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Pubertal ovarian hormone exposure reduces the number of myelinated axons in the splenium of the rat corpus callosum

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Abstract

The size of the female rat corpus callosum decreases in response to pubertal ovarian hormone exposure, but the underlying changes in axonal composition have not been examined. In the current study, animals underwent ovariectomy or sham surgery at day 20, and the number of myelinated and unmyelinated axons were examined in young adulthood (2 mo.) using electron microscopy. Ovariectomized animals had a greater number of myelinated axons compared to intact animals, while total axon number was not affected. Ovarian hormone exposure seems to limit the number of axons that become myelinated in the splenium, while not affecting the number of axons crossing through the region.

Keywords

estrogen; progesterone; myelin; oligodendrocyte; adolescence; corticosteroids

The corpus callosum is sexually dimorphic in the rat, with males having a larger total corpus callosum size than females (Berrebi et al, 1988; Fitch et al, 1991; Bimonte et al., 2000a; 2000b; 2000c; Denenberg et al., 1991). The splenium, defined as the posterior 20% of the total callosal length, carries axons from the visual cortex (Kim et al., 1996). Sex differences in the size of the splenium (Nunez and Juraska, 1998) are due to a greater number of myelinated axons within the splenium of males though there are no differences in total axon number (Kim et al., 1996). Differences in myelinated axon number are established by young adulthood (day 60), but are not seen in prepubertal animals examined at 25 days of age (Kim and Juraska, 1997). On the other hand, females have more total axons at day 25. However, because of continued axon elimination in females this difference is abolished by day 60 (Kim and Juraska, 1997). Thus, the onset of puberty around day 35 may affect both myelination and axon withdrawal.

Prepubertal ovariectomy (OVX) at day 25 has been shown to increase the overall size of the corpus callosum in adulthood (Bimonte et al., 2000a, 2000b, 2000c), while OVX at day 70 is without effect (Bimonte et al, 2000c). The underlying structural changes responsible for this increase in size have not been examined. It is possible that the removal of ovarian hormones before puberty reduces axon elimination or increases the number of axons that get myelinated, resulting in a greater callosal size. In the primary visual cortex, which projects axons through

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the splenium, females who undergo prepubertal OVX have more neurons than intact females in adulthood (Nunez et al. 2002), making it possible that OVX is reducing cell death in the visual cortex. This could prevent the continued axon elimination seen in females between days 25 and 60. In the present study, the numbers of myelinated and unmyelinated axons were quantified using electron microscopy in OVX and sham-operated rats, in order to examine the effects of pubertal ovarian hormones on axonal composition in the splenium of the corpus callosum.

Subjects were 14 female Long-Evans hooded rats, descended from Simonsen Laboratory stock and born in the Department of Psychology's breeding colony at the University of Illinois. All procedures were approved by the IACUC at the University of Illinois. Animals were weaned at 25 days and then double or triple housed in standard cages. The light-dark cycle was 12 hours, with food and water provided *ad libitum*.

All ovariectomies (n=8) and sham surgeries (n=6) occurred at postnatal day 20, with puberty typically occurring around 35 days of age. Surgeries were performed under isoflurane anesthesia and animals were given carprofen (5mg/kg) to relieve post-operative pain.

Between 59–62 days of age, animals were weighed, deeply anesthetized with sodium pentobarbital and perfused intracardially with Ringer's solution followed by a buffered solution of 1% glutaraldehyde/4% paraformaldehyde. As described by Kim et al. (1996), the brain was weighed and cut midsagittally, and one blockface was then stained with osmium tetroxide *en block* to measure the overall length of the corpus callosum. Tissue from the other blockface was embedded for electron microscopy and 1µm sections were cut and stained with toluidine blue. An outline of the splenium (posterior 1/5 of the total length) was traced under the camera lucida at 40X, and ImageJ software (Version 1.28, 2002) was used to quantify area.

Silver/gold ultrathin sections (60–90nm) were cut using a Leica Ultracut UCT, stained with uranyl acetate and lead citrate, and digital images were collected at 11,500X using a Phillips CM200 transmission electron microscope. Due to the regional variability in the axonal composition of the splenium (Kim et al., 1996), micrographs were taken throughout its entire dorsal-ventral and anterior-posterior extent using 6 evenly spaced columns. The number of micrographs per column (6–15) varied with the width of the splenium, such that 50–55 micrographs (each 2.7 µm × 2.7 µm) were collected per animal.

The numbers of myelinated and unmyelinated axons were counted in each micrograph. Axons were distinguished based on the presence of aligned microtubules while glia were differentiated by their more irregular shape and lack of microtubules (Figure 1). Some oligodendrocytes also exhibit dark cytoplasm while astrocytes contain ribosomes and pale cytoplasm. Axon density was multiplied by area of the splenium to obtain axon number for each animal. Axon diameter was measured for a minimum of 200 axons of each type (myelinated, unmyelinated) from micrographs spaced throughout the splenium. The smallest axon diameter was measured and did not include the myelin sheath for myelinated axons. Separate one-way analyses of variance were completed for each measure (myelinated, unmyelinated, and total axon number, splenial area, axon diameters) using Systat (Version 11, 2004).

Comparisons of body weight showed a trend for an effect of ovarian hormone exposure (p=.089; mean for OVX= 237g, sham= 211g). No differences were found for brain weight.

Pubertal ovarian hormone exposure significantly affected the number of myelinated axons in the splenium (p=.012; Figure 2a), with OVX animals having 12.8% more myelinated axons compared to sham animals. OVX was without effect on the number of unmyelinated axons or total axon number (Figure 2b and c). The diameter of myelinated and unmyelinated axons did

not differ between groups. There was a trend for an effect of ovarian hormone exposure on area of the splenium ($p=.079$, OVX > sham by 8.4%).

Ovarian hormone exposure resulted in fewer axons becoming myelinated in the splenium. This effect is likely to be the cellular basis for sex differences and ovarian hormone effects on the size of the splenium and the whole callosum itself (Berrebi et al, 1988; Fitch et al, 1991; Bimonte et al., 2000a, 2000b, 2000c; Denenberg et al., 1991; Nunez and Juraska, 1998). It also appears that pubertal ovarian steroids are the cause of sex differences in myelinated axons in the splenium (Kim et al., 1996).

The present study is in agreement with previous preliminary data from our laboratory indicating increases in myelination following pre-pubertal ovariectomy in a broader age range of adult females (Pych et al., 2001). Though unmyelinated axons were not examined in the preliminary study, unmyelinated axon number was quantified in the present study and did not change with ovarian hormone exposure. Therefore, the continued elimination of axons previously demonstrated in females between days 25 and 60 (Kim and Juraska, 1997) does not seem to be dependent on pubertal ovarian hormones. Although OVX results in a greater number of neurons in the primary visual cortex compared to intact females (Nunez et al., 2002), it does not increase the number of axons crossing through the splenium.

The effects of OVX on the number of myelinated axons in the splenium of females may be due to hormonal influences on oligodendrocytes. Sex differences in oligodendrocytes have been demonstrated *in vivo*, where females have a reduced density of oligodendrocytes in the corpus callosum compared to males, though females also have a higher number of proliferating oligodendrocytes (Cerghet et al, 2006). This suggests that the lifespan of oligodendrocytes in females is reduced, which could limit the number of axons that become myelinated. *In vitro*, estrogen delays the exit of oligodendrocytes from the cell cycle which results in higher numbers of proliferating oligodendrocytes in cell cultures derived from female tissue (Marin-Husstege et al., 2004). However, the possible role of ovarian hormones on the sex differences in oligodendrocyte survival *in vivo* remains unexplored. In the present study, removal of estrogen may have reduced oligodendrocyte proliferation but enhanced survival, which could allow for more oligodendrocytes in OVX females to survive and myelinate additional axons. In addition, progesterone can affect oligodendrocytes and myelination. However progesterone has been shown to promote myelination in the central and peripheral nervous system (Ghoumari et al., 2005; De Nicola et al., 2006; Garcia-Segura and Melcangi, 2006) making its role unclear in the present study, where the removal of progesterone and estrogen increased the number of axons that became myelinated.

The interaction of estrogen with corticosteroids is also a possible route for ovarian hormones to influence the development of myelination. Glucocorticoid levels are elevated in females rats compared to males, even at resting conditions (Critchlow et al., 1963), and corticosterone levels are reduced in OVX female rats (Figueiredo et al., 2007). Animals that undergo adrenalectomy at postnatal day 11 or 25 show increased brain weight and greater amounts of myelin compared to intact animals (Meyer, 1983; Meyer and Fairman, 1985; Meyer, 1987; Devenport and Devenport, 1983; Yehuda and Meyer, 1991), suggesting that corticosteroids have an inhibitory effect on myelination. In addition, remyelination by oligodendrocytes in the central nervous system is reduced by corticosteroid exposure (Chari et al., 2006). Direct effects of stress or corticosteroids on the size of the corpus callosum have not been examined in rats. However, there is evidence in humans that severe childhood stress, such as neglect, can result in a smaller corpus callosum (Teicher et al., 2004).

Estrogen also interacts with many growth factors such as insulin-like growth factor-I (reviews by Cardona-Gómez et al., 2002; Cardona-Gómez et al., 2001), brain-derived neurotrophic

factor (Gibbs, 1998; Jezierski and Sohrabji, 2000; also review by Sohrabji and Lewis, 2006), neurotrophin-4 (Jezierski and Sohrabji, 2000), and neurotrophin-3 (Bimonte-Nelson et al., 2004), which could affect myelination. However, the effects of estrogen on growth factor expression differ greatly depending on the age and region examined (Jezierski and Sohrabji, 2000; Scully and Otten, 1995). The interaction of estrogen with growth factors has not been examined in adolescence or in the corpus callosum, making it difficult to draw conclusions regarding the effects on myelination seen in the present study. Further work is necessary to integrate the complicated pattern of effects of ovarian hormones, corticosteroids, and growth factors to determine the mechanism through which pubertal ovarian hormone exposure may affect cell proliferation, survival, differentiation, or growth in the splenium to result in the reduced number of axons that become myelinated.

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Figure 1. Electron micrograph from the splenium of the rat corpus callosum showing myelinated and unmyelinated axons. * indicates myelinated axons, while ^ points to unmyelinated axons and + indicates glial processes. Scale bar= 1 micron.

Figure 2.

The number of myelinated axons (a) was significantly affected by ovarian hormone exposure ($p=.012$), while unmyelinated axons (b), and total axons (c) in the splenium of the rat corpus callosum were not different in young adult animals that were ovariectomized or underwent sham surgery at 20 days of age.