XY sex chromosome complement, compared with XX, in the CNS confers greater neurodegeneration during experimental autoimmune encephalomyelitis

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Women are more susceptible to multiple sclerosis (MS) and have more robust immune responses than men. However, men with MS tend to demonstrate a more progressive disease course than women, suggesting a disconnect between the severity of an immune attack and the CNS response to a given immune attack. We have previously shown in an MS model, experimental autoimmune encephalomyelitis, that autoantigen-sensitized XX lymph node cells, compared with XY, are more encephalitogenic. These studies demonstrated an effect of sex chromosomes in the induction of immune responses, but did not address a potential role of sex chromosomes in the CNS response to immune-mediated injury. Here, we examined this possibility using XX versus XY bone marrow chimeras reconstituted with a common immune system of one sex chromosomal type. We found that experimental autoimmune encephalomyelitis mice with an XY sex chromosome complement in the CNS, compared with XX, demonstrated greater clinical disease severity with more neuropathology in the spinal cord, cerebellum, and cerebral cortex. A candidate gene on the X chromosome, toll-like receptor 7, was then examined. Toll-like receptor 7 expression in cortical neurons was higher in mice with XY compared with mice with XX CNS, consistent with the known neurodegenerative role for toll-like receptor 7 in neurons. These results suggest that sex chromosome effects on neurodegeneration in the CNS run counter to effects on immune responses, and may bear relevance to the clinical enigma of greater MS susceptibility in women but faster disability progression in men. This is a demonstration of a direct effect of sex chromosome complement on neurodegeneration in a neurological disease.

sex differences | XY genes | Tlr7

Most autoimmune diseases affect females more than males in both humans and mice. In addition to multiple sclerosis (MS), other examples include systemic lupus erythematosus (SLE), rheumatoid arthritis, and Hashimoto thyroiditis (1). This enhanced susceptibility in females compared with males has also been observed in animal models such as experimental autoimmune encephalomyelitis (EAE) in Swiss Jim Lambert (SJL) mice, spontaneous SLE, chemically induced lupus, adjuvant arthritis, and thyroiditis (1). Most studies on sex differences in autoimmune diseases have focused on sex hormones (2). However, a role for sex hormones does not exclude a contribution of sex chromosomes to the female bias in susceptibility to these autoimmune diseases. Further, although MS is more prevalent in females with more robust immune responses, some data have suggested that men with MS demonstrate a more progressive neurodegenerative course (3–8) (reviewed in ref. 2). Thus, we hypothesized that there could be sex-related factors that have opposing effects on the immune system versus the CNS, causing female MS patients to have more robust peripheral immune responses, but a more resilient CNS response to injury. A comprehensive understanding of sex differences could lead to therapies in sex-biased autoimmune or neurodegenerative diseases.

In our previously published work, we found that sex chromosome complement affected EAE susceptibility. We used SJL transgenic mice, known as four-core genotypes (FCGs), in which the sex-determining region of the Y chromosome (*Sry*) has been deleted from the Y chromosome (denoted Y^-). XY^- mice with an autosomal *Sry* transgene, denoted XY^- (*Sry*+), are fertile and are fathers to ovary-bearing XX and XY^- females. Comparisons between XX and XY^- mice revealed effects of sex chromosomes not confounded by differences in types of gonadal hormones, as they are both gonadally female. When proteolipid protein (PLP) 139–151 sensitized lymph node cells (LNCs) derived from either XX or XY^- immunized mice were adoptively transferred into a common recipient, there was a dramatic difference in EAE severity, with those receiving XX cells having much more severe EAE than those receiving XY^- cells (9). This demonstrated a sex chromosome effect on the induction of encephalitogenic immune responses during immunization of adult mice with PLP 139–151. However, it remained unknown whether there were sex chromosome effects in the CNS response to injury.

Previous work suggests that sex chromosomes may play a role in CNS plasticity. Gene expression array studies in mice and humans have shown that a significantly higher proportion of genes on sex chromosomes, compared with genes on autosomes, are preferentially expressed in the brain compared with other somatic tissues (10, 11). Additionally, neurons with different sex

Significance

Women are more susceptible to multiple sclerosis (MS), but men demonstrate a more progressive disease course. In the MS model, experimental autoimmune encephalomyelitis (EAE), XX as compared with XY, conferred greater encephalitogenic responses. Here, we examined effects of sex chromosomes in the CNS using bone marrow chimeras with XX versus XY CNS and immune systems of the same sex chromosomal type. EAE mice with XY CNS, compared with XX, demonstrated more severe clinical disease with more neurodegeneration in the spinal cord, cerebellum, and cerebral cortex. Expression of the X gene *toll-like receptor 7*, known to induce neuronal damage, was increased in XY mice. These results may bring insight into why men progress faster during a disease characterized by increased susceptibility in women.

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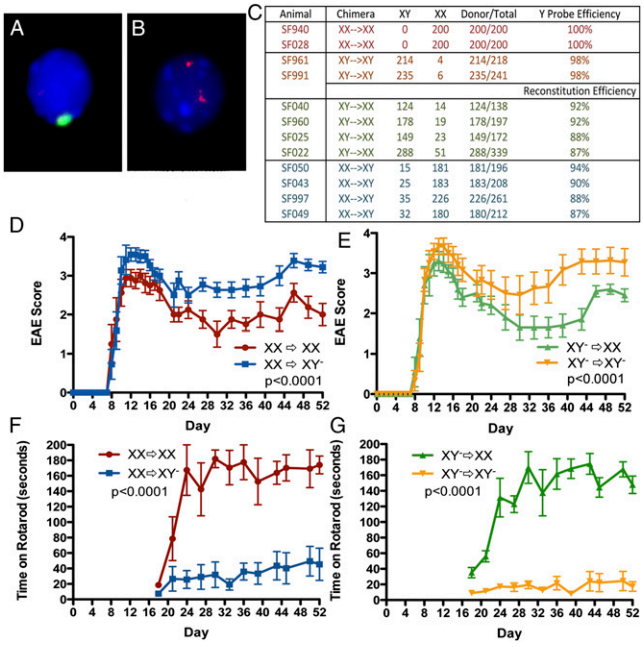
chromosome complements develop differently, regardless of gonadal sex (12). These findings are consistent with the observations of anatomically dimorphic brain regions between females and males in the human and mouse (13–15). Although many hypothesize that sex hormones may contribute to the sexual dimorphism in a number of neurological diseases, such as Parkinson disease, Alzheimer's disease, and schizophrenia, a role for sex chromosomes has not been excluded (16). To test whether sex chromosomes may directly influence the CNS response to injury, we generated bone marrow chimeras (BMCs) of XX and XY$^-$ gonadal female mice in which the sex chromosome complement of the reconstituted immune system was varied independently of that in the brain. This allowed us to examine sex chromosome effects in the CNS during EAE without confounding effects of differences in sex chromosome complement in the immune system.

Results

Reconstitution Efficiency in the FCG BMCs Model. We generated FCG BMCs in the following combinations: (*i*) XX immune system, XX CNS; (*ii*) XX immune system, XY$^-$ CNS; (*iii*) XY$^-$ immune system, XX CNS; and (*iv*) XY$^-$ immune system, XY$^-$ CNS. We first determined the reconstitution efficiency of the BMCs by marking X and Y chromosomes in splenocytes using DNA fluorescence in situ hybridization. As a positive control, XX chimeric mice reconstituted with XX bone marrow (BM) cells (XX→XX; donor BM genotype before arrow and recipient after arrow) and XY$^-$ chimeric mice reconstituted with XY$^-$ immune cells (XY$^-$→XY$^-$) were labeled with an X chromosome (red) and Y chromosome (green) probe mixture. Both X and Y labeling was present in DAPI$^+$ cells in XY$^-$→XY$^-$ chimeras (Fig. 1*A*), whereas Y labeling was absent in DAPI$^+$ cells in XX→XX chimeras (Fig. 1*B*). The reconstitution percentage was then determined to be in the range of 87–94% (Fig. 1*C*) in chimeric mice with XX CNS reconstituted with XY$^-$ BM cells (XY$^-$→XX) and chimeric mice with XY$^-$ CNS reconstituted with XX BM cells (XX→XY$^-$).

XY CNS, Compared with XX, Confers Greater Disease Severity. To determine the role of sex chromosome complement in the CNS independent of sex chromosome effects on the immune system, we compared PLP 139–151 peptide-induced active EAE in SJL BMCs that had been reconstituted with the same immune system and had a common hormonal background (all females), but differed in their sex chromosome complement in the CNS (XX→XX vs. XX→XY$^-$, and XY$^-$→XX vs. XY$^-$→XY$^-$). As shown in Fig. 1 *D* and *E*, mice with XY$^-$ sex chromosome complement in the CNS, compared with XX, had worse standard EAE disease severity scores late in EAE. Additionally, mice were subjected to rotarod testing, a more sensitive measure of coordination. Consistent with EAE scores, mice with XY$^-$ CNS were able to stay on the rotarod for fewer seconds compared with mice with XX CNS (Fig. 1 *F* and *G*). Notably, this sex difference in the CNS runs counter to those found in the induction phase of adoptive EAE in SJL mice where autoantigen-specific XX immune cells were more encephalitogenic than XY$^-$. Together, this indicates that sex chromosome effects in disease can be tissue-specific.

To determine whether the results were limited to one strain of mouse, we next used C57BL/6 mice of the FCGs. We compared myelin oligodendrocyte glycoprotein 35–55 peptide-induced active EAE in C57BL/6 BMCs that had been reconstituted with the same immune system and had a common hormonal background (all females), but differed in their sex chromosome complement in the CNS as above. We observed similar results in the C57BL/6 background as in the SJL background, whereby mice with XY$^-$ CNS, compared with XX, had worse EAE scores (Fig. S1 *A* and *B*) and rotarod performance (Fig. S1 *C* and *D*). Together, data in two different mouse strains demonstrate that chimeras with XY$^-$ CNS, compared with XX, have more clinical disability late in EAE.



Animal	Chimera	XY	XX	Donor/Total	Y Probe Efficiency
SF940	XX-->XX	0	200	200/200	100%
SF028	XX-->XX	0	200	200/200	100%
SF961	XY-->XY	214	4	214/218	98%
SF991	XY-->XY	235	6	235/241	98%
					Reconstitution Efficiency
SF040	XY-->XX	124	14	124/138	92%
SF960	XY-->XX	178	19	178/197	92%
SF025	XY-->XX	149	23	149/172	88%
SF022	XY-->XX	288	51	288/339	87%
SF050	XX-->XY	15	181	181/196	94%
SF043	XX-->XY	25	183	183/208	90%
SF997	XX-->XY	35	226	226/261	88%
SF049	XX-->XY	32	180	180/212	87%

Fig. 1. SJL mice with XY CNS, compared with XX CNS, have greater clinical EAE disease severity. (*A–C*) Representative 100× captures of splenocytes from XY$^-$→XY$^-$ (*A*; notations detail BM donor before arrow and irradiated recipient after arrow) and XX→XX (*B*) mice stained for X (red) and Y (green) probes and DAPI (blue) nuclear stain. A single red and green label identified XY, whereas two red labels and no green labels identified XX. Nearly all DAPI$^+$ XY$^-$→XY$^-$ cells had one red and one green label, whereas most DAPI$^+$ XX→XX cells had two red and no green labels. (*C*) Chart summarizing the probe staining efficiency in representative XX→XX (red) and XY$^-$→XY$^-$ (orange) BMCs and the reconstitution efficiency of XY$^-$→XX (green) and XX→XY$^-$ (blue) BMCs. The reconstitution efficiency was calculated by taking the proportion of donor cells in a sample (donor/total × 100) normalized by the staining efficiency of the Y probe in a known control (98% in XY$^-$→XY$^-$). For example, using sample SJL female 040 (SF040), reconstitution efficiency = (124/138) × 100/98 = 92%. By this method, the reconstitution efficiency ranged from 87–94%. (*D–G*) BMCs, XY genotype in the host CNS, compared with XX, had greater disease severity at late time points (i.e., had higher EAE scores) [$P < 0.0001$, XX→XX ($n = 9$) vs. XX→XY$^-$ ($n = 12$), and $P < 0.0001$, XY$^-$→XX ($n = 12$) vs. XY$^-$→XY$^-$ ($n = 13$), repeated measures one-way ANOVA with Bonferroni post hoc test] and had worse rotarod performance (i.e., spent less time on the rotarod, lower scores) ($P < 0.0001$, XX→XX vs. XX→XY$^-$, and $P < 0.0001$, XY$^-$→XX vs. XY$^-$→XY$^-$, repeated measures one-way ANOVA with Bonferroni post hoc test). Data are displayed as mean clinical scores ± SEM. Data are representative of three repeated experiments.

XY CNS, Compared with XX, Demonstrates Greater Spinal Cord and Cerebellar Pathology in EAE. At the endpoint of disease, we examined the chimeric SJL EAE mice for neuropathology related to ambulation and coordination, in the spinal cord and cerebellum, respectively. Consistent with clinical disability results, mice with XY$^-$ CNS, compared with XX, had more demyelination (Fig. 2 *A* vs. *B* and *C* vs. *D*) and greater axonal loss (Fig. 2 *E* vs. *F* and *G* vs. *H*) in the spinal cords when assessed by anti–myelin-basic protein (MBP) antibody staining and anti–neurofilament-200 (NF200), respectively. Cerebellar pathology was examined by quantifying the degree of white matter demyelination with anti-MBP antibody, and the number of healthy Purkinje cell bodies stained with anti–Calbindin D28-K. Mice with XY$^-$ CNS, compared with XX, had more demyelination in the white matter of the cerebellum (Fig. 2 *I* vs. *J* and *K* vs. *L*) and lower numbers of healthy Purkinje cells characterized by full-bodied, balloon-like cell bodies with clearly visible dendritic arborization (Fig. 2 *M* vs. *N* and *O* vs. *P*).

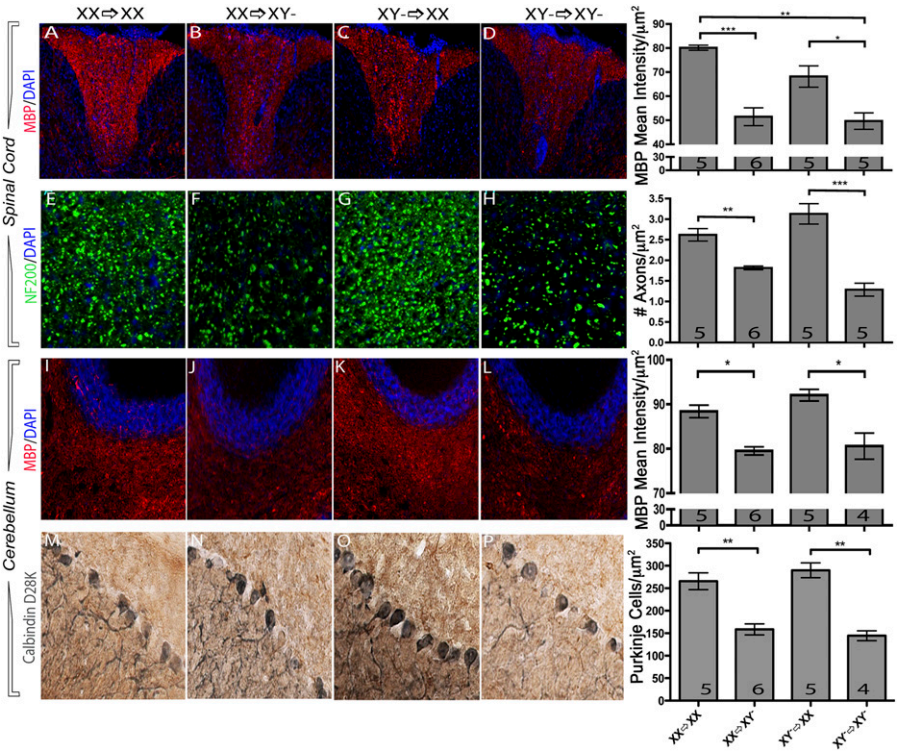


Fig. 2. Mice with XY CNS, compared with XX CNS, have greater spinal cord and cerebellar pathology. (*A–D*) Representative 10× captures of myelin stained with MBP (red) in the dorsal column of the thoracic spinal cord. SJL mice with XY CNS have less myelin staining intensity compared with mice with XX CNS in the setting of the same immune system ($P < 0.001$, XX→XX vs. XX→XY$^-$, and $P < 0.05$, XY$^-$→XX vs. XY$^-$→XY$^-$, two-way ANOVA, *Top Right*). Tissues were counterstained with DAPI (blue). (*E–H*) Representative 40× captures of axons stained with NF200 (green) and DAPI at the lateral funiculus of the thoracic spinal cord at day 52 of active EAE. Mice with XY CNS have fewer number of axons compared with mice with XX CNS ($P < 0.05$, XX→XX vs. XX→XY$^-$, and $P < 0.001$, XY$^-$→XX vs. XY$^-$→XY$^-$, two-way ANOVA, *Second Row Right*). (*I–L*) Representative 10× captures of midsaggital cerebellar white matter stained with MBP (red) and granule cell layer stained with DAPI (blue). Mice with XY CNS have less MBP staining intensity in the cerebellar white matter compared with mice with XX CNS ($P < 0.05$, XY$^-$→XX vs. XY$^-$→XY$^-$, and XX→XX vs. XX→XY$^-$, two-way ANOVA, *Third Row Right*). (*M–P*) Representative 20× captures of Purkinje cells stained with Calbindin-D28K (black) in the midsaggital plane of the cerebellum. Mice with XY CNS have less organized and fewer number of Purkinje cells compared with mice with XX CNS in the setting of the same immune system ($P < 0.01$ for XY$^-$→XX vs. XY$^-$→XY$^-$, and XX→XX vs. XX→XY$^-$, two-way ANOVA, *Bottom Right*). Graphs are displayed as mean ± SEM $*P = 0.05$, $**P < 0.01$, $***P < 0.001$. Data are representative of two repeated experiments.

XY CNS, Compared with XX, Demonstrates Greater Synaptic Loss in the Cerebral Cortex in EAE. We next examined the integrity of the cerebral cortex, focusing on the primary somatosensory strip representing the hindlimbs (S1HLs; Franklin & Paxinos, Bregma –1.23 to –1.31 mm, plates 41–42, ref. 17) and adjacent primary motor cortex (M1) because these regions process afferent sensory information from the spinal cord and convey efferent motor behavior that are continuously modified by cerebellar input. Because cortical synaptic loss is one of the earliest signs of neurodegeneration in several neurological diseases including EAE (18–21), we assessed pre- and postsynaptic loss with antibodies to Synapsin 1 and postsynaptic density protein 95 (PSD-95), respectively. Mice with XY CNS, compared with XX, had less PSD-95 expression in the S1HL and M1 region (Fig. 3 *A* vs. *B*, *C* vs. *D*, and *E*). Mice with XY CNS, compared with XX, also demonstrated a trend of lower Synapsin 1 expression, but this did not reach statistical significance (Fig. 3*F*).

XY CNS, Compared with XX, Has More Tlr7-Expressing Neurons in EAE. Because the molecular pattern recognition receptor *toll-like receptor 7* (*Tlr7*) is X-linked and its signaling in neurons has been shown to cause neurodegeneration (22), we examined the levels of Tlr7 expression in the cerebral cortex. Tlr7 was abundantly expressed in the cerebral cortex of EAE mice (Fig. 4 *A–E*), particularly in cortical neurons in layers I–VI (Fig. 4 *F–H*). Notably, mice with XY CNS, compared with XX, had higher percentages of Tlr7-expressing cortical neurons, as evidenced by Tlr7 and neuronal nuclei (NeuN) colocalization (Fig. 4 *F–I*). Because Tlr7 has a widely known role in innate immune responses in antigen-presenting cells of the immune system and Tlr7 signaling in microglia can lead to neurodegeneration, we also examined Tlr7 in microglia. Interestingly, Tlr7 was minimally expressed in microglia in the cerebral cortical layers I–VI (Fig. 4 *J–L*), and there were no sex chromosome effects on Tlr7 expression in microglia (Fig. 4*M*). Together our data suggest that increased Tlr7 expression in neurons of XY$^-$ mice leads to more neuronal degeneration compared with XX during EAE. This conclusion would be consistent with observations by others that neuronal Tlr7 expression can cause neurodegeneration (22). However, further experiments using neuronal *Tlr7* conditional knockouts backcrossed onto the FCG sex chromosome model would be required to show a causal relationship between increased Tlr7 in neurons and increased neurodegeneration in the XY$^-$ CNS.

Sex Chromosome Effects in the CNS Are Not Confounded by Sex Chromosome Effects in the Immune System of Chimeras. To rule out an effect of sex chromosomes in the immune system in our comparisons, spinal cords were assessed for the quantity of infiltrating T cells and macrophages based on anti-CD3 and anti-CD68 antibody staining, respectively. There were no effects of sex chromosomes in the CNS on CD3$^+$ T-cell (Fig. S2 *A* and *B*) or CD68$^+$ macrophage infiltration (Fig. S2 *C* and *D*).

Then, we examined cytokine production in ex vivo autoantigen-stimulated splenocytes. There were no differences between any groups in IFNg, TNFa, IL-6, IL-2, IL-12p40, IL-17, IL-27, IL-4, IL-5, IL-10, and IL-13 cytokine levels, as well as matrix metalloproteinase-9 (Fig. S3).

Our experimental design also allowed us to examine the role of sex chromosomes in the immune system without confounding variation in sex chromosomes in the CNS. We compared clinical EAE data between BMC reconstituted with XX versus XY immune systems in the setting of the same CNS (XX→XX vs. XY$^-$→XX, and XX→XY$^-$ vs. XY$^-$→XY$^-$). Interestingly, these comparisons revealed no difference in clinical EAE when assessing for effects of sex chromosome complement in the immune system in these chimeras (Fig. S4 *A* and *B*).

Differential Tlr7 Expression Does Not Explain Sex Chromosome Effects in the Immune System During Adaptive Immune Responses. We next asked whether differential expression of the same sex chromosome

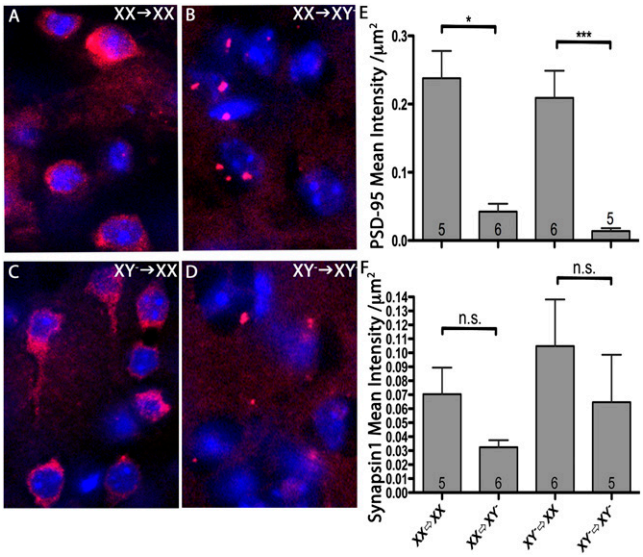


Fig. 3. Mice with XY CNS, compared with XX CNS, have greater synaptic loss in the cerebral cortex. (*A–D*) Representative 60× captures of PSD-95 protein staining (red) in cortical neurons of the S1HL region of the cerebral cortex. Tissues were counterstained with DAPI (blue). (*E*) Mice with XY CNS have less PSD-95 staining intensity compared with mice with XX CNS ($P = 0.05$, XX→XX vs. XX→XY$^-$, and $P < 0.001$, XY$^-$→XX vs. XY$^-$→XY$^-$, two-way ANOVA). (*F*) Trend of less Synapsin 1 staining in mice with XY CNS compared with mice with XX CNS.

gene that is related to worse neurodegeneration in XY$^-$ mice might also be responsible for the increase in adaptive immune responses in XX mice that were previously observed in our publication (9). Specifically, we wondered whether the former is an effect of higher Tlr7 expression in neurons of XY$^-$ mice and the latter an effect of higher Tlr7 expression in immune cells of XX mice. Notably, the place to look for differential expression of Tlr7 playing a role in sex chromosome effects in the immune system would be where we had previously found an effect of sex chromosomes in the immune system, namely in LNCs from autoantigen-immunized adult mice (9). We would not want to assess Tlr7 in the immune system of BM cells developed in irradiated hosts, as there was no effect of sex chromosomes in those immune cells. Thus, we repeated the adoptive EAE results from our previous paper (9), whereby adoptively transferred autoantigen-stimulated XX LNCs conferred greater encephalitogenicity compared with XY$^-$ LNCs (Fig. S5*A*). In parallel, separate aliquots of these same LNCs were examined for Tlr7 expression including subpopulations of T lymphocytes, B lymphocytes, and macrophages. We found that Tlr7 expression levels were no different between LNCs derived from XX versus XY$^-$ in any immune cell subset (Fig. S5*B*). Given the numerous genes expressed on sex chromosomes, particularly the X chromosome, it is not surprising that a single gene (*Tlr7*) would not be responsible for both the effect of sex chromosomes on the CNS and the effect of sex chromosomes on adaptive immune responses. Other candidate genes on X, including *forkhead box P3* and *CD40 ligand*, are now being pursued for playing a role in sex chromosome effects on adaptive immune responses.

Discussion

This report demonstrates an effect of sex chromosome complement in the CNS response to injury. Specifically, having an XY CNS, as opposed to XX, results in more axonal and neuronal loss and greater demyelination in the spinal cord and cerebellum during an immune-mediated injury. An XY CNS compared with XX also resulted in greater synaptic loss in the cerebral cortex during EAE, particularly in the somatosensory and primary motor strip. Synaptic loss is one of the earliest signs of neurodegeneration in several neurological disorders, particularly Alzheimer's and Huntington disease, and in EAE (18–21, 23, 24).

Much of the literature on the origins of sex-biased neurological diseases has focused on the role of sex steroid hormones, particularly the role of estrogen and testosterone in neuroprotection (20, 21, 25–28). The increased female bias for MS could theoretically result from deleterious effects of physiologic levels of female sex hormones or protective effects of male sex hormones. In the MS model, no studies have shown deleterious effects of endogenous estrogens as would be evidenced by improved EAE in ovariectomized versus sham-operated mice. Rather, one group showed that endogenous levels of estradiol protect from EAE as ovariectomized were worse than sham-operated groups (29), whereas other groups found no effect of ovariectomy (30, 31). Very high pregnancy levels of estrogens are clearly protective in EAE (31, 32), but there is a dose effect whereby lower doses, similar to those present during the menstrual cycle, may not be high enough to provide significant protection (31, reviewed in ref. 2). In contrast, endogenous circulating levels of testosterone in males have clearly been shown to be protective in EAE, with numerous groups having shown that castrated young males show worse EAE scores in mouse strains characterized by a female bias (33, reviewed in ref. 2). Together, there is no evidence that endogenous circulating levels of estrogens are deleterious in females, but there is evidence that endogenous levels of testosterone may be protective in males. Thus, testosterone-mediated protection could contribute to sex differences in EAE. However, this protective effect of endogenous testosterone in males does not exclude an additional role of sex chromosomes.

Our finding of sex chromosome complement effects in the CNS during EAE has implications for the role of sex chromosomes in MS as well as other neurological disorders with a sex bias, such as Alzheimer's disease, Parkinson disease, schizophrenia, and stroke. Our results suggest that other sex-related factors can play a role and have been underappreciated. Indeed, a large proportion of sex chromosome genes, particularly those on the X chromosome, are involved in brain development and function (10). Our current studies suggest that there are sex chromosome genes expressed in the CNS in mouse and humans that may affect the progression of sex-biased neurological diseases.

Ultimately, in intact mice and humans, sex differences will be due to the contribution of both sex hormones and sex chromosomes. Here, it is fascinating that the XY sex chromosome complement is associated with more neurodegeneration in EAE, as testosterone treatment is known to be neuroprotective in both EAE and cuprizone-induced chronic demyelination (20, 34). A yin-yang, or compensatory, effect between sex hormones and sex chromosomes has been previously postulated and found in other systems (35, 36). Together, it is tempting to speculate that the XY sex chromosome complement may drive greater neurodegeneration in CNS diseases, but this is held in check in young males with high levels of testosterone. This protection is eventually lost, however, as testosterone levels begin to wane gradually during andropause, which initially starts at approximately age 30 in humans.

Our comparison of XX versus XY reveals differences in the nonpseudoautosomal regions (non-PARs) of X versus Y, as opposed to genes on the terminal recombining PARs that are shared between X and Y. Because this is a relative comparison, a gene on non-PAR Y could lead to more neurodegeneration or a gene on non-PAR X could lead to CNS resilience. The first possibility is somewhat unlikely, as there are very few genes on non-PAR Y that relate to processes other than reproduction and most sex chromosome effects published to date are X chromosome effects (37, 38).

Regarding an effect of a gene on non-PAR X promoting resilience, this could occur in two ways. First, a gene on the X chromosome that escapes X inactivation would be expressed

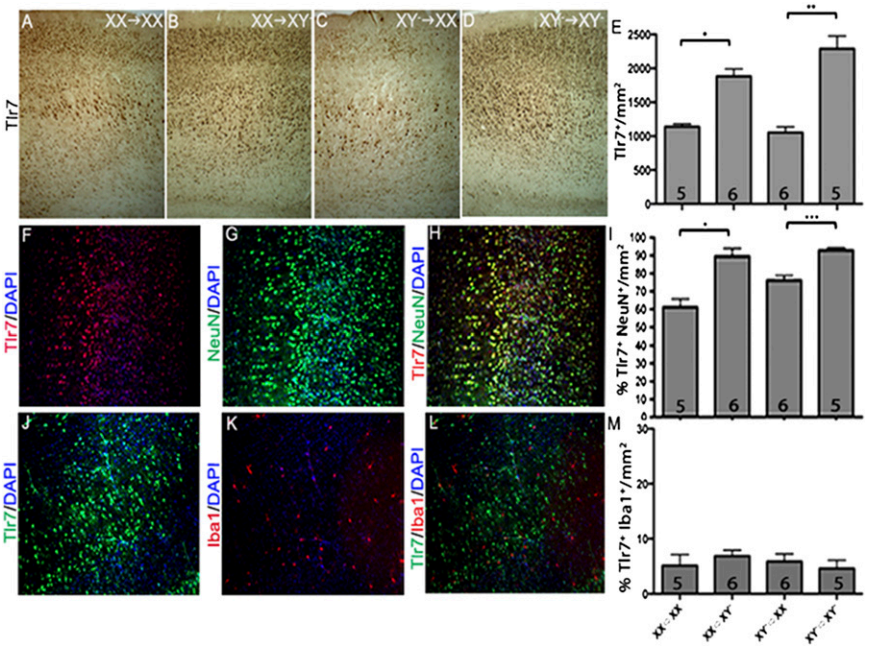


Fig. 4. Mice with XY CNS, compared with XX CNS, have more Tlr7-expressing cortical neurons in EAE. (*A–D*) Representative 10× capture of coronal sections of cerebral cortical layers I–VI stained with Tlr7 in XX→XX, XX→XY$^-$, XY$^-$→XX, and XY$^-$→XY$^-$ BMCs. (*E*) SJL mice with XY CNS, compared with XX, have more Tlr7$^+$-expressing cells in the cerebral cortex ($P < 0.05$, XX→XX vs. XX→XY$^-$, and $P < 0.01$, XY$^-$→XX vs. XY$^-$→XY$^-$, two-way ANOVA). (*F–H*) Representative 10× confocal capture of a coronal section of cerebral cortical layers I–VI stained with Tlr7 (red) and NeuN (green), where *F* represents red channel only, *G* represents the green channel only, and *H* represents a merged image. Tissues were counterstained with DAPI (blue). (*I*) SJL mice with XY CNS, compared with XX, have a higher percentage of Tlr7$^+$-expressing cortical neurons ($P < 0.05$, XX→XX vs. XX→XY$^-$, and $P < 0.001$, XY$^-$→XX vs. XY$^-$→XY$^-$, two-way ANOVA). (*J–K*) Representative 10× confocal capture of a coronal section of cerebral cortical layers I–VI stained with Tlr7 (green) and ionized calcium-binding adapter molecule 1 (Iba1) (red), where *J* represents green channel only, *K* represents the red channel only, and *L* represents a merged image. Tissues were counterstained with DAPI (blue). (*M*) There were few Tlr7$^+$ microglia in cortical layers I–VI, and no differences in the number of Tlr7$^+$ microglia were observed between all groups.

at increased dosage (twice in females vs. once in males), and this X gene could promote neuroprotection. If differential Tlr7 expression indeed causes neurodegeneration, our data showing higher Tlr7 expression in XY CNS compared with XX are not consistent with X dosage. Had *Tlr7* escaped X inactivation in XX mice, we would have observed the opposite results, with XX mice having higher levels of Tlr7 compared with XY$^-$. Second, a gene on the X chromosome that is X inactivated but has differential imprinting (paternal vs. maternal) would be differentially expressed in X^{m}X^p versus X^{m}Y mice (39, 40). Specifically, all cells from XY$^-$ mice express the maternal imprint, whereas half of cells in XX mice express the maternal imprint and half the paternal imprint. Such unique mosaicism in X gene expression occurs in XX individuals due to the random inactivation of either the maternal or paternal X on a cell-to-cell basis. In our model, mice with one copy of the X chromosome (XY) have greater expression of the X gene *Tlr7* in neurons than mice with two copies of the X chromosome (XX). This indicates that differential *Tlr7* expression is not due to X dosage but is more likely due to parental imprinting. Specifically, the paternally imprinted X chromosome has reduced *Tlr7* expression, resulting in less Tlr7 in X^{m}X^p compared with X^{m}Y mice. Consistent with this hypothesis, another gene on X, *Xlr3b*, has been shown to be affected by imprinting, is expressed in frontal cortex and hippocampus, and plays a role in cognitive function in mice (41). Specifically, the paternal imprint has reduced *Xlr3b* expression, with higher levels of expression driven by the maternal imprint inducing neurodegeneration.

Our data demonstrating no sex chromosome effects in the immune system of BMCs in the setting of the same CNS further extend our previously published findings of an effect of XX, compared with XY, on autoantigen-stimulated LNCs being more encephalitogenic in the induction phase of adoptive EAE. In the present data, sex chromosome effects were not observed in hematopoietic stem cells derived from BMs of 5-wk-old XX versus XY$^-$ mice used to reconstitute age-matched host immune systems 7 wk before active EAE induction. In contrast, previously, sex chromosome effects were observed in XX versus XY lymphocytes derived from lymph nodes of 8-wk-old PLP-immunized mice that were then stimulated ex vivo with autoantigen and adoptively transferred to naive recipients (9). Together, these two complementary findings show that the earlier observation of a sex chromosome effect on the immune system, specifically on the adaptive immune response, is not inherently present in BM cells of juvenile mice, but rather becomes evident either after these BM cells undergo maturation or upon immunization to induce the adaptive immune response. Further studies defining when and how sex chromosomes affect the immune system are now warranted.

In summary, sex chromosomes may independently affect the CNS response to a given immune-mediated injury, potentially bearing some relevance on the fact that despite increased MS susceptibility in females, males tend to have more disability progression (2–8). Further, our data indicate that it is important to consider a potential role for sex chromosome genes in future studies of sex differences in neurological diseases. Finally, the model described herein will be an important tool in examining sex differences in a variety of CNS diseases, as most CNS diseases have an immune component.

Materials and Methods

Animals. Mesenchyme forkhead 1 XY$^-$ (*Sry*+) males (Y$^-$ chromosome of 129 origin) were backcrossed with WT SJL and C57BL/6 females for over 20 generations to obtain litters consisting of the following genotypes: gonadal female XX and XY$^-$ and gonadal male XX (*Sry*+) and XY$^-$ (*Sry*+) (42). Only gonadal females were used in these studies.

Surgery. All donor and recipient mice were ovariectomized at 4 wk old as described (33).

BMC. BM cells were isolated from both femurs and tibias of age-matched, 5-wk-old, ovariectomized donors and depleted of T and B cells using anti-thymus cell antigen 1.2 (Thy1.2) and anti-CD19 microbeads, respectively, and AutoMACS Magnetic Cell Separator (Miltenyi Biotec) according to the manufacturer's instruction. Thy1.2$^-$ and CD19$^-$ cells were transferred i.v. into 4-wk-old irradiated recipients by tail vein injection at the concentration of 1.5×10^7 cells/0.2 mL injectable grade PBS per animal, which were irradiated with 850 rads. Detailed methods are described in *SI Materials and Methods*.

EAE. Seven weeks after reconstitution, at age 12 wk, active EAE ensued and animals were monitored daily for EAE signs based on a standard EAE 0–5 scale scoring system, as previously described (9).

Histological Preparation. Mice were anesthetized and perfused, and tissue was collected as previously described (43).

Cell Culture and Cytokine Analysis. Spleens were quickly extracted during perfusion and processed for cell culture as previous described (43).

Immunofluorescence and Chromagen. Tissue sections for immunofluorescence and chromagen were treated as previously described (43). All antibodies used and detailed methods are described in *SI Materials and Methods*.

Fluorescence in Situ Hybridization. Fluorescence in situ hybridization was performed on splenocytes to identify X and Y chromosomes using the probe mix RAB9B (XqF1)/WC Y (Kreatech Diagnostics) according to the manufacturer's instructions, described in detail in *SI Materials and Methods*. Under a 40× objective, a single color red under the Cy5 channel and a single color green under the FITC channel within a $DAPI^+$ cell identified XY, whereas two red signals identified XX.

Reconstitution Efficiency. To assess reconstitution efficiency, 150 or more splenocytes taken from XX→XY^- and XY^-→XX chimeras were identified under a 40× objective as either XX or XY according to fluorescence in situ hybridization labeling. The percentage reconstitution was calculated based on the number of donor cells present in the total cells counted, normalized to the efficiency of the Y probe stain.

Microscopy and Quantification. Stained sections were photographed and quantified as previously described (43). The detailed protocol can be found in *SI Materials and Methods*.

Statistical Analysis. EAE severity significance was determined by repeated measures one-way ANOVA (Prism5). Immunohistochemical and cytokine data were analyzed by one-way ANOVA, and post hoc analysis was performed on F-stat values and significance was determined at the 95% confidence interval.

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