genotypes (XXM & XYF) had an intermediate degree of dysregulation, suggesting that this sex difference is being mediated by both sex chromosomes and sex hormones.

Building on this work, we asked what immune cells may be mediating these sex effects. We investigate microglia, the CNS resident immune cells, in WT males and females to determine the contribution of these cells to the inflammatory environment. We found few sex differences in microglial characteristics including morphology and density. Unexpectedly, we provide evidence to suggest that sexually differential cytokines are preferentially expressed in astrocytes in EAE rather than microglia. Furthermore, the total number of astrocytes are upregulated in the CeA of females and not males in EAE, indicating that proliferative astrogliosis may underlie the sex-specific immune response in this region. This work suggests that not only does the degree to which EAE produces an inflammatory state within the CeA differ based on biological sex, but that different cell populations may mediate this response in each sex.

Chapter 3: Sex differences in the Immune Response of the CeA in EAE

3.1. Introduction

MS is an autoimmune disease generally characterized by CNS axon demyelination, leading to a progressive loss of function until eventual death. Over the course of the disease, more than 50% of patients will suffer from chronic pain, an affliction that represents a significant source of reduced quality of life in those that are already suffering (Österberg et al., 2005). Currently, the available therapeutics fail to adequately remedy this problem, so chronic pain remains a major concern in this population (Forbes et al., 2006; Hadjimichael et al., 2007).

As in many autoimmune diseases, MS is more common in women than it is in men (Whitacre C., 2001). On the other hand, pain appears to be evenly distributed between men and women suffering from MS (Hirsh et al., 2009). However, this lack of sex difference in pain phenotypes may belie differences in the underlying mechanisms that facilitate pain in each sex. Within the spinal cord, different immune cells have been shown to facilitate neuropathic pain in males and females, relying predominately on microglia and infiltrating immune cells, respectively (Sorge et al., 2015). Since this discovery, more studies have revealed more neuroimmune sex differences in both the DRG and the spinal cord in neuropathic pain (Berta et al., 2016; Lopes et al., 2017; Luo et al., 2019; Luo et al., 2021). Prior to presentation of motor deficits, the animal model of MS, EAE, mimics the neuropathic pain phenotype of the clinical population (Olechowski et al., 2009). Within the DRG (Friedman et al., 2019; Maguire et al., 2022) and the spinal cord (Catuneanu et al., 2019; Wiedrick et al., 2021), neuroimmune sex differences have been found previously in EAE, demonstrating dissociable mechanisms of pain facilitation within these pain relevant regions.

While the spinal cord has been extensively studied in EAE, the brain has seldom been considered for its role in chronic pain in this disease. The collection of brain regions that become active following the sensation of pain includes several disparate brain regions involved in both the sensory and emotional aspects of pain (Garcia-Larrea & Peyron, 2013). Given the known roles of the amygdala in the recognition and processing of unpleasant information, it is perhaps unsurprising that it is a member of this network. Within the clinical population, lesions within the amygdala have been associated with deficits in fear and anger recognition, but pain has yet to be studied in association with amygdalar lesions (Pitteri et al., 2019). Although several subdivisions of the amygdala are involved in the processing of painful

information, the CeA has garnered significant attention in the past decade for its ability to modulate pain. The CeA receives both processed and unprocessed pain information that is then sent to a variety of regions, including the brainstem, hypothalamus, and basal forebrain, that then modulate behavioural responses to this noxious information (Neugebauer et al., 2015; Neugebauer et al., 2020). Two neuronal cell populations have recently been showcased as having two opposing functions on pain, wherein activation of protein kinase C δ positive neurons is pronociceptive and the activation of somatostatin neurons is antinociceptive (Wilson et al., 2019). However, the authors, as well as others, have shown that in chronic pain, these cell populations become dysregulated, potentially contributing to the chronification of pain (Adke et al., 2021; Dworsky-Fried et al., 2022; Lin et al., 2022). How this dysregulation occurs is currently unknown, although current evidence supports the involvement of the immune system.

Microglial activation within the CeA has previously been shown to be associated with increased visceral pain in rats (Yuan et al., 2020; Yuan et al., 2022). In a previous study from our lab, we demonstrated that EAE induced similar microglial morphological alterations at disease onset, the time of peak nociceptive hypersensitivity (Dworsky-Fried et al., 2022). This EAE-induced microglial activation within the CeA was associated with a reduction in the efficacy of morphine in female mice, but not in males. Investigating this further, Dworsky-Fried et al. (2022) directly stimulated CeA microglia via microinjection of lipopolysaccharide (LPS) and found the same sex effect, suggesting that microglia within this region are acting differently in males and females. At EAE onset, immune dysregulation within the DRG has been shown to be significantly greater in females at EAE disease onset with very little change in males, so we hypothesized that the female immune response within the CeA would be significantly greater than males and that this sex difference in immune dysregulation would primarily come from microglia (Friedman et al., 2019).

Using the FCG model, we show the greatest degree of cytokine dysregulation within the amygdala of XXF EAE mice and the least dysregulation in the amygdala of XYM mice. The XYF and XXM genotypes demonstrate moderate amounts of dysregulation in comparison, suggesting a role of both sex hormones and sex chromosome genes in this effect. To investigate the role of microglia, we first assessed microglial morphology and density within the CeA, finding little difference in response between sexes. We next assessed the colocalization of CeA microglia with several cytokines including two of the

most highly differentially regulated cytokines from our protein assay, CXCL1, and CXCL10. We find minimal colocalization of these cytokines with microglia, finding instead that they colocalized to a greater extent with astrocytes. Furthermore, the number of *Gfap*+ cells in the CeA was increased only in females, indicating that astrogliosis may underlie the sex-specific immune response in this region.

3.2. Materials & Methods

3.2.1. Animals

Four Core Genotype:

Male C57Bl/6J mice with the *Sry* gene (129-derived), responsible for testes development, translocated from the Y chromosome to chromosome 3 (provided by Dr. Arthur Arnold, UCLA) were bred with WT C57Bl/6J females to generate four-core genotype (FCG) mice. This permits the production of four groups of mice: gonadal females and gonadal males with either XX or XY sex chromosomes, allowing the dissociation of hormonal vs chromosomal sex effects. Mice were backcrossed onto a C57Bl/6J background for a minimum of eighteen generations. FCG mice ranged between 15-50 weeks in age.

Cx3cr1^{CreER};Ai9:

B6.129P2(Cg)-Cx3cr1<tm2.1(cre/ERT2)Litt> mice (Cx3cr1^{CreER}; Jackson Laboratory) express Cre-ERT2 under the *Cx3cr1* promoter, allowing for tamoxifen-induced Cre expression within macrophages. These mice were backcrossed with C57Bl/6J for at least 12 generations. Cx3cr1^{CreER} mice were bred with Ai9 mice (129S6/SvEvTac x C57Bl/6NCrl origin, backcrossed with C57Bl/6J for at least one generation) to generate Cx3cr1^{CreER};Ai9 mice that express TdTomato in Cx3cr1+ cells following tamoxifen administration. Male and female Cx3cr1^{CreER};Ai9 mice were injected with tamoxifen at post-natal day 13 for two days to induce lasting Cre expression specific to resident tissue macrophages in the CNS (microglia). Control animals received a hind limb incision at 12 weeks of age, one week prior to euthanasia.

EAE Induction:

To induce EAE, reagents provided by Hooke Laboratories (Cat. #: EK-2110) were used. Mice were subcutaneously administered myelin oligodendrocyte glycoprotein amino acids 35-55 (MOG_{35-55}; 50 µg/animal) emulsified in an inflammatory agent, Complete Freund's Adjuvant (CFA; 50 µL). Mice were given an intraperitoneal injection of pertussis toxin (100 ng) on the day of immunization and again 24 hours later. Control mice received subcutaneous injections of CFA and the subsequent intraperitoneal pertussis injections at the same times as the EAE mice.

Animal Ethics:

All animal procedures were conducted in compliance with the Canadian Council on Animal Care Guidelines and Policies, with approval from the University of Health Sciences Animal Care and Use Committee at the University of Alberta. Mice were housed in standard wire-top cages (1-5 per cage) in a temperature- and humidity-controlled environment with water and food was available *ad libitum*. Mice were kept on a 12-hour light/dark cycle and all experiments were performed during the light cycle.

3.2.2. Post-Mortem

Luminex Multiplex Immunoassay:

Once a FCG EAE mouse reached disease onset, the mouse and a genotype-matched CFA control were euthanized using pentobarbital, and brains were immediately removed following saline (0.9% NaCl) flush. Brains were placed in a brain block and 2 mm punches corresponding to the amygdala and primary somatosensory cortex were collected and flash frozen on dry ice then placed in a -80°C freezer for storage. Punches were homogenized in tissue extraction reagent (Thermo Fisher Scientific, Burlington, ON, Canada) and the concentration of each sample was standardized to 2 mg/ml. Samples were sent to Eve Technologies (Calgary, AB, Canada) where a Luminex multiplex immunoassay was performed to observe the concentration of 45 different cytokines within each sample.

RNAscope FISH:

Animals were euthanized with pentobarbital and intracardially perfused with ice cold saline (0.9% NaCl). Brains were immediately flash frozen on dry ice into O.C.T. cryomolds. Brains were cut into 12 µm sections onto slides that were placed in a -80°C freezer. Slides were immersed in 4% PFA at 4°C for 15 minutes followed by 4 subsequent dehydrations in ethanol at room temperature for 5 minutes each (50%, 70%, 100%, 100%). Slides were allowed to air-dry for 5 minutes at room temperature and a hydrophobic barrier was drawn around the tissue using an Immedge® Hydrophobic Barrier PAP Pen. RNAscope Protease III (ACDbio) was added to each slide and the tissue was allowed to incubate for 30 minutes at room temperature. Slides were then washed in 1X phosphate-buffered saline twice. One hundred µL of an RNAscope probe (ACDbio) cocktail for targeted mRNA (*Gfap* to label astrocytes, *Itgam* to label

microglia, *Cxcl1*, *Cxcl10*, *Il17*, *Il1β*, & *Tnfα*) was added to each slide and the slides were left to incubate for two hours at 40°C. Slides were washed in 1X Wash Buffer (ACDbio) twice, then amplification reagents 1-4 were added sequentially with washes between each reagent incubation using 1X Wash Buffer. The slides were cover slipped using Prolong Gold with DAPI. Tissue was imaged using a Zeiss Axio Imager microscope with a 20X objective lens (Carl Zeiss, Oberkochen, Germany). Cells positive for fluorescent expression of cytokines were manually counted and colocalization with ITGAM and GFAP was assessed manually. Positive cells were determined by the presence of 3 or more puncta. Total cell counts of *Itgam*+ and *Gfap*+ cells were counted using QuPath (v0.4.4) (Bankhead et al., 2017).

Microglial Morphology:

Female (N=13) and male (N=11) naïve and EAE C57Bl/6J Cx3cr1^{CreER};Ai9 mice aged 10-12 weeks at the beginning of experimentation were euthanized and transcardially perfused with ice-cold 4% PFA. Brains were collected and post-fixed in 4% PFA for 48 hours then cryoprotected in 30% sucrose. Brains were then placed into O.C.T. cryomolds and sliced at 20 µm. Microglia within the CeA were imaged at 40X using Z stacks (Zeiss LSM710 Confocal microscope) and Bitplane Imaris (9.9.0) was used to generate 3D reconstructions of microglial morphology. Soma (area, sphericity, volume) and process (branch points, maximum length, total length) characteristics were analyzed. Tissue processing and analyses performed by Ethan Chen.

Immunohistochemistry (IHC):

Male and female C57Bl/6J mice aged 10-12 weeks at the beginning of EAE induction in CFA control or EAE groups were deeply anesthetized at EAE disease onset and intracardially perfused and post-fixed with ice-cold 4% PFA. Following cryoprotection in 30% sucrose, brains were frozen into O.C.T. cryomolds. Brains were cut into 20 µm sections onto slides that were placed in a -80°C freezer. Tissue was then blocked (NDS/NGS) and primary antibodies were left on the tissue to incubate overnight at room temperature. Primary antibodies and concentrations were as follows: rat anti-cluster of differentiation 4 (CD4; 1:200; Bio-Rad Laboratories, ON, Canada), rat anti-cluster of differentiation 45 (CD45; 1:100; BD Pharmingen, San Diego, CA, USA), and rabbit anti-ionized calcium binding adaptor molecule (IBA-1; 1:500; Wako, Richmond, VA, USA). On the second day, secondary antibodies were added: donkey anti-rabbit Alexa Fluor 488 (1:200; Thermo Fisher Scientific, Carlsbad, CA, USA) was

used for IBA-1 and donkey anti-rat Alexa Fluor 647 (1:200; Invitrogen, Eugene, OR, USA) was used for immunolabelling of CD4 and CD45. Tissue was then mounted using Prolong Gold antifade with DAPI and cover slipped. Images were taken using a Zeiss Axio Imager microscope with a 20X objective lens (Carl Zeiss, Oberkochen, Germany).

3.2.3. Statistics

Normality and Variance:

Data were assessed for normality prior to statistical analyses in two ways. Qualitative analysis of normality using QQ plot was the primary means of determining normality. If the normality of the data was ambiguous based on the QQ plot, Shapiro-Wilk test for normality of the residuals was used to decide normality. Non-parametric data was analyzed using Mann-Whitney test to compare ranks.

Parametric data was analyzed for equal variance using qualitative assessment of the homoscedasticity plot and quantitative assessment via F test. Parametric data with significantly different variances were analyzed using unpaired t test with Welch's correction.

Post-mortem:

Statistical analyses of all post-mortem analyses except for comparisons of Luminex cytokine analyses generating volcano plots were performed on GraphPad Prism (9.5.1). Comparisons of 4 independent groups were performed using 2-way ANOVAs and comparisons of 2 independent groups were performed using independent sample t-tests. Multiple comparisons tests were run using the Holm-Šídák test. Data are presented as mean ± the SEM, and significance thresholds were set at $p < 0.05$.

Luminex multiplex cytokine analyses generating volcano plots were performed using the rstatix package on R (4.2.2) by Julia Nickols. Comparisons of differential cytokine expression in CFA controls and EAE animals in the Luminex multiplex immunoassay were performed using the Mann-Whitney U test with FDR correction for multiple comparisons. Significance thresholds were set at $q < 0.05$.

3.3. Results

3.3.1. Female gonadal or chromosome complement is associated with greater dysregulation of inflammation within the amygdala

To begin characterizing the inflammatory milieu within the amygdala, we performed a Luminex on tissue punches from the subcortical region around the CeA in FCG mice at EAE disease onset. Comparing all EAE and CFA animals, we found that there was significant dysregulation of cytokines in the amygdalar punches. Five cytokines were downregulated (CX3CL1, CXCL2, IL10, IL13, and VEGF) and 9 cytokines were found to be upregulated (CCL11, CCL12, CCL19, CCL22, CXCL1, CXCL9, CXCL10, G-CSF, and TIMP-1; **Fig. 2B**). When comparing the 4 genotypes, we saw the greatest dysregulation of cytokines in the XXF genotype, wherein 8 cytokines were dysregulated compared to XXF CFA controls (CCL11, CCL12, CCL19, CCL22, CXCL1, CXCL10, EPO, and VEGF; **Fig. 2C**). The XYM genotype showed the least amount of dysregulation, with only CCL11 being significantly upregulated (**Fig. 2F**). Within the XXM and XYF genotypes, 6 (CCL2, CCL11, CCL12, CX3CL1, CXCL1, and CXCL10; **Fig. 2D**) and 4 (CCL12, CX3CL1, CXCL10, and VEGF; **Fig. 2E**) cytokines were dysregulated, respectively. To ensure that greater cytokine production in CFA control males did not obscure an inflammatory profile within the EAE males, comparisons of CFA control cytokine concentrations were performed amongst the four genotypes and showed that males did not have a greater expression of cytokines in our control animals (**Fig. S8**)

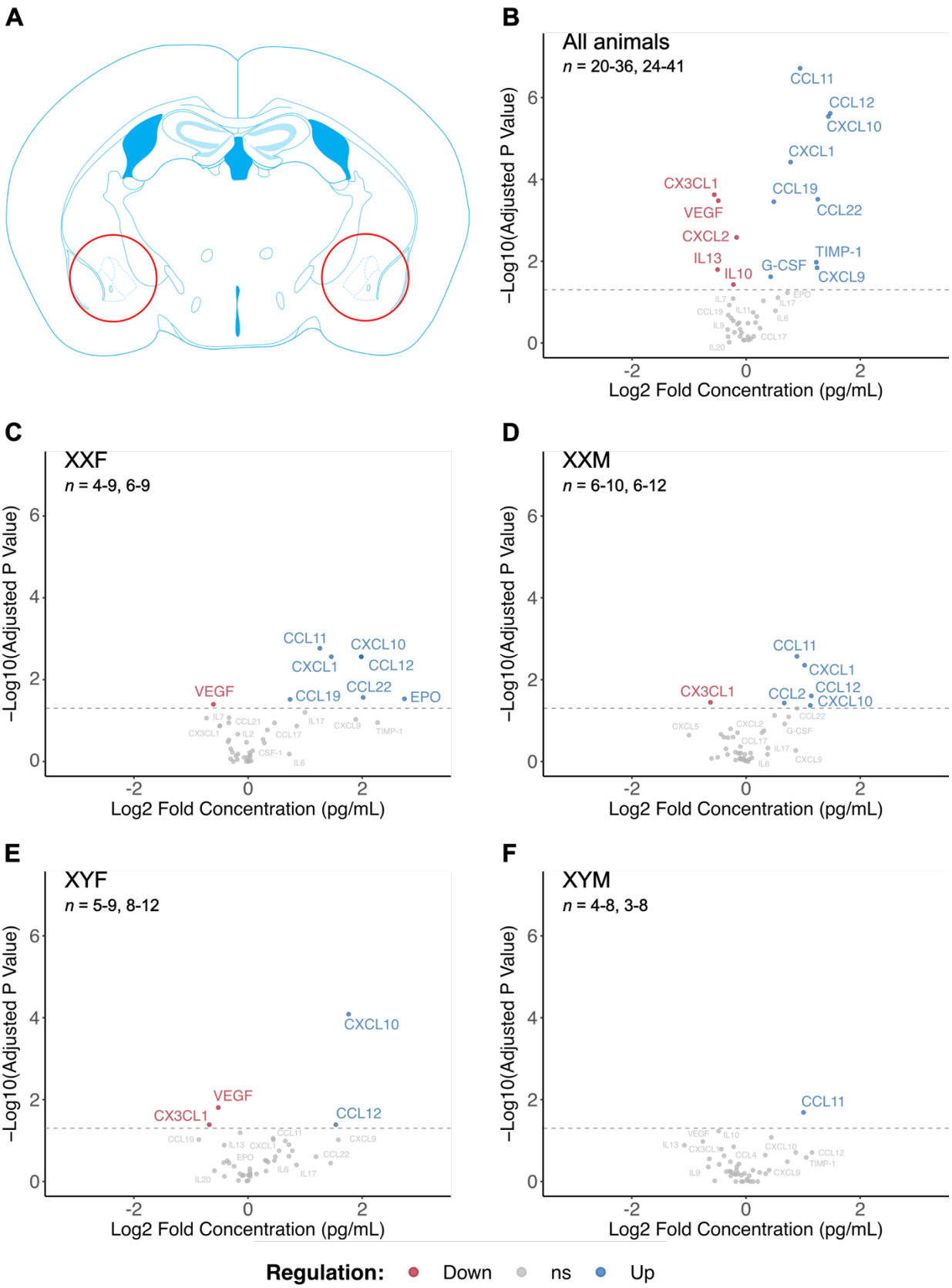
A
B
All animals
n = 20-36, 24-41
CCL11
CCL12
CXCL10
CXCL1
CX3CL1
CCL19
CCL22
VEGF
CXCL2
TIMP-1
IL13
G-CSF
IL10
CXCL9
−Log10(Adjusted P Value)
Log2 Fold Concentration (pg/mL)
C
XXF
n = 4-9, 6-9
D
XXM
n = 6-10, 6-12
E
XYF
n = 5-9, 8-12
F
XYM
n = 4-8, 3-8
Regulation: Down ns Up

Fig. 2: FCG females display greater cytokine dysregulation in the amygdala in EAE at disease onset. (A) Schematic depiction of the location of tissue punch. Tissue punches were centered around the CeA, denoted by the dotted lines. Tissue punches were homogenized, and cytokine concentrations were assessed via Luminex multuplex immunoassay. **(B)** Compared to CFA controls, EAE induces significant cytokine dysregulation in the amygdala. **(C-F)** The amygdala in XXF displays the greatest degree of cytokine dysregulation compared to the other genotypes and the XYM genotype displays the least cytokine dysregulation. Data are presented as change in EAE compared to CFA controls. Significantly downregulated cytokines are shown in red, upregulated cytokines are shown in blue. Horizontal dotted lines on volcano plots represent the significance threshold set at $p < 0.05$.

3.3.2. Male and female microglia differ minimally in EAE

To delve into potential differences in microglial response to EAE within the CeA, microglial morphology was measured in female and male EAE and naïve control mice. Microglial soma area, volume, and sphericity were measured, in addition to process bifurcations, total length, and longest arborization. Soma volume was greater in female microglia regardless of disease (**Fig. 3B**, effect of sex: F(1,20) = 4.464, ***p = 0.0474***; effect of disease: F(1,20) = 1.164, ***p = 0.2935***; interaction effect of sex and disease: F(1,20) = 0.01191, ***p = 0.9142***). Total process length was higher for female microglia than male microglia, again regardless of disease (**Fig. 3F,** effect of sex: F(1,20) = 5.654, ***p = 0.0275***; effect of disease: F(1,20) = 3.114e-005, ***p = 0.9956***; interaction effect of disease and sex: F(1,20) = 3.515, ***p = 0.0755***) When comparing process bifurcations, a significant interaction effect was found between EAE and sex, showing that male microglia have a reduction in process bifurcations in EAE, but female microglia do not (**Fig. 3E**, effect of sex: F(1,20) = 7.430, ***p = 0.0130***; effect of disease: F(1,20) = 0.7002, ***p = 0.4126***; interaction effect between disease and sex: F(1,20) = 4.497, ***p = 0.0466***). All other measures were not significantly different between EAE and CFA controls or between males and females. This data suggests that there are some, although few, differences in terms of the microglial morphological response to EAE in the CeA of male and female mice.

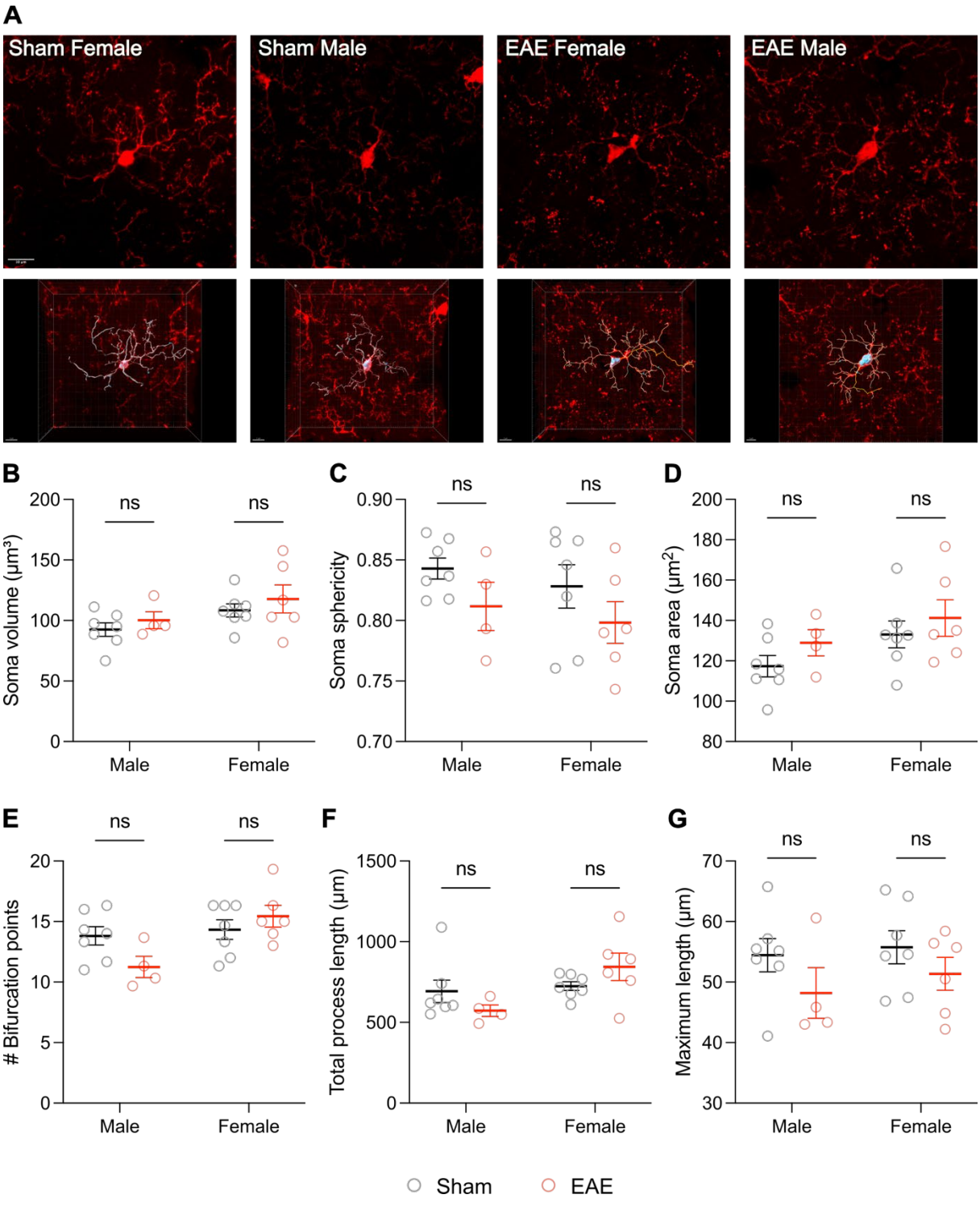
A
Sham Female
Sham Male
EAE Female
EAE Male
B
Soma volume (µm³)
200
150
100
50
0
ns
ns
Male
Female
C
Soma sphericity
0.90
0.85
0.80
0.75
0.70
ns
ns
Male
Female
D
Soma area (µm²)
200
180
160
140
120
100
80
ns
ns
Male
Female
E
Bifurcation points
20
15
10
5
0
ns
ns
Male
Female
F
Total process length (µm)
1500
1000
500
0
ns
ns
Male
Female
G
Maximum length (µm)
70
60
50
40
30
ns
ns
Male
Female
Sham
EAE

Fig. 3: Microglial morphological response to EAE in the CeA differs minimally between sexes in C57Bl/6J Cx3cr1^{creER};Ai9 mice. **(A)** Representative images (top) and 3D reconstructions (bottom) created in IMARIS of CeA microglia in sham and EAE mice. **(B)** Female microglial volume is greater than male microglia but is not altered by EAE. **(C)** Microglial soma sphericity is not different between females and males or shams and controls. **(D)** Microglial soma area is not different between females and males or shams and controls. **(E)** Male microglia display a reduction in the number of process branch points in EAE, but female microglia do not. **(F)** Female microglia have a greater total process length than male microglia, but without an effect of EAE. **(G)** The maximum process lengths are unchanged in EAE and not different between males and females. Due to digital zoom when imaging, scales are image specific; analyses on Imaris took this into account. Data presented as mean ± SEM.

To assess another feature of CeA microglia in EAE, we assessed microglial density using both IHC and RNAscope FISH. IBA-1+ cells were not increased in EAE compared to CFA controls (**Fig. 4B**, t(6.281) = 0.3926, ***p = 0.7076***) and *Itgam*+ cells were not upregulated in the CeA of EAE mice (**Fig. 4D**, t(16) = 0.6759, ***p = 0.5087***). No effects of sex were found.

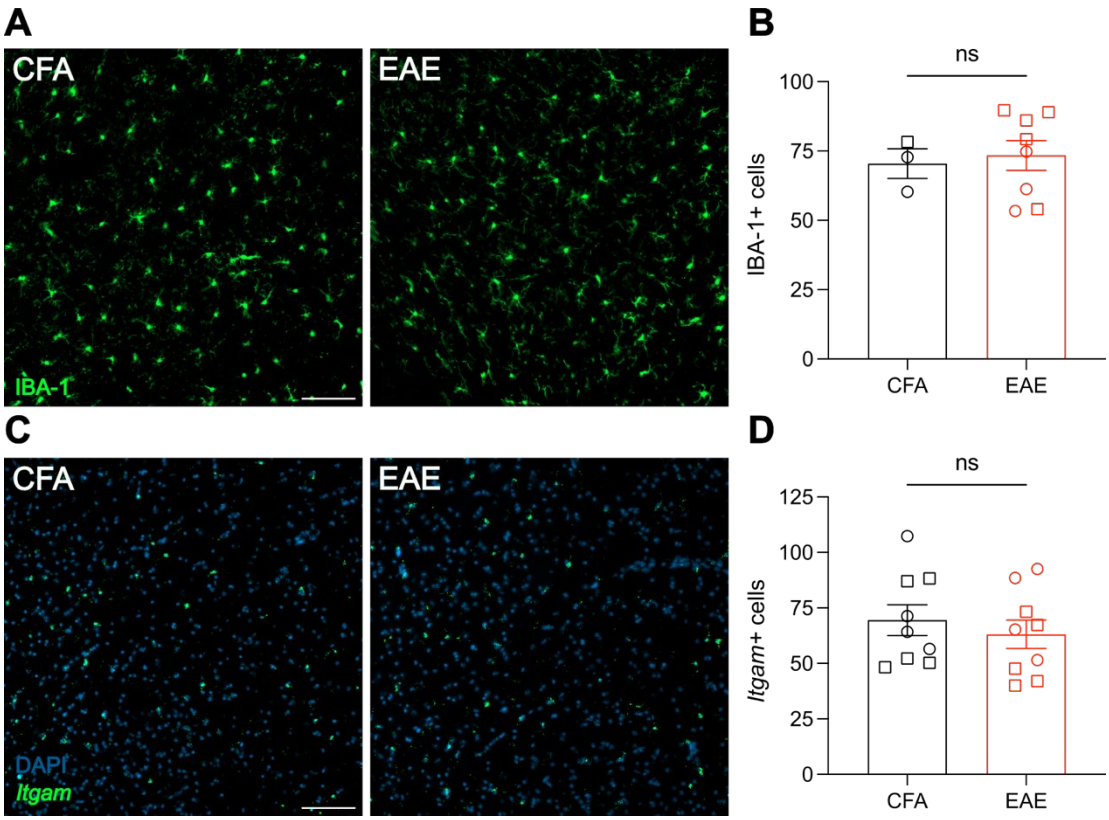

Fig. 4: Microglial density within the CeA does not change in EAE in WT C57Bl/6J mice. (A) Representative images of IBA-1 **(green)** staining via IHC. **(B)** Total IBA-1+ cells within the CeA do not change in EAE compared to controls. **(C)** Representative images of *Itgam* **(green)** staining via RNAscope. **(D)** Total *Itgam*+ cells within the CeA do not change in EAE. No effects of sex were observed, so sexes are pooled. Females are square, males are circles. Scale bars = 100 μm. Data presented as mean ± SEM.

3.3.3. *Il1β* and *Tnfα* predominately colocalize with microglia in EAE, but *Cxcl1* and *Cxcl10* do not

Microglia are frequently associated with pro-inflammatory responses, so we asked if there were sex differences in microglial expression of classical pro-inflammatory cytokines often associated with microglia, *Il1β* and *Tnfα*. Consistent with the results of the Luminex, we found that the total number of *Il1β*+ cells was not upregulated in EAE compared to CFA controls (**Fig. 5B**, U = 14, ***p = 0.1091***). However, *Il1β*+*Itgam*+ cells were found to be significantly upregulated in EAE (**Fig. 5C,** U = 10**, *p = 0.0274***), and the colocalization between *Il1β*+ cells with *Itgam*+ cells was not significantly different between CFA controls and EAE (**Fig. 5D,** t = 2.213, ***p = 0.0597***). *Tnfα*+ cells were upregulated in EAE (**Fig. 5F**, U = 9, ***p = 0.0019***) and *Tnfα*+*Itgam*+ cells were similarly found to be upregulated (**Fig. 5G**, U = 10.50, ***p = 0.0030***). No difference was found in colocalization of *Tnfα*+ cells and *Itgam*+ cells between CFA controls and EAE (**Fig.5H**, t(15) = 0.2265, ***p = 0.8239***). No significant interaction effect of sex and disease was found in any analysis, so sexes are pooled. These results indicate that pro-inflammatory cytokines, particularly *Tnfα*, are upregulated within the CeA at EAE onset and that microglia are the primary producers of these cytokines. However, this upregulation is not sex dependent. The mRNA of the pro-inflammatory cytokine IL17 was also stained, but no *Il17*+ cells were found in CFA or EAE (data not shown).

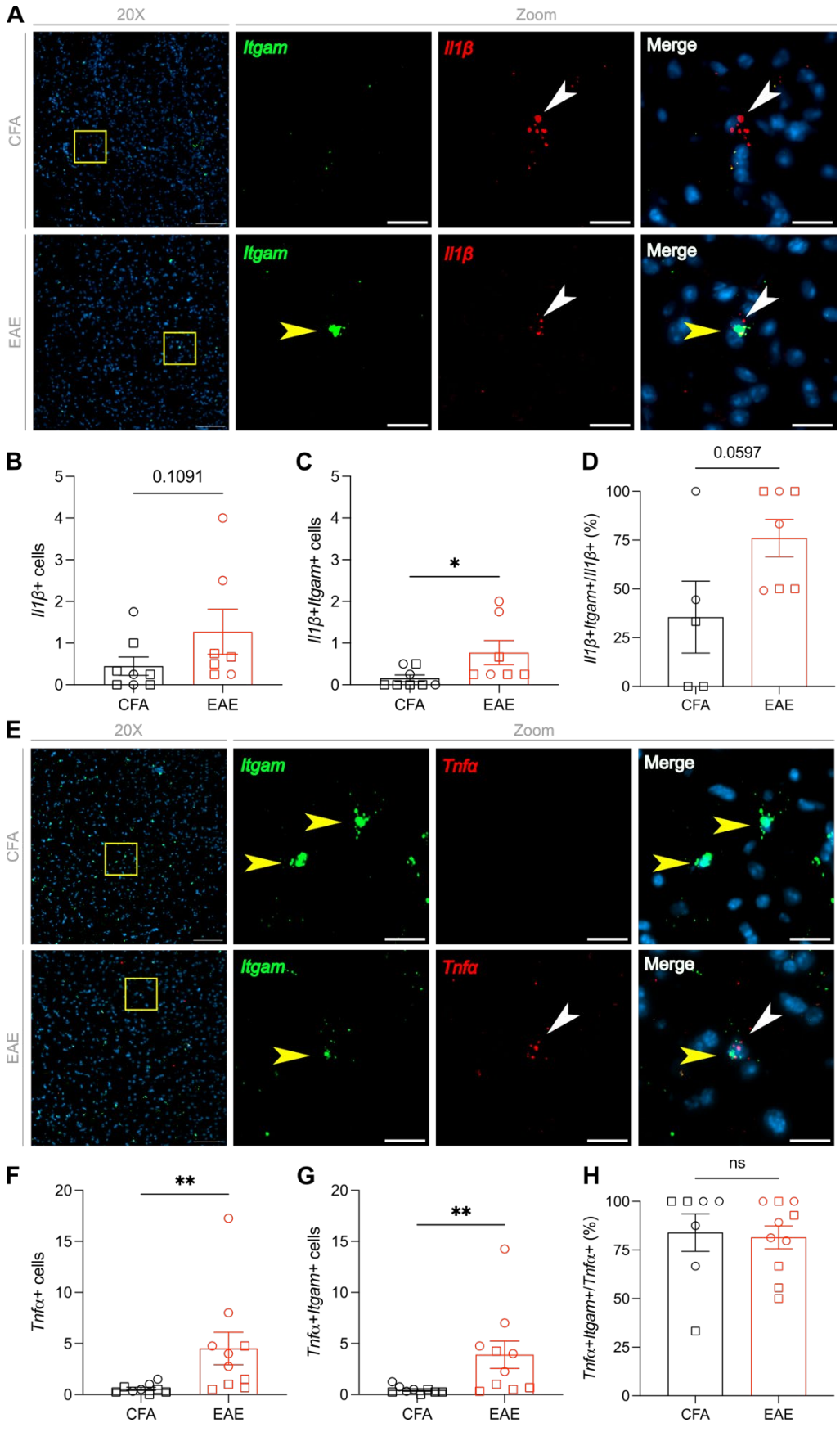
A
20X
Zoom
CFA
EAE
Itgam
Il1β
Merge
B
Il1β+ cells
0.1091
CFA
EAE
C
Il1β+Itgam+ cells
*
D
Il1β+Itgam+/Il1β+ (%)
0.0597
E
Tnfα
F
Tnfα+ cells
**
G
Tnfα+Itgam+ cells
H
Tnfα+Itgam+/Tnfα+ (%)
ns

Fig. 5: Pro-inflammatory cytokines predominately colocalize with microglia in the CeA in EAE. (A) Representative images of *Il1β* **(red)** and *Itgam* **(green)** RNAscope in the CeA in CFA and EAE. **(B)** Total *Il1β*+ cells are unchanged in the CeA in EAE. **(C)** Total *Il1β*+*Itgam*+ cells are upregulated in EAE. **(D)** Colocalization of *Il1β* with *Itgam*+ cells is not significantly upregulated in EAE within the CeA. **(E)** Representative images of *Tnfα* **(red)** and *Itgam* **(green)** RNAscope in the CeA in CFA and EAE. **(F)** Total *Tnfα*+ cells are significantly upregulated in EAE. **(G)** Total *Tnfα*+*Itgam*+ cells are upregulated in EAE. **(D)** Colocalization of *Tnfα and Itgam*+ cells is not significantly upregulated in EAE. Yellow arrows denote *Itgam*+ cells. White arrows denote cytokine+ cells. No effects of sex were observed, so sexes are pooled. Females are square, males are circles. 20X images have a scale bar = 100 μm. Zoomed images have a scale bar = 20 μm. Data presented as mean ± SEM.

To determine if microglia were the primary producers of chemokines shown to be differentially dysregulated in XX and/or gonadal females, *Cxcl1* and *Cxcl10* were stained alongside the microglial marker, *Itgam,* in the CeA. Within the CeA, *Cxcl1*+ cells were increased in EAE compared to CFA controls (**Fig. 6B**, U = 9.500, ***p = 0.0305***). *Cxcl1*+*Itgam*+ cells were not upregulated in EAE (**Fig. 6C**, U = 23, ***p = 0.5736***) and colocalization of *Cxcl1*+ cells and *Itgam*+ cells did not increase in EAE (**Fig. 6D**, t(12) = 0.6705, ***p = 0.5152***). *Cxcl10*+ cells were not upregulated in the CeA in EAE (**Fig. 6F**, t(14) = 0.4107, ***p = 0.6875***), nor were *Cxcl10*+*Itgam*+ cells (**Fig. 6G**, U = 24, ***p = 0.4383***) or *Cxcl10*+ colocalization with *Itgam*+ cells (**Fig. 6H,** t(11) = 1.398, ***p = 0.1897***). No interaction effect of sex and disease was found in any analysis, so sexes were pooled. The lack of sex effect in total positive cell counts of *Il1*β, *Tnf*α, *Cxcl1*, or *Cxcl10* cannot be explained by influence of other regions (BLA or IC) within the tissue punches from our Luminex (**Fig. S9**).

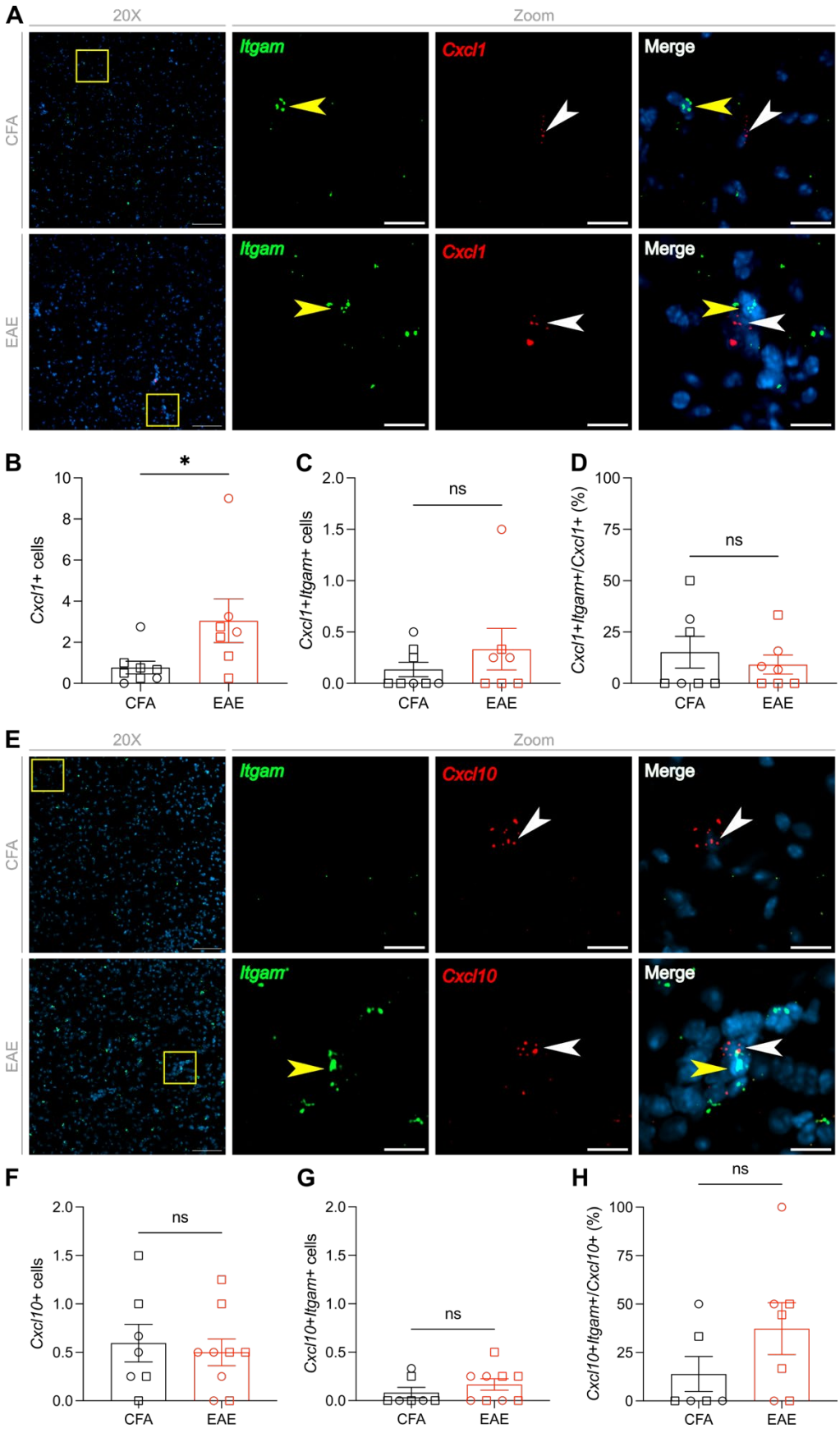
A
20X
Zoom
CFA
EAE
Itgam
Cxcl1
Merge
B
Cxcl1+ cells
*
CFA
EAE
C
Cxcl1+Itgam+ cells
ns
D
Cxcl1+Itgam+/Cxcl1+ (%)
ns
E
Cxcl10
F
Cxcl10+ cells
G
Cxcl10+Itgam+ cells
H
Cxcl10+Itgam+/Cxcl10+ (%)

Fig. 6: *Cxcl1* and *Cxcl10* colocalize at low levels with microglia in the CeA. (A) Representative images of *Cxcl1* **(red)** and *Itgam* **(green)** RNAscope in the CeA in CFA and EAE. **(B)** Total *Cxcl1*+ cells are upregulated in the CeA in EAE. **(C)** Total *Cxcl1*+*Itgam*+ cells are not upregulated in EAE. **(D)** Colocalization of *Cxcl1* with *Itgam*+ cells is not upregulated in EAE within the CeA. **(E)** Representative images of *Cxcl10* **(red)** and *Itgam* **(green)** RNAscope in the CeA in CFA and EAE. **(F)** Total *Cxcl10*+ cells are unchanged in EAE. **(G)** Total *Cxcl1*+*Itgam*+ cells are unchanged in EAE. **(D)** Colocalization of *Cxcl10 and Itgam*+ cells is not significantly upregulated in EAE. Yellow arrows denote *Itgam*+ cells. White arrows denote cytokine+ cells. Sexes are pooled. Females are square, males are circles. 20X images have a scale bar = 100 μm. Zoomed images have a scale bar = 20 μm. Data presented as mean ± SEM.

3.3.4. *Cxcl1* and *Cxcl10* primarily colocalize with *Gfap*+ astrocytes in the CeA

Colocalization of *Cxcl1* and *Cxcl10* with microglia was minimal and not upregulated in EAE, so we decided to investigate the colocalization of these chemokines with GFAP+ astrocytes. Consistent with our results in WT mice, using FCG mice we found an upregulation of *Cxcl1*+ cells in the CeA (**Fig. 7B**, t(10.96) = 3.767, ***p = 0.0031***) but not *Cxcl10*+ cells (**Fig. 7F**, U = 36.50, ***p = 0.7725***). There was an increase in *Cxcl1*+*Gfap*+ cells in EAE (**Fig. 7C**, U = 10.50, ***p = 0.0049***); no difference was seen in *Cxcl10*+*Gfap*+ cells (**Fig. 7G**, U = 20, ***p = 0.0713***). In EAE, there were significantly more *Cxcl1*+ cells colocalized with *Gfap*+ cells than in CFA controls and this effect was sex dependent where XYM animals had significantly greater upregulation of cells that colocalized than females did (**Fig. 7D,** effect of disease: F(1,12) = 7.004, ***p = 0.0213***; effect of sex: F(1,12) = 0.6207, ***p = 0.4461***; interaction effect of sex and disease: F(1,12) = 4.959, ***p = 0.0459***). No difference was observed between CFA and EAE in terms of *Cxcl10* colocalization with *Gfap*+ cells (**Fig. 7H**, t(14) = 1.141, ***p = 0.2732***). Unless otherwise stated, no effect of sex was found and therefore sex was pooled for analyses.

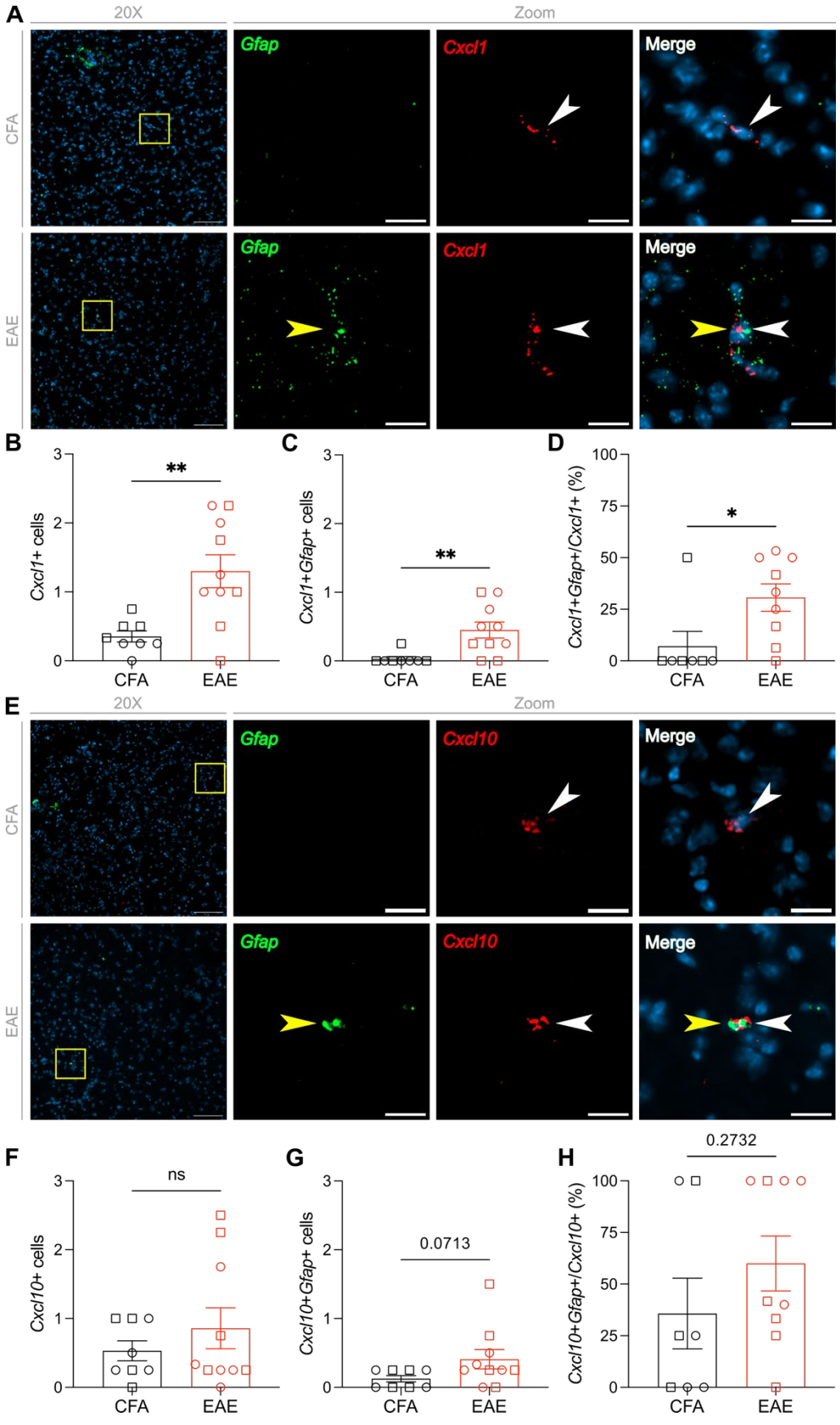
A
20X
Zoom
CFA
EAE
Gfap
Cxcl1
Merge
B
Cxcl1+ cells
**
CFA
EAE
C
Cxcl1+Gfap+ cells
**
D
Cxcl1+Gfap+/Cxcl1+ (%)
*
E
Cxcl10
F
Cxcl10+ cells
ns
G
Cxcl10+Gfap+ cells
0.0713
H
Cxcl10+Gfap+/Cxcl10+ (%)
0.2732

Fig. 7: *Cxcl1* and *Cxcl10* colocalize at greater levels with *Gfap*+ astrocytes in the CeA. (A) Representative images of *Cxcl1* **(red)** and *Gfap* **(green)** RNAscope in the CeA in CFA and EAE. **(B)** Total *Cxcl1*+ cells are upregulated in the CeA in EAE. **(C)** Total *Cxcl1*+*Gfap*+ cells are upregulated in the CeA in EAE. **(D)** colocalization of *Cxcl1*+ cells with astrocytes is increased in EAE. **(E)** Representative images of *Cxcl10* **(red)** and *Itgam* **(green)** RNAscope in the CeA in CFA and EAE. **(F)** Total *Cxcl10*+ cells are not upregulated in the CeA in EAE. **(G)** *Cxcl10*+*Gfap*+ cells are not upregulated in EAE. **(H)** Colocalization of *Cxcl10* and *Gfap*+ cells are not upregulated in EAE. Yellow arrows denote *Gfap*+ cells. White arrows denote cytokine+ cells. As an interaction effect of sex is only seen in *Cxcl1* colocalization with *Gfap*, sexes are pooled. Females are square, males are circles. 20X images have a scale bar = 100 μm. Zoomed images have a scale bar = 20 μm. Data presented as mean ± SEM.

3.3.5. *Gfap*+ astrocytes are increased in the CeA of XXF mice in EAE, but not in XYM mice

Astrogliosis is often characterized by increased proliferation of astrocytes (Anderson et al., 2014). To further investigate the astrocytic response within the CeA in EAE, we measured total *Gfap*+ cell counts within the CeA of XXF and XYM EAE and CFA control mice. More *Gfap*+ cells were found in the CeA of EAE XXF mice, but not in XYM mice (**Fig. 8B**, interaction effect between sex and disease: $F(1,14) = 8.383$, ***p = 0.0118***). These results indicate that there may be increased astrogliosis within the CeA of females in EAE, but not in males.

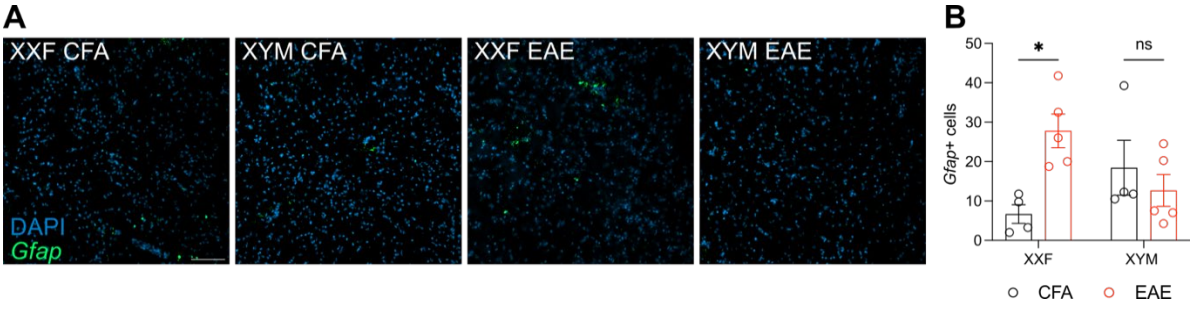

Fig. 8: *Gfap*+ astrocytes display increased density in EAE XXF mice, but not in EAE XYM mice. (A) Representative images of *Gfap* **(green)** staining in the CeA. **(B)** *Gfap*+ cells are upregulated in the CeA of XXF mice, but not XYM mice. Scale bar = 100 μm. Data presented as mean ± SEM.

3.3.6. Few immune cells infiltrate into the CeA at EAE disease onset

Within the spinal cord, infiltration of leukocytes has been observed in neuropathic pain models and EAE (Draleau et al., 2014; Dworsky-Fried et al., 2022). As we did not observe complete colocalization between cytokines and microglia or astrocytes we decided to investigate the possibility of infiltrating immune cells in the CeA. To investigate the source of the dysregulated cytokines within the brains of WT C57Bl/6J mice, we performed IHC to assess the presence of infiltrating leukocytes using the pan-leukocyte marker, CD45. As microglia express low levels of CD45, tissue was also stained for IBA-1 and cells that expressed both markers were deemed to be non-infiltrating. No difference was observed between CFA controls and EAE animals within the CeA (**Fig. 9B**, t(8.935) = 1.998, ***p = 0.0770***), indicating that infiltrating immune cells are unlikely to be contributing to the cytokine environment within the CeA.

As we found a trend towards increased infiltrating immune cells within the CeA, we decided to probe what cells might be infiltrating. EAE is predominately believed to be a T cell-driven disease and CD4+ T cells have been shown to infiltrate into the spinal cord to a greater extent than other immune cells at early disease timepoints (Barthelmes et al., 2016). As such, we stained for CD4+ cells to investigate whether CD4+ T cells were infiltrating. No differences were observed between CFA and EAE in the CeA (**Fig.9C**, U = 16.50, ***p = 0.8593***). This indicates that there may be infiltrating immune cells within the parenchyma of the brain at EAE onset, although the infiltrating immune cells are unlikely to be CD4+ T cells.

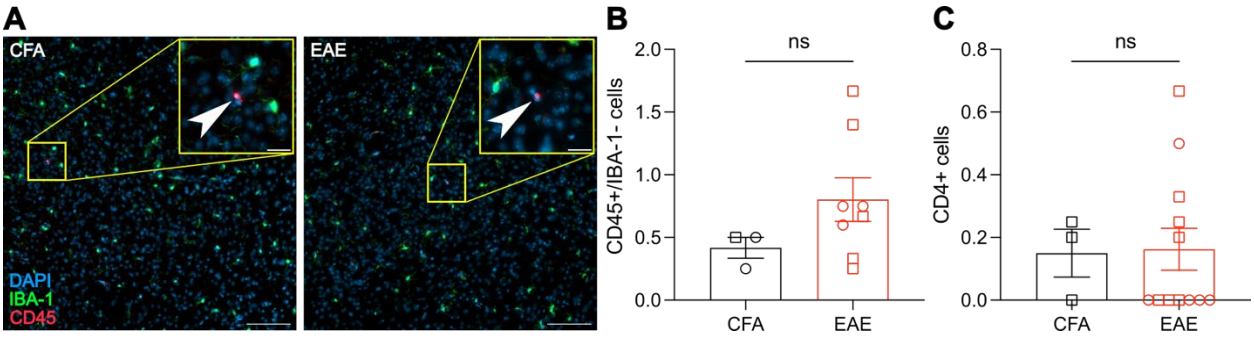

Fig. 9: Minimal leukocyte infiltration in CeA at EAE onset. (A) Representative images of CD45 **(red)**, a pan-leukocyte marker and IBA-1, a marker for microglia, **(green)** IHC staining in the CeA of WT C57Bl/6J mice. White arrows denote CD45+/IBA-1- cells, indicating infiltrating immune cells. **(B)** Cells positive for CD45 but negative for IBA-1 are not significantly upregulated in the CeA in EAE. **(C)** CD4+ cells are not upregulated in the CeA. Females are squares, males are circles. Zoomed out scale bar = 100 μm. Zoomed in scale bar = 20 μm. Data presented as mean ± SEM.

3.4. Discussion

3.4.1. EAE induces cytokine dysregulation within the amygdala

In our initial foray into evaluating the immune sequelae of EAE in the amygdala, we found a significant degree of dysregulation of cytokines. The majority of studied cytokines can be grouped into 4 main categories based on their primary known functions: chemokines, angiogenic factors, and pro- or anti-inflammatory cytokines. Most of the dysregulated cytokines were chemokines (composing 9 of 14 dysregulated cytokines), and 1 cytokine from each of the other groups were found to be decreased in EAE. Additionally, granulocyte colony-stimulating factor (G-CSF) and tissue inhibitor matrix metalloproteinase 1 (TIMP-1) were found to be upregulated in EAE. This suggests that the inflammatory environment of the amygdala in EAE is dominated by chemokine communicators.

The primary known function of chemokines is to communicate with immune cells to facilitate the migration from one region to another. Traditionally, this effect is chemoattractive, wherein a chemokine will bind to its receptor on an immune cell, facilitating the movement of that immune cell to the source of that chemokine (Hughes & Nibbs, 2018). For example, CCL11, found to be ubiquitously upregulated in the amygdala of EAE animals in this study, binds preferentially to CCR3, which is expressed primarily on eosinophils but also to a lesser degree on neutrophils, facilitating the migration of these immune cells (Heath et al., 1997). Although we did not find a statistically significant increase in infiltrating immune cells within the CeA, this was likely driven by a low sample size in our CFA control group, and it is likely that there are low levels of infiltrating immune cells within the CeA in EAE animals at disease onset. Potential infiltrating immune cells are discussed below in §3.4.6.

Chemokine function has been explored for many decades, and while promotion of chemotaxis is the most well-defined function of chemokines, there are a plethora of alternative functions that chemokines can have on immune cells (López-Cotarel et al., 2017). Additionally, chemokines can play a role in neuromodulation and vascular remodelling via non-immune cell mediated effects (Salvador et al., 2021; Schober A, 2008). For example, CXCR3, the primary receptor for interferon-induced chemokines, CXCL9 and CXCL10, which were found to be upregulated in this study, has also been shown to be upregulated on neurons in the DRG and spinal cord following spinal nerve ligation in male subjects (Jiang et al., 2017; Kong et al., 2021). Both studies show that CXCL10-CXCR3 binding increases the firing rate of neurons that express the receptor, contributing to the neuropathic pain elicited by the nerve ligation,

presenting the possibility that CXCL10 in the CeA in EAE may be directly acting on neurons. Conversely, in the hippocampus of viral infected male mice, endothelial-and epithelial-derived CXCL10 acts on neurons to inhibit long term potentiation, suggesting that the effects of CXCL10 may be region specific and can induce either increased or decreased neuronal activity depending on location (Blank et al., 2016). As no studies have assessed the role of CXCL10 in the CeA, the exact function of CXCL10 on neurons in this region, if there are any, is unknown. It is also worth noting that CXCR3 is an X-linked gene that escapes X-inactivation, adding further intrigue into this pathway in our current experiment evaluating sex differences. As the scope of our study did not include receptors, the precise function of dysregulated chemokines remains an interesting yet unelucidated question.

Although the majority of dysregulated cytokines were chemokines, there were some consistently dysregulated ligands that were not. VEGF was found to be downregulated in our comparison of all CFA and EAE animals, but this effect also seems to be mediated by gonadal females as we only saw significant downregulation of VEGF in XXF and XYF EAE animals compared to CFA controls, but not XXM or XYM. Interestingly, in 2018, Rothammer et al. found that knockdown of microglial VEGF was able to ameliorate EAE disease severity. The authors also showed in culture that microglial VEGF induced a greater pro-inflammatory response from astrocytes and resulted in greater toxicity towards neurons and oligodendrocytes, suggesting that VEGF may be an important mediator of microglial-astrocyte communication facilitating EAE disease severity. The amygdala has no known role in EAE disease onset, and we did not find VEGF downregulation within our somatosensory punches, suggesting that this is not a pan-CNS response. Aside from cell-to-cell communication, VEGF also plays an important role in angiogenesis (Melincovici et al., 2018). No studies to date have investigated the BBB in the amygdala in EAE; however, two studies have investigated the BBB permeability in the amygdala in other conditions. Xu et al. (2019) found that in male Sprague Dawley rats, chronic restraint stress induced enhanced blood brain barrier permeability within the amygdala. Interestingly, Vore et al. (2022) investigated the effect of adolescent intermittent ethanol exposure in male and female Sprague Dawley rats and found that females did not exhibit increased BBB permeability, while males did, although not in the amygdala, suggesting region, sex, and condition specificity to this effect. These studies suggest that specific pathological conditions can induce enhanced BBB permeability in the amygdala in a sex-dependent way, implicating a

potential link to explain our VEGF findings, as decreased VEGF in female EAE animals may facilitate enhanced blood brain barrier permeability which then augments the infiltration of inflammatory mediators. This is further addressed in §3.4.5 and §3.4.6.

3.4.2. Female phenotypes and genotypes are associated with greater inflammation within the amygdala

A major finding of our current study is that within the amygdala there is greater dysregulation of cytokines in EAE mice with either XX chromosome complement and/or ovaries compared to EAE mice with XY chromosome complement or testes. This is consistent with the DRG in EAE at disease onset, where it has previously been shown that female mice have significantly greater transcriptional dysregulation of the immune system in EAE than male mice (Friedman et al., 2019).

This exaggerated immune dysregulation in EAE females is, however, not universal within the CNS. In the spinal cord of EAE mice at disease onset, Catuneanu et al. (2019) showed no sex differences in immune cell infiltration or microglial morphology in the dorsal horn of the spinal cord; instead, astrogliosis and axonal damage seemed to be increased more in male spinal cords. Although the authors of that study found no sex difference in demyelination within the dorsal horn, Wiedrick et al. (2021) found significantly more demyelination in the lumbar spinal cord of female EAE mice than males at 20 days post-induction. Wiedrick et al. further elaborated on their spinal cord analyses with a comprehensive chemokine/receptor RT-PCR experiment wherein they found that within EAE, greater dysregulation occurred in males than in females. Interestingly, the authors evaluated naïve chemokine levels in addition to CFA controls and EAE animals and found that at a baseline, female mice had ~25% greater chemokine expression compared to males. Chemokine dysregulation in the CFA group compared to the naïve baselines revealed that the males had stronger response to CFA, reacting 50% more to CFA than females. When comparing CFA to EAE mice then, it appears they have relatively similar disease changes, which masks the differences in change from naïve baselines. This has implications for our current study as the implicit assumption was that the male and female responses to CFA were comparable, which may not be true, particularly given our behavioural results suggesting that the gonadal females may have an exaggerated immune response (**Fig. 1B,E**; **Fig. S2**). As such, there was the

possibility that our Luminex data shows greater dysregulation in mice with XX chromosomes and gonadal females in the EAE group compared to the CFA group because the CFA and EAE groups of XY and gonadal males were both already elevated compared to what would be naïve conditions. To address this, we compared the concentrations of cytokines from our Luminex in the CFA control groups. **Fig. S8** shows the nine cytokine comparisons of the CFA FCG mice for the Luminex data where we found a significant effect. Of the 45 cytokines analyzed in our CFA animals, CCL4 was higher in gonadal females, IL9 and CXCL10 were higher in XY animals, and 6 other ligands had a significant interaction effect (CCL11, CCL21, CCL22, IL-10, IL-11, & EPO). Of these, CCL11, CCL22, and EPO had the highest expression in the XYF genotype, ameliorating concern that these cytokines might be being masked by a high CFA baseline in the male mice. Only IL-10 had a significantly greater expression in the XYM CFA mice, but we did not observe dysregulation of IL-10 in any specific genotype, so while it is possible that IL-10 may have some masked sex effects, they are likely to be minor. This data suggests that the baseline sex differences of our CFA controls likely do not contribute to the sex effects that we see when comparing EAE response in each genotype. Further studies should be performed to assess the naïve inflammatory expression within the amygdala of FCG mice to evaluate whether naïve baseline differences play a significant role in disease state inflammation.

Altogether, the results from our Luminex multiplex immunoassay indicate that in EAE there is significant dysregulation within the amygdala and that animals with XX chromosome complement or ovaries experience greater dysregulation in cytokine protein expression than animals with XY chromosomes or testes. This provides evidence to support our hypothesis that the female inflammatory environment within the CeA is more dysregulated in EAE.

3.4.3. Microglia largely do not exhibit sex differences in the CeA in EAE

Given the known roles of microglia in cytokine production and the sex differences observed in Dworsky-Fried et al. (2022) following direct LPS administration to the CeA, we asked if microglia were the primary facilitators of our observed sex differences. Changes in soma characteristics are an established method of assessing microglial reactivity following induction of chronic pain (Taylor et al., 2017). Microglial changes within the CeA have been shown in conditions of stress-induced visceral pain (Yuan et al., 2021;

Yuan et al., 2022) and EAE (Dworsky-Fried et al., 2022). In EAE, microglial cell body size has been shown to change similarly between males and females, but only males see a significant change in the number of cell branches (Dworsky-Fried et al., 2022). To try to further expand on this work, we utilized a Cx3cr1^{CreER};Ai9 mouse model where CX3CR1+ cells constitutively expressed TdTomato, allowing for the visualization of microglia within the CNS at a high resolution. By selectively providing tamoxifen at days 14-16, we also avoid labelling infiltrating macrophages (Plemel et al., 2020). In terms of response to EAE, we found very few differences in microglial morphological responses between females and males. Our findings largely recapitulate the work of Dworsky-Fried et al. (2022) wherein soma changes are sex-independent, but we do find a difference in one characteristic of microglial processes: the number of bifurcations. Akin to the findings of the previous study, the number of bifurcations decreased in male microglia in EAE, but not in females. This may signify a modest functional difference between male and female microglia within the CeA. As the majority of our measurements had no sex differences though, the microglial response within the CeA between males and females in response to EAE may not be substantially different.

To further characterize the microglial response to EAE, we quantified microglial cell counts using both IHC and RNAscope. In both assays, we did not observe a significant difference in total microglial cell counts within the CeA, nor did we observe an effect of sex. Although no studies have assessed the effect of chronic pain on CeA microglia proliferation, microglia have been shown to proliferate in various pain models within the spinal cord (Jiang et al., 2022a; Tsujikawa et al., 2023), basolateral amygdala (Jiang et al., 2022b), and somatosensory cortex (Wang et al., 2019). In EAE, microglia have been shown to proliferate in the spinal cord (Hammer et al., 2015), although no studies have reported on microglial proliferation in EAE within the brain, indicating that our results within the CeA are novel. Our results suggest that proliferation is not a feature of the microglial response to EAE in the CeA. Together with our morphological analyses, it appears that the microglial response to EAE in the CeA may not be the primary driver for sex differences in the inflammatory environment. However, given the highly complex functions of microglia, the physical features may not be representative of the role that these cells play in inflammation. To assess the inflammatory functions of microglia in the CeA more directly, we utilized RNAscope to

evaluate the expression of pro-inflammatory cytokines and sexually dimorphic chemokines within microglia.

3.4.4. Microglia upregulate mRNA expression of pro-inflammatory cytokines within the CeA without sex effect in EAE

Within the CNS, microglia have been shown to produce several pro-inflammatory cytokines, such as IL1β and TNFα (Smith et al., 2012). Although we did not find upregulation of IL1β in the amygdala, consistent with the findings of Chanaday & Roth (2016) in the frontal cortex of rats at EAE onset, we asked if, at the transcriptional level, microglia were producing different levels of these pro-inflammatory cytokines. Consistent with our Luminex results, we did not find an overall upregulation of *Il1β*+ cells, though we did find an upregulation of *Il1β*+*Itgam*+ cells, suggesting that CeA microglia are upregulating IL1β, albeit modestly. The lack of change in our Luminex in IL1β may be explained by the heavy processing of IL1β that occurs prior to being a functional mediator of communication; we may see an increase in IL1β mRNA, but the molecule isn't being translated and processed to its final form. Alternatively, excess IL1β protein may be cleared extremely quickly to bring the expression back to CFA control levels. Regardless, it appears that in EAE, microglia within the CeA upregulate the expression of *Il1β*. However, our findings suggest that this is independent of sex, as we do not find differences between our males and females. This is similar to our findings in TNFα.

Although we were unable to detect TNFα in our Luminex, TNFα mRNA was readily detectable in our RNAscope assay. We saw a significant upregulation of *Tnfα*+ cells in the CeA in EAE, the majority of which being microglia. The discrepancy between our Luminex and RNAscope experiments likely speaks to a failure in our ability to appropriately measure TNFα concentration given our inability to measure it in both the CFA and EAE groups, though further studies should be performed to verify this speculation. The high, but not total, colocalization of *Tnfα* with microglia is consistent with previous reports of microglia being the primary producers of TNFα in the CNS (Gonzalez Caldito N, 2023). As we found with *Il1β*, the changes in mRNA expression of TNFα did not differ between males and females, suggesting that the pro-inflammatory cytokines produced by microglia may not be dependent on sex. As we did not evaluate post-

transcriptional differences, there may be sex-dependent changes in protein expression that we are unable to assess, although given our lack of sex effects in our Luminex assay, this is unlikely for these classical pro-inflammatory cytokines. On the other hand, several cytokines from our Luminex were found to be upregulated in either only gonadal females or animals with XX sex chromosome complement. As such, we asked if these cytokines were being produced by microglia in a sex dependent way.

3.4.5. Astrocytes potentially mediate the sex-dependent differences in inflammatory milieu of the CeA in EAE

We selected two chemokines to investigate: CXCL1, which was upregulated in the amygdala of animals with XX sex chromosome complement but not XY, and CXCL10, which was upregulated in the amygdala of XXF, XXM, and XYF mice, though to a greater extent in gonadal females. Both of these cytokines have been implicated in pain (Aloyouny et al., 2020; Silva et al., 2017) and in EAE (Dhaiban et al., 2020; Grist et al., 2018), although only CXCL1 has been studied for its role in pain in EAE (Zhang et al., 2019a). Despite known differences in pain and MS, the majority of works to date have neglected to evaluate the influence of sex on these cytokines and their functions. Following intraperitoneal injection of LPS, an endotoxin that activates TLR4, into CD1 mice, females display a greater upregulation of both CXCL1 and CXCL10 within the liver than males do, showing that these cytokines are regulated differently between sexes (Dunstan et al., 2024). Further, sex effects of CXCL1 in pain have been demonstrated in the DRG using a murine model of chemotherapy-induced peripheral neuropathy (Luo et al., 2019). This study found that TLR9 on macrophages was important in the expression of CXCL1 in response to paclitaxel treatment in males, but not in females. The canonical receptor of CXCL1, CXCR2, has also been shown to be influenced in sex dependent ways. Using a model of low back pain caused by intervertebral disc degeneration, Lee et al. (2022) found that female mice that were allowed to run had a significant upregulation of CXCR2, an effect not seen in males. CXCL10, on the other hand, has largely been unexplored for potential sex differences, although its receptor, CXCR3 is X-linked and can escape X-inactivation, particularly in T cells, resulting in greater expression of this receptor in female T cells (Oghumu et al., 2019). Neither of these chemokines have been extensively studied within the amygdala, however.

The only studies to date that investigate CXCL1 or CXCL10 in the amygdala have looked at chronic stress or alcohol withdrawal. While investigating stress-induced depression-like behaviour in mice, Li et al. (2018) found a significant upregulation of CXCL1 in the amygdala of male mice. Following cessation of chronic ethanol consumption in male C57Bl/6J mice, McCarthy et al. (2018) found a downregulation of *Cxcl10* in the amygdala, though they did not report protein concentration. Neither study investigated the effect in female mice, nor investigated pain, presenting a clear deficit in the literature.

We first quantified the total *Cxcl1*+ cells and found, independent of sex, an upregulation of cells in EAE within the CeA. However, in contrast to what we found in our Luminex, when we assessed the total *Cxcl10*+ cells we did not observe an upregulation in EAE in either sex. This implicates a role in post-transcriptional functions that could facilitate the sex differences seen in our Luminex, but also potentially of the pro-inflammatory cytokines that we studied. Cytokine mRNA is cleared relatively quickly due to an AU sequence near the 3' end, but the speed of clearance still varies, and this may contribute to our observed differences (Shaw & Kamen, 1986). It has previously been shown in mouse cultured fibroblasts and bone-derived macrophages that the stability of cytokine mRNA, including *Tnf*, *Cxcl1*, and *Cxcl10*, have different half-lives, which could explain our confounding findings depending on the exact timeline by which cytokine dysregulation is occurring (Hao & Baltimore, 2009). This has also not been studied for sex differences, so this may be a sex-dependent process. Alternatively, the differences that we are observing in our Luminex data may be driven at the translational level. The lack of clear chromosomal or hormonal effects within our Luminex suggests an interaction between multiple pathways that warrants further investigation to elucidate the contribution of each sex-related pathway to dysregulation of the immune system. For example, the sex chromosome-encoded RNA helicases DDX3X, which escapes X chromosome-inactivation, and its Y-chromosome correlate, DDX3Y, have recently been shown to be sexually dimorphic in their ability to repress translation, wherein cultured cells with DDX3Y and DDX3X, as would be found in males, have greater repression of translation than cells with two copies of the DDX3X gene (Shen et al., 2022). Loss of DDX3X in mice preferentially reduces circulating lymphoid cells *in vivo*, and *in vitro* reduction of DDX3X in macrophages reduces the RNA upregulation of various cytokines such as IL-1, IL-6, and CXCL10 in the presence of microbial insult (Szappanos et al., 2018). DDX3X has also been shown to mediate microglial inflammatory responses to LPS and spinal cord injury

in female mice (Wang et al., 2022) and this effect has been replicated in cultured astrocytes where DDX3X expression was increased alongside GFAP in response to P2Y1R ligand binding, though the authors did not disclose the sex of the cell line (Alam et al., 2023). Together, this evidence places DDX3 genes in prime position to be a potential mediator for the sex differences in neuroinflammation within the CeA that we observed.

Following our assessment of total *Cxcl1* and *Cxcl10* positive cells, we investigated what cells were producing these cytokines. We found very low colocalization of both cytokines with *Itgam*, our microglial marker, in either CFA or EAE condition and no effect of disease. Instead, we found a significant upregulation of *Cxcl1+Gfap+* cells in EAE and better colocalization of *Cxcl10+* cells with *Gfap+* cells in both CFA and EAE, suggesting that astrocytes upregulate expression of *Cxcl1* in the CeA in EAE and more astrocytes express *Cxcl10* than microglia. An upregulation of astrocyte-mediated inflammation in EAE within the CeA would be consistent with other works that have shown astrocyte upregulation of immune genes in the spinal cord (Itoh et al., 2017). Although we still do not find sex differences in these experiments, our finding that astrocytes seem to be more important in both of these sex-dependent cytokines indicates that astrocytes may underlie the sex differences in inflammation from our Luminex. This argument is further supported by our finding that *Gfap+* astrocytes are only upregulated in EAE of XXF mice in the CeA, but not XYM mice. Together, this data suggests that reactive astrogliosis in EAE is greater within the CeA of female mice, which may then facilitate a greater inflammatory response.

Astrocyte involvement in MS and EAE pathogenesis is well established (Kunkl et al., 2022; Yi et al., 2019), as is their involvement in chronic pain conditions (Ji et al., 2019; Lu et al., 2022). Several studies have assessed the roles of spinal astrocytes on neuropathic pain during the early stage of EAE and found increased astrocyte reactivity at EAE disease onset (Ding et al., 2022; Dutra et al., 2013; Segal et al., 2020). To directly investigate the role of spinal astrocytes in EAE-induced pain, Ding et al. (2022) performed intrathecal injections of the astrocytotoxin, L-α-aminoadipate, to inhibit astrocyte reactivity during the presymptomatic stage of the disease when EAE animals were demonstrating mechanical hypersensitivity. The authors found that inhibition of astrocyte reactivity facilitated a recovery of nociceptive thresholds, highlighting the importance of astrocytes in EAE-induced pain. Interestingly, the authors also cleared microglia from the spinal cord using PLX and found no change in nociceptive

thresholds. However, all mice used in this experiment were female; given the current dogma of the importance of microglia in the facilitation of pain in male mice, experiments such as these may be ignoring sex differences in the involvement of astrocytes in EAE-induced pain.

To that end, sex differences in astrocyte response to EAE have been presented previously. Spence et al. (2011) demonstrated that conditional knockout of estrogen receptor from astrocytes, but not neurons, diminished the protective effects of estrogen on EAE disease severity, underscoring the importance of sex hormones in astrocyte function. Another study from the Voskuhl Lab revealed sex differences in the astrocytic transcriptomic response to EAE within the optic nerve (Tassoni et al., 2019). At early and chronic stages of the EAE disease course, the authors show significant upregulation of GFAP within the optic nerve and this upregulation is associated with an increase in complement component proteins, namely C3, a complement protein associated with neurotoxic astrocytes. However, this effect was only seen in females; male astrocytes of the optic nerve had significantly greater upregulation of THBS1, a neuroprotective factor. In line with these findings, only female mice had a significant reduction of axonal density within the optic nerve and females also experienced significantly greater retinal ganglion cell loss than males. Clearly there are sex-dependent mechanisms in the astrocytic response to EAE, though the full extent of these differences has yet to be revealed.

The function of astrocytes within the CeA has not been fully elucidated, and the studies that have been performed do not adequately address the inflammatory role of astrocytes in chronic pain in both sexes. Using a spared nerve injury model, Sagalajev et al. (2018) attempted to show the function of unilateral (right) CeA astrocytes by specific inhibition using either gap-junction decouplers or D-amino acid oxidase inhibitors. They found no effect on mechanical hypersensitivity of administering either drug to the CeA, although they also note that both drugs have several off-target effects and only male animals were used. Similarly, Wahis et al. (2021) showed that bilateral activation of oxytocin receptor-expressing astrocytes within the CeA of male and female C57Bl/6J mice induced an increase in inhibitory post-synaptic currents in CeA neurons, although they found no effect on mechanical hypersensitivity within either their spared nerve injury cohort or their controls. These findings suggest that astrocytes in the CeA are mediating other effects independent of pain-signalling. However, it should be noted that exogenous activation of astrocytes does not replicate physiological conditions and the contribution of astrocytes to

the inflammatory environment within chronic pain states, including MS and EAE, is more complex than simply activated or not activated.

While an effect on pain or nociception has not been found, astrocyte activity within the CeA has been found to influence fear and anxiety. Martin-Fernandez et al. (2017) bilaterally activated CeA astrocytes of male C57Bl/6J mice using Gq-DREADDs and found an increase in inhibitory signalling from neurons. This increased inhibitory drive of the CeA was associated with decreased fear responses, but no alteration of anxiety behaviour as measured by the elevated plus maze. These findings were largely recapitulated by Wiktorowska et al. in 2021, where the authors found that ablating the glucocorticoid receptor from CeA astrocytes of C57Bl/6N mice reduced fear responses and decreased anxiety as measured by open field. However, it should be noted that the authors did not report the sex of the mice used in these experiments. As chronic pain models, including EAE (Majidi-Zolbanin et al., 2015; Trifunovic et al., 2021), have been shown to influence anxiety and stress responses in mice, astrocytes within the CeA may be more involved in the multifaceted cognitive and emotional aspects of pain than in the sensory or nociceptive aspects.

There are several caveats to this component of the current project. First, GFAP has been shown to be poorly expressed in subcortical grey matter (Zhang et al., 2019b). As such, we are likely not staining all astrocytes within the CeA in our analysis and therefore cannot discern whether there is pan-reactivity of astrocytes in the CeA of EAE females or whether it is a specific subset of astrocytes that express GFAP. Given the reported difficulty in labelling astrocytes within this region, using multiple probes or genetic lines may be warranted to get a more ubiquitous assessment of astrocytes in this region (Koh et al., 2017; Marowsky et al., 2009). Another limitation is that while several cytokines were dysregulated in the amygdala, we only assessed the colocalization of *Cxcl1* and *Cxcl10* with *Gfap*+ astrocytes and did not characterize the origins of the majority of dysregulated cytokines; this could potentially be addressed by comprehensive single cell RNAseq to detect which cells display an upregulation of each cytokine in the CeA of EAE mice. Similarly, greater colocalization of cytokine mRNA with astrocytes does not equate to more release from astrocytes, so it is possible that the astrocytes may not be upregulating the release of the protein products of these mRNA. Lastly, as the experiments investigating astrocytes were only performed in FCG tissue, there is the possibility that this effect is not observed in WT animals. Although

we have found largely consistent results between the two mouse strains, this experiment should be reproduced in WT animals to ensure that there are no differences between strains.

This project provides a brief glimpse into the potential role of astrocytes in the inflammatory environment of the CeA in EAE, but we still do not understand the role that these cells are performing. In EAE, astrocytes can serve both beneficial (Spence et al., 2011; Voskuhl et al., 2009) or deleterious effects (Wheeler et al., 2020). Therefore, it is important to recognize that although we have observed some hints of astrocyte involvement in immune sex differences, more work must be done to determine the actual contribution of astrocytes to the inflammatory environment of the CeA in EAE. This could be addressed via conditional knockouts in the CeA of female and male EAE animals to determine the influence these cells have at the behavioural level. Given the reported sequential influence of microglia on astrocytes in males (Li et al., 2022; Liddelow et al., 2017; Villarreal et al., 2021), it is possible that microglia facilitate an initial immune response via release of inflammatory mediators such as TNFα which then induce a sex-specific effect on astrocytes within the CeA. This sex difference in astrocytes could result from differential expression of either TNF receptors which then mediate different downstream pathways. Astrocytes have been shown to express both TNF receptor types (Yan et al., 2003), but sex differences in astrocyte TNF response have not been assessed. Neurons within the DRG expressing type 1 TNF receptors display an inherent sex bias in signalling pathways where TNFα signalling induces P38 activation whereas in females it preferentially induces the NF-kB pathway, which may mimic the sex diergism in astrocytic response to TNFα (Maguire et al., 2022). However, as we did not perform manipulation experiments of these pathways, it is also possible that astrocyte reactivity in the CeA in EAE is independent of microglial signalling, necessitating further experimentation.

In addition to cytokine production, another primary known function of astrocytes is the maintenance of the BBB (Daneman & Prat, 2015). In 2019, Haruwaka et al. demonstrated that prolonged inflammatory conditions in male mice resulted in increased microglial phagocytosis of astrocytic end-feet. It is possible that males and females in EAE have different degrees of increased BBB permeability due to pathological astrocytic responses. Although we did not find a sex difference in infiltrating immune cells into the CeA in EAE, it is important to note that we have a low sample size in this analysis, limiting our ability to evaluate this. It is also possible that at this early disease timepoint that immune cells are

influencing the CeA parenchyma environment via extravasation of exuded cytokines from within the lumen of blood vessels into the parenchyma without themselves infiltrating. Additionally, as we also only looked at the onset timepoint, we may be observing the beginnings of blood brain barrier degradation, which may be exaggerated in females due to a greater degree of astrogliosis. This could be addressed by a FIT-C or Evans Blue assay to assess blood brain permeability at the time of disease onset. It appears that astrocytes may be major participants in the neuroimmune response within the CeA in EAE, though further research will be needed to determine the precise roles that astrocytes are fulfilling.

3.4.6. There are minimal infiltrating immune cells in the CeA at EAE disease onset

As the aim of this project was to investigate the inflammatory environment of the amygdala in EAE, we assessed whether peripheral immune cells were infiltrating into the CeA and found some suggestion that this was occurring at low levels. EAE has been shown to promote the infiltration of immune cells, particularly CD4+ T cells, into the parenchyma of the spinal cord of both males and females (Mifflin et al., 2017; Nacka-Aleksic et al., 2015). Several of the chemokines we found to be upregulated in our amygdala punches have been shown to be able to facilitate migration of CD4+ cells (Blauth et al., 2015; Khaw et al., 2021; Park et al., 2014; Rapp et al., 2019; Zhang et al., 2018). However, we were unable to find any CD4+ cells within the parenchyma of EAE brains within any region, indicating that the infiltrating immune cells are not T helper cells. Additionally, we did not find upregulation of cytokines known to be released by CD4+ T cells such as IFNγ, IL-2, IL-4, IL-5, IL-10, or IL-13 (Romagnani S, 1999). Lastly, we received further evidence of this with our assessment of IL17 mRNA, an interleukin known to be produced by CD4+ Th17 cells, wherein we did not find any *Il17*+ cells in any region analyzed. These results do not preclude, however, the possibility that other lymphoid cells such as CD8+ T cells or B cells are infiltrating, although these immune cells have been shown to infiltrate into the spinal cord later in the disease than CD4+ cells do, suggesting it is unlikely these cells are infiltrating at this early timepoint (Barthelmes et al., 2016).

Although we did not confirm which immune cells were infiltrating, we can speculate based on previous data and which cytokines were upregulated. There are several potential infiltrating immune cells, including mast cells, dendritic cells, monocytes, eosinophils, and neutrophils. Much of the research to

date around mast cells in EAE have pertained to the relationship of resident mast cells in the meninges, where mast cells regulate T cell activity and infiltration (Russi et al., 2016; Sayed et al., 2010). While mast cells seem to infiltrate into the brain parenchyma of patients with MS at perivascular locations and within active lesions, no studies have reported infiltrating mast cells in the brain of EAE animals, making mast cells an unlikely candidate for infiltration of the CeA or other subcortical structures (Russi et al., 2016). Dendritic cells are antigen presenting cells important for the activation of T cells in EAE to elicit demyelination. Using EAE in female CD11c-eYFP mice, Clarkson et al. (2014) found a great concentration of dendritic cells populating the area around the ventricles, but very few dendritic cells were found within subcortical regions, indicating that dendritic cells are also unlikely to be infiltrating into the CeA.

As only CD45+/IBA-1- cells were counted to determine infiltrating immune cells, infiltrating macrophages would not have been counted in our assay due to their expression of IBA-1 (**Fig. 9A**). However, it is possible that monocytes are infiltrating. Within the spinal cord of female C57Bl/6J mice, Wang et al. (2021) showed that at peak disease (15 days post-induction), monocyte and macrophage cells infiltrated into the spinal cord to an even greater extent than CD4+ cells. Further, monocyte infiltration into the spinal cord has been shown to be important in EAE disease progression, highlighting that not only does monocyte infiltration occur in EAE, but that it is important for behavioural phenotypes (Ajami et al., 2011). Within the brain, CCR2+ monocytes have been shown to infiltrate at EAE disease peak (Saederup et al., 2010), although this may not be representative of the degree of infiltration at disease onset based on the findings of Ajami et al. (2011) which saw zero monocyte infiltration into the spinal cord at onset. Additionally, monocyte recruitment into the CNS is highly linked to CCL2-CCR2 signalling (Mahad & Ransohoff, 2003). Given that we found CCL2 upregulation in XXM EAE mice only, there does not appear to be a ubiquitous upregulation of the major chemokine signalling pathway to attract monocytes, decreasing the likelihood that monocytes are infiltrating into the CeA at disease onset.

Based on the dysregulated cytokines that we observed in our FCG mice in EAE, two cell types are most likely infiltrating into the amygdala at disease onset. Given the known role of CCL11 in eosinophil migration and the universal upregulation we found in our Luminex, the infiltrating immune cells could be eosinophils. In female Wistar rats, cells expressing CCR3, a marker for eosinophils, were found

to be upregulated in the hippocampal fimbria of EAE animals compared to controls during the acute stage of the disease, showcasing that infiltration of eosinophils does occur in the brain of EAE animals (Shou et al., 2019). As we found that CCL11 was the only upregulated cytokine in all four genotypes and we did not see a sex difference in amount of infiltrating immune cells in EAE, this leaves eosinophils as the most likely candidate of infiltrating immune cells. The other most likely candidate cell type are neutrophils. Neutrophils are known to produce many of the chemokines that we found upregulated in our study like CXCL1 and CXCL10, signifying a potential contributor to the inflammatory soup that we observe in the EAE amygdala (Scapini et al., 2000). Neutrophils have also been found within the brain following disease onset in C57Bl/6J mice (Jiang et al., 2016). ELR-positive chemokines, such as CXCL1 and CXCL2, specifically induce migration of neutrophils; our analyses of all animals showed upregulation of CXCL1 and downregulation of CXCL2. Comparing different genotypes, the CXCL1 upregulation was only seen in animals with XX chromosome complement, suggesting that the degree of infiltration of neutrophils may be different between female and male mice, which we do not see in our assay. Another piece of evidence is that Simmons et al. (2014) showed that IL-17 promoted and IFNγ reduced neutrophil recruitment into the brain of EAE mice 1-3 days post-onset. Given that we were largely unable to find upregulated IL-17 in EAE brains, this decreases the likelihood that neutrophils are the infiltrating immune cells.

Our data indicates that eosinophils or neutrophils are the most likely cell types that could be infiltrating into the amygdala at EAE onset, with monocytes as another potential cell type. It is worth noting that we did not check for infiltrating immune cells within the FCG brains. Given the few differences we found in the Luminex assays performed on the FCG and WT amygdala, and the observation that the differences present may be driven by neuronal-microglial communicators, it is unlikely, though not impossible, that there are different immune cells infiltrating between strains. Depending on which immune cells are infiltrating at this early disease point could play an important role in the inflammatory environment within the amygdala that may relate to pain. Moving forward this could be addressed by single cell flow cytometry of amygdala tissue at disease onset to determine the presence of different cell types. Currently, we find evidence to suggest that infiltrating immune cells may be contributing to the inflammatory environment within the CeA at disease onset, though they likely play a minor role, implicating resident immunocompetent cells as the primary drivers of this effect.

Conclusion

This thesis presents data pertaining to the use of the FCG model to study EAE as well as the sex differences in the immune response within the amygdala in EAE. We demonstrate that FCG mice respond in a similar manner to WT mice during the presymptomatic period, both behaviourally and immunologically. Utilizing the FCG model to investigate sex differences in the immune system, we demonstrate that female mice display greater immune dysregulation than males do within the amygdala. To investigate the source of these sex differences, we perform morphological, density, and colocalization assays of microglia in the CeA of males and females and find that microglia display minimal sex differences and low colocalization with sexually differential cytokines. Instead, we present evidence to support that astrocytes may mediate these sex differences within the CeA. This work suggests that not only does the degree to which EAE produces an inflammatory state within the CeA differ based on biological sex, but that different cell populations may mediate this response in each sex. Consequently, chronic pain in men and women with MS may arise via different mechanisms and sex may be a critical consideration in the development of potential therapies for pain in MS.

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Appendix

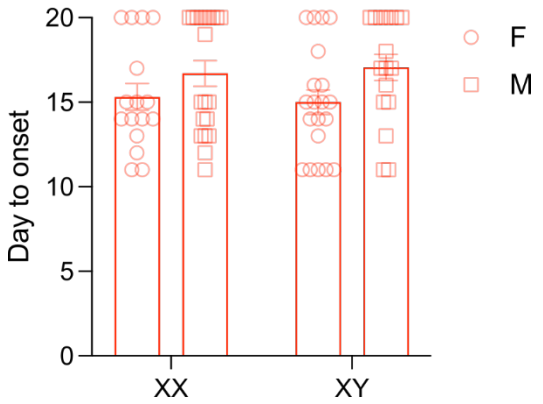

Fig. S1: Gonadal females reach disease onset earlier than gonadal males in EAE, regardless of chromosome complement.

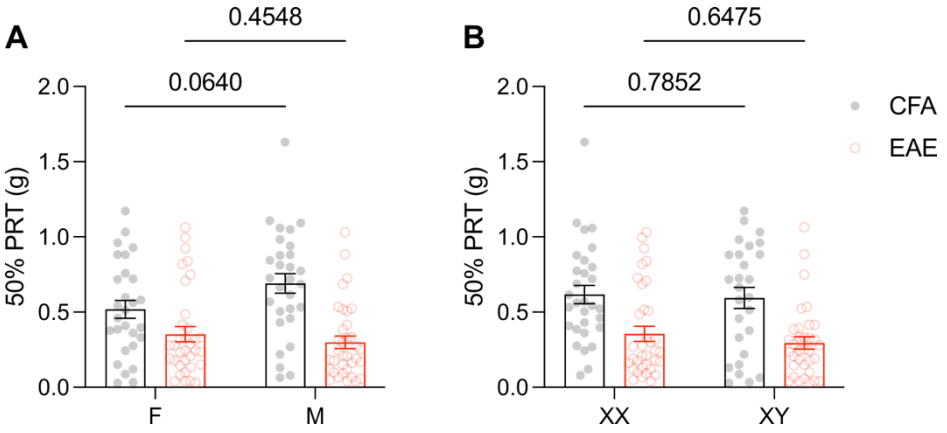

Fig. S2: Gonadal females have reduced nociceptive thresholds in response to CFA control injection. (A) At disease onset, gonadal females in the control CFA group have lower mechanical thresholds than gonadal males. No difference between EAE mechanical thresholds were found. **(B)** At disease onset, no difference between XX or XY mice was found in either CFA control or EAE conditions.

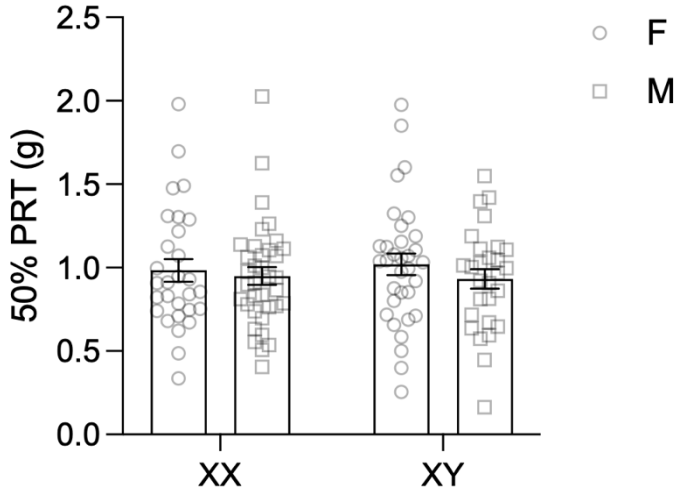

Fig. S3: Baseline mechanical nociceptive thresholds of FCG mice do not differ.

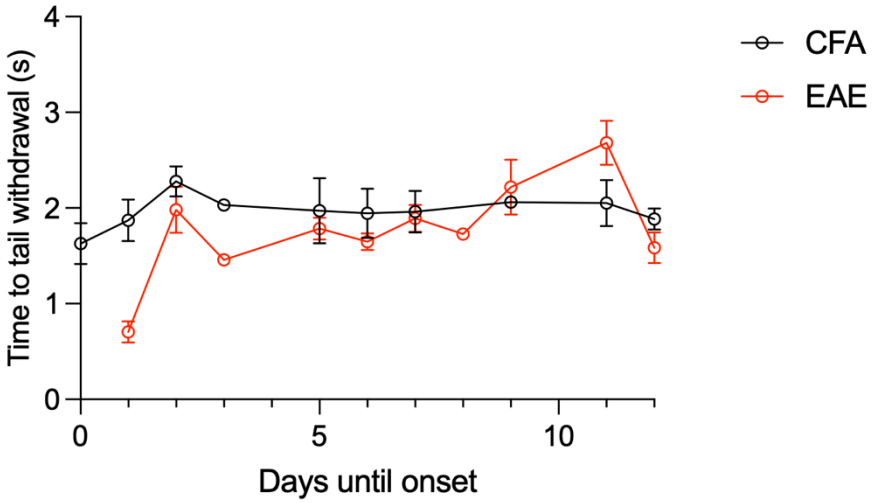

Fig. S4: Thermal nociceptive thresholds decline in FCG EAE mice 1 day prior to disease onset.

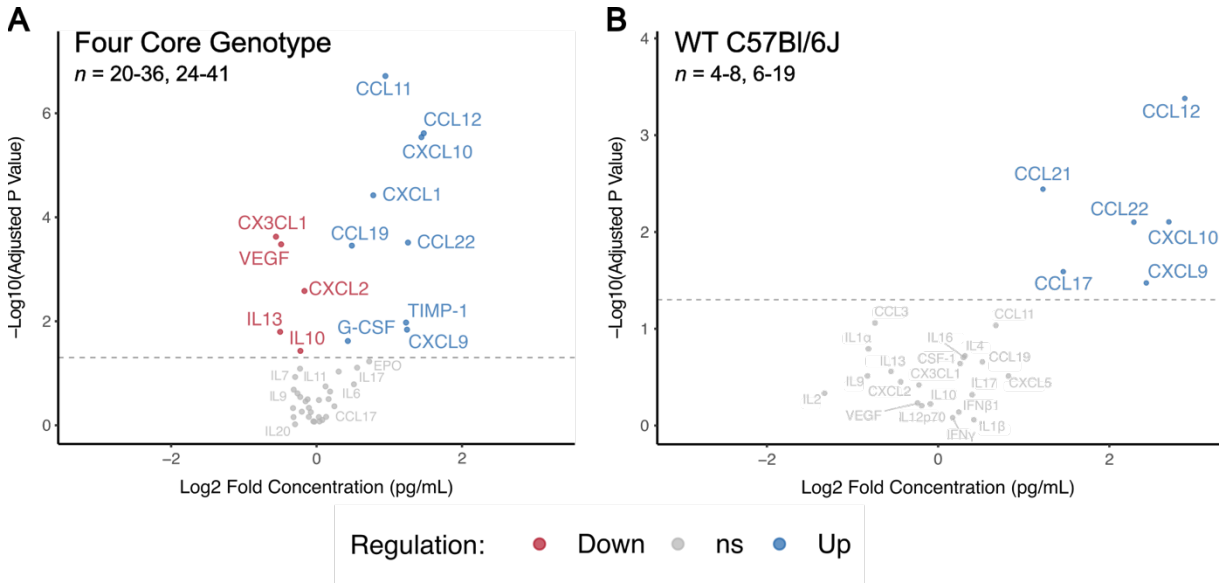

Fig. S5: EAE cytokine dysregulation is similar within the amygdala between FCG mice and WT C57Bl/6J mice. (A) Cytokine dysregulation within the amygdala of FCG mice in EAE compared to CFA controls. All sexes are pooled. **(B)** Cytokine dysregulation within the amygdala of WT C57Bl/6J mice. Sexes are pooled. Horizontal dotted lines on volcano plots represent the significance threshold set at $p < 0.05$.

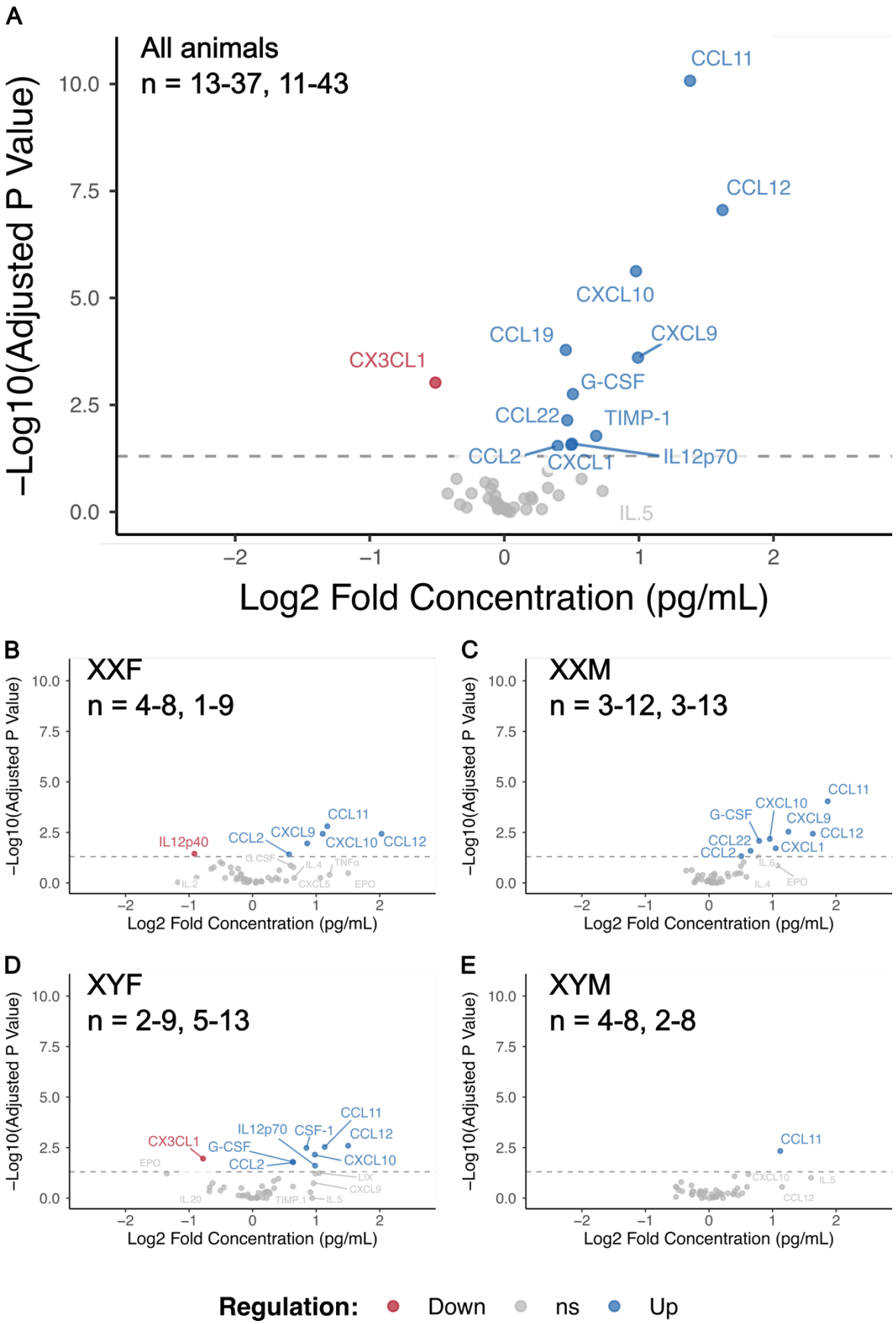
A
All animals
n = 13-37, 11-43
−Log10(Adjusted P Value)
Log2 Fold Concentration (pg/mL)
CCL11
CCL12
CXCL10
CCL19
CXCL9
CX3CL1
G-CSF
CCL22
TIMP-1
CCL2
CXCL1
IL12p70
IL.5
B
XXF
n = 4-8, 1-9
IL12p40
C
XXM
n = 3-12, 3-13
D
XYF
n = 2-9, 5-13
CSF-1
E
XYM
n = 4-8, 2-8
Regulation:
Down
ns
Up

Fig. S6: EAE induces significant cytokine dysregulation in the somatosensory cortex of FCG mice. **(A)** Dysregulated cytokines within the somatosensory cortex of all FCG mice in EAE compared to CFA controls. **(B-E)** XXM and XYF genotypes display greater cytokine dysregulation than the XXF genotype, which has greater dysregulation than XYM mice. Horizontal dotted lines on volcano plots represent the significance threshold set at $p < 0.05$.

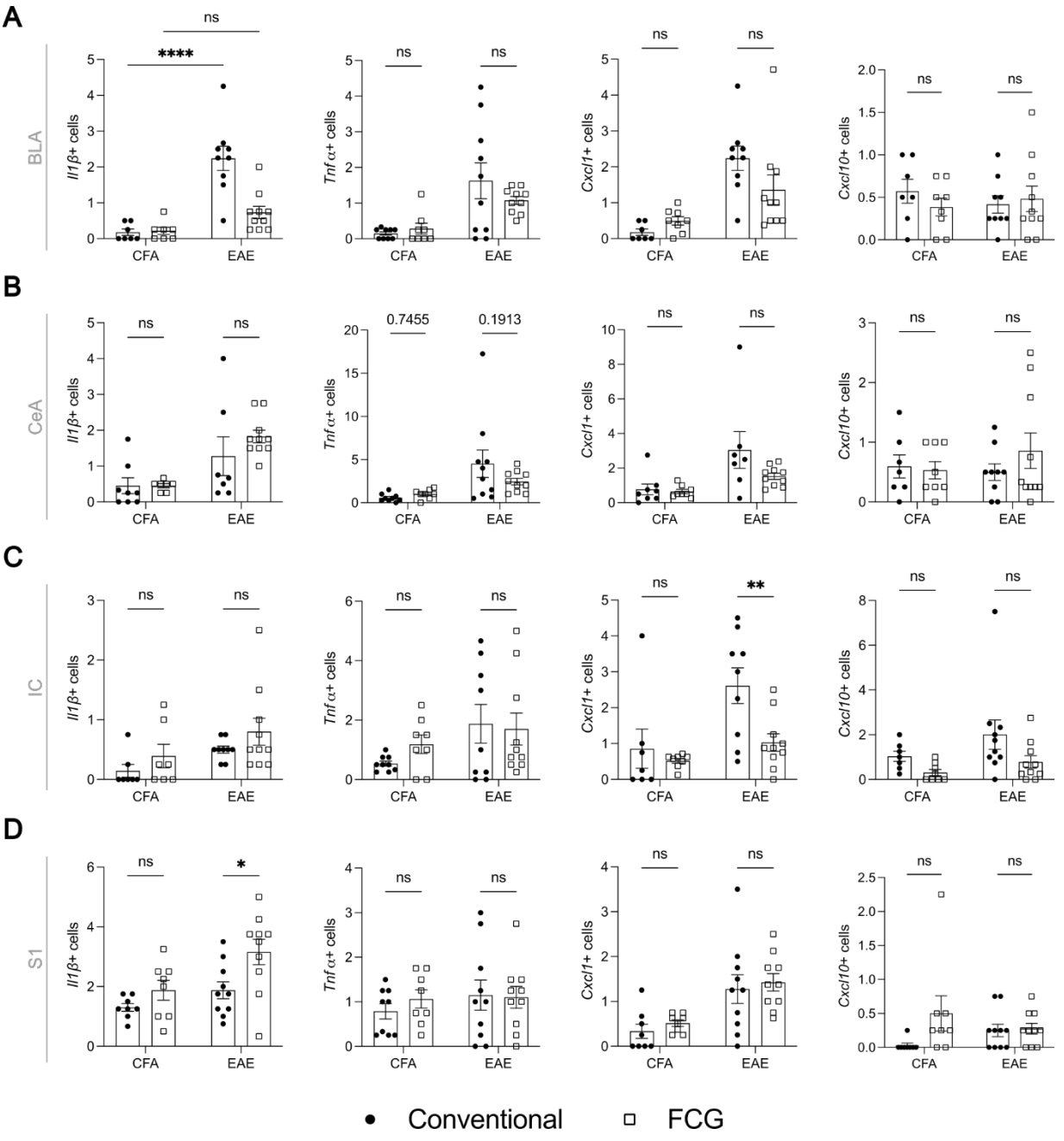

Fig. S7: The transcriptional profiles of FCG and WT mice display few differences in response to EAE. (A) Within the BLA, WT EAE mice upregulate *Il1β*+ cells compared to CFA controls, but this upregulation is not seen within the BLA of FCG mice. No other strain differences are observed in the BLA. **(B)** No strain differences are observed in the transcriptional profile within the CeA. **(C)** Within the IC, *Cxcl1*+ and *Cxcl10*+ cells are expressed in more cells in WT mice than in FCG mice. No differences were observed in *Il1β*+ or *Tnfα*+ cells within the IC between strains. **(D)** Within the somatosensory cortex, significantly more *Il1β*+ cells were found in FCG mice than in WT mice, regardless of disease. No other significant strain differences were observed in the somatosensory cortex.

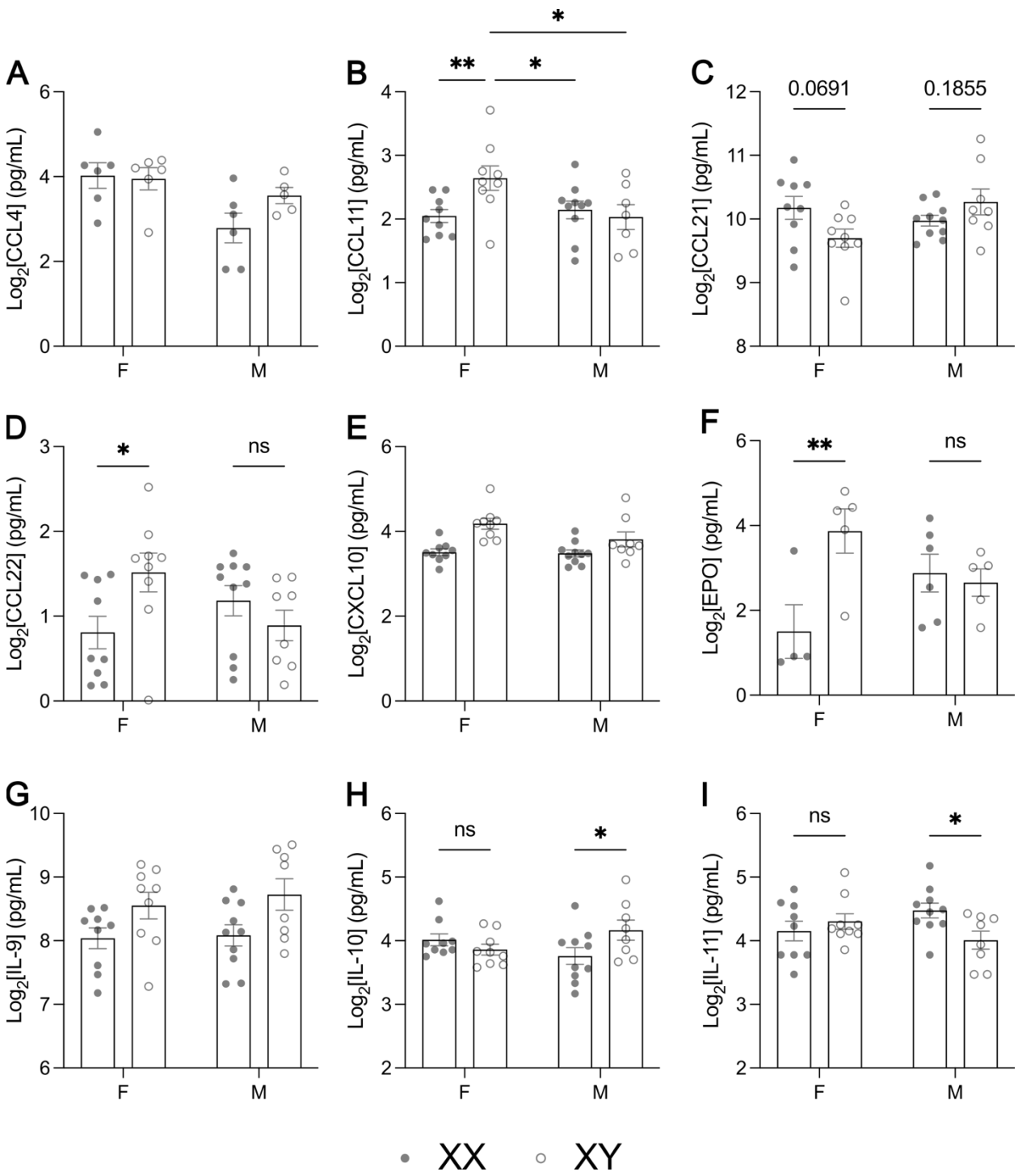
A
B
C
D
E
F
G
H
I
Log2[CCL4] (pg/mL)
Log2[CCL11] (pg/mL)
Log2[CCL21] (pg/mL)
Log2[CCL22] (pg/mL)
Log2[CXCL10] (pg/mL)
Log2[EPO] (pg/mL)
Log2[IL-9] (pg/mL)
Log2[IL-10] (pg/mL)
Log2[IL-11] (pg/mL)
F
M
*
**
ns
0.0691
0.1855
XX
XY

Fig. S8: Greater cytokine expression in CFA controls of males does not hide an inflammatory profile in the amygdala of EAE males. (A) Gonadal female controls express greater CCL4 than male controls in the amygdala. **(B)** XYF controls have greater CCL11 expression in the amygdala than other genotypes. **(C)** XYF controls have lower CCL21 expression in the amygdala than other genotypes. **(D)** XYF controls have higher expression of CCL22 in the amygdala than other genotypes. **(E)** XY controls have significantly greater expression of CXCL10 in the amygdala than XX controls. **(F)** XXF controls have lower EPO than XYF controls. **(G)** XY controls have higher IL-9 expression in the amygdala than XX controls. **(H)** XYF controls express the same amount of IL-10 as XXF controls, but XYM controls have higher expression of IL-10 than XXM mice in the amygdala. **(I)** XYM controls express lower IL-11 than other genotypes.

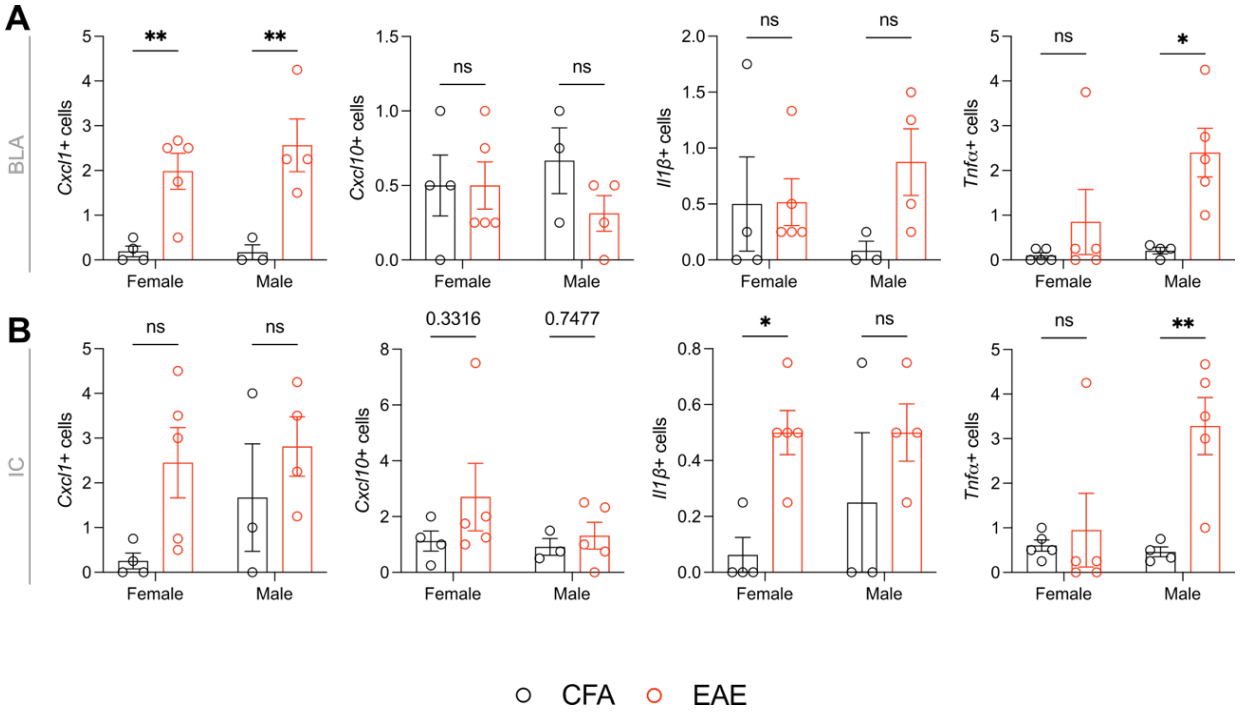

Fig. S9: Differential expression of cytokine positive cells within the BLA or the IC does not explain the sex differences observed within the Luminex multiplex immunoassay of the amygdala. RNAscope data for *Cxcl1, Cxcl10, Il1β,* and *Tnfα* positive cells in the BLA and the IC of WT males and females. **(A)** Within the BLA, WT females do not have greater upregulation of cytokine expressing cells than WT males in EAE. **(B)** Within the IC, WT females do not have greater upregulation of cytokine expressing cells than WT males in EAE.