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Sexual dimorphism in the white matter of rodents

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Abstract

Sexual dimorphism of astrocytes and neurons is well documented in many brain and spinal cord structures. Sexual dimorphism of oligodendrocytes (Olg) and myelin has received less attention. We recently showed that density of Olg in corpus callosum, fornix, and spinal cord of wild-type male rodents are more densely packed than in females; myelin proteins and myelin gene expression is likewise greater in males than in female rodents. However, glial cell proliferation and cell death were two times greater in female corpus callosum.

Endogenous sex hormones, specifically lack of androgens, produce an Olg female phenotype in castrated male mouse. In vitro studies using Olg culture also showed differences between males and females Olg survival and signaling pathways in response to sexual hormones. Sexual dimorphism of white matter tracts and glia in rodents indicates the necessity for controlling gender in experimental studies of neurodegenerative disorders. Most importantly, our studies suggest that hormones may contribute to sexual dimorphism observed in certain human diseases including multiple sclerosis.

Keywords

sexual dimorphism; oligodendrocytes; glia; proliferation; sex hormones

BACKGROUND

Sexual dimorphism in the brain was demonstrated for the first time in 1976 by Nottebohm and Arnold in the vocal control area of the songbird [1]. This report prompted a series of studies in rodents and humans that focused on sexual dimorphism of neurons and astrocytes in the hypothalamus and hippocampus [2,3,4]. In men, the amygdala, hypothalamus, and fronto-medial cortex have larger volume relative to cerebral volume [5]. Using MRI, morphological

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differences have been mostly reported in cortical gray matter volume [6,7] with human females having larger volumes of areas associated with language functions [8,9]. A few studies have assessed the white matter differences between sexes and reported that men, compared to women, have larger overall white matter relative to cerebral size [6,5] while others groups showed that women have larger white matter volumes involved in interhemisphere connectivity [10,11]. Postmortem studies of sexual dimorphism reported a larger genu of the corpus callosum and part of the anterior body [12] in men than women. These findings suggest that fibers are larger in males than in females.

In recent years, sexual dimorphism in animals was explained by the action of different sex hormones on brain development. Both testosterone and estrogen were implicated in sexual differentiation. In fetal and early postnatal period there is high concentration of sex steroid receptors in cortical regions including corpus callosum [13] which recedes postnatally, a differential localization between androgen and estrogen receptors [14,15] suggesting differentiation in density and size of specific neurons where aromatization takes place, and a drop in cortical estrogen receptors and levels of messenger RNA postnatally suggesting that estradiol may modulate cortical differentiation [16,17].

Sexual dimorphism of Olg and central myelin has received less attention. Central white matter of CNS contains myelinated and unmyelinated fibers and glia cells. A few studies examined the existence of sexual differences in the white matter tracts, and showed that white matter volume [7], especially in the corpus callosum [18] is increased in males. At the microscopic level, an increase in the number of myelinated axons in the splenium of male rat corpus callosum compared to female rat has been described [19,20]. Similar findings were found in the genu of corpus callosum [21]. Females have more unmyelinated fibers than males. Using electron microscopy and stereological principles, an increase in size and myelination of corpus callosum between adulthood and middle-age in rats was reported [22]. A more recent study in rats using similar methodology confirmed that white matter volume, the volume of myelinated fibers and the volume of the myelin sheaths in young male rats are significantly larger than those of young female but in middle-aged animals the ratio was reversed [23]. It was suggested that differences in levels of steroid hormones in developing animals may be responsible for sex differences in myelination of axons due to potential increase in expression of myelin proteins in the central nervous system by steroid hormones [24,20]. Furthermore a protective effect on myelin of sex steroids was reported not only in developing white matter but also in injured white matter. For example, estradiol has a dose-dependent protection effect against oxygen-induced apoptotic cell death in primary oligodendrocytes [25], progesterone restored myelination and increased density of oligodendrocytes progenitors in spinal cord injury model [26], and also progesterone in combination with estradiol in experimental autoimmune encephalitis (EAE), a model of multiple sclerosis restored myelin proteins expression [27].

Previous studies hint that the volume of myelin is less, and fewer Olg are present in females compared to males. In our previous histological and immunocytochemical studies of neuroglia in the spinal cord, we suspected apparent differences in numbers of neuroglia between males and females [28] but never quantified these observations.

In this brief review of our work and work by others we describe molecular and biochemical differences between sexes at level of oligodendrocytes in rodents and show that exogenous hormones influence survival of oligodendrocytes in vivo and vitro with greater changes in proliferation and cell death in females than males.

SEXUAL DIMORPHISM OF OLIGODENDROCYTES

Using two different methods, immunohistochemistry and in situ hybridization, we quantified the number of Olg in male and female rodents. Using an antibody directed to carbonic

anhydrase-II (CA II), which is a protein isoform expressed only in Olg, we found in certain white matter tracts, corpus callosum, fornix (Fig 1) and ventral funiculus of spinal cord a 22-35% greater number of oligodendrocytes in males compared with females rodents [29]. Using in situ hybridization to assess expression of a different Olg protein's (proteolipid protein) message, an approximate 30% increase in cell number was found in male's corpus callosum and fornix (Table 1) confirming difference in density of Olg between sexes seen with CA-II immunohistochemistry. The difference in Olg number was more striking in younger rodents where males had 40% more Olg compared to females.

The increased density of Olg prompted us to assess myelin-specific proteins and myelin gene expression as Olg are the myelin producing cells. The most abundant myelin protein is myelin basic protein (MBP) accounting for 40% [30]. Total MBP protein levels, summing all four isoforms was found to be 20-25% greater in male brain and spinal cord of mice. Although the total MBP expression was higher in male, the 21,5 kDa isoform was more abundant in female by approximately 30-35%. The role of 21.5 kDa isoform is not known, but is transported to nucleus [31], raising suspicion that it regulates transcription differently between sexes. In male rodents, the level of Olg proteins messenger (proteolipid protein and CA-II), determined by real time PCR, was 170 and 320%, respectively, higher in male suggesting increase in gene and protein expression in Olg in male compared to female. As a result of increased density of Olg in male white matter tracts we expected to find an increase in cell proliferation in males. Surprisingly, we found two times greater proliferation of glia cells in female corpus callosum than in males from late postnatal to aged animals. 85-93% of proliferating cells were astrocytes and Olg. To better explain increased proliferation of glia cells in females, but lower total cell number than in males, a higher percentage of Olg must die in females than in males. Indeed, cell death was assessed using immunostaining of glia cells with active caspase-3 (a marker for apoptosis) and revealed a 50% higher percentage of cell death in females compared with males. Moreover assessing u-calpain expression, a protease implicated in degradation of myelin [32,33], we found it was two times greater in female spinal cord and brain, supporting previous findings of increased cell death of Olg. Because proliferation and cell death are increased in females, Olg in female rodents probably have a shorter life span and a more rapidly turnover than male Olg.

We clearly found sexual differences in male and female rodents not only in density of Olg but also in Olg's myelin protein and message expression, and proliferation and cell death of glia cells.

HORMONAL ROLE IN SEXUAL DIMORPHISM OF OLIGODENDROCYTES: IN VIVO EVIDENCE

We and others showed the presence of steroid receptors in Olg; the estrogen receptors (ER) α and β [34,35,36], androgen [37] and progesterone receptors [38]. Moreover Olg can synthesize progesterone from pregnenolone, but they do not express enzymes necessary to synthesis estradiol or testosterone as do astrocytes [39]. However, in tissue cultures Olg proliferation, maturation and cell death depend on estrogen concentration [40,41] while progesterone has various effects in vivo [42] and in vitro where an increase in cellular branching was reported [40,41]. Hormonal influence on proliferation of Olg in vivo has not been studied before, but castration and/or treatment with exogenous hormones showed that testosterone and estradiol are protective or decrease severity of disease in EAE [43,44]. It is hypothesized that reduction of disease severity in EAE is due to decreased of proinflammatory cytokine production with estradiol treatment [44].

To test the effect of sexual hormones in rodents Olg, castrated males at 6 months were injected in our lab with bromodeoxyuridine (BrdU) one week before they were 9 months old and

compared with 9 month old male and female mice that were also injected with BrdU. Cells incorporate BrdU only while in S phase of the cell cycle, approximately 6-8 hours for glia cells [45] being a useful marker for cell proliferation. The number of BrdU positive cells, doubled in the castrated male, matching the female phenotype. In the same time the number of Olg (CA-II positive cells) in corpus callosum and fornix decreased compared with unoperated males. (Figs 2,3). These findings of increased glia cell proliferation and decreased density of Olg in castrated mice imply that glia cell death is also increased in castrated mice and show that primary sex hormones, presumably testosterone, influence proliferation and probably cell death of Olg. It is also possible that loss of androgens could not be compensated by local production of neurosteroids.

HORMONAL ROLE IN SEXUAL DIMORPHISM OF OLIGODENDROCYTES: IN VITRO EVIDENCE

Recently, we examined the direct effects of sex hormones on Olg using enriched Olg primary cultures from male and female mice. Different concentrations of progesterone (P2), estrogen (E2) and dihydrotestosterone (DHT), either alone or in various combinations, were added to enriched Olg cultures to determine their effects upon total cell numbers, proliferation, and cell death [46]. Female fluctuation of P2 concentration is well known and depends on the phase of estrous cycle [47] as well as pregnancy [48], supporting theoretical contribution of P2 to Olg sexual dimorphism. When hormones were added to Olg culture, P2 significantly increased the number of Olg in both sexes (but more so in female cultures), E2 had a minor effect on Olg number and DHT reduced Olg numbers in both sexes. When P2 was combined with DHT, the two hormones mitigated the reduction in Olg numbers caused by DHT alone; the combination of P2 with E2 lead to a decrease in the number of Olg, especially in females. Further, the changes in Olg number is attributable to regulation of cell death and not to proliferation since hormones did not alter incorporation of BrdU in cells [46]. Indeed, the number of caspase-3+ cells decreased in direct proportion to the increase in the total number of cells. The regulation of Olg death is likely dependent upon different signaling pathways as in vivo studies show that DHT is either protective or detrimental to neurons depending on differences in the signaling pathways activated by the intracellular and the membrane androgen receptors [49]. We examined protein kinase B (Akt), MAPK (Mitogen Activated Protein Kinase) and mammalian target of rapamycin (mTOR) pathways because they are abundant in Olg [50,51], activated by hormones, and they are the most frequently studied pathways in relation to survival and proliferation of cells. Akt activation phosphorylates downstream proteins, mTOR being one of them which affects further proliferation or cell survival [52,53]. P2 in our Olg cultures up-regulated pAkt more in females than males correlating with the effects of P2 on Olg numbers. Akt influences cell survival and proliferation by downstream mTOR activation via transcriptional regulation [52,53]. Indeed, in our system P2 treatment increased pmTOR manifold, indicating the role of Akt/mTOR pathway in Olg survival. Blocking the Akt pathway with a PI3 Kinase inhibitor abolished the effects of P2 further confirming the influence of this pathway in regulating Olg survival. In contrast, and as expected from reducing the number of Olg, DHT led to reduction of pAKT in both sexes. Combination of hormones showed trends in the activation of signaling proteins and while not significant, the P2 and DHT combination reduced pAkt levels by half and increased pMAPK44 by about a third [46]. Both MAPK 42/44 is activated in excitotoxic cell death, induced in Olg by glutamate and in pro-inflammatory processes mediating neurodegenerative diseases [54,55]. The timing and duration of MAPK activation is likely to be important in determining the extent to which Olg's die [56,57] but this parameter has not been thoroughly examined. Our and others results suggest complexity of hormonal interactions and signaling pathways.

The picture is more complicated in vivo where interaction between Olg and hormones is not only influenced by endogenous hormones but also by a myriad of other factors such as

neurosteroid synthesis, interaction between neurons and glial cells and most likely growth factors. Moreover, expression of P2 receptors in brain have been shown to be induced by estrogen or testosterone, both in vivo and in vitro, suggesting that P2 receptors may influence sexual differentiation of certain brain structures [58,59].

IMPLICATIONS OF SEXUAL DIMORPHISM IN MULTIPLE SCLEROSIS

We show in rodents for the first time that the density of Olg is greater in males than in females but more importantly we show that these differences are dynamically changing in mature rodents. These differences are partly under the influence of sex hormone activity although the factors responsible for sexual dimorphism most likely involve a combination of hormones as well as other factors. Nevertheless, for both in vivo and in vitro studies, we show the necessity of using proper gender controls in neuro-experimental studies. Since quantitative differences in terms of Olg numbers, myelin proteins, etc. between control and experimental groups are often less than 100% and the differences between male and female outcome measurements are in a similar range, comparison of experimental to control values, unless all animals are of the same sex, might simply be due to sex differences. While the topic of this study was not multiple sclerosis (MS), these studies raise further questions and open venues of research in trying to explain differences between males and females in MS. It has been demonstrated that men have more destructive CNS lesions than women [56] and diffuse axonal loss is more prevalent in men with MS [57]. In another study, the lowest level of testosterone in women correlated with increases in number of brain lesions [58]; other studies showed that during pregnancy the relapse rate and disability progression decreases [59]. Understanding the presence of CNS sexual dimorphism at the cellular level and how hormones influence proliferation and death of Olg may reveal that hormones not only modulate the immune system but also show how glial cells contribute to the differences in disease phenotype between male and female MS patients. Its relevance is now more important in view of underway clinical studies that use sex hormones to altering the course of the disease.

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Fig 1.

Oligodendrocytes in age matched C57BL/6 mouse male (A) and female (B) in brain, at the level of rostral fornix were immunostained with carbonic anhydrase-II (CA-II) antibody. Carbonic anhydrase-II isoform is specific for Olgs identified by their small, round cell bodies from which emanate thin processes. Only Olgs whose nuclei were visible in sections were counted using a Leitz Laborlux microscope equipped with a 1 cm sq grid in the eyepiece. There were more carbonic anhydrase positive (CA-II⁺) cells in males compared with females (see Cerghet et al., 2006 for quantification). Bar = 20µm.

Fig. 2.

To determine proliferation of glial cells, age matched (9 month old C57BL/6 strain) female, male, and castrated male mice were injected intraperitoneally with BrdU for three consecutive days and were sacrificed one week after the first injection. Brain sections (matched at the same anatomical level) were used for immunostaining with BrdU antibody and visualized with a fluorescent secondary antibody. BrdU positive cells were counted blindly in consecutive sections in the entire corpus callosum using a Leica microscope. The number of BrdU⁺ cells in female rodents is roughly twice that of unoperated males. However, the number of BrdU⁺ cells in female rodents is similar to that of castrated males, suggesting castration disinhibits the effects of male hormones on Olg proliferation. ** $p < 0.001$.

Fig. 3.

To determine the density of Olg brain section from age matched female, male and castrated male mice were immunostained with carbonic-anhydrase-II antibody. Carbonic anhydrase positive cells (CA-II⁺) were counted in corpus callosum and fornix. Differences in CA-II⁺ cell number is expressed as percentage increase of cells in unoperated males and castrated males compared with females rodents. Quantitative data revealed a decrease in the number of oligodendrocytes (CA-II⁺ cells) in castrated males compared with unoperated males. *p<0.05; **p<0.001; ***p<0.0001

Table 1

Percentage increases in oligodendrocytes number by two methods: immunohistochemistry (CA-II+ cells) and by in situ hybridization (proteolipid protein mRNA+ cells) in corpus callosum (CC), fornix (FX) and ventral column of spinal cord (VF). Density of oligodendrocytes is increased in white matter tracts in male compared to female and is not species dependent in rodents.

	Immunohistochemistry	In situ hybridization
Mouse CC	18-35%	30%
Mouse FX	20-34%	30-35%
Mouse VF	14-30%	31-37%
Rat CC	33%	
Rat FX	40%	