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Synaptic alterations and immune response are sexually dimorphic in a non-pertussis toxin model of experimental autoimmune encephalomyelitis

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Abstract

Multiple sclerosis is an autoimmune disorder of the central nervous system (CNS) characterized by locomotor impairments, cognitive deficits, affective disorders, and chronic pain. Females are predominately affected by MS compared to males and develop motor symptoms earlier. However, key symptoms affect all patients regardless of sex. Previous studies have shown that demyelination and axonal damage play key roles in symptom development, but it is unclear why sex differences exist in MS onset, and effective symptom treatment is still lacking. We here used a non-pertussis toxin (nPTX) experimental autoimmune encephalomyelitis (EAE) model in C57BL/6 mice, to explore chronic symptoms and sex differences in CNS autoimmunity. We observed that, like in humans, female mice developed motor disease earlier than males. Further, changes in pre- and post-synaptic protein expression levels were observed in a sexually dimorphic manner with an overall shift towards excitatory signaling. Our data suggest that this shift towards excitatory signaling is achieved through different mechanisms in males and females. Altogether, our study helps to better understand sex-specific disease mechanisms to ultimately develop better diagnostic and treatment tools.

Keywords

Multiple sclerosis; Sex difference; EAE; Excitatory signaling; Synaptopathy; Excitotoxicity; Chronic neuropathic pain; Central nervous system; Inflammation; Leukocyte

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Competing interests
None

INTRODUCTION

Multiple sclerosis is an autoimmune disorder of the central nervous system (CNS) that predominately afflicts females at a 3 to 1 ratio compared to males (Constantinescu et al., 2011; Voskuhl et al., 2018). When males do develop the disease, they present with motor symptoms at a later age and deteriorate at a more progressive rate (Constantinescu et al., 2011). MS patients typically present with motor, sensory, and cognitive deficits associated with disease progression. Specific complications include optic neuritis, limb weakness, cognitive impairments, depression, and chronic neuropathic pain (CNP) (Bermejo et al., 2010; Mitchell et al., 2005; O'Connor et al., 2008). CNP is prevalent in 50–86% of MS patients and dramatically reduces the quality of life of patients (Bermejo et al., 2010; Murphy et al., 2017; O'Connor et al., 2008). Unexplainable pain symptoms often precede motor deficits and can be an early sign of MS that can be confirmed with a MRI scan (Fallata et al., 2017; Gass et al., 1997). Hallmarks of disease pathology include demyelinated lesions, inflammation, and axonal damage, and more recently, grey matter synaptopathy, which involves the dysfunction or loss of synapses (Catuneanu et al., 2019; Centonze et al., 2009; Ciccarelli et al., 2014; Compston and Coles, 2008; Mandolesi et al., 2015).

EAE, an animal model for MS, displays some of the key pathologies of the disease including focal lesions of demyelination, axonal damage, synaptopathy, and inflammation through active sensitization to myelin antigens, such as myelin oligodendrocyte glycoprotein (Centonze et al., 2009; Constantinescu et al., 2011; Pitt et al., 2000). While most studies have focused on motor disease, an increased interest is now given to the sensory complications and the associated neuropathology, especially in areas that are involved in pain processing and chronicity of neuropathic pain.

Recent studies have shown that an increased sensitivity to mechanical stimuli precedes motor disease development in EAE (Yuan et al., 2012). In addition, changes in somatosensory cortex synaptic proteins have been reported in EAE, in female mice, prior to the onset of motor deficits (Potter et al., 2016). Synaptic alterations have been demonstrated in chronic MS and EAE, indicative of potential changes taking place in excitatory and inhibitory signaling. In MS patients, a reduction in proteins such as synaptophysin and post-synaptic density protein 95 (PSD95) that are critical for synaptic integrity and maintenance has been observed in the hippocampus despite minimal neuronal loss (Dutta et al., 2011). For example, glutamate transporter and receptor expression alterations occur that cause excitotoxicity and inflammatory damage of inhibitory GABAergic interneurons leading to a shift towards an excitatory state (Groom et al., 2003; Nistico et al., 2013; Pitt et al., 2000). Specifically, increased glutamate-mediated transmission due to release of pro-inflammatory cytokines causes hyperexcitation and possibly neurodegeneration (Mandolesi et al., 2015). Such shifts in nociceptive CNS sites towards increased excitation and reduction in perineuronal nets (PNN) have been implicated in promoting CNP (Potter et al., 2016).

A common component of EAE is pertussis toxin (PTX), which is a potent adjuvant used to increase immune cell activation and permeability of the blood-brain barrier (BBB) to allow for greater immune cell infiltration for disease induction (Bermejo et al., 2010; Linthicum,

1982). PTX has also been shown to alter the immune response by decreasing regulatory T-cell numbers and suppressing their function (Cassan et al., 2006; Chen et al., 2006). In addition, Blankenhorn and colleagues have shown that PTX overrides certain genetic checkpoints changing the overall pathogenesis of the disease causing progression to activate similar genetic loci in female and male EAE (Blankenhorn et al., 2000; Mogil and Chanda, 2005). By altering disease pathogenesis through the influence of PTX, potential sex differences may be masked and not accurately reflect differences between females and males when studying changes in various protein expression.

In the present study, we are using an EAE model that does not use PTX (nPTX) to assess the sex-specific behavioral and pathogenic changes occurring in the spinal cord as well as in the cortex and hippocampus. These particular regions were chosen due to their critical roles in locomotion and pain perception. In particular, we elucidated sex-specific differences in EAE pathogenesis that phenotypically are similar, but are mechanistically different. We hypothesized that sexually dimorphic protein expression may lead to similar behavioral deficits that have different underlying molecular contributors in females and males. Such dimorphism would support the need to develop treatment options taking sex specificity into account.

MATERIALS AND METHODS

Mice

Adult wild-type (WT) C57BL/6J female and male (3–4 months old) mice were purchased from The Jackson Laboratory and were allowed to acclimate in the animal facility at Drexel University for at least 1 week prior to the start of experiments. Mice were housed in groups of 5 in a 12hour day/night cycle and had access to food and water *ad libitum*. All animal experiments under protocol #20627 were approved by Drexel University's Institutional Animal Care and Use Committee (IACUC).

EAE Induction and Motor scoring

Mice were immunized using subcutaneous injections containing an emulsion of 100µg of MOG_{35–55} peptide and complete Freund's adjuvant (CFA; InvivoGen, San Diego, California) supplemented with 200µg of heat-inactivated *Mycobacterium tuberculosis* H37Ra in CFA as previously described (Krementsov et al., 2014). These injections were administered 50µL each to the posterior right and left flanks. All mice received a booster MOG/CFA injection 7 days after the first injection. The CFA control group of mice received the same immunization without the MOG_{35–55} peptide. Mice were evaluated on a daily basis for both body weight changes and signs of motor deficits. Motor scores were assigned based on the following: 0 – no motor deficits, 1 – loss of tail tone, 2 – fully flaccid tail, 3 – hind-limb paralysis, 4 – forelimb paralysis, 5 – moribund, 6 – death (Brambilla et al., 2011).

Pain Assessment

Mechanical allodynia was assessed using the Von Frey test as described previously (Dellarole et al., 2014). Briefly, Von Frey hair monofilaments were used to assess sensitivity to a mechanical stimulus administered to the hindpaw of each mouse. Mice were placed in

individual clear plexiglass chambers elevated on a mesh-wire platform and allowed to acclimate for 30 minutes. The von Frey hair filaments were applied to the plantar surface of each hindpaw using the up-down method of analysis as previously described (Chaplan et al., 1994). If no reaction is seen to the administered monofilament, a monofilament with a slightly greater diameter is used. This is done for a series of 5 trials once the first positive pain response is noted. The paw withdrawal threshold is then measured in grams (g) of force, with thresholds typically ranging from 0.0 to 2.0 g. Hindpaw withdrawal was only considered a pain response if paired with at least one or more of the following actions: looking at the hindpaw, stimulus avoidance behavior, tucking the tail under stimulated hindpaw, and/or licking the stimulated hindpaw, which are indicative of cognitive awareness of the stimulus as being painful. Baseline testing was done a week prior to EAE induction and then pain was assessed on a weekly basis.

Isolation of Leukocytes from Spinal Cord and Flow Cytometry Staining

Mice were sacrificed 31 days post-immunization (dpi), perfused with PBS and spinal cords were harvested and kept in cold MACS buffer. All samples were homogenized and dissociated through a 70µm filter and washed in ice-cold MACS buffer (PBS pH7.2, 0.5% BSA, 2mM EDTA). Single-cell suspensions were centrifuged at 4°C for 10 min at 300 *g*. Myelin was removed by incubation of the samples with myelin removal beads II for 15 min at 4°C, and purification through a LD column (Miltenyi Biotec, Bergisch Gladbach, Germany). The myelin-free cell suspensions were then centrifuged for 10 min at 300 *x g* and resuspended in MACS buffer at a concentration of 10⁷ cells/mL. The percentages of macrophages and microglia were determined by flow cytometric analysis using FITC-anti-CD45 and PE/Cy7-anti-CD11b (Miltenyi Biotec, 1:100). Data were acquired using a BD FACS Canto (BD Biosciences, San Jose, CA) and analyzed with FlowJo (TreeStar, Ashland, OR).

Luxol Fast Blue Staining

Animals were perfused at 31 dpi with 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS) at 30 dpi. Brain and lumbar spinal cord were embedded in paraffin and microtome-cut into 10µm thick sections and placed onto slides. Sections were deparaffinized and hydrated in 95% EtOH. The slides were immersed in Luxol fast blue solution and incubated at 56°–60°C overnight. The slides were then immersed in 55% EtOH to remove excess stain and rinsed in dH₂O. Differentiation was done through a quick immersion in a lithium carbonate solution followed by 70% EtOH until grey and white matter can be distinguished. Sections were then dehydrated and mounted with ClearVue™ Mountant XYL (Thermo Scientific).

Whole Tissue Protein Extract

At 31 dpi animals were deeply anesthetized with Ketamine (215mg/kg) and Xylazine (43mg/kg), and perfused with phosphate buffered saline (PBS) until blood was thoroughly removed from the circulatory system. Tissue was then isolated from the cortex, hippocampus, and lumbar spinal cord and quickly frozen on dry ice. Tissue was homogenized in RIPA buffer (10mM sodium phosphate buffer pH 7.2, 150mM NaCl, 2mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease

inhibitor cocktail (Santa Cruz, Dallas, TX) and phosphatase inhibitors (Biovision, Milpitas, CA), and incubated at 4°C for 30 min. on a rocker. The samples were then centrifuged at 4°C for 15 minutes at 13,200RPM. Protein concentrations were determined using the *DCTM* protein assay (Biorad, Hercules, CA).

Synaptosome Protein Extract

Brain—Animals were anesthetized at 31 dpi with Euthasol (0.2mL/kg) until unresponsive to toe pinch and perfused with HEPES perfusion buffered sucrose solution (2mM HEPES pH 7.4, 320mM sucrose, 2mM EGTA, 10mM Na pyrophosphate, 1mM Na orthovanadate, 10mM NaF, 0.1mM PMSF) and cortex and hippocampus were collected and quickly frozen on dry ice. The cortical and hippocampal tissues were homogenized in HEPES homogenization buffered sucrose solution (2mM HEPES pH 7.4, 320mM sucrose, 2mM EGTA, 10mM Na pyrophosphate, 1mM Na orthovanadate, 10mM NaF, 1µg/mL aprotinin, 1µg/mL leupeptin, 0.1mM PMSF) and centrifuged at 1,000xg for 10 minutes at 4°C. The supernatant (S1) was collected and the 1st pellet (P1) was kept as a protein/synaptosome preparation purity control (Supplemental Fig.1). The supernatant S1 was then spun at 17,000xg for 15 min at 4°C. Supernatant 2 (S2) was collected and pellet 2 (P2) representing the synaptosomal fraction was resuspended in 120µl of HEPES homogenization buffer.

Spinal Cord—Since the spinal cord contains a high amount of white matter that can interfere with the isolation of synaptosomes, 40µl of myelin removal beads were added to the S1 fractions, and incubated for 15 minutes at 4°C on a rocker. The samples were then centrifuged at 5,000 x g for 10 minutes at 4°C. The myelin-depleted S1 supernatants were then processed as above, and the resulting synaptosomal P2 fractions were resuspended in 100µl of HEPES homogenization buffer.

Western blotting

Protein resolution was done using sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 8–15% gels, transferred to nitrocellulose (Turbo blot, Biorad) and blocked in 5% non-fat milk (Biorad) in 1x TBS-T (10mM Tris-HCL pH 7.5, 150mM NaCl, 0.1% Tween-20). Membranes were probed with antibodies against: myelin basic protein (MBP, rat, 1:15,000, Millipore, Burlington, MA); 2'3' cyclic nucleotide 3' phosphodiesterase (CNPase, rabbit, 1:1000 Cell Signaling, Danvers, MA); ionized calcium binding adaptor molecule 1 (Iba1, rabbit, 1:750, Proteintech, Rosemont, IL); glial fibrillary acidic protein (GFAP, mouse, 1:1000, Santa Cruz), post-synaptic density protein 95 (PSD95, mouse, 1:5000, BioLegend, San Diego, CA); vesicular glutamate transporter 1 (VGLUT1, mouse, 1:1000, Neuromab, Davis, CA); gephyrin (mouse, 1:1000, Santa Cruz); glutamic acid decarboxylase 65 (GAD65, rabbit, 1:1000, Millipore); Glutamate aspartate transporter (GLAST, rabbit, 1:1000, Cell Signaling), and gamma-aminobutyric acid type A receptor (GABA_AR, rabbit, 1:1000, Sigma-Aldrich, St. Louis, MO), followed by horseradish peroxidase-conjugated species-specific secondary antibodies. Proteins of interest were visualized with enhanced chemiluminescence (ECL, Pierce, Waltham, MA) and band intensity was quantified using Quantity One® software (Biorad). Data were normalized for each sample to the total amount of protein loaded through visualization with Ponceau S solution (Sigma) and expressed as percent of naïve group (Supplemental Fig.2).

Statistical analysis

Statistical analysis of EAE motor scores were conducted using two-way ANOVA and per day analysis using Bonferroni's multiple comparisons test. Cumulative disease index was analyzed using Mann-Whitney test. Two-way ANOVA was used to assess changes in withdrawal thresholds. Western blots and flow cytometry were analyzed using t-test for single comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

nPTX EAE-induced allodynia and motor deficits occur in both female and male mice

Due to the sex-based differences in MS, we first compared motor and sensory complications in female and male EAE mice. We analyzed both motor function and sensitivity to a mechanical stimulus after immunization. Female and male C57BL/6 mice developed motor scores of 2.0 and showed signs of hindlimb and lower trunk paralysis (Fig. 1A). Between 16 to 23 dpi, motor scores were significantly higher in females than in males (Fig. 1A). We also calculated the cumulative disease index (CDI) between day 14 and day 31 post-EAE induction was determined as a measure of disease severity, which was significantly less in males compared to females (Fig. 1B). In addition, the day of motor symptoms onset, defined as two consecutive days with a score of at least 2, occurred earlier in females than in males (Fig. 1A,B).

When assessing mechanical allodynia, CFA was used as a control in order to identify when transient inflammatory pain was not the primary underlying mechanism for hindpaw withdrawal and was instead due to disease induced pain (Gregory et al., 2013; Olechowski et al., 2009). As expected, CFA mice developed inflammatory pain and at 8 dpi could be distinguished from MOG/CFA induced neuropathic pain (Fig. 1C,D). Both females and males similarly developed EAE-induced mechanical allodynia that remained for the duration of the experiment (Fig. 1C,D; Female CFA $n=9$, Female MOG/CFA $n=25$; Male CFA $n=9$, Male MOG/CFA $n=25$).

nPTX EAE-induced glial changes and leukocyte infiltration

Astrocytes and microglia regulate inflammation and are involved in the maintenance of healthy synapses in the CNS (Claycomb et al., 2013; Eroglu and Barres, 2010; Liu and Hong, 2003; Perea et al., 2009). Astrocyte activation, associated with a corresponding increase in GFAP, in response to disease onset and progression can induce cytokine production (Jukkola et al., 2013). Similar protein levels of GFAP were observed between naïve female and male mice in both the spinal cord and the brain (Supplemental fig. 3A,B,C). However, following EAE-induction a significant increase in GFAP expression was observed only in the male lumbar spinal cord (Fig. 2A). There was a reduction in cortical GFAP expression for both females and males (Fig. 2B). There was no significant change in GFAP expression in EAE-hippocampal tissue compared to naïves (Fig. 2C). To evaluate microglial/macrophage activation we investigated changes in Iba1 expression, as increased levels are associated with MS and EAE disease progression (Sloka et al., 2013). In naïve mice, there were no sex differences in Iba1 expression both spinally and supraspinally (Supplemental fig. 3D,E,F). Upon EAE induction, a significant increase in Iba1 expression

was observed in the lumbar spinal cord of both females and males, with a more robust increase seen in the males (Fig. 2D). There was also a significant increase in cortical Iba1 for both sexes (Fig. 2E). No significant changes in Iba1 were observed in the hippocampus (Fig. 2F). These data indicate elevated glial response in the spinal cord of both females and males.

Flow cytometry was used to analyze the distribution of leukocyte populations in the spinal cord of female and male mice. Our data showed that a significantly greater fraction of cells expressing the leukocyte common antigen CD45 were present in the spinal cord of male mice, compared to females (Fig. 3A,B). We next compared the fractions of infiltrating CD45^{hi} immune cells and CNS-resident CD45^{low} microglia. Whereas no sex differences were seen in microglial cells, we observed a significantly higher percentage of spinal cord infiltrating peripheral macrophages in males (Fig. 3C,D). No sex-specific changes were observed in infiltrating CD3⁺T cells or CD45R(B220) B cells (data not shown). These data indicate that there was a greater degree of monocyte-derived macrophage mediated immune activity occurring in males compared to females suggestive of sex specific differences in immune responses.

Demyelination occurs in the lumbar spinal cord and cortex of female and male mice

Changes in myelin integrity within the brain and spinal cord were investigated using LFB staining and western blot analysis for MBP and CNPase, two integral myelin proteins. Analysis of LFB staining showed prominent demyelination in the ventral horn of the spinal cord and somatosensory cortex in both female and male mice, however reduced LFB staining was not prominent in the hippocampus (Fig. 4A,B,C). In naïve mice, females and males expressed similar levels of MBP and CNPase in the lumbar spinal cord (Supplemental fig. 4A,D). There were no differences in naïve CNPase supraspinal expression, however MBP levels were significantly lower in the male cortex and hippocampus compared to females (Supplemental fig. 4B,C,E,F). Correlating with the LFB data, a significant reduction in cortical MBP and CNPase expression was observed post-EAE induction at 31 dpi for both female and male mice (Fig. 4A,E,H). CNPase expression was also significantly reduced in the spinal cord of both females and males (Fig. 4G). There appeared to also be a decrease in MBP expression in the male spinal cord; however, this decrease was not significant (Fig. 4D; #p=0.05). No significant changes were observed in hippocampal myelin-associated proteins for both females and males (Fig. 4F,I).

Changes in synaptic proteins are sex specific

Synaptopathy is observed in both EAE and MS and is becoming more widely recognized as a primary MS pathology, in conjunction with demyelination and axonal damage that can precede them (Centonze et al., 2009; Mandolesi et al., 2015). To begin evaluating synaptic differences between male and female mice, we assessed the expression levels of post-synaptic scaffolding proteins in synaptosomal preparations from cortex, hippocampus, and spinal cord, comparing naïve animals first and then EAE mice at 31 dpi. PSD95 and gephyrin are scaffold proteins that are present at excitatory and inhibitory post-synaptic membranes, respectively. Naive expression of PSD95 was similar between female and male spinal cord and cortex, however, males had significantly lower PSD95 expression levels in

the hippocampus (Supplemental fig. 5A,B,C). Naïve expression of gephyrin was significantly lower in the male cortex, but no differences were seen in the lumbar cord or hippocampus (Supplemental fig. 5D,E,F). Following EAE, no significant changes in levels of PSD95 were observed in males or females (Fig. 5A,B,C). However, EAE females showed significantly reduced gephyrin expression in the cortex, but not males (Fig. 5E). In the hippocampus there was a significant decrease in gephyrin expression in males, but no changes in females (Fig. 5F). There were no significant changes in gephyrin in the lumbar spinal cord for both sexes (Fig. 5D). In summary, EAE induced sex specific changes in gephyrin but had no impact on PSD95.

Glutamate receptor and transporter expression is impacted sex-specifically in the lumbar cord and supraspinally

Enhanced glutamate production and signaling through its receptors have been observed in MS and EAE brains (Bolton and Paul, 2006; Groom et al., 2003; Sulkowski et al., 2014). An excitotoxic environment, through increased glutamate signaling and accumulation in disease states, has been linked to axonal damage and contributes to synaptopathy (Piani et al., 1991; Potter et al., 2016; Sapolsky, 2000). As expected, NMDAR1 protein levels were significantly increased in both male and female lumbar spinal cord synaptosomes in chronic EAE mice (Fig. 6A). Naïve males expressed less synaptic NMDAR1 in the spinal cord than females (Supplemental fig. 6A). Supraspinally, NMDAR1 expression was similar between naïve females and males and remained unaltered in the cortex and hippocampus post-EAE induction (Fig. 6B,C; Supplemental fig. 6B,C). Vesicular glutamate transporter 1 (VGLUT1) is involved in the filling of synaptic vesicles with glutamate. Striking differences in the regulation of VGLUT1 at the synapse following EAE were observed in males and females lumbar spinal cord. While VGLUT1 protein levels were significantly reduced by over 50% in females, they were significantly increased by over 2400% in males (Fig. 6D). In naïve spinal cord synaptosomes, VGLUT1 was significantly lower in males compared to females (Supplemental Fig. 6D). Supraspinally, VGLUT1 was significantly increased in EAE female hippocampus only (Fig. 6F), with no significant changes under naïve conditions (Supplemental Fig. 6E,F). Excitotoxic amino acid transporter 1 (EAAT1/ GLAST) is a glutamate transporter expressed on perisynaptic astrocytes in the spinal cord, and may be involved in the regulation of nociceptive behavior (Tao et al., 2005). In the brain, EAAT1 deregulation leads to mental disorders (Paula-Lima et al., 2013). In naïve animals supraspinal expression of GLAST was similar between females and males, however, expression was greater in the male lumbar cord compared to female (Supplemental Fig. 6G,H,I). Following chronic EAE, GLAST expression in females remained unchanged in both spinal and supraspinal regions (Fig. 6G,H,I). However, in the male spinal cord and cortex GLAST levels were significantly decreased (Fig. 6G,H). In the naïve hippocampus comparable GLAST expression levels were seen in both sexes and no significant changes were observed post-EAE induction (Supplemental Fig. 6I; Fig. 6I).

Sexually dimorphic changes in spinal and hippocampal GABAergic synthetic enzyme GAD65 and GABA_A receptor

Inhibitory synaptic transmission is essential for neuronal activity, informational processing, and network synchronization (Bhat et al., 2010). In MS patients, proteins characteristic of

inhibitory synaptic networks have been shown to be diminished in the blood of patients, which promotes disease progression and may mirror what is occurring in the CNS with a compromised BBB (Demakova et al., 2003). An important protein involved in catalyzing the production of inhibitory neurotransmitter GABA is GABAergic synthetic enzyme GAD65. In naïve mice levels of GAD65 were similar between females and males in the lumbar spinal cord and cortex, however, males expressed less in the hippocampus compared to females (Supplemental Fig. 7A,B,C). Expression of GAD65 was significantly reduced in the male lumbar cord, cortex, and hippocampus post-EAE induction (Fig. 7A,B,C). However, expression levels were only reduced in the cortex for females with no changes seen in GAD65 expression in the lumbar cord or hippocampus (Fig. 7A,B,C). GABA_AR, a critical inhibitory receptor for GABA, was expressed at lower levels in the spinal cord and higher levels in the cortex of males compared to females under naïve conditions, but hippocampal expression levels were similar (Supplemental Fig. 7D,E,F). Post-EAE GABA_AR expression remained unaltered in lumbar cord and hippocampal tissue for both sexes (Fig. 7D,F). In contrast, post-EAE GABA_AR expression was significantly reduced in the cortex for both sexes (Fig. 7E).

DISCUSSION

Non-PTX EAE induction mimics pain and motor symptoms of human disease course

Historically, EAE induction has included the use of PTX to increase immune cell activation and permeability of the BBB. However, previous studies have demonstrated that PTX can mask potential sex differences associated with disease progression and can impact Treg population numbers and functionality (Blankenhorn et al., 2000; Cassan et al., 2006; Chen et al., 2006). PTX-EAE susceptibility genetically acts through the locus *ee9* for both sexes, which influences infiltration of mononuclear cells in the spinal cord and motor symptom severity. Both females and males develop motor disease; however, whereas females typically develop both mechanical and cold hypersensitivity, males only develop hypersensitivity to various stimuli in certain models of EAE and they differ between strains of mice (Lu et al., 2012). This is suggestive of PTX's potential to alter disease course in a differential manner dependent on the sex and genetic line of the animal (Aicher et al., 2004; Rahn et al., 2014). However, when EAE is induced without PTX, sexual dimorphism is seen in the activation of a variety of genetic loci associated with disease development and severity (Blankenhorn et al., 2000). The behavioral deficits and presence of synaptopathy from our study reflects a clinical disease course that is more appropriately reflective of human MS progression. Approximately 70% of MS patients experience chronic pain regardless of sex and our study demonstrates that males develop chronic pain along with their female cohort. In addition, while both female and male C57BL/6 mice develop motor symptoms, males have delayed motor symptom onset. Overall, using this EAE model we are able to more closely mimic human MS onset and progression, with male mice developing motor symptomology later than females in the nPTX model; while both females and males develop chronic pain that precedes motor symptoms similar to humans (Olechowski et al., 2009). Our data show that the level of pain was independent of disease severity as the tactile pain was established before any obvious motor symptoms and did not change with increasing motor deficits, and persisted for the duration of the experiment. Our data would support the idea developed by

Wang and colleagues that peripheral nerve injury is contributing to pain in EAE (Wang et al., 2017).

Glial activation and immune response in spinal tissue is greater in males

Increased glial responses and immune cell infiltration within the CNS are associated with an inflammatory environment, further contributing to its persistence through secretion of pro-inflammatory molecules that can lead to compromised myelin and synaptic integrity (Grasselli et al., 2013; Jukkola et al., 2013). In a healthy state sex differences exist in immune cell profiles, such as in supraspinal microglia transcriptomes and functionality that point towards intrinsic sex differences that may influence immunological consequences of disease (Guneykaya et al., 2018). Immune responses are elicited in both the female and male spinal cord however a large population of monocyte-derived macrophages dominates the EAE immune response in males. Glial and immune responses in the lumbar spinal cord were more prevalent in male mice in regards to both macrophage and astrocyte reactivity. Pronounced leukocyte infiltration and monocyte-derived macrophage activity in the male cord is indicative of a more robust innate-mediated immune response. Macrophage infiltration from the periphery into the CNS is minimal under normal physiological conditions; however, greater infiltration has been shown to occur during peak EAE disease coinciding with increased inflammation and symptoms (Jiang et al., 2014). Increased Iba1 and reduced GFAP expression is seen cortically for both sexes that is suggestive of an inflammatory environment that could be modulating the activity of the astrocyte population due to increased presence of pro-inflammatory cytokines and chemokines. These changes in glial populations within the spinal cord and cortex may contribute to the presence and/or chronicity of observed behavioral symptoms.

Excitatory-inhibitory imbalances occur in lumbar spinal cord with sexually dimorphic mechanisms for suppressing inhibitory signaling

Excitotoxicity through overproduction of glutamate or impaired uptake has been shown to occur in MS and EAE due, in part, to chronic inflammation (Bellizzi and Gelbard, 2013; Piani et al., 1991; Pitt et al., 2000). Increased NMDAR1 expression in the female and male lumbar cord is indicative of an excitotoxic environment, which could be in response to microglial activation and secretion of pro-inflammatory cytokines like TNF- α and IL-1 β by infiltrating macrophages (Grasselli et al., 2013; Smith et al., 2012). Studies have shown that the release of inflammatory cytokines by microglia and macrophages plays a crucial role in enhanced glutamate transmission that is seen in EAE and MS (Centonze et al., 2009; Jiang et al., 2014; Rossi et al., 2012). Our data suggests that there is enhanced glutamatergic signaling and an excitotoxic environment in both the female and male lumbar spinal cord that differ mechanistically. In the lumbar cord of females, the data points towards enhanced glutamate transmission and accumulation at the synaptic cleft with increased NMDAR1 signaling and reduced removal of glutamate by VGLUT1. Males demonstrate a similar excitatory/inhibitory imbalance, but not just due to changes in excitatory-related proteins.

An excitatory-inhibitory imbalance is not only caused by increased excitatory signaling, but may also be caused by a decrease in inhibitory synaptic transmission through the reduction of GABA-associated proteins. In addition to increased NMDAR1 expression, GAD65 is

reduced in males, which suggests that there is a reduction in GABA synthesis further favoring an excitatory shift. Previous research has demonstrated that epigenetic suppression of GAD65 in mice, through the gene *Gad2*, causes persistent pain (Zhang et al., 2011). Not only is there reduced GABA synthesis, there is also impaired removal of glutamate from the synaptic cleft as suggested by the reduction of GLAST in the male spinal cord and this impaired glutamate removal has been shown to occur in previous EAE studies and inflammatory studies (Korn et al., 2005; Vercellino et al., 2007). These findings suggest that removal of glutamate from the synaptic cleft is impaired and synthesis of GABA along with its synaptic release is reduced.

Pronounced demyelination of the cortex promotes impaired synaptic signaling/function

In MS, grey matter demyelination and synaptopathy have been shown to contribute to permanent neurological disabilities and reduced motor function associated with disease progression and over 50% of MS patients experience cognitive dysfunction (Calabrese et al., 2013; Chiaravalloti and DeLuca, 2008). We observed extensive demyelination within the somatosensory cortex of both female and male EAE mice, along with decreased expression of myelin-associated proteins. In addition, there was a cortical reduction of proteins that regulate glutamate expression and excitatory signaling suggestive of altered neuronal activity and functionality (Table 1).

Reduction of the inhibitory scaffolding and receptor proteins, GABA producing synthetic enzyme, and glutamate transporters all promote an excitotoxic environment. Increased excitatory activity in the cortex, specifically the anterior cingulate cortex (ACC), has been associated with impaired sensory perception and chronic pain through enhanced glutamate release and receptor response (Kang et al., 2013; Sawamoto et al., 2000; Zhuo, 2007). The ACC is heavily implicated in persistent pain states due to its role in modulation of affective states and emotional responses to corresponding pain (Hart et al., 2003). Cortical synaptopathy during chronic EAE appears to target inhibitory-synaptic signaling proteins and impedes the removal of excess glutamate through either direct reduction in GLAST or due to the possible loss of functional astrocyte populations as suggested by decreased GFAP expression. Robust demyelination of the cortex, along with synaptopathy, contributes to reduced inhibitory signaling, a potential driver for pain chronicity and reduced motor function in EAE. The non-PTX EAE model may be more effective for studying grey matter pathology, including supraspinal demyelination, since we observe greater similarities with the grey matter pathology occurring in MS patients.

Sexually dimorphic hippocampal shift towards excitatory signaling

Previous studies have established the role of the hippocampus in affective disorders, such as depression and anxiety, from which approximately 50% of MS patients suffer. Excessive exposure to excitatory neurotransmitters in this region of the brain has been shown to compromise the circuitry (Abele et al., 1990). In our study, we show that in chronic EAE females, vesicular uptake of glutamate for transport in the hippocampus is increased, which may be indicative of a compensatory mechanism to offset glutamate excitotoxicity that is already occurring in supraspinal regions. However, there are no other changes seen that would suggest an excitatory-inhibitory imbalance in this region. In the chronic EAE male

hippocampus, there is a reduction in both gephyrin, a scaffold protein anchoring inhibitory receptors to the synaptic membrane and GAD65, a protein involved in the production of GABA neurotransmitter (Table 1).

CONCLUSION

Overall, despite similar phenotypic presentation of EAE symptoms in females and males at a chronic time point, sexually dimorphic modulation in expression of critical glial and synaptic proteins is indicative of molecularly distinct mechanisms of disease development. Further research is needed concerning EAE and sex specificity in glial, myelin, and synaptic protein expression along with sex differences in non-diseased states. Our data indicates that sex differences in expression of proteins involved in myelin stability, immune response, and synaptic integrity are not only altered upon EAE induction, differences exist as well in healthy female and male mice. Observed differences in baseline expression of such proteins in naïve mice may give some indication of why diseases that involve autoimmunity, inflammation, and/or chronic pain are more prevalent in one sex compared to the other. Therefore, our data shows disease-induced biochemical changes are sexually dimorphic emphasizing the need to explore female and male disease-mediated mechanisms of inflammation and chronic pain in order to develop targeted therapies with increased efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- nPTX EAE reflects some aspects female and male MS motor and sensory deficits
- Behavioral deficits are seen in both female and male EAE
- Macrophage-mediated innate immune response is stronger in males
- Synaptic protein expression are sexually dimorphic, but favor excitatory signaling

Figure 1: Behavioral pathology occurs post-nPTX EAE induction in female and male mice. (A) Female and male mice develop motor deficits post-EAE induction (Two-way ANOVA; female n=25, male n=25; per day analysis; **p<0.01, ***p<0.001). (B) Clinical parameters of nPTX-EAE. The day of motor deficit onset was defined as the first day when a mouse scores at least 2 for two consecutive days. The cumulative disease index (CDI) which was calculated as the sum of clinical scores between days 14 and 31, represents a measure for disease severity over time (CDI; Mann-Whitney; ***p=0.0006). (C) Female MOG/CFA mice show significantly decreased hindpaw withdrawal threshold compared to female CFA controls (Two-way ANOVA; CFA n=9, MOG/CFA n=25; ****p<0.0001). (D) Male MOG/CFA mice show significantly decreased hindpaw withdrawal threshold compared to male CFA controls (Two-way ANOVA; CFA n=9, MOG/CFA n=25; ****p<0.0001).

Figure 2: Modulation of glial proteins occurs in spinal and cortical regions following nPTX EAE induction.

Western blots analyses comparing the levels of GFAP (A-C) and IBA1 (D-F) between naïve and 31 dpi EAE for each sex, in the spinal cord, cortex and hippocampus. Bar graphs represent the mean $\pm$ s.e.m. of $n=5-6$ /group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Figure 3: nPTX EAE-induced leukocyte infiltration occurs in the spinal cord of both female and male mice.

(A) CD45⁺ cells were gated based on total cells collected from the spinal cord in both female and male mice; Gating was done between CD45⁺ and CD11b⁺ cells to separate high and low populations for distinguishing macrophage and microglia populations (Representative dot plots for gating strategy are shown). (B) Leukocyte infiltration into the spinal cord occurs in both female and male mice, but there are significantly more CD45⁺ cells in males than females. (C) There is no significant difference in the proportion of CD45^{low} CD11b⁺ microglial cells between females and males. (D) There is a significantly greater macrophage population (CD45^{high}CD11b⁺) in males compared to females. Bar graphs represent the mean $\pm$ s.e.m. of $n = 4$ females, and $n = 6$ males. ** $p < 0.01$

Figure 4: Demyelination of the lumbar spinal cord and cortex occur in female and male mice. (A) Representative histological sections stained for Luxol fast blue (LFB) showing areas of demyelination in the female and male ventral lumbar spinal cord and somatosensory cortex (Scale Bar=100µm). (B) Diagram showing the orientation and portion of the lumbar spinal cord imaged for LFB staining. (C) Diagram showing the orientation and portion of the brain imaged for LFB staining. (D-F) Western blot analyses comparing the levels of MBP between naïve and 31 dpi EAE for each sex in the spinal cord, cortex and hippocampus. (G-I) Western blots analyses comparing the levels of CNPase between naïve and 31 dpi EAE for each sex in the spinal cord, cortex and hippocampus. Bar graphs represent the mean $\pm$ s.e.m. of n=5–6/group. **p<0.01, ***p<0.001, ****p<0.0001, #p=0.05

Figure 5: Sexually dimorphic changes are observed in inhibitory post-synaptic scaffolding protein gephyrin.

Western blots analyses comparing the synaptic levels of PSD95 (A-C) and Gephyrin (D-F) between naïve and chronic EAE (31dpi) for each sex in the spinal cord, cortex and hippocampus. Bar graphs represent the mean $\pm$ s.e.m. of $n=5$ /group. * $p<0.05$

Figure 6: Sexually dimorphic EAE-induced changes are observed in lumbar cord and supraspinally in female and male mice.

Western blots analyses comparing the synaptic levels of NMDAR1 (A-C) and VGLUT1 (D-F) between naïve and chronic EAE (31dpi) for each sex in the spinal cord, cortex and hippocampus. (G-I) Western blots analyses comparing the levels of GLAST in whole cell lysates between naïve and chronic EAE (31dpi) for each sex in the spinal cord, cortex and hippocampus. Bar graphs represent the mean $\pm$ s.e.m. of $n=5-6$ /group. * $p<0.05$, ** $p<0.01$

Figure 7: Sexually dimorphic changes in GABAergic synthetic enzyme in the lumbar spinal cord and hippocampus.

Western blots analyses comparing the synaptic levels of GAD65 (A-C) and GABA_A receptor (D-F) between naïve and 31 dpi EAE for each sex in the spinal cord, cortex and hippocampus. Bar graphs represent the mean $\pm$ s.e.m. of n=5–6/group. *p<0.05, **p<0.01

Table 1:
Synaptic changes are differentially expressed in lumbar cord and supraspinally between female and male EAE mice.

Summation of the synaptic data collected from western blotting analysis with excitatory proteins designated in red and inhibitory proteins designated in blue.

	Lumbar Spinal Cord		Cortex	Hippocampus
Females (EAE vs Naïve)	↑ NMDAR1	↓ VGLUT1	↓ Gephyrin GAD65 GABA _A R	↑ VGLUT1
Males (EAE vs Naïve)	↑ NMDAR1 VGLUT1	↓ GLAST GAD65	↓ GLAST GAD65 GABA _A R	↓ Gephyrin GAD65