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Sexual Dimorphism of Oligodendrocytes is Mediated by Differential Regulation of Signaling Pathways

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Abstract

Sexual dimorphism of white matter has not been considered important, the assumption being that sex hormones are not essential for glial development. We recently showed exogenous hormones in vivo differentially regulate in male and female rodents the lifespan of oligodendrocytes (Olg) and amount of myelin (Cerghet et al., 2006). To determine which hormones regulate male and female Olg development, we prepared enriched Olg cultures grown in serum free medium with estrogen (E2), progesterone (P2), and dihydrotestosterone (DHT) or their combinations. P2 significantly increased the number of Olg in both sexes but more so in females; E2 had minor effects on Olg numbers; and DHT reduced Olg numbers in both sexes but more so in females. Combinations of hormones affected Olg numbers differently than single hormones. The change in Olg numbers was not due to changes in proliferation but rather survival. P2 increased pAKT many-fold but MAPK levels were unchanged, indicating activation of the Akt pathway by P2 is sufficient to regulate Olg differentiation. DHT reduced pAkt in both sexes but differentially increased pMAPK in males and decreased it in females. Stressing Olg reveals that both sexes are protected by P2 but females are slightly better protected than males. Females always showed greater differences than males regarding changes in Olg numbers and in signaling molecules. Given the greater fluctuation of neurosteroids in women than in men and the higher incidence of multiple sclerosis (MS) in women, these sexually dimorphic differences may contribute to differences in male and female MS lesions.

Keywords

Oligodendrocytes; progesterone; estrogen; testosterone; Akt; Mapk

Introduction

Sexual dimorphism has been demonstrated in grey matter, particularly the hypothalamus (Mong and McCarthy, 1999) and hippocampus (Means and Dent, 1991; Zaidel et al., 1994). While studying neurons, researchers noticed differences in the numbers of myelinated and unmyelinated axons between sexes (Mack et al., 1995). Studies have shown an increase in the number and density of myelinated axons in the splenium of male rat corpus callosum compared to females (Kim et al., 1996). Observations from Skoff's lab (Ghandour and Skoff, 1988; Knapp et al., 1990) hint that sexual dimorphism also exists between the number of Olg and myelin protein expression. More recently, we demonstrated that Olg density and myelin basic proteins are greater in males compared to females at all ages (Cerghet et al., 2006). Our studies also showed greater proliferation and Olg death in females, indicating Olg in females have a shorter life span and a higher turnover rate compared to males.

Interestingly, the profile of Olg in castrated male mice gradually matched the profile of females indicating exogenous steroid hormones, presumably testosterone, regulated Olg number. However, the CNS also synthesizes neurosteroids from cholesterol, suggesting an interplay between exogenously and endogenously produced steroids (Stoffel-Wagner, 2001; Schumacher et al., 2004).

Very few studies have examined the direct effect of these neuroactive steroids upon glia versus indirect effects caused by neuronal mediated activation of glia. Testosterone enhanced the excitotoxic damage caused by treating Olg with AMPA or kainic acid (Caruso et al., 2004) while 17- β estradiol protects Olg from cell death (Takao et al., 2004). E2 differentially activated the MAPK pathway in male and female astrocytes, and male astrocytes exhibited less death than females (Zhang et al., 2002). These studies clearly indicate that sex hormones have major influences on glial number, proliferation, and morphology. Activation of signaling cascades by sex hormones in neurons have been examined in numerous studies (Behl, 2002) but is poorly understood for glia. More importantly, the question of whether hormones differentially activate signaling pathways in Olg that lead to functional differences of male and female Olg has not been investigated. These neurosteroids function via genomic (De Nicola et al., 2003; Patchev et al., 2004) or rapid non-genomic mechanisms (Beyer and Hutchison, 1997) that utilize various signaling pathways (Bhat and Zhang, 1996; Azcoitia et al., 1999). We focused upon the Akt (protein kinase B), MAPK (Mitogen Activated Protein Kinase), and mTOR (mammalian Target Of Rapamycin) pathways because these pathways are activated by hormones in most cells and are present in Olg (Flores et al., 2000; Keshamouni et al., 2002).

Our studies show striking differences between males and females in regards to Olg survival and signaling pathways in response to hormones, confirming our *in vivo* studies that show females show greater changes than males in regards to proliferation and death. From a practical perspective, they demonstrate that sex is a critical variable to be considered in planning and evaluating *in vivo* and *in vitro* studies of Olg development.

Materials and Methods

Two-3 day old B6CBA male and female mice (Jackson Labs, Bar Harbor ME) were used for all cell culture experiments. They were sexed based on the presence or absence of a blackened zone in males and females, respectively, between genitalia and base of the tail. Male mice also have an obvious greater anogenital distance compared to females (Vandenbergh and Huggett, 1995).

Enriched oligodendrocyte cultures

Primary glial cultures were prepared via routine lab procedures (Knapp et al., 1987) from male and female mouse brains that were separately grown in 100mm Petri dishes (2 brains/dish) that were coated with 100 μ g/ml polylysine (Sigma, St. Louis, MO). They were grown in DMEM media (Gibco-Invitrogen, Carlsbad, CA) containing 10% FBS (Gibco) and 1% antibiotic and antimycotic drugs (Gibco-Invitrogen) for 9–10 days at which time the cells became confluent. Then, the upper layer of cells containing mostly Olg precursors and Olg were dislodged by gently blowing the cells with media using a 10cc syringe attached to a 21-gauge needle. The cells were collected with the media and then plated onto another uncoated 100mm Petri dish to allow microglia and astrocytes to adhere to the dish. After 30 minutes, floating cells, which are mostly Olg precursors and Olg, were collected with a 5cc pipette, into a 15cc centrifuge tube, spun down, and counted using a haemocytometer; then an equal number of cells (about 7000 cells/coverslip) from male and female Olg were plated onto 20 μ g/ml polylysine coated 12mm coverslips. From one Petri dish of primary cultures we obtain enough Olg progenitors/Olg for 24 coverslips. These cultures contain

more than 95% Olg (determined by double immunostaining for A007 and GFAP) between 3 and 5 days, the time frame used for the studies. As phenol red is believed to have estrogenic properties, phenol red free medium (GIBCO-Invitrogen) was used for all experiments. Oligodendrocyte cultures for all experiments were grown in DMEM without phenol red or serum, but supplemented with selenium (10nM), transferrin (50µg/ml), insulin (5µg/ml) (all from Sigma, Saint Louis, MO), and ABAM (GIBCO-Invitrogen). After 1 day, cultures were treated with 1, 2.5, 5, 10, 50, 100 and 500nM concentrations of E2, P2 or DHT (Sigma); the number of cells in the coverslips were counted after 3 days of treatment with the hormones (see diagram, Fig. 1), by immunostaining with A007, a marker for post-mitotic immature (A007+/PLP-) and mature Olg (A007+/PLP+) (Bansal et al., 1992).

Immunocytochemistry

Oligodendrocytes were immunostained live with the A007 marker and then fixed with 70% ethanol. Expression was visualized with a donkey anti-mouse IgM Texas Red secondary (1:500) (Gibco-Invitrogen). Nuclei were stained with bisbenzamide (1:1000 dilution). Only Olg with healthy, dispersed nuclear chromatin were counted along the two maximum diameters of the coverslip. Oligodendrocytes with clumped chromatin or absent nuclei (a frequent occurrence) were not counted.

For cell proliferation studies, BrdU (10nM) was added with hormones to coverslips and cells were immunostained live for A007 at 2, 24, or 72 hrs as described above; they were processed for BrdU immunostaining (Yang and Skoff, 1997; Saluja et al., 2001) using a monoclonal IgG (1:100) (Beckton Dickinson, Franklin Lakes, NJ) and visualized with an anti-mouse Alexa green secondary (1:500) (Gibco-Invitrogen).

For caspase immunocytochemistry, a polyclonal antibody against cleaved caspase-3 (1:100) (Cell Signaling Technology, Beverly, MA) was visualized with an anti-rabbit Alexa green secondary antibody (1:1000) (Gibco-Invitrogen).

Microscopy

A007+ Olg were counted along the two longest diameters of the coverslip under 20× objective and 10× eyepiece with a Leica DM IRB phase fluorescent microscope. The number of Olg counted using this method is 70% of the area of the coverslip. Results are expressed as a percentage to the male control using the formula: % of cells = number of cells in treated group/number of cells in male control X100.

To determine generation of new Olg, both BrdU+/A007+ and BrdU-/A007+ cells were counted along the two maximum diameters of the coverslip under 40× objective and 10× eyepiece. Results were expressed as % of proliferating Olg = number of BrdU+ and A007+ cells/total number of A007+ cells X100. The total number of A007+ cells along with cleaved caspase-3+ cells was counted and the percentage of dying cells calculated using the formula above. Because large numbers of A007+/cleaved caspase-3+ cells are located within the boundaries of the two maximum diameters and it was necessary to use a 40× objective to clearly visualize chromatin, twenty random fields were counted for each treatment group and the experiment was repeated twice.

Western blotting

Enriched Olg cultures were prepared from primary glial cultures as described above but were re-plated in polylysine coated T25 flasks. Hormones were added after one day in culture. Three days after treatment with the hormones, protein was extracted from cells by homogenization in a lysis buffer [50 mM Tris, pH 7.4, 1 mM dithiothreitol (DDT), and 0.1 mM EDTA] with protease inhibitors (Zhang et al., 2004) and denatured by heating to 95°C

for 10 minutes. Protein concentration was measured using an Eppendorf bio-photometer (Eppendorf, Westbury, NY). 100µg protein was loaded into a 10% polyacrylamide gel followed by electroblotting onto PVDF transfer membrane. Blots were incubated with pAkt, Akt, pMAPK, MAPK, pmTOR, mTOR all at 1:1000 dilution except for GSK3β (Cell Signaling); followed by incubation with anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase (1:5000) (Jackson Laboratory, Bar Harbor ME) and were developed with the Chemicon chemiluminescence kit (Chemicon, Tamecula, CA). All blots were stripped and reprobed for β-actin (1:5000)(Sigma) and optical density analysis was performed using Image Quant (Molecular Dynamics, Sunnyvale, CA).

Inhibition of PI3 kinase

50µM LY 294002 (Cell Signaling Technology) was added to enriched Olg cultures 1 hour before adding the hormones and throughout the duration of hormonal treatment (4 days). After 4 days, coverslips were immunostained for A007, and the number of Olg counted along the two longest diameters of the coverslip. Results were expressed as a ratio to male control.

Statistical Analysis

One way Anova was used for Fig. 3–Fig. 6 and Student's t-test for Fig. 7–Fig. 11, Fig. 14.

Results

Density of Olg in control cultures

In virtually every experiment (Fig. 3–Fig. 5, Fig. 12–Fig. 13), we found that the number of Olg derived from females was approximately 10–15% greater than those derived from males 4 days after preparing the enriched cultures. The number of Olg progenitors (mitotic) and Olg (post-mitotic) plated onto coverslips was the same for both sexes and carefully controlled (see Discussion for explanations).

Effects of hormones on the density of Olg

We quantified the number of Olg in enriched cultures that were treated for 3 days with different concentrations of P2, E2 or DHT (Fig. 2–Fig. 3). Three days after hormonal treatment, the number of Olg counted per coverslip ranged from about 125 to 320 (see Materials and Methods), providing adequate numbers of cells for reproducible cell counts and statistics. P2 increased the number of Olg for both males and females at 2.5 and 5nM concentrations (Fig. 3A). With 2.5nM P2, the numbers of Olg significantly increased about 40–50% in males and 90–100% in females when compared to untreated male controls. At this concentration, the increase in Olg in females compared to males in the group was significant. At 5nM P2, Olg increased more than 2 fold in both males and females with females having 20% more Olg than males in the group. At 10nM and higher P2, the number of Olg returned to control or lower numbers.

In contrast to P2, E2 had little effect upon the numbers of Olg at doses below 50 nM in both sexes but appear toxic above 50nm (Fig. 3B). Although E2 had little effect on numbers of A007+ cells, we observed clusters of 10–100 cells that resemble oligospheres (Vitry et al., 1999). The number and size of these clusters in cultures dramatically increased when treated with 50nm E2 in females, indicating that E2 may differentially regulate proliferation of neural precursors between sexes (data not shown).

The male hormone DHT reduced the numbers of A007+ Olg 10–35% at all concentrations tested compared to the controls in both sexes. This effect however is more pronounced in DHT treated females compared to female controls (Fig 3C). Because T4 may be converted

to E2 obfuscating interpretation of data, DHT is not converted to E2 permitting evaluation of its direct effects upon Olg.

Effect of hormonal combinations on Olg density in males and females

Because P2 synergizes with E2 to regulate the ratio of proliferation to apoptosis (Seeger et al., 2005) by altering the levels of P2 receptor immunoreactivity in the brain (Dufourney et al., 1997), we examined the effects of hormonal combinations in our culture system. In contrast to the administration of P2 concentrations that led to Olg increases, the same concentrations of P2 (2.5 and 5nM) combined with 10 and 50nM E2 caused a reduction in the number of Olg in both sexes. The effect was generally greater in females, and 5nM P2 and 10nM E2 produced a highly significant decrease in Olg number in females. Lowering the concentration of E2 to 1nM and 5nM with 2.5nM and 5nM P2 caused a slight increase in Olg numbers (Fig. 4A). These results demonstrate that regulation of Olg numbers is exquisitely sensitive to exogenous hormonal concentrations. When DHT was added together with P2, the positive effects of P2 was abolished and, instead, Olg density was reduced 25–30% when treated with 10nM DHT and 5nM P2. However, P2 reduces the negative effect of 1nM DHT concentration in both sexes though it was not statistically significant (Fig. 4B).

Regulation of proliferation and cell death of Olg by hormones

The increases or decreases in Olg numbers in response to hormonal treatment could be due to an alteration in proliferation and/or cell death. To determine influence of neuroactive steroids on proliferation, cultures were treated with hormones in the presence of BrdU. Coverslips were immunostained at 2, 24 or 72 hrs after treatment for BrdU and A007. Results are expressed as a percentage to the total number of Olg. With P2 treatment, the percentage of BrdU+ Olg at any time point was the same as controls (Fig. 5A–B). The number of Olg that incorporated BrdU increased from 5–10% at 2 hrs to about 30–40% at 24 hrs in both P2 and controls. The low percentage of BrdU+/A007+ cells at 2 hours is predicted because cells are in the S or G2 phase and A007+ cells are mainly post-mitotic (Knapp and Skoff, 1991). The number of BrdU+ Olg remained the same from 24 to 72 hrs in both sexes, indicating few A007- Olg proliferated and differentiated into A007+ Olg in this 48 hour interval. With 5 nM P2 treatment, the total number of Olg significantly increased at 72 hrs compared to the controls, implying that P2 increases the survival of differentiated Olg. (Fig. 5B).

With E2, there was a significant reduction in the number of BrdU+ Olg in response to treatment with 50nM E2 at 24 hrs in both males and females compared to controls. This difference was not noticed at 2 hrs and was abolished at 72 hrs indicating that E2 at this concentration might delay the differentiation of Olg because we counted only the cells that have proliferated and differentiated at each time point. No significant changes were noted in the total number of Olg with E2 at any time point tested Fig. 5C, D). This data set matches the data that shows E2 has little effect upon the total Olg numbers (Fig. 3).

Because neuroactive steroids tested did not alter BrdU incorporation, we next looked at differences in cell death in response to treatment with hormones. With 2.5 and 5nM P2, the number of cleaved caspase3+ cells in both sexes was reduced 10–15% compared to controls. This finding indicates that the increase in the density of Olg in response to P2 is mainly due to its effect on survival of Olg (Fig. 6A). In contrast to P2, E2 produced no significant changes in Olg death except for a mild reduction in the number of dying Olg in females with 10nM estrogen (Fig. 6B). DHT treatment increased the number of dying Olg about 15–30% at all concentrations in both males and females. Moreover, the response with 10nM DHT was significantly higher in the females compared to males (Fig. 6C). The increases or

decreases in the numbers of dying Olg are inversely proportional to the decreases and increases in Olg numbers, respectively.

Although P2 by itself reduced the number of caspase3+ Olg, E2 and P2 in combination increased Olg cell death at concentrations that did not have any effect when added alone (Fig. 6D). Reducing the concentrations of E2 abolished this effect, correlating well with the total cell numbers.

Regulation of Akt and MAPK pathways in Olg by hormones

Because Akt and MAPK are the two pathways most frequently studied in relation to survival and proliferation, we examined the activation of these pathways in our culture system in response to P2, E2 and DHT. Enriched Olg cultures were plated in 25cc flasks and treated with P2 (2.5, 5nM), E2 (10, 50nM) or DHT (10nM), concentrations that had the maximum effect on Olg density. After 3 days of hormonal treatment, protein was extracted from the cells and Western blot analysis performed for the amount of phosphorylated and total proteins in the Akt and MAPK pathway. P2 treatment activated the Akt pathway that correlated with the effects of P2 on Olg density. Olg derived from males had a 2× and 4–5× increase in the amount of pAkt with 2.5nM and 5nM P2, respectively. The amount of activated Akt was always higher in females compared to males even in the controls, similar to the changes seen in Olg density in response to P2 treatment (Fig. 7A). No significant changes were seen in the total amount of Akt in response to P2 treatment (data not shown). No significant changes were observed in the total or pMAPK (pMAPK42/44) in response to P2 treatment either in males or females, indicating that pMAPK's are not required for regulating Olg numbers in response to P2 (Fig. 7B–D).

In contrast to P2, treatment with 10 or 50nM E2 in males did not show significant increases in pAkt; however, it caused a significant up-regulation of pAkt in females compared to males given the same amount of P2 (Fig. 8A). Interestingly, this same concentration led to a 10–20% increase in the number of Olg in females. No significant changes were observed in MAPK pathway in response to E2 treatment either in the activated form or the total protein (Fig. 8B–C).

While P2 and E2 treatment caused an upregulation of pAkt, DHT treatment, not surprisingly, significantly reduced the amount of pAkt especially in females (Fig. 9A). Surprisingly, DHT differentially affected the MAPK pathway in males and females. It caused an increase in amount of pMAPK42/44 in treated males compared to untreated males. DHT led to a decrease in the pMAPK42/44 in treated females compared to untreated females given 10nM DHT (Fig. 9B–C).

The combinations of P2 and E2 or P2 and DHT produced modest but not significant changes in pAkt that matches the changes in Olg numbers. They reconfirm the above data that activation of pAkt is sufficient in itself to regulate cell numbers (Fig. 10).

Regulation of signaling proteins downstream of Akt by P2 and E2

Because major changes were noted in the Akt pathway in response to P2 and DHT, we examined regulation of signaling proteins downstream of Akt. As Akt regulates cell survival by blocking GSK3 β and/or by the activation of mTOR pathway via regulation of transcription, we looked at both GSK3 β and pmTOR in response to P2 and E2.

5nM P2 treatment caused an increase in the amount of pmTOR in both sexes, indicating that Akt/mTOR pathway contributes to the changes seen in Olg density in response to P2. Surprisingly, pmTOR increases manifold with 10 and 50nM E2 treatment in both males and females but more in females compared to males (Fig. 11). Because Olg numbers were only

modestly increased with E2, the up-regulation of mTOR suggests it has other functions besides regulating Olg numbers. No changes were seen in total GSK3 β levels in response to either E2 or P2 (data not shown) implying that the effects seen on Olg with the addition of P2 may be due to the activation of mTOR pathway via the activation of Akt.

Inhibition of PI3 kinase reverses the effects of P2

To confirm that the changes observed in Olg numbers in response to P2 activate the pAkt pathway, we blocked the activation of Akt with the PI3 kinase inhibitor (LY294002). Blockade of the phosphorylation of Akt reversed the effects of 2.5nM P2 upon Olg numbers. The effects with 5nM P2 and the inhibitor did not totally reverse Olg numbers. However, it caused a significant reduction in the Olg numbers when compared to numbers of Olg without the inhibitor in P2 treated cultures (Fig. 12). The inhibitor by itself did not seem to have any significant effect on the control cultures either in males or females. Western blot analysis of Olg cultures treated with 5nM P2 along with 50 μ M PI3 Kinase inhibitor LY294002 showed a reduction in the amount of pAkt less than control levels (data not shown).

Effects of growth factor withdrawal on Olg

Our results definitively show differential responses of male and female Olg to neuroactive steroids in terms of cell numbers and activation of signaling pathways. To relate this information to in vivo injury models such as MS or TBI, we began to investigate whether male and female Olg respond differentially to stress. A simple experiment is to withdraw insulin because it causes cell death in Olg cultures and in vivo (Ye et al., 1995; Shankar et al., 2006). Oligodendrocyte cultures were grown for 3 days and the insulin withdrawn from the culture media for 24hrs. After 24 hrs of insulin withdrawal, the numbers of Olg were counted. The number of Olg was significantly reduced about 30% in the males; Olg were reduced 20% but not significantly in females. Addition of 5nM P2 restored normal levels of Olg in males; in females, the number of Olg increased 20% in comparison to untreated females (Fig. 13). As predicted, (Fig. 14) pAkt levels in both sexes decreased in insulin's absence, matching the decrease in Olg numbers. However, the addition of P2 to the insulin deprived cells led to a modest pAkt increase but still below predicted normal levels. pMAPK42 levels were essentially the same in both sexes in response to insulin withdrawal and remained low even with P2 (Fig. 14 A, B). pMAPK44 levels in insulin deprived were lower compared to controls, and agrees with the DHT data showing both decreases in pMAPK44 and Olg numbers. However, the addition of P2 did not increase pMAPK44, as might be predicted based upon the increase in Olg numbers. This data suggests regulation and modulation of other signaling pathways are activated in the absence of insulin.

Discussion

Effects of neuroactive steroids on oligodendrocytes

Our in vivo studies showed that the lifespan and proliferation of Olg is partially regulated by castration (Cerghet et al., 2006). To determine which neuroactive steroids contribute to this phenotypic change, enriched Olg cultures were treated with different concentrations of P2, E2, or DHT for 3 days. After 3 days, the vast majority of Olg progenitors became post-mitotic (Fig. 5) and had differentiated into MBP+ cells. We routinely observed in these older, control cultures that there was always 10–15% more Olg on coverslips derived from females versus males even though we carefully plated the same number of Olg. A similar sexually dimorphic difference in the number of Olg was attributed to serum (Marin-Husstege et al., 2004). However, our culture system is devoid of serum and growth factors except for insulin, suggesting pre-existing conditions contribute to the sexual dimorphism. At the time of plating (2–3 days postnatal), glial progenitors have been exposed to the pre-

and neonatal surge of exogenous androgens and, possibly, estrogens (Mack et al., 1996; Bimonte et al., 2000). Another intriguing possibility is that genetically determined factors predispose Olg to generate these sexually dimorphic differences. Numerous studies now show sexually dimorphic differences in different cell types before exposure to hormones. Muscle stem cells derived from females have higher regenerative capacity than those derived from males even before exposure to hormones (Deasy et al., 2007). Male pre-implantation embryos, including humans, develop faster than females (Avery et al., 1992; Dumoulin et al., 2005). Whatever the causative factors for Olg sexual dimorphism, we find cultured female Olg differ from males in many different parameters whether neurosteroids are absent or present.

Oligodendrocytes are exquisitely sensitive to concentrations of neurosteroids, females much more so than males. Progesterone, at low concentrations, significantly increased the number of Olg in females as much as 50% compared to males. Progesterone, at increasingly higher concentrations, reduced Olg numbers compared to controls, with Olg derived from females showing greater sensitivity. While the differences in Olg numbers between males and females may seem minor, the data is based upon a short 3-day survival. If such differences play out in vivo, the sexually dimorphic regulation of Olg numbers might be quite dynamic. The concentrations of neurosteroids we used were based upon previous in vitro Olg studies (eg., Caruso et al., 2004; Marin-Husstege et al., 2004) and our preliminary studies in which we tested a wide range of concentrations. It is difficult to relate the concentrations of P2 that are effective in culture with in vivo sera and brain levels. P2 concentration in mouse sera depends upon the phase of estrous cycle. In one mouse study (Ganguly et al., 2007), P2 ranged from 30–68 nM depending upon the cycle stage and in another study (Anupriwan et al., 2008), P2 was greater than 100 nM during pseudo-pregnancy but decreased to undetectable levels after ovulation. In rat brain, P2 levels appear to be 4× greater than in serum, presumably due to local steroidogenic synthesis (Schumacher et al., 2004). Because P2 levels fluctuate in female brains and are undoubtedly different than in male brains, P2 is one hormone that likely contributes to the sexual dimorphism of Olg numbers in mice brains. In vivo, of course, the brain is exposed to different neurosteroids that may negate or facilitate regulation of Olg numbers. We show combinations of neurosteroids also affected cultured Olg numbers, and this finding strongly suggests that neurosteroid combinations have an in vivo effect.

We found P2 is sufficient to maintain Olg in a state of differentiation (Fig. 6) but it is also protective because the addition of P2, when added to culture media lacking insulin, protects Olg from death (Fig. 13). Progesterone improves remyelination in a toxin-induced model of demyelination, and was more effective in females than in males (Li et al., 2006; Schumacher et al., 2007). Because P2 accelerates neuronal differentiation, it is unclear whether it directly or indirectly affects Olg.

In contrast to P2, DHT modestly reduced Olg numbers at lower concentrations and highly (20–35%) reduced Olg numbers at higher concentrations. This finding is in agreement with another study (Caruso et al., 2004) in which T4 was toxic to Olg and enhanced the excitotoxic damage caused by treating them with AMPA or kainic acid. In vivo, its effects are poorly understood as DHT either protects or damages neurons, depending on whether it acts via a membrane receptor or an intracellular receptor, either through the MAPK or Akt pathway (Gatson and Singh, 2007).

E2 had little effect on Olg numbers of both sexes at low concentrations; at higher concentrations, it led to a modest reduction in Olg numbers. This finding may seem surprising because E2 plays a major regulatory role in proliferation of many cell types. Our failure to demonstrate an effect of E2 upon Olg is most likely due to the late stage of

differentiation of Olg progenitors when E2 was applied. Our enriched Olg cultures contain clusters of cells lightly attached to the bed layer. The number and size of the clusters increased 2–3 fold in response to E2, with females showing a greater increase than males (data not shown). Many cells in the clusters were NG2+ and nestin+, strongly suggesting E2 affects early stage Olg progenitors and neural precursors, and this finding confirms E2 is biologically active in our system.

Effects of hormonal combinations

Surprisingly, the combination of P2 and E2 at concentrations, at which P2 produced increases in Olg numbers, led to decreases in Olg numbers. However, the combination of P2 and E2 at low concentrations, at which P2 had no effect upon Olg numbers, led to increases in Olg numbers. The combination of P2 with low DHT concentrations led to a modest, although not significant reduction, in cell death compared to DHT alone. Clearly, P2 synergizes with E2 and DHT to regulate Olg differentiation and death. This set of experiments shows (1) the exquisite sensitivity of Olg to concentrations of neurosteroids and (2) these neurosteroids act in concert. We still cannot paint a clear picture as to how neurosteroid combinations affect Olg differentiation; other neuronal studies that used hormonal combinations to evaluate their neuroprotective effects have also yielded variable results that depended on the age of the animal, dose of hormones, and treatment before or after injury (Bramlett, 2005).

Proliferation and death of oligodendrocytes

The changes in Olg numbers caused by P2 and DHT are due to regulation of cell death. None of the hormones altered the rate of incorporation of BrdU into A007-/+ cells 2 hours after incubation. This finding indicates these hormones do not stimulate A007- Olg progenitors and A007+ post-mitotic Olg to re-enter S phase in greater numbers than controls. E2, however, delayed the exit of Olg precursors from the cell cycle in response to mitogen withdrawal, in agreement with a previous study using a different paradigm (Marin-Husstege et al., 2004). It is unlikely these E2 stimulated Olg underwent another round of cell division because total numbers of A007+ Olg did not increase above control levels after the 3 day treatment.

Hormonal regulation of Olg death by hormones is evident because the numbers of caspase-3+ cells changes inversely to the change in Olg numbers. The percent changes in the numbers of caspase-3+ cells do not directly match the percent changes in numbers of Olg but this is predictable because dying cells are caspase-3+ for a short period of time.

Olg derived from females responded more vigorously than males with respect to Olg generation and death in response to hormones. These differences may be viewed as protective or deleterious. In a preliminary study, the withdrawal of insulin for 24 hours without P2 decreased Olg numbers 25% in both sexes but with the addition of P2 the number of male Olg returned to their control levels whereas the number of female Olg increased above their control values. This preliminary study demonstrates the protective effect of P2 when Olg are stressed, and it suggests that P2 has other protective roles in female Olg.

Signaling pathways activated by hormones

Predictably, P2 up-regulated pAkt as much as six-fold in both sexes with females usually having more pAkt than males. Increases in pAkt generally correlate with increases in Olg numbers in a 2.5:1 ratio: a fivefold increase in pAkt corresponds to a twofold increase in Olg. Surprisingly, P2 did not alter MAPK levels in either sex, indicating that activation of MAPKs above their normal levels are not required for Olg differentiation/maintenance in

our system. Akt activation, in turn, phosphorylates either mTOR or GSK3 β that affects proliferation or survival of cells (Hay, 2005; Chong et al., 2007). As expected, P2 treatment increased pmTOR, indicating that mTOR activation is a likely modulator of Olg differentiation. Total GSK3 β , predictably, was unaffected. Activation of Akt by P2, blocked by the PI3 kinase inhibitor LY294002, significantly decreased Olg density. pAkt, in the presence of P2 and inhibitor, was less than control levels but total Akt was unchanged indicating that P2 specifically activates the Akt pathway via phosphorylation (Flores et al., 2000; Pang et al., 2007).

E2 did not lead to changes in pMAPK pathway, even though it activates this pathway in neurons and plays a major role in cell proliferation/cell death (Hayashi et al., 2007; Numakawa et al., 2007). The lack of an effect upon MAPKs may be due to the fact that E2 had little effect upon Olg numbers; more likely, MAPKs are activated in Olg when cell death pathways are activated as shown by DHT.

DHT led to a reduction of pAkt in both sexes, correlating with the reduction in the number of Olg. In contrast to P2 and E2 where pMAPKs were unchanged, DHT altered pMAPKs in a sexually dimorphic manner. DHT caused an increase in activation of MAPKs in males but a reduction in females, suggesting MAPKs function to protect Olg from death. Activation of MAPKs as well as complete suppression of MAPK pathway has been described in cell death in Olg precursors (Horiuchi et al., 2006). MAPKs 42/44 are activated in excitotoxic Olg death induced by glutamate (Rosin et al., 2004) and in pro-inflammatory processes mediating neurodegenerative diseases (Nikodemova et al., 2006). Because downstream targets of MAPK are sensitive to the duration of MAPK activation (Marshall, 1995; Cook et al., 1999), timing and duration of MAPK activation may dictate the extent to which Olg's die. Our studies suggest that MAPKs become activated when apoptosis kicks into place but whether it ameliorates or exacerbates Olg death is still unclear.

Although combinations of these hormones did not show significant changes in either activated or total amount of signaling proteins, certain trends emerged. The combination of P2 and DHT led to a reduction in pAkt levels and an increase in pMAPK44 levels similar to those seen with the treatment of DHT alone. The combination of 5nM P2 with 10nM E2 led to an increase in Olg numbers in males and a decrease in females with no changes in MAPK pathways. We predicted that MAPKs should increase in females, inversely corresponding to the decrease in female cell numbers. These studies show the complexity of interactions between Akt and MAPK pathways, and they also imply additional signaling mechanisms are activated.

These neurosteroids activate signaling pathways by binding to classical nuclear receptors and to membrane receptors associated with caveolae in plasma membranes; the latter is responsible for signaling through Akt (Honda et al., 2000) and MAPK pathways (Migliaccio et al., 1996; Singer et al 1999). We and others have shown these neurosteroid receptors are present in Olg, but when genomic or non-genomic pathways are activated is unclear. Indeed, P2, E2 and DHT are further metabolized to act via their own respective hormone receptors and also other receptors. For example, P2 is metabolized to 5 α dihydroxyprogesterone that acts via the progesterone receptor (PR) and to 3 α , 5 α tetrahydroprogesterone or allopregnanalone that exerts its effect via the GABA receptor. Although these P2 metabolites increased myelination via the GABA receptors, the effect was completely abolished in PR knockout mice indicating that PR receptors are essential for P2 and its metabolic action (Ghoumari 2003). Estrogen metabolites also act via ER dependent and independent mechanisms (Liu and Bachmann, 1998).

These sexually dimorphic differences described here may also be directly relevant to differences in female and male MS lesions. Men have more destructive CNS lesions than women (Weatherby et al., 2000). Moreover, diffuse axonal loss, even in normal-appearing white matter, is more prevalent in men with MS (Miller and Leary, 2007). In one MS study, serum levels of sex hormones, T4 especially, are different compared to controls (Tomassini et al., 2005); in another study, high E2 and low P2 levels correlated with increased numbers of gadolinium enhancing lesions (Bansil et al., 1999). Treatment with T4 in males with MS improved the cognitive functions but no changes were noticed in the number of gadolinium enhanced lesions (Sicotte et al., 2007). Interpretation of these studies is complicated because the differential effects of T4 on MS lesions may due to its conversion to E2 or DHT. The aforesaid studies should not be construed to imply that levels of sex hormones are the root cause of MS but they play exacerbating or ameliorating roles. Certainly, sexually dimorphic differences of the immune system account for some of these CNS differences but further consideration needs to be given to intrinsic CNS sexually dimorphic glial differences and how they may contribute to differences in MS lesions.

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Figure 1.
Diagram illustrates timeline for preparation of enriched Olg cultures and hormonal treatment.

Figure 2.

A007 immunostaining of enriched Olg cultures from controls (a,d) or after treatment with 2.5nM (b,e) or 5nM P2 (c,f) from either males (a–c) or females (d–f). Mature Olg elaborate large membrane sheets that resemble unfurled myelin sheaths. Differences in Olg numbers between males and females are not noticeably different in these representative low magnification pictures.

Figure 3.

Quantification of the A007+ cells in cultures treated with different concentrations of P2 (A), E2 (B), or DHT (C) derived from males and females. 2.5nM P2 shows increases in the number of Olgs in females and 5nM P2 shows increases in both males and females. DHT shows a modest reduction in the number of Olgs. The data is expressed as a percentage change compared to male control (male control is set at 100%). The data is an average of 2–3 experiments and in each experiment 2–3 coverslips was counted for each treatment condition. Values represent mean + SEM. * indicates significant difference compared to male control; # significant difference compared to female control respectively; + significant difference between males and females within same treatment group. *P< 0.05, ** P < 0.01. +P<0.05. #P<0.05.

Figure 4. Percent difference in the number of A007+ cells in cultures treated with different combinations of P2, E2 (A) and P2, DHT (B). Data is expressed as a percentage compared to the male control. Data is an average of 2–3 experiments and in each experiment 2–3 coverslips were counted for each treatment condition. * indicates significant difference compared to male controls; + significant difference between males and females within same treatment group. *P<0.05, + P<0.01.

Figure 5.

Percent of BrdU+/A007+ cells and total A007+ cells in enriched Olg cultures treated with different concentrations of P2 (A, C) and E2 (C, D) after 2, 24 and 72 hrs of hormonal treatment. Data is the average from 3 coverslips at each time point from 3 experiments. * $P < 0.05$.

Figure 6.

Percentage of dying cells in enriched Olg cultures treated with different concentrations of P2 (A), E2 (B), DHT (C) or combinations of P2 with E2. Cleaved caspase-3+ cells are expressed as a percentage of total A007+ cells. Data is an average of 2 experiments with cells counted from 20 fields for each treatment group in an experiment. * indicates significant difference compared to male control; + significant difference between males and females within the group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. + $P < 0.001$.

Figure 7.

Relative densities of pAkt (A) and pMAPK42/44 (B, C) in enriched Olg cultures treated with different concentrations of P2. The ratio of relative density of pAkt or pMAPK to β actin was calculated. The values are the mean + SEM of 3 different samples, each sample collected from cultures made from 6–8 brains. * $P < 0.05$ compared to male control (D) Western blot of pAkt, pMAPK and β actin in control and P2 treated cultures.

Figure 8.

Relative densities of pAkt (A) and pMAPK42/44 (B, C) in enriched Olg cultures treated with different concentrations of E2 expressed as a ratio to β actin. *P<0.05 compared to male control, +P<0.05 comparing males to females

Figure 9.

Relative densities of pAkt (A) and pMAPK42/44 (B, C) of Olg cultures treated with different concentrations of DHT expressed as a ratio to β actin. * $P < 0.05$ compared to male control; + $P < 0.05$ between males and females of the same treatment group.

Figure 10.
Relative densities of pAkt (A) and pMAPK42/44 (B, C) in Olg cultures treated with different combinations of P2 with E2.

Figure 11.

Quantitative analysis of relative densities of p-mTOR in enriched Olg cultures treated with different concentrations of P2 (A) and E2 (B) expressed as a ratio to β actin. * $P < 0.05$ compared to male control. (C) Western blot of pmTOR and β actin for P2 and E2.

Figure 12.

Quantification of the A007+ cells in cultures derived from males and females treated with different concentrations of P2 + PI3 kinase inhibitor LY 294002. The effects of P2 on enriched Olg cultures are abolished by the addition of the inhibitor. The data is expressed as a percentage compared to male control. The data is an average of 2–3 experiments; in each experiment 2–3 coverslips were counted for each treatment condition. One-way ANOVA was used to compare each group with the male control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to male control. Two tailed t-test assuming equal variance was used to compare each group treated with or without the inhibitor. + $P < 0.05$ within a treatment group with or without the inhibitor.

Figure 13.

Percentage of A007+ cells in cultures derived from males and females deprived of insulin and supplemented with P2. *P<0.05 compared to male control.

Figure 14.

Relative densities of pAkt (A) and pMAPK42/44 (B–C) of Olg cultures deprived of insulin and treated with 5nM P2 and expressed as a ratio to β actin. * indicates significant difference compared to male control with a P value of < 0.05.