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Testosterone-mediated neuroprotection through the androgen receptor in human primary neurons

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Abstract

Estrogen is an active neuroprotectant and is presently investigated as a potential therapy against Alzheimer's disease for women. To determine if male hormones could also be neuroprotective, we investigated the effect of testosterone, methyltestosterone, and epitestosterone at physiological concentrations on primary cultures of human neurons induced to undergo apoptosis by serum deprivation. Serum deprivation significantly induces neuronal apoptosis in a protracted fashion. As expected, physiological concentrations of 17-β-estradiol and transcriptionally inactive 17-α-estradiol protect neurons against apoptosis. Similar to 17-β-estradiol, physiological concentrations of testosterone are also neuroprotective. Androgen receptors are present at 8 ± 2 fmol/mg protein in the neuron cultures. The non-aromatizable androgen, mibolerone, is also neuroprotective and aromatase inhibitor, 4-androsten-4-OL-3,17-dione, does not prevent testosterone-mediated neuroprotection. In contrast, anti-androgen, flutamide, eliminates testosterone-mediated neuroprotection. Testosterone analog, methyltestosterone, showed androgen receptor-dependent neuroprotection that was delayed in time indicating that a metabolite may be the active agent. The endogenous anti-androgen, epitestosterone, also showed a slight neuroprotective effect but not through the androgen receptor. These results indicate that androgens induce neuroprotection directly through the androgen receptor. These data suggest that androgens may also be of therapeutic value against Alzheimer's disease in aging males.

Keywords: Alzheimer's disease, androgen, apoptosis, estrogen, human primary neurons.

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Epidemiological studies have shown that decreasing levels of estrogen is a risk factor for Alzheimer's disease and hormone replacement therapy (HRT) offers protection against Alzheimer's disease (Paganini-Hill 1996; Schneider *et al*. 1996; Tang *et al*. 1996; Schneider *et al*. 1997). In animal models and cell cultures, estrogen reverses the behavioral and biochemical changes in ovariectomized rats (Simpkins *et al*. 1997) and enhances neuritic outgrowth and survival (Woolley and McEwen 1993, 1994; McEwen and Woolley 1994; Brinton *et al*. 1997; Woolley *et al*. 1997; McEwen *et al*. 1999). Estrogen acts through genomic transactivation and non-genomic pathways (reviewed by Woolley 1999). Genomic events include up-regulation of brain-derived neurotrophic factor, nerve growth factor (NGF), epidermal growth factor (Birge 1996) and of Bcl-2 proteins (Dubal *et al.* 1999; Pike 1999). Estrogen also modulates p53 activity and cell fate (Wade *et al*. 1999). Non-genomic events involve signal transduction, and it has been shown that estrogen activates the mitogen-activated protein kinase cascade in the cerebral cortex (Singh *et al*. 1999, 2000a; Toran-Allerand 2000a,b). In addition, estrogen decreases the amount of amyloid-β peptide produced in neurons (Jaffe *et al*. 1994; Xu *et al*. 1998) and can protect against amyloid-β peptide-mediated neurotoxicity (Goodman and Mattson 1996; Behl *et al*. 1997). Others propose that estrogen acts as an antioxidant, although it is unlikely that physiological

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Abbreviations used: HRT, hormone replacement therapy; NGF, nerve growth factor; TUNEL, TdT-mediated dUTP nick-end labeling.

levels of estrogen will have antioxidant activity (Behl *et al*. 1997; Moosmann and Behl 1999).

In contrast, little is known about the neuroprotective role of androgens in the aging CNS. Men in their sixties are usually less prone to Alzheimer's disease than women of the same age (Molsa *et al*. 1982; Jorm *et al*. 1987). However, androgens eventually decrease with age (Flood *et al*. 1995; Vermeulen 1991). Testosterone replacement therapy improves depression, and verbal and spatial memory in aging men (Sternbach 1998). At the molecular level, testosterone is shown to increase NGF and p75-nerve growth factor receptor and to decrease Alzheimer's amyloid-β peptide in primary rat cortical neurons (Tirassa *et al*. 1997; Gouras *et al*. 2000). Therefore, decreasing levels of testosterone could account for men's increasing susceptibility to Alzheimer's disease with age (Molsa *et al*. 1982).

Although at much lower levels, androgens are also present in women and decrease with age (Rako 1998). Decreasing androgen levels are associated with a number of post-menopausal conditions such as osteoporosis, depression, reduced muscle and bone mass and increased visceral fat (Davis 1999). Evaluation of testosterone in neuronal cell lines failed to reveal a neuroprotective role (Green *et al*. 1997). In the present study, we assess the role of physiological concentrations of androgens on serum deprivation-mediated apoptosis of human primary CNS neuron cultures. We find that testosterone protects neurons against serum deprivation by acting through androgen receptors.

Methods and materials

Neuronal culture

Human fetal brain tissue (12–16 weeks) was obtained in accordance with the guidelines established by the Medical Research Council and approved by the Institutional Review Board of McGill University. Neurons were isolated and cultured as previously described (LeBlanc 1995). To summarize, brain tissue was minced in phosphate buffered saline and dissociated with 0.25% trypsin (Gibco-BRL, Rockville, MD, USA). The cells were subsequently treated with 10% serum and 0.1 mg/mL deoxyribonuclease I (Roche Molecular Biochemical, Indianapolis, IN, USA) and the resulting homogenate filtered through 130- and 70-μm nylon mesh. The neurons were plated at 3×10^6 cells/mL on poly-L-lysine (Sigma Chemicals, St Louis, MO, USA) coated ACLAR™ (33C; 5 mm; Allied Chemical Corp., Pottsville, PA, USA) coverslips and cultured *in vitro* for 10 days. The media contains phenol-free minimal essential media in Earle's balanced salt solution containing 0.225% sodium bicarbonate, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1% dextrose, 1 × antibiotic Pen-Strep (all products from Gibco-BRL) and 5% decomplemented fetal bovine serum (HyClone, Logan, UT, USA). In complete serum containing media, the basal amount of testosterone is present at 9 pM and estrogen is at 18 pM.

Neuronal treatment

The neurons were serum-deprived in the absence or presence of 2 nM 17-α-estradiol or 17-β-estradiol, 4 nM testosterone enanthate, epitestosterone or methyltestosterone (the concentration represents peak physiological levels in reproductive age women and men). All hormones were purchased from Sigma except methyltestosterone obtained at United States Pharmacopeia (Rockville, MD, USA). Testosterone enanthate was used because the ester increases the duration and action of testosterone. Testosterone enanthate will not bind the androgen receptor unless the ester is hydrolyzed. The testosterone enanthate is hydrolyzed into testosterone in the neuronal cultures as evidenced by the antagonistic effect of flutamide. The chemical structure of these compounds is shown in Fig. 1. The media was changed every 48 h. The hormones were dissolved in various stock concentrations in 100% ethanol and added to the media to give final concentrations of 2 and 4 nM or the indicated dose with equivalent amounts of ethanol. Control serum-deprived neurons receive the equivalent amount of ethanol. Similarly, mibolerone, flutamide and aromatase inhibitor were

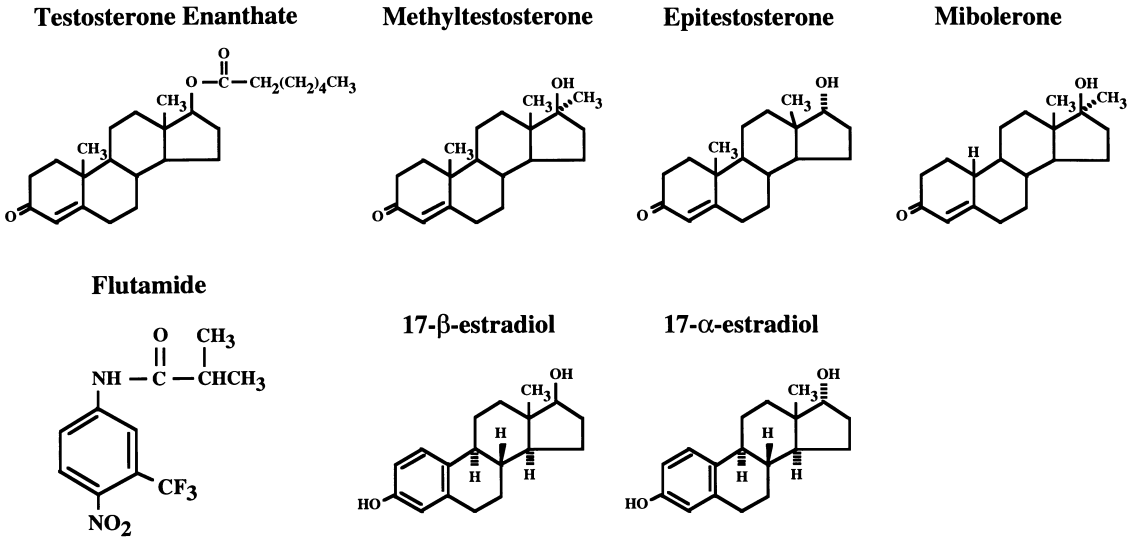

Fig. 1 Chemical structure of androgen, estrogens and flutamide.

dissolved in ethanol and added to the media to give final concentrations of 3 nM mibolerone (DuPont NEN, Boston, MA, USA), 2 μM and 20 μM flutamide (Sigma), or 5 ng/mL and 50 ng/mL 4-androsten-4-OL-3, 17-dione (Sigma). At the end of the treatment, coverslips were fixed with 4% paraformaldehyde, 4% sucrose in phosphate buffered solution (Harlow and Lane 1988).

Determination of androgen receptors

Androgen receptors were identified by incubating 6 nM [^{3}H]mibolerone (DuPont NEN; Spec. Act. 85 Ci/mmol) with 6×10^6 neurons to measure total binding. Non-specific binding was assessed by competing the binding of [^{3}H]mibolerone with a 200-fold excess cold mibolerone as previously described (Kaufman *et al*. 1993). Specific binding was determined by subtracting non-specific from total binding and dividing by the protein concentration as determined by the Lowry assay (Lowry *et al*. 1951).

Determination of neuronal cell death by TUNEL

Fixed neurons were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Cell death was detected by TUNEL (TdT-mediated dUTP nick-end labeling) using the Cell Death Kit I (Roche Molecular Biochemicals) as described by the manufacturer. All cells were counterstained with 100 ng/mL propidium iodide (Pharmingen, Mississauga, Ontario, Canada) to allow confirmation of the apoptotic morphology of the cells and to detect the total number of cells present under fluorescence microscopy. The percentage of neuronal cell death was determined by screening five areas of each coverslip (a minimum of 500 cells) and comparing the total number of TUNEL-positive (green fluorescence) and morphologically apoptotic cells over the total number of cells (red fluorescence) present in each sample. The neuronal cell death was confirmed in representative experiments of each assay with 3-[4,5-dimethylthazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reducing assays. Briefly, neurons were plated directly into 24-well plates and studied for MTT reduction using the Cell Proliferation Kit 1 (MTT) as described by the manufacturer (Roche Molecular Biochemicals; data not shown).

A two-tailed Student's *t*-test for unpaired samples was used for comparison between the level of neuronal cell death in serum-deprived neurons and that in serum-deprived neurons treated with hormones. *p*-values of < 0.05 were used as indicative of statistical significance.

Results

Androgens are as neuroprotective as estrogens against serum-deprivation-mediated apoptosis of human primary neurons

Estrogen is hypothesized to play an important role against Alzheimer's disease in women (Birge 1996). Women receiving HRT are less susceptible to Alzheimer's disease (Tang *et al*. 1996). As HRT often contains androgens (Gelfand 1992) and men's susceptibility to Alzheimer's disease increases with age in parallel with reduced levels of androgens (Vermeulen 1991), we investigated the role of testosterone enanthate, methyltestosterone and epitestosterone in neuroprotection against serum deprivation in primary cultures of human neurons.

We have shown that these human neurons undergo a protracted form of cell death with active recombinant caspases (Zhang *et al*. 2000). Serum deprivation also induces a protracted cell death but there is a significant amount of neuronal cell death by serum deprivation within 24 h ($p < 0.005$) (Fig. 2a). The addition of physiological

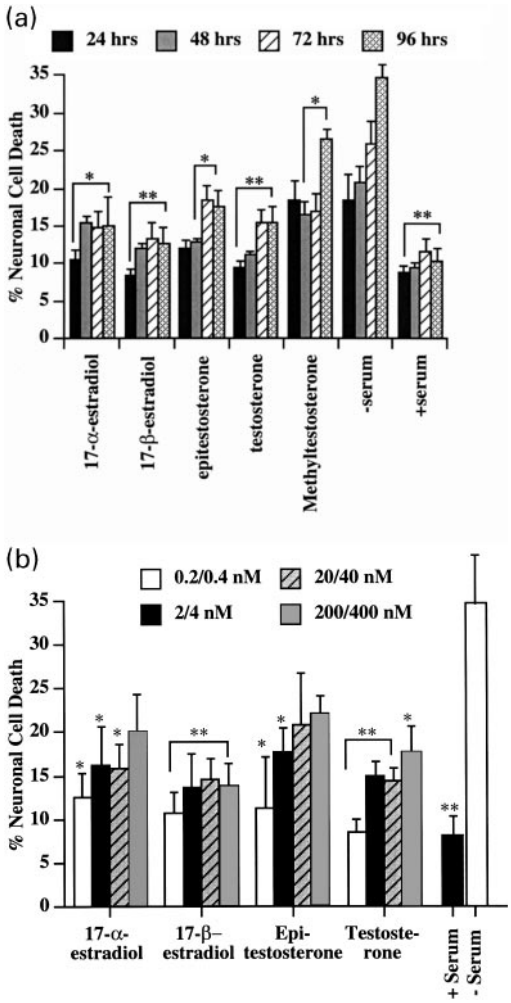

Fig. 2 Androgens offer neuroprotection against serum deprivation-mediated apoptosis. (a) Time study of neuroprotection by 4 nM testosterone, epitestosterone or methyltestosterone and 2 nM 17-β-estradiol or 17-α-estradiol treatment at 24, 48, 72 and 96 h of serum deprivation. The level of apoptosis in hormone-treated neurons is expressed as percentage neuronal cell death detected by propidium iodide and TUNEL staining. Data represents the mean and SEM of experiments of 10 independent neuronal cultures for all except methyltestosterone ($n = 5$). (b) Dose response effect of each hormone on neuronal protection. Data represents the mean and SEM of eight independent experiments. $^*p < 0.05$, $^{**}p < 0.005$ indicate the significance of the difference between serum deprived neurons in the absence and in the presence of hormone.

concentrations of 4 nM testosterone enanthate to serum-deprived neurons eliminates apoptosis completely for 24 and 48 h and significantly reduces apoptosis at 72 and 96 h of treatment. In contrast, methyltestosterone did not significantly inhibit apoptosis at 24 h, but did show a 20% reduction in apoptosis between 48 and 96 h of treatment ($p < 0.05$). The anti-androgen, epitestosterone also had no statistically significant effect at 24 h but reduced apoptosis by 20–40% from 48 to 96 h of treatment ($p > 0.05$). As shown (Behl *et al*. 1997; Green *et al*. 1997; Pike 1999; Green and Simpkins 2000), 2 nM physiological concentrations of 17-β- ($p < 0.005$) and 17-α−estradiol ($p < 0.05$) were also neuroprotective from 24 to 96 h of treatment.

Addition of 10 times less or between 10 and 100 times more hormone to serum-deprived neurons shows that the lower 0.2 or 0.4 nM concentration are slightly more neuroprotective than the 2 and 4 nM concentration (Fig. 2b). Increasing the levels of hormone slightly reduced the neuroprotective effect in 17-α-estradiol and androgen treated cultures. These results indicate that the neuroprotective effect of androgens and estrogens at physiological concentrations is likely one that is receptor mediated and rule out a possible antioxidant function in neuroprotection. It is possible that the higher concentrations of hormones are slightly toxic to neurons thereby reducing the neuroprotective effect. Most importantly, the significant neuroprotective effect observed with the lowest dose of hormone indicates the strong neuropotency of these hormones.

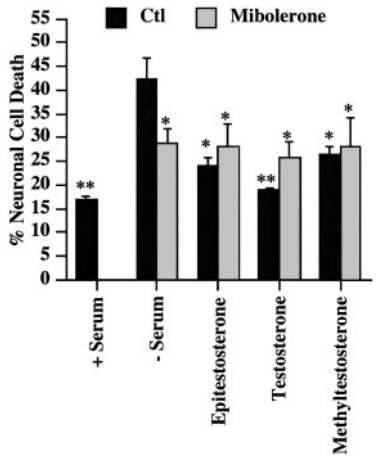

Fig. 3 Mibolerone protects against serum deprivation-mediated neuronal apoptosis. Serum deprived neurons were treated with 4 nM epitestosterone, methyltestosterone or testosterone in the presence or absence of 3 nM mibolerone for 96 h. Data represents the mean and SEM from three independent neuronal preparations. *$p < 0.05$, **$p < 0.005$ indicate the significance of the difference between serum deprived neurons in the absence and in the presence of hormone.

Androgens directly protect neurons and do not require aromatization into estrogens

To determine if the neuroprotective role of androgens was directly through androgen receptors or through aromatization into estrogens (Balthazart and Ball 1998), we first determined if androgen receptors were present in our cultures using non-aromatizable [^{3}H]mibolerone in a binding assay. Our results show that androgen receptors are present at 8 ± 2 fmoles/mg protein. We then assessed the effect of the non-aromatizable androgen, mibolerone, on neuroprotection (Fig. 3). We find that similar to testosterone, mibolerone significantly protects neurons against serum deprivation even after 96 h of serum deprivation. The neuroprotective effect of mibolerone is not as strong as that of testosterone. Similarly, methyltestosterone also shows neuroprotection at a lower level than in testosterone. Comparison of the chemical structure of these compounds (Fig. 1) indicates that the presence of the 17-methyl group decreases the neuropotency of androgens. The addition of mibolerone to testosterone, epitestosterone or methyltestosterone does not increase neuroprotection in cells treated with hormone in absence of mibolerone suggesting that both mibolerone and natural androgens act through the same receptor. The affinity of mibolerone is at least 100-fold higher than testosterone, epitestosterone or methyltestosterone (Wilson and French 1976; Turcotte *et al*. 1988). Therefore, mibolerone binding to the androgen receptor would compete out the other androgens as evidenced by the levels of neuroprotection consistent with a mibolerone-specific effect. Together, these results indicate that androgens can be directly neuroprotective without being aromatized to estrogen.

To confirm that testosterone is not acting by aromatization into estrogen, we added a cell permeable aromatase inhibitor, 4-androsten-4-OL-3,17-dione, to the testosterone-treated neurons (Fig. 4). The results show that the aromatase inhibitor does not have a significant effect on neuronal cell death by serum deprivation ($p > 0.8$). In the presence of testosterone, 4-androsten-4-OL-3,17-dione, does not prevent testosterone-mediated neuronal protection ($p > 0.4$). These results confirm the direct action of testosterone rather than an indirect effect through aromatization into estrogens.

To determine if activation of the androgen receptor results in the neuroprotective action of testosterone, we assessed the effect of a non-steroid pure anti-androgen, flutamide (Simard *et al*. 1986) on testosterone-mediated neuroprotection (Fig. 5). Flutamide alone at either 2 or 20 μM does not have a significant effect on neuronal survival or cell death. However, flutamide significantly abolishes testosterone-mediated neuroprotection. Together with the mibolerone and aromatase inhibitor studies, these results strongly suggest that the neuroprotective function of testosterone occurs through the androgen receptor. Flutamide also significantly inhibits the methyltestosterone-mediated neuroprotection. The effect is not as significant as seen with testosterone. However, methyltestosterone is also less neuroprotective than testosterone either because of the 17-methyl

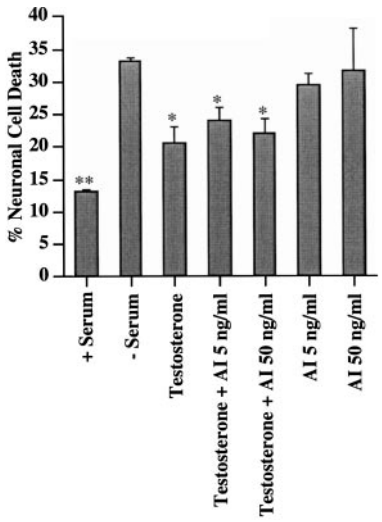

Fig. 4 Aromatase inhibitor, 4-androsten-4-OL-3,17-dione, does not inhibit testosterone neuroprotection. Serum deprived neurons were treated in the absence or presence of 4 nM testosterone and 5 and 50 ng/mL aromatase inhibitor, 4-androsten-4-OL-3, 17-dione (AI), and kept in culture for 96 h. Neuronal apoptosis was measured as described and expressed relative to control serum deprived neurons (arbitrarily placed at 100%). Data represents mean and SEM of three independent experiments. $*p < 0.01$, $**p < 0.005$ indicate the significance of the difference between serum deprived neurons in the absence and in the presence of hormone/drug.

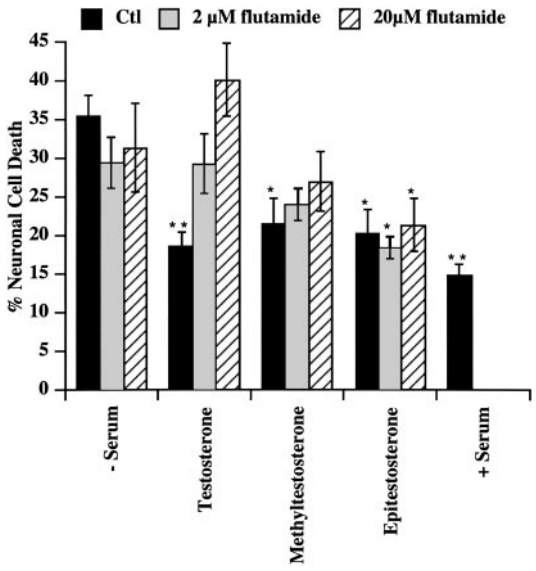

Fig. 5 Flutamide prevents testosterone-mediated neuroprotection. Serum-deprived neurons treated with 4 nM androgens in the absence or presence of 2 and 20 μM flutamide for 96 h. Data represents the mean and SEM of four independent experiments. $*p < 0.05$, $**p < 0.002$ indicates the significance of the difference between neurons that are serum deprived and those treated with hormone. Comparison of hormone and flutamide treated cells were made to serum deprivation in the presence of flutamide.

group as discussed later or the lower affinity of methyltestosterone for the androgen receptor (Wiita *et al*. 1995). Alternatively, the slow neuroprotective effect of methyltestosterone, which is only observed after 48 h of treatment, indicates that metabolites of methyltestosterone may be produced over time and promote neuroprotection through both androgen receptor dependent (antagonized by flutamide) and androgen receptor independent (not antagonized by flutamide) mechanisms.

In contrast, flutamide could not inhibit the endogenous anti-androgen, epitestosterone-mediated neuroprotection indicating that the neuroprotection of epitestosterone is independent of androgen receptors. Together, these results confirm that the neuroprotective effect of testosterone depends on an interaction with androgen receptors and can be competed out with antagonists.

Discussion

While much attention has been given to the role of 17-β-estradiol against Alzheimer's disease, little is known about the molecular mechanism of androgens in neuroprotection. Testosterone propionate prevents developmental neuronal loss in the medial preoptic nucleus of males or sex-reversed female rats (Dodson and Gorski 1993). Androgens can increase the volume, neuron number and synapses of developing rat superior cervical ganglion (Wright *et al*. 1991). In aging, there is little evidence that androgens regulate neuronal survival. However, testosterone deficiency in males is associated with conditions that indicate CNS neuronal dysfunction such as depression, anxiety and memory loss (Sternbach 1998). Furthermore, replacement therapy significantly improves these symptoms. Here we show that androgens offer as much neuroprotection against growth factor deprivation mediated neuronal apoptosis of CNS differentiated human neurons as 17-β-estradiol. Neuroprotective effects both occur at physiological concentrations. Therefore, we conclude that neurons are as responsive to androgens as estrogens with respect to neuronal survival.

We show that physiological levels of testosterone protect against serum deprivation-mediated neuronal apoptosis through interaction with androgen receptors. We confirmed the presence of androgen receptors in the human neuron cultures. We show that the non-aromatizable form of androgen, mibolerone, induces neuroprotection similar to testosterone. Mibolerone is a highly specific synthetic androgen that binds the androgen receptor with 100-fold higher affinity than the natural androgen, testosterone (Wilson and French 1976; Traish *et al*. 1986; Turcotte *et al*. 1988; Markiewicz and Gurpide 1997). Furthermore, aromatase inhibitor, 4-androsten-4-OL-3,17-dione, does not block testosterone-mediated neuroprotection. However, the neuroprotective effect of testosterone is blocked by

the pure synthetic anti-androgen, flutamide (Simard *et al*. 1986; Namer 1988; Brogden and Chrisp 1991; Labrie 1993; Markiewicz and Gurpide 1997; Singh *et al*. 2000b). Therefore, the neuroprotective effect of testosterone is mediated through androgen receptors.

The neuroprotective effect of testosterone is 100% up to 48 h after serum deprivation. Thereafter, there is increasing neuronal apoptosis, even in the presence of testosterone, although the levels are generally 60% lower than in absence of hormone. Since we change the media every 48 h, turnover of testosterone cannot be responsible for the less protective effect. It is more likely that cumulative insult caused by continuous serum deprivation is responsible for the inability of testosterone to neuroprotect completely in time. As shown in other systems, inhibiting cell death with one compound may not be sufficient for the treatment of neurodegenerative diseases and combination therapies including both cell death inhibitors and pro-survival factors may be necessary to completely suppress neuronal cell death.

The neuroprotective effect of the weak endogenous anti-androgen, epitestosterone (Nuck and Lucky 1987; Starka *et al*. 1989; Starka *et al*. 1991) is surprising. This is clearly not the case for the other anti-androgen, flutamide. Furthermore, flutamide did not antagonize the epitestosterone effect, indicating that epitestosterone unlike mibolerone, testosterone and methyltestosterone, cannot act through the androgen receptor. Epitestosterone is aromatized into 17-α-estradiol (Finkelstein *et al*. 1981). Given that the neuroprotective effect of epitestosterone occurs only after 48 h of treatment, it is likely that the neuroprotective effect is mediated through aromatization into 17-α-estradiol. As shown by others, and us here, the transcriptionally inactive 17-α-estradiol is also neuroprotective (Green *et al*. 1997). It is proposed that 17-α-estradiol mediates neuroprotection through signal transduction rather than through a genomic pathway.

The fact that methyltestosterone and mibolerone are not as neuroprotective as testosterone indicates that the structure of the steroid may be very important in mediating the neuroprotective effect of androgens. Comparison of the chemical structure of the three compound shows that mibolerone and methyltestosterone share a 17-methyl group that is absent in testosterone. Possibly, this methyl group accounts for the lesser neuropotency of methyltestosterone and mibolerone.

At this time, we do not know the exact mechanism of action of androgens in these neurons. Like estrogen, androgens are nuclear receptor proteins that can activate gene transcription or act through signal transduction. The neuroprotective effect of estrogen is known to act through the estrogen receptor and to activate both genomic and non-genomic pathways of neuronal protection (Woolley 1999). Through the genomic pathway, estrogen up-regulates Bcl-2 levels (Dubal et al. 1999; Pike 1999) and it is possible that increased Bcl-2 levels enhance neuronal protection against serum deprivation. Whether androgens regulate gene expression or activity of survival genes in a manner similar to estrogens remains to be determined but this is a likely mechanism to explain the neuroprotective nature of androgens, which are well known to support growth and survival of androgen responsive tissues.

Our results in primary cultures of human neurons contrast with those observed in the estrogen responsive, human SK-N-SH, neuronal cell line (Green *et al*. 1997). Androgens are metabolized by these cells and can affect proliferation (Maggi *et al*. 1998). However, it is not unexpected to find differences between primary neurons and neuroblastoma cell lines as these differ in many ways such as in the state of differentiation, cell growth and cell death. *In vivo*, androgen receptors are expressed in the temporal, frontal and hippocampal regions of the brain (Puy *et al*. 1995; Finley and Kritzer 1999). Androgen receptors are selectively localized to neuronal subtypes, and immunoreactivity appears specific to pyramidal neurons in primate prefrontal cortex (Finley and Kritzer 1999). The number of androgen receptors does not differ in male and female rat or monkey brains (Clancy *et al*. 1992). It is possible that the neuroprotective effect of testosterone through androgen receptors observed in human neurons in culture could also occur in the human brain.

Our findings raise the possibility that androgens could help in the treatment of Alzheimer's disease in a manner similar to estrogen-replacement therapy in women. Symptoms associated with the decreasing levels of androgen in both men and women are alleviated by hormone replacement therapy. The content of androgens in women's HRT should be taken into consideration in epidemiologic studies on the effect of HRT against Alzheimer's disease. Regardless of the mechanism, these results show an important new lead in the treatment of neurodegenerative diseases. Androgens may prove to be an effective treatment for aging males and offer neuroprotection against Alzheimer's disease. However, the time of treatment is likely very important to have a beneficial effect. In post-mortem human hippocampus, the amount of androgen receptors significantly decrease with age in the CA1 region (Tohgi *et al*. 1995). Therefore, decreasing amounts of androgen receptors are likely to result in these cells becoming non-responsive to androgens. Similar to the inability of HRT to act against Alzheimer's disease when given to post-menopausal women (Mulnard *et al*. 2000), it is unlikely that treatment with androgens after down-regulation of androgen receptors in the brain will be helpful.

Acknowledegments

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