

Neurosteroid Hormones Modulate the Differentiation of Adult Human Multipotent Mesenchymal Stromal Cells

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Abstract

Objective: To examine the effect of neurosteroids on human multipotent mesenchymal stromal cell (hMSCs) neuroglial differentiation.

Materials and methods: Human MSCs were isolated and expanded *in vitro*. The expression of neurosteroid receptors by hMSCs was examined using immunocytochemistry and the effect of neurosteroids on the proliferation and differentiation of hMSCs was investigated using immunocytochemistry and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) survival assay.

Results: Human MSCs express receptors for neurosteroids. Nestin expression is decreased by neurosteroids. Neurosteroids also exert differential effects depending on the gender of the hMSC donor; dihydrotestosterone (DHT) was responsible for maximal A2B5 expression in hMSCs from male donors whereas 17- β estradiol (E2) exerted the greatest effect on differentiation of 'female' hMSCs. Maximal effects of E2 and DHT were observed at a concentration of 100nM, progesterone (PROG) at 250nM. The neurosteroid-induced increase in oligodendroglial differentiation was abrogated by specific receptor antagonists for E2, PROG and DHT. High concentrations of neurosteroids were toxic for hMSCs, irrespective of gender.

Conclusions: These results suggest a significant role for neurosteroid hormones in the differentiation of hMSCs and may have important implications for the development of MSC transplantation therapy for multiple sclerosis and in autoimmune diseases.

Keywords: Neurosteroids; Human multipotent mesenchymal stromal cells; Oligodendrocytes; Cell differentiation; Multiple Sclerosis

Introduction

Neurosteroids are those steroid hormones which, independent of their origin, are capable of modifying neural activities [1]. They can act through classical/genomic or rapid/non-genomic pathways within the CNS. Since the discovery of *de novo* synthesis of dehydroepiandrosterone sulphate (DHEAS) from cholesterol in the brain tissue by Baulieu in 1981 (so-called neurosteroid synthesis) [2], the direct synthesis of the majority of the steroid hormones such as pregnenolone, progesterone and sex steroids, estradiol, testosterone and dihydrotestosterone and the presence of key enzymes for steroid synthesis have been demonstrated in CNS tissue [3,4]. Neurosteroids are produced in particular in myelinating glial cells, though also in astrocytes and in neurons [5]. Within the CNS, neurosteroids appear to exert a variety of effects [6,7], not least on proliferation and differentiation of cells of neural lineage.

More specifically, a number of authors have reported the presence of neurosteroid hormone receptors on oligodendrocytes (OLG). These are the cells responsible for synthesising and maintaining myelin in the CNS, and they form, together with myelin, the principal target for immune damage in Multiple Sclerosis (MS), the commonest acquired disease causing neurological disability in young adults. MS has a pronounced gender bias (more than twice as common in females), and a number of sex steroid changes have been documented in this disease. Significant neurosteroid effects on oligodendrocytes [8-11], on oligodendrocyte progenitors [12], and on pre-progenitors [13,14], have been reported. Neurosteroids also exert effects on neural stem cells [15].

Adult bone marrow-derived multipotent mesenchymal stromal cells (MSCs) are multipotent cells that can differentiate into various cell phenotypes including hepatic cells, skeletal and cardiac myocytes, as well as bone, cartilage and fat cells [16-19]. Bone marrow -derived cells have been shown to express neuroectodermal markers [20,21], and can differentiate into cells with both the phenotype and function of myelinating glia [22,23], findings which have prompted much interest in the potential development of new therapeutic strategies in neurodegenerative diseases [24].

We hypothesized that neurosteroid hormones were likely to exert significant effects on the biology and behaviour of MSCs. Here we investigate this possibility by studying the expression of neurosteroid receptors on human MSCs (hMSCs), and the effects of the neurosteroid hormones 17 β -estradiol (E2), progesterone (PROG) and dihydrotestosterone (DHT) on the differentiation of hMSCs *in vitro*, studying in particular the expression of nestin, which is seen

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in neural stem cells but not their more differentiated progeny, and of A2B5, expressed by oligodendrocyte progenitors *in vitro* but not by uncommitted neural stem cells.

Materials and Methods

Isolation and culture of hMSCs

Local ethics committee approval for the collection of hMSCs from patients undergoing total hip replacement was obtained. Patients were formally consented for marrow donation. Mononuclear cells were isolated using a density gradient (Lymphoprep, Axis-Shield PoC AS) and incubated with 0.15M ammonium chloride, 0.01M potassium bicarbonate and 0.15mM EDTA in ddH₂O at 4°C for 10 minutes to lyse red blood cells. MSCs were plated at a density of 4x10⁵/cm² and incubated with standard hMSC medium [Dulbecco's modified eagle's medium (D5523, Sigma) supplemented with 10% foetal bovine serum (FBS, StemCell Technologies Inc., Canada, 06471)]. Cultures were incubated in a humidified, 5% CO₂ atmosphere at 37°C and the medium exchanged every 5-7 days. Upon reaching a minimum of 80% confluence, adherent cells were passaged with trypsin (Trypsin-EDTA, BE17-161E, Cambrex) and replated at 0.5x10⁶ cells per T75 flask. Cells beyond second passage conform to the defining criteria for MSCs as published by the International Society for Cellular Therapy position statement [25] (results not illustrated) and were plated at 1x10³/cm² onto PLL-coated (P5899, Sigma) glass coverslips in 4-well chamber slides containing standard hMSC medium. The medium was exchanged at 24hrs and supplemented with E2 (E2758, Sigma), PROG (P7556, Sigma) or DHT (A8380, Sigma). DHT was used because it is not a substrate for aromatase conversion to estrogen. Stock solutions in ethanol of E2, PROG and DHT (1000nM) were diluted into hMSC cultures at 0.01nM, 0.1nM, 1nM, 5nM, 10nM, 50nM, 100nM, 250nM, 500nM and 1000nM concentrations. The final concentration of ethanol in culture medium was <0.03%. The optimal concentration of neurosteroid was determined using a single, male marrow. The effects of this optimal hormone concentration were further examined using a female and additional male marrow.

Detection of steroid hormone receptors on hMSCs

Adult hMSCs from both male and female donors were plated in standard hMSC culture medium and fixed with 4% paraformaldehyde (Alrich 15,812-7) in potassium buffered saline (PBS) (Sigma P4417) for 5 minutes. Cells were permeabilized with ice-cold methanol for 10 minutes at -20°C. Prior to application of primary antibody [anti-estrogen receptor- α (anti-ER α) (mc-20, Santa Cruz Biotechnology, Inc., CA, 1 μ g/ml), anti-estrogen receptor- (anti-ER β) (AB1410, Chemicon, UK, 1:300), anti-progesterone receptor (anti-PR) (MAB462, Chemicon, UK, 1:1000), anti-androgen receptor (anti-AR) (PG-21, Chemicon, UK, 1:100)] cells were incubated with PBS supplemented with 5% normal goat serum (NGS; S-1000, Vector Laboratories Inc., CA) for 30 minutes at room temperature. Cells were incubated with appropriate secondary antibodies (AF488 goat anti-rabbit IgG or AF488 goat anti-mouse IgG_{2a}, Molecular Probes, Invitrogen, UK, 1:1000). In all cases, isotype control staining experiments were carried out and little discernible non-specific or background staining was observed (results not illustrated). The nuclei were counterstained with Hoescht. Coverslips were mounted onto glass slides with Vectashield (H1000, Vector Lab., CA).

Effects of neurosteroids on *in vitro* oligodendroglial lineage differentiation of hMSCs as detected by immunocytochemistry

Adult hMSCs from a male donor were maintained for 7 days in media containing various concentrations of E2, PROG or DHT and stained for early oligodendroglial cell markers. Cells were double-stained for A2B5 (MAB1416, R&D Systems, UK, 2 μ g/ml) and nestin (MAB5326, Chemicon, UK, 1:400). A2B5 staining was performed live; cells were first incubated in a humidified chamber for 30 minutes at 37°C with 5%NGS and subsequently with anti-A2B5 antibody. Cells were then processed for intracellular staining (see above). We concentrated on the expression of A2B5, an accepted marker oligodendroglial lineage and differentiation, at least in cell culture studies [26], and a powerful indicator of oligodendrocyte progenitor differentiation potential, as others have used this marker in sex steroid-oligodendrocyte lineage studies [27].

Co-expression of steroid hormone receptors and A2B5

Adult hMSCs were cultured on PLL-coated coverslips with the optimal concentration of E2, PROG and DHT. On day 7, coverslips were double-stained with antibodies against the relevant receptor and A2B5.

Specificity of steroid hormone effect

To investigate whether the observed effects of steroid hormones were mediated through the relevant receptors ICI 182,780 (1000nM, Tocris, UK), mifepristone (10,000nM, M8046, Sigma, UK) and flutamide (100nM, F9397, Sigma, UK) were used as selective receptor antagonists for E2, PROG and DHT respectively [28-30].

Expression of other oligodendrocyte precursor cell markers

Adult hMSCs cultured in standard medium supplemented with 100nM E2, 250nM PROG or 100nM DHT were stained with anti-PDGF α R and anti-NG2 antibodies at 7 days.

MTT proliferation assay

Cell survival was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) survival assay (M5655, Sigma, UK). Viable cells with active mitochondria reduce MTT to form a visible dark blue formazan reaction product. Briefly, hMSCs were incubated with 1mg/ml of MTT in standard culture medium for 1 hour at 37°C. The supernatant was aspirated and the formazan product dissolved in isopropanol. A Multiskan Ascent spectrophotometer was used to measure the specific absorbance at 540nm.

Cell counts

Stained cells were visualized and manually counted in pre-designated areas with Leica FW4000-TZ fluorescent microscope and photographed with Leica DFC350-FX digital camera with the assistance of Leica FW4000 fluorescent imaging software. The number of total cells was evaluated by counting Hoescht-positive nuclei.

Statistics

SPSS statistical package (SPSS 13.0) was used for statistical analysis; one-way analysis of variance (ANOVA) and post-hoc multiple comparisons (Dunnett's multiple comparison test). Results from the various steroid hormone concentrations were compared with those from cells treated with standard (hormone-deficient) medium. Values of p<0.05 were considered significant.

Results

Adult hMSCs express neurosteroid hormone receptors

Adult hMSCs both from male and female donors were stained for the expression of neurosteroid hormone receptors. Expression was uniformly high in all hMSC cultures examined and no significant effect of gender was observed. Expression of ER α was seen preferentially in the cell nucleus, but cytoplasmic staining was also observed (Figure 1a). ER β expression appeared to be localized to the cytoplasm mainly (also as expected; Figure 1b). PR and AR expression was seen predominantly in the cell nucleus (Figure 1c and 1d). Addition of steroid hormone to the culture medium did not discernibly change the visible intensity

or distribution of steroid hormone receptor immunostaining (Figure 1e-h).

Neurosteroid hormones enhance the oligodendroglial progenitor differentiation of hMSCs and inhibit cell proliferation

Treatment with various concentrations of neurosteroid hormones revealed significant effects on the differentiation of male donor-derived hMSCs. All three neurosteroids reduced the expression of the neural stem cell lineage marker nestin. This effect was seen for DHT over a concentration range of 1-250nM ($p < 0.01$), for E2 at greater than 0.1nM and less than 1000nM ($p < 0.05$) and for PROG at all concentrations

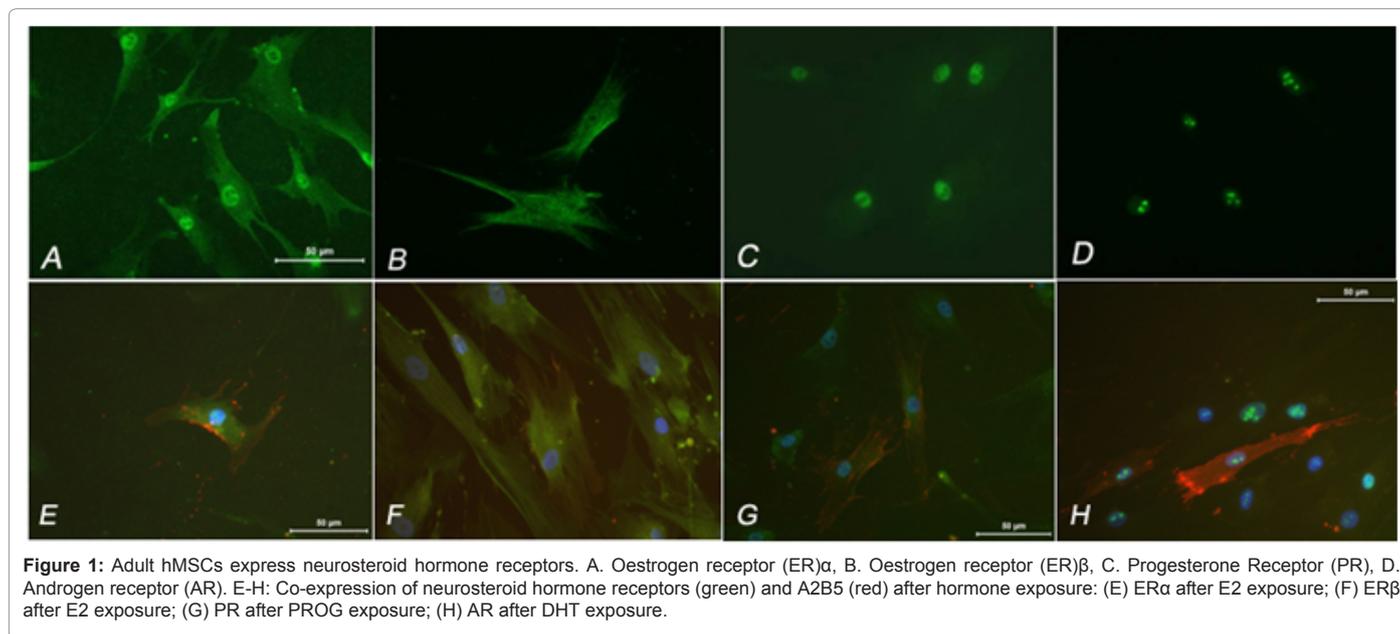


Figure 1: Adult hMSCs express neurosteroid hormone receptors. A. Oestrogen receptor (ER) α , B. Oestrogen receptor (ER) β , C. Progesterone Receptor (PR), D. Androgen receptor (AR). E-H: Co-expression of neurosteroid hormone receptors (green) and A2B5 (red) after hormone exposure: (E) ER α after E2 exposure; (F) ER β after E2 exposure; (G) PR after PROG exposure; (H) AR after DHT exposure.

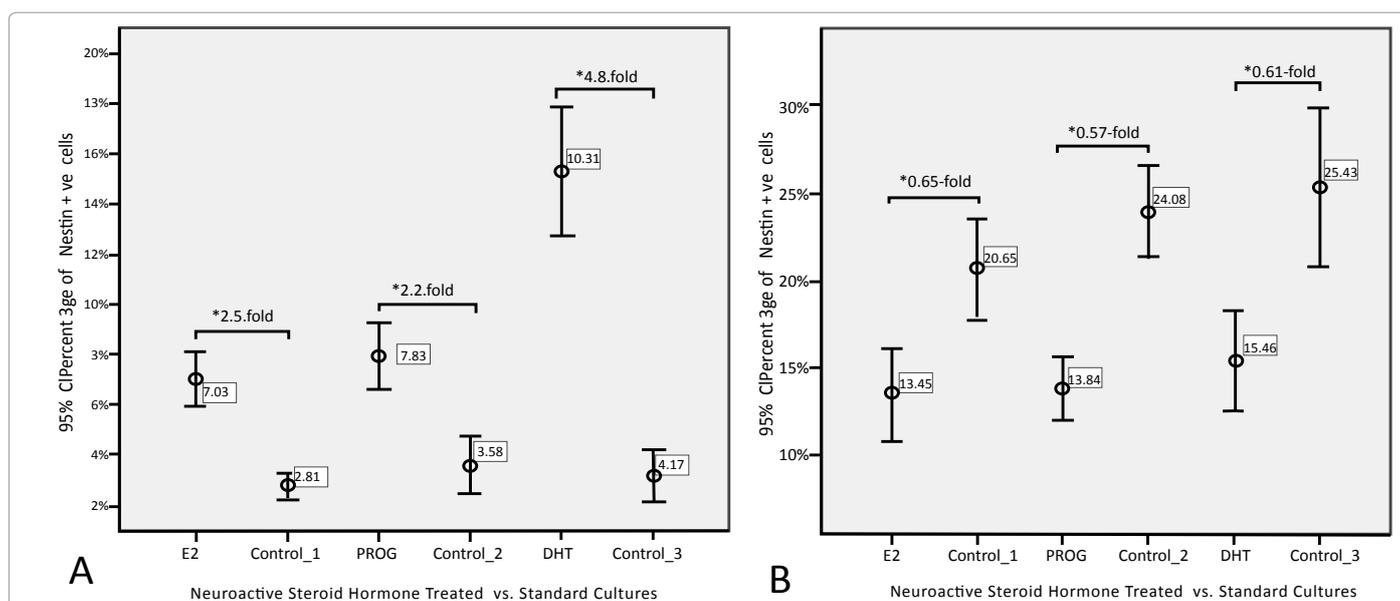


Figure 2: The effect of neurosteroid hormones on the oligodendroglial differentiation of adult hMSCs. At the most effective concentrations (i.e. 100nM for E2 and DHT and 250nM for PROG; * $p < 0.01$), ratios for the increased expression of A2B5 +ve cells (A) and the decreased expression of nestin +ve cells (B) were calculated in comparison with hormone-deficient (standard) cultures.

examined ($p < 0.01$). Addition of ethanol alone (0.03%) to MSC cultures did not alter the expression of nestin.

DHT, E2 and PROG enhanced the expression of A2B5, a marker of oligodendrocyte progenitors, in all applied doses (Figure 1e-h, Figure 2a), although this effect was not statistically significant at very low concentrations (0.01nM) of E2 and PROG, or at high concentrations of PROG (1000nM). The most effective concentrations were 100nM, 250nM and 100nM for E2, PROG and DHT, respectively ($p < 0.01$). Addition of ethanol alone did not change the expression of A2B5. At these most active concentrations, DHT increased A2B5-positive cell numbers 4.8-fold, whereas, E2 and PROG increased the relative expression of A2B5 by 2.5- and 2.2-fold respectively (Figure 2a). Nestin expression, by contrast, was decreased by 0.65-, 0.57- and 0.61-fold with treatment with E2, PROG and DHT respectively (Figure 2b).

The effects of the optimal hormone concentration were further examined using additional male and female marrows. The results for the male marrow replicated those found in the original study; nestin expression was reduced in steroid-treated cultures and DHT, E2 and PROG increased A2B5 expression by 5.3-, 3.3-, and 4.1-fold respectively. Nestin expression was decreased in the female marrow by culture with neurosteroids, but only E2 administration increased A2B5 expression significantly (2.4-fold).

The neurosteroid-induced decrease in nestin expression, together with the increase in the proportion of hMSCs expressing A2B5, is consistent with differentiation. Although total cell number decreased only with very high concentrations of neurosteroids, we cannot speculate on whether differential expansion and apoptosis of sub-populations of cells has occurred on the basis of the experiments performed to date.

Co-administration of neurosteroid receptor antagonists inhibits the effect on differentiation

Administration of specific receptor antagonists, ICI 182,780, mifepristone and flutamide, together with E2, PROG and DHT, abolished the previously observed changes in nestin and A2B5

expression, indicating that the ER α , ER β , PR and ARs have functional roles in the oligodendroglial differentiation of adult hMSCs. On day 7, the proportion of cells double-labeled with antibodies against nestin and A2B5 were not significantly different compared to cultures treated with standard, hormone-deficient medium (Figure 3).

Expression of other oligodendrocyte precursor cell markers

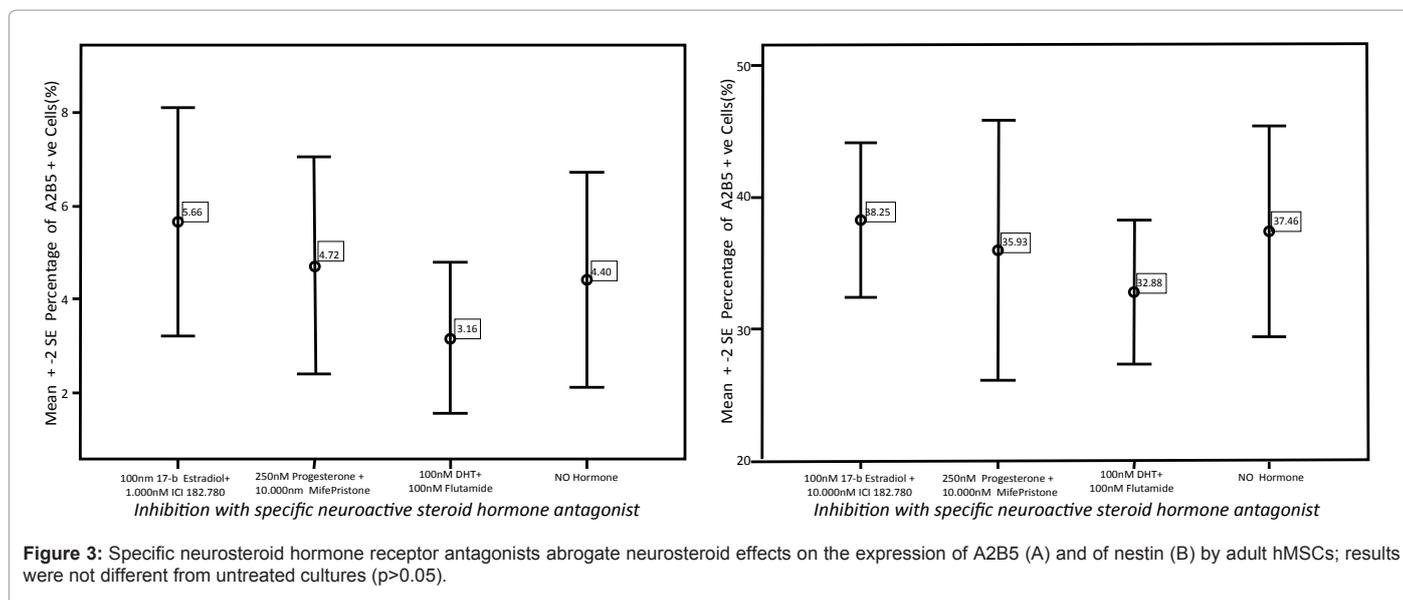
On day 7, hMSCs cultured in standard medium supplemented with 100nM E2, 250nM PROG or 100nM DHT were stained for the presence of the alternative oligodendrocyte progenitor markers PDGF α R and NG2. Hormone-treated cultures had increased numbers of cells expressing the PDGF α R as compared to standard cultures, although this did not reach statistical significance. Unexpectedly, all cells were positive for NG2; no difference was observed between hormone treated and standard cultures.

Neurosteroid hormones exert lethal effects on mesenchymal stem cells at higher concentrations

It became evident during these studies that higher concentrations of all three neurosteroid hormones resulted in a decrease in cell numbers in adult human MSCs *in vitro*. MTT assays revealed that concentrations of PROG and DHT of 1000nM or greater, exhibited a significant toxic effect on hMSCs, resulting in cell death, which was comparable to that of paraformaldehyde administration. Co-culture with high concentrations of E2 showed a trend towards decreased cell viability but this was not significant (Figure 4).

Discussion

Bone marrow (BM) stroma contains multipotent progenitors that appear to be able to give rise to mature cells of mesenchymal and non-mesenchymal lineages, including neurons and glia [31-33]. BM-derived cells, presumably immature precursors or stem cells, can enter the brain and differentiate into neural cells in both mice [34,35] and humans [36]. Intra-lesional or intravenous delivery of adult BM-derived cells enhances remyelination in the rat spinal cord [37-39]. Such cells are therefore very promising candidates for transplantation



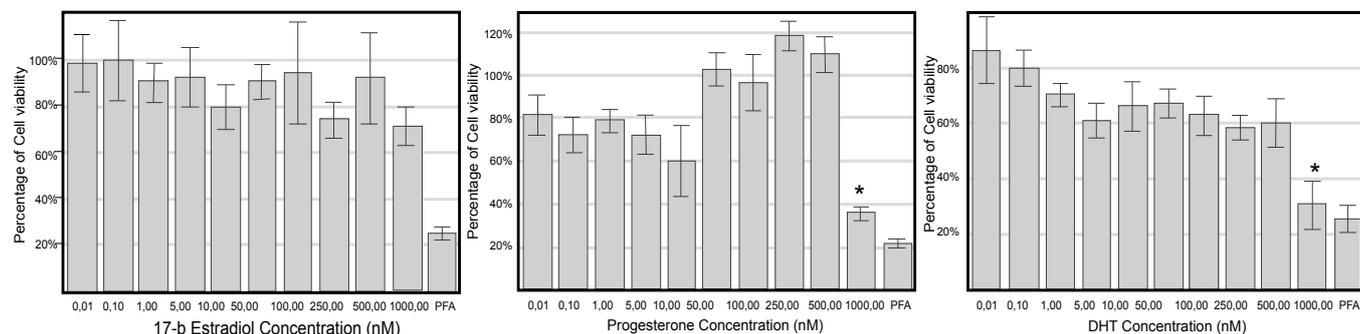


Figure 4: High concentrations of neurosteroids are toxic to adult hMSCs. This was most marked when hMSCs were incubated with high concentrations (1000nM) of PROG and DHT (cell numbers were compared to PFA administration, $p > 0.05$) but there was also a negative trend with increasing concentrations of E2. Error bars show 95.0% CI of mean.

therapy; they are relatively accessible, autologous, and not complicated by the ethical issues surrounding the use of embryonic stem cells. BM-derived cells have been used for decades in the treatment of hematological malignancy. The extensive safety data collected over this period has facilitated the rapid transition from laboratory to clinic, and early phase clinical trials employing bone marrow-derived cells in the treatment of neurological disease have been completed [40,41].

In MS, both the incidence and prevalence in women are over twice that in men [42]. Other autoimmune diseases also show a female predominance, including rheumatoid arthritis, psoriasis and thyroiditis. The phenotype of MS in male patients tends to be more severe; males are more likely to have primary progressive MS, in which disability is relentlessly progressive.

A disruption of the hypothalamo-pituitary-gonadal axis and diminished production of sex hormones has been reported in both MS and the animal model experimental allergic encephalomyelitis (EAE). In addition, MS patients are known to have lower serum concentrations of oestrone sulphate than healthy controls, although this does not correlate with disease severity [43]. Male mice with EAE, and male MS patients, have low serum testosterone levels [44]. Oestrogens modulate the activity of MS during pregnancy [45,46] in association with an immune shift from Th-1 to T helper type-2 (Th-2) [47]. Treatment with the predominant estrogen in pregnancy, oestriol, temporarily decreases the number of enhancing lesions on brain MRI of relapsing remitting MS patients [48]. This effect has also been observed in rodent EAE where pregnancy suppresses both the disease onset [49] and relapses [50]. Gonadectomy increases the severity of EAE irrespective of gender [51,52], whereas treatment with oestrogens or androgens suppressed the disease [53-56].

Estrogen may mediate its effects by binding to the estrogen receptors ER α and ER β , and a newly recognized ER-X. Zhang et al. reported the presence of both ER α and ER β in an OLG cell line and in enriched OLG cultures [57], and showed that ER α receptors are located in the OLG nucleus, whereas ER β receptors are cytoplasmic. Adding oestriol to glial cultures enhanced differentiation of OLGs [58]. Estrogen significantly decreases the cytotoxicity of the peroxynitrite generator 3-(4-morpholinyl)-sydnominine (SIN-1) in both immature and mature OLGs in a dose-dependent manner and is neuroprotective for OLGs [59]. Estrogen increases CNS myelination in the rat [60].

PROG, synthesized by Schwann cells, promotes the formation of

new PNS myelin sheaths [61,62]. PROG also stimulates myelination by OLGs in the CNS. When added to organotypic slice cultures of rat and mouse cerebellum, PROG accelerated myelin formation, irrespective of gender, via the intracellular receptor (PR); its effect was blocked by the PR antagonist mifepristone (RU486), and PROG had no effect on myelination in cerebellar slice cultures from PR knockout mice [63]. In the CNS of old male rats, PROG promoted remyelination of axons by OLGs after toxic demyelination [64]. PROG is synthesized only in the early stages of oligodendrocyte precursor cell (OPC) differentiation, but is highly metabolized by OLGs. In addition, the neurosteroid, 3 α , 5 β -THPROG, is principally formed by OPCs [65].

The effects of androgens in the nervous system are less well characterized. Testosterone may be neuroprotective, either directly or indirectly via its aromatization to estradiol [66]. The role of androgens, specifically testosterone, in the pathophysiology of demyelinating diseases of CNS is rather controversial. While women are more susceptible to MS, men are more likely to have progressive and severe disease [67]. Although several reports declare that testosterone is protective in EAE models [68], Caruso et al. demonstrated that testosterone makes OLGs more vulnerable to excitotoxic death; the toxic effects of kainite or AMPA and cyclothiazide were amplified after the administration of testosterone into short-term pure cultures of OLGs (4 days *in vitro*) [69].

In summary therefore, studies of MS epidemiology and myelination biology indicate that female sex steroids are associated with an ameliorating effect on the course of MS, and exert a positive influence on the myelinating and remyelinating activity of CNS glia. A number of observers have, not surprisingly, linked these observations [70,71]. Our findings enhance our understanding of the role of neurosteroids in cell differentiation and, more specifically, represent the first evidence that adult stem cells are clearly neurosteroid responsive, neurosteroids decreasing proliferation and nestin expression, and encouraging the oligodendroglial differentiation of hMSCs. These observations raise the possibility that in the future, as we come to a broader and deeper understanding of the role of these molecules in stem cell development, currently available neurosteroids may be used therapeutically to enhance spontaneous (or extraneous) stem cell repair processes. Conversely, the toxic effect we found at higher doses serves as a potentially valuable caution that inappropriate dosing might not

simply have neutral effects, but may be positively detrimental to repair and regeneration in the central nervous system.

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