The neural androgen receptor: a therapeutic target for myelin repair in chronic demyelination

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Myelin regeneration is a major therapeutic goal in demyelinating diseases, and the failure to remyelinate rapidly has profound consequences for the health of axons and for brain function. However, there is no efficient treatment for stimulating myelin repair, and current therapies are limited to anti-inflammatory agents. Males are less likely to develop multiple sclerosis than females, but often have a more severe disease course and reach disability milestones at an earlier age than females, and these observations have spurred interest in the potential protective effects of androgens. Here, we demonstrate that testosterone treatment efficiently stimulates the formation of new myelin and reverses myelin damage in chronic demyelinated brain lesions, resulting from the long-term administration of cuprizone, which is toxic for oligodendrocytes. In addition to the strong effect of testosterone on myelin repair, the number of activated astrocytes and microglial cells returned to low control levels, indicating a reduction of neuroinflammatory responses. We also identify the neural androgen receptor as a novel therapeutic target for myelin recovery. After the acute demyelination of cerebellar slices in organotypic culture, the remyelinating actions of testosterone could be mimicked by 5α-dihydrotestosterone, a metabolite that is not converted to oestrogens, and blocked by the androgen receptor antagonist flutamide. Testosterone treatment also failed to promote remyelination after chronic cuprizone-induced demyelination in mice with a non-functional androgen receptor. Importantly, testosterone did not stimulate the formation of new myelin sheaths after specific knockout of the androgen receptor in neurons and macroglial cells. Thus, the neural brain androgen receptor is required for the remyelination effect of testosterone, whereas the presence of the receptor in microglia and in peripheral tissues is not sufficient to enhance remyelination. The potent synthetic testosterone analogue 7α-methyl-19-nortestosterone, which has been developed for long-term male contraception and androgen replacement therapy in hypogonadal males and does not stimulate prostate growth, also efficiently promoted myelin repair. These data establish the...
efficacy of androgens as remyelinating agents and qualify the brain androgen receptor as a promising drug target for remyelination therapy, thus providing the preclinical rationale for a novel therapeutic use of androgens in males with multiple sclerosis.

Keywords: myelin; remyelination; multiple sclerosis; testosterone; androgen receptor

Abbreviations: ARNesCre = androgen receptor; EGFP = enhanced green fluorescent protein; 5α-DHT = 5α-dihydrotestosterone

Introduction

The past decade has offered significant advances in the treatment of multiple sclerosis with the development of new immunomodulatory drugs, anti-inflammatory agents and neutralizing antibodies (Kieseier and Stuve, 2011). However, although reducing CNS lesions by modulating immune attacks remains the first-line therapy for multiple sclerosis, there is an urgent need for additional treatments to promote repair of the damaged myelin. There is indeed ample evidence that the failure of myelin repair is a major contributing factor to the outcome of multiple sclerosis and to its evolution into a progressive disorder (Martino et al., 2010). A therapeutic option, which has recently gained much interest, would be to boost the endogenous capacity of the brain to repair myelin damage. Myelin can indeed be self-repaired during the early stages of multiple sclerosis, but with the progression of the disease, the capacity of myelin recovery decreases, possibly because of the failure of oligodendrocyte precursor cell recruitment and differentiation (Franklin and ffrench-Constant, 2008; Kuhlmann et al., 2008; Piaton et al., 2009). The remyelination of axons indeed requires the generation of new oligodendrocytes from oligodendrocyte precursor cells.

Testosterone has a beneficial influence on experimental autoimmune encephalomyelitis, a widely used disease model for the immune-mediated and inflammatory aspects of multiple sclerosis (Palaszynski et al., 2004). It is likely that the immunomodulatory, anti-inflammatory and neuroprotective actions of testosterone contribute to this effect (Liva and Voskuhl, 2001; Malkin et al., 2004; Page et al., 2006; Fargo et al., 2009). These observations were the basis for a clinical trial in which the effects of testosterone supplementation were studied in males with relapsing-remitting multiple sclerosis for 1 year. The outcomes, anti-inflammatory effects and improved cognitive performance were encouraging, and additional preclinical investigations on the actions of testosterone may provide support for further clinical trials (Sicotte et al., 2007; Gold et al., 2008).

Whether testosterone therapy also promotes myelin repair has not been investigated. Addressing this important question requires an experimental model that induces the demyelination of axons in a highly reproducible manner and allows accurate quantification of their remyelination. Cuprizone-induced demyelination of brain white matter tracts is a well-characterized and widely accepted animal model that fulfils these requirements and is particularly suitable for testing remyelination therapies in the absence of confounding adaptive immune responses (Matsushima and Morell, 2001; Kipp et al., 2009; van der Star et al., 2012). In this model, addition of the copper chelator cuprizone to the diet of mice results in the death of oligodendrocytes and the demyelination of brain white matter tracts. Although the cuprizone model does not accurately mimic multiple sclerosis as a disease, there are similarities between cuprizone-induced chronic demyelination and multiple sclerosis lesions, characterized by the apoptosis of oligodendrocytes. Moreover, the model has been extremely useful for studying the biology of remyelination and for identifying factors and signalling mechanisms involved in this complex process (Blakemore and Franklin, 2008; Harsan et al., 2008; Kipp et al., 2009).

Its reproducibility and experimental accessibility make the cuprizone model particularly valuable for studying the effects of testosterone on myelin repair and for identifying its signalling mechanisms. Actions of testosterone on neural targets can indeed be mediated by the androgen receptor, or they may involve its conversion to oestradiol by the brain aromatase (García-Segura et al., 2003). Testosterone is also converted to the potent non-aromatizable androgen 5α-dihydrotestosterone (5α-DHT), required for male reproductive tract development and prostate growth (Askew et al., 2007). Both testosterone and 5α-DHT bind to the androgen receptor as a ligand-inducible transcription factor or interact with components of growth factor signalling, but they can also induce second messenger signal transduction cascades by acting via distinct cell-surface receptors (Heemers and Tindall, 2007).

Here we show that androgen therapy promotes myelin repair in severe demyelinated lesions where spontaneous remyelination is no longer possible. Chronic brain demyelination and oligodendrocyte depletion were induced in mice by cuprizone intoxication for 12 weeks (Matsushima and Morell, 2001; Mason et al., 2004; Harsan et al., 2008). Such a situation closely resembles chronic multiple sclerosis lesions, characterized by the failure of spontaneous remyelination (van der Star et al., 2012). In addition, the remyelinating effect of testosterone was shown after acute lyssolecithin-induced demyelination in cultured cerebellar slices. We also demonstrate a key role of the androgen receptor in the remyelinating actions of testosterone by use of a selective antagonist and of mice carrying the testicular feminization mutation (ARtm mice) (Merlet et al., 2007). Moreover, we identified the brain androgen receptor as a target for testosterone by its specific deletion in neural cells (ARNesCre mice) (Raskin et al., 2009). Importantly, the testosterone analogue and potent androgen receptor agonist 7α-methyl-19-nortestosterone, which is not a substrate for the 5α-reductase and thus spares the prostate, also efficiently promoted myelin repair (Anderson et al., 2003).
Materials and methods

Mice

Wild-type C57/Bl6 intact and castrated/ovariectomized male and female mice were purchased from the Janvier Breeding Centre (France). The total number of wild-type mice used was 120 (eight intact males, eight intact females, 68 castrated males and 36 ovariectomized females). Transgenic mice expressing enhanced green fluorescent protein (EGFP) driven by the mouse myelin proteolipid protein gene promoter (Pppl-EGFP mice) were provided by the Cleveland Clinic Foundation. In these mice (12 castrated males), the fluorescent protein is selectively expressed in cells of the oligodendrocyte lineage at all developmental stages, from the chondroitin sulfate proteoglycan (NG2) NG2+ precursors to mature oligodendrocytes (Mallon et al., 2002). 

AR^tm mice, which carry a naturally inactivating mutation of the androgen receptor (20 intact males), were obtained from the Commissariat à l’Energie Atomique (France). They were genotyped by real-time TaqMan® allelic discrimination (ABI Prism 7000; Applied Biosystems). polymerase chain reaction (PCR) primers (forward 5’-ACG AGG CAG CAGCAT ACC A-3’ and reverse 5’-TAG TCC AAT GGG AGG CAG CAGCAT ACC A-3’) and two internal probes, one specific to the wild-type sequence associated with a VIC™ (ABT, Lifetech) fluorochrome (5’-CGC ACC CCC CGC C-3’) and the other specific to the mutated sequence associated with a 6-carboxyfluorescein (FAM) fluorochrome (5’-CCG CAC CCC CGC C-3’) were used in genotyping (Merlet et al., 2007). Transgenic AR^NesCre mice were obtained by crossing floxed androgen receptor mice (AR^flox) (De Gendt et al., 2004) with transgenic mice expressing Cre recombine driven by the promoter and the nervous system-specific enhancer of rat nestin (Nes), selectively active in neuronal and glial precursor cells (Tronche et al., 1999). The analysis of the resulting AR^flox Nes-Cre mice (AR^NesCre mice) has been described elsewhere (Raskin et al., 2009). The Cre-mediated deletion of the AR^flo allele was indeed only found in neuronal tissues, and AR^NesCre males have normal development of the urogenital tract and are able to produce offspring (20 castrated males were used).

Treatments

Except for the AR^tm mice, which were used intact, mice were castrated around the age of 8 weeks. After an acclimatization period of ~2 weeks, they were placed on a diet containing 0.2% cuprizone (bis-cyclohexanone-oxaldihydrazone, Sigma), mixed into milled chow and available ad libitum for 12 weeks. Food was replaced every 48 h. Mice then received a cuprizone-free diet for the following 6–9 weeks until brain sampling (Harsan et al., 2008). After cuprizone withdrawal, mice received a subcutaneous Silastic® implant (inner diameter: 1.57 mm; outer diameter: 2.41 mm; length: 10 mm; Dow Corning) for 6–9 weeks, which was empty (controls) or filled either with crys-talline testosterone (Sigma), 5α-DHT (Sigma) or 7α-methyl-19-nortestosterone (Population Council). These implants produced constant levels of the steroids for the entire treatment period. All aspects of animal care were approved by the regional authorities.

Determination of steroid levels

Plasma and brain levels of testosterone and 5α-DHT were measured by gas chromatography/mass spectrometry as previously described (Liere et al., 2009). Plasma levels of 7α-methyl-19-nortestosterone were determined by a specific radioimmunoassay (Kumar et al., 1997).

Immunohistochemistry

Mice were deeply anaesthetized with a mixture of ketamine–xylazine and then fixed by transcardiac perfusion with 4% freshly prepared paraformaldehyde solution in PBS (0.1 M, pH 7.5). Brains were then removed and postfixed with the same paraformaldehyde solution for at least 2 days. Sagittal vibratome sections (50 μm) were prepared from one hemisphere and stored in 24-well plates containing PBS and 0.001% sodium azide solution at 4°C until further processing. The other brain hemisphere was processed for paraffin sectioning. Paraffin blocks were cut into 4–5 μm sections with a microtome (Leica).

Immunohistochemistry, using the free-floating vibratome sections, was performed as previously described (Harsan et al., 2008). Briefly, non-specific binding sites in tissue sections were blocked (VECTASTAIN® kit, Vector Laboratory), followed by overnight incubation with different primary antibodies against carbonic anhydrase II (CA II), myelin basic protein (MBP) and S100 protein (prepared in the laboratory of S.G.), glial fibrillary acidic protein (GFAP, Dako), ionized calcium binding adaptor molecule 1 (Iba1, Wako), NG2, Olig2 (Millipore), phosphorylated neurofilaments (SMI-31, Abcam) and 5-bromo-2’-deoxyuridine (BrdU, Roche). Sections were then incubated with biotinated secondary antibody and avidin/biotinylated enzyme complex (ABC) mixture (VECTASTAIN® kit). Colour was developed by the Vector®SK VIP peroxidase substrate (VIP). Fluorescent conjugated antibodies (Alexa Fluor® 488 or 546, Invitrogen) were also used.

For myelin basic protein immunostaining, paraffin-embedded sections were subjected to antigen retrieval by boiling in 10 mM sodium citrate buffer (pH 6.0) containing 0.05% Tween 20 at 98°C for 10 min in a microwave oven and were then allowed to cool in buffer at room temperature for 20 min. Sections were rinsed for 10 min in Tris-buffered saline at pH 6.0, followed by several washes in PBS containing 0.03% Triton™ X-100, blocking by VECTASTAIN® kit and incubation with primary antibody MBP for 2 h. This was followed by incubation with secondary antibody (1 h), ABC complex formation (40 min) and colour development (5 min). Tissue sections were washed, mounted permanently, photographed and further analysed using National Institute of Health (NIH) ImageJ® software.

Semi-thin plastic sections and electron microscopy

Anaesthetized mice were perfused with 2% paraformaldehyde and 2% glutaraldehyde (Sigma). Sagittal vibratome brain sections (200 μm) were prepared and kept overnight in the same fixative. Tissue sections were further postfixed in 0.03% Triton™ X-100, blocking by VECTASTAIN® kit and incubation with primary antibody MBP for 2 h. This was followed by incubation with secondary antibody (1 h), ABC complex formation (40 min) and colour development (5 min). Tissue sections were washed, mounted permanently, photographed and further analysed using National Institute of Health (NIH) ImageJ® software.

Organotypic cerebellar slice cultures

Postnatal Day 10 Sprague–Dawley rat pups (Janvier) were used (n = 120). Cerebellar parasagittal slices (350-μm thick) were cut on a Macilwain tissue chopper and transferred onto Millipore membranes of 30 mm diameter with a 0.4 μm pore size (Millicell, Millipore). Slices were maintained in culture on the membranes in six-well plates containing 1 ml of medium at 35°C in a 5% carbon dioxide (CO2) humidified atmosphere (Ghomiari et al., 2005). To induce demyelination,
the medium was removed from the wells after 7 days in culture and fresh medium containing 0.5 mg/ml lysophosphatidyl choline (Sigma) was added to the cultures for 17–18 h (Birgbauer et al., 2004; Hussain et al., 2011). The medium was then removed and the slices were incubated for four additional days in the absence or presence of 10 μM testosterone, 10 μM flutamide or 1 μM 5α-DHT. Steroids were dissolved in 0.01% ethanol and control cultures were treated with vehicle alone.

Myelin basic protein staining in the slices was measured using a confocal Zeiss LSM 410 (Carl Zeiss Inc) image analysing system. Images were acquired with a non-confocal configuration (488 nm and 543 nm excitations). For each measurement, a region of interest was located around the deep nuclei zone and the apical ends of each lobule, according to our observation that myelination begins at the deep nuclei zone in the cerebellum and thereafter progresses in the white matter along the axons. The myelin basic protein staining intensity was measured in these areas, using NIH ImageJ software.

**Immunohistological analysis**

Immunostaining densities (myelin basic protein, glial fibrillary acidic protein), myelin basic protein immunofluorescence and EGFP fluorescence intensity were analysed using NIH Image J. They were quantified on a continuous scale of 0–255 (darkest). To minimize differences among the respective measurements, we set as control an arbitrary level of staining. Staining density was evaluated as per cent (light pixels/light + dark pixels). Results are expressed as percentage of control ( = 100%). For counting cells, 12 vibratome slices from each brain and three regions of the corpus callosum from each slice were photographed. Then, four to six areas, each of 10000 μm², were analysed. Cells were counted by using NIH ImageJ software.

**Statistical analysis**

Group differences were analysed using two-way or one-way ANOVA followed by Newman–Keuls post hoc tests. Data are presented as mean ± standard error of the mean (SEM), and asterisks indicate significance as follows: ***P < 0.001; **P < 0.01; *P < 0.05. Statistica 64, version 10 (StatSoft) was used for analysis.

**Results**

**Recovery from chronic demyelination by testosterone therapy**

To assess the effect of testosterone therapy on myelin repair and to exclude the confounding effects of endogenous gonadal steroid hormones, we used castrated male and female C57Bl/6 mice. We previously reported that the number of oligodendrocytes in the corpus callosum and other myelinated fibre tracts is greater in males than females. We show here that > 20 weeks after removal of the gonads, a difference between sexes affecting the number of CA II immunoreactive oligodendrocytes remained (Fig. 1A and B). Feeding cuprizone to castrated mice for 12 weeks induced severe depletion of CA II+ oligodendrocytes and demyelination in the corpus callosum of both males and females, and this effect was not reversed 6 weeks after cuprizone removal from the diet in animals receiving empty subcutaneous Silastic® implants (Fig. 1). Even as late as 12 weeks after cuprizone withdrawal, no significant remyelination was observed (not shown), confirming the induction of a chronic demyelinating lesion (Harsan et al., 2008). However, when mice received subcutaneous Silastic® implants filled with testosterone for 6 weeks, the oligodendrocyte number and myelin basic protein immunostaining were restored in the corpus callosum of both sexes (Fig. 1). The implants produced plasma levels of 4.34 ± 0.5 ng/ml of testosterone, as determined by gas chromatography/mass spectrometry, corresponding to normal basal levels (Nyby, 2008). Brain levels of testosterone (3.16 ± 0.3 ng/g) reflected plasma levels as the hormone readily crosses the blood–brain barrier.

Endogenous levels of testosterone were found to be low in uncastrated male mice exposed for 12 weeks to cuprizone and group-housed (plasma: 0.66 ± 0.4 ng/ml; brain: 0.77 ± 0.42 ng/g). This may explain why no spontaneous remyelination was observed 6 weeks after cuprizone withdrawal. Treatment with Silastic® testosterone implants indeed efficiently restored oligodendrocytes within the corpus callosum of uncastrated males (control: 8 ± 0.5, cuprizone + empty implants: 0.9 ± 0.2; cuprizone + testosterone: 7.6 ± 0.3 oligodendrocytes/10 000 μm², means ± SEM, F(2, 15) = 54, P < 0.001, n = 6 per group).

We then investigated the remyelinating effect of testosterone in transgenic Plp-EGFP mice, expressing the EGFP in oligodendrocytes and myelin under the control of the myelin proteolipid protein gene promoter (Mallon et al., 2002). Following 12 weeks of exposure to cuprizone, EGFP fluorescence on brain sections was reduced by 85%, attesting to the depletion of oligodendrocytes and myelin loss throughout the brain (Fig. 2). Six weeks of testosterone treatment was sufficient to significantly increase the number of EGFP+ oligodendrocytes and myelin in the brain.

Testosterone-dependent remyelination was further assessed on semi-thin plastic-embedded sections and by electron microscopy (Fig. 3). The majority of cuprizone-demyelinated axons were remyelinated after 6 weeks of testosterone treatment, whereas axons of mice receiving empty implants remained demyelinated (Fig. 3B). Importantly, the mean diameter of corpus callosum axons was reduced by ~70% after cuprizone-induced demyelination, but returned to normal size after testosterone treatment (Fig. 3C). Although the density of corpus callosum axons tended to be decreased by cuprizone, testosterone had no effect on this parameter (Fig. 3D). The number of axons was quantified on SMI-31 immunostained sections, as this antibody reacts with phosphorylated neurofilament and broadly labels both thick and thin axons (Deboy et al., 2007).

**Remyelination involves the proliferation and maturation of oligodendrocyte precursor cells**

The remyelination of axons requires the generation of new oligodendrocytes from oligodendrocyte precursor cells (Chang et al., 2002; Franklin and ffrench-Constant, 2008). Oligodendrocyte precursor cells are distributed throughout the normal adult brain (Richardson et al., 2011). They are recruited to areas of demyelination, where they proliferate and differentiate into myelin-forming cells (Chang et al., 2000; Tripathi et al., 2010). We first
examined whether testosterone treatment promotes the recruitment of Olig2 immunoreactive cells within the chronically demyelinated corpus callosum. This transcription factor, which is expressed during early development and in the adult brain, is involved in the specification and differentiation of oligodendrocytes (Ligon et al., 2006; Maire et al., 2010). Olig2+ cells, abundant within the corpus callosum of control male mice, were markedly depleted by cuprizone (Fig. 4A and C). Following 6 weeks of testosterone treatment, the corpus callosum was repopulated by Olig2+ cells, and their density was even higher than in controls.

Testosterone treatment also promoted the recruitment of NG2+ oligodendrocyte precursor cells after cuprizone-induced demyelination. The corpus callosum of control mice only contained a small number of NG2+ cells. After 12 weeks of cuprizone insult followed by the insertion of an empty Silastic® implant under the skin for 6 weeks, the density of NG2+ cells was even further reduced. In contrast, treatment with testosterone-filled implants for 6 weeks resulted in an important recruitment of NG2+ oligodendrocyte precursor cells and a 5-fold increase in oligodendrocyte precursor cell density (Fig. 4B and D).

After 6 weeks of testosterone treatment, cell nuclear Olig2 staining within the corpus callosum co-localized with NG2+ cell processes and CA II immunoreactivity, thus attesting to the identity of oligodendroglial cells (Fig. 4E–G). Moreover, after 6 weeks of testosterone treatment, the majority of dividing cells identified 24 h after incorporation of the proliferation marker BrdU, belonged to the oligodendroglial lineage, as confirmed by co-immunolabelling with CA II (Fig. 4H–J). However, at this already advanced stage of remyelination, only a limited number of oligodendroglial cells continued to proliferate. Taken together, these findings strongly suggest that testosterone may promote the proliferation of oligodendrocyte precursor cells and their subsequent differentiation into oligodendrocytes.

Effects of testosterone on astrocytes and microglia

The activation and proliferation of astrocytes and microglial cells are part of the neuroinflammatory responses observed in demyelinating lesions and are neuropathological hallmarks of multiple
sclerosis. We thus examined the influence of testosterone on astrocytes and microglial cells during myelin repair. Cuprizone-induced demyelination resulted in strong reactive astrogliosis within the male mouse corpus callosum, as revealed by a marked increase in glial fibrillary acidic protein immunolabelling (Fig. 5A and C). A significant increase in the number of astrocytes was confirmed by counting S100+ cells (controls: 1.3 \( \pm \) 0.1; cuprizone: 3.2 \( \pm \) 0.4 cells/0.01 mm\(^2\), \( P < 0.01\); \( n = 5–6 \) per group). At 6 weeks after cuprizone withdrawal, levels of glial fibrillary acidic protein were similar in mice receiving a testosterone implant when compared with those receiving an empty implant. Thus, testosterone did not affect astrocyte activation during the first 6 weeks of treatment. However, glial fibrillary acidic protein immunostaining significantly declined after 9 weeks of testosterone treatment, whereas it remained elevated in empty implant-treated mice (Fig. 5A and C). As for astrocytes, the number of activated Iba1+ microglial cells was strongly increased during demyelination and stayed elevated after 6 weeks of testosterone administration, but returned to control levels after 9 weeks of testosterone (Fig. 5B and D).

**Pharmacological evidence for remyelination signalling of testosterone through the androgen receptor**

Pharmacological effects of testosterone on remyelination were assessed in cerebellar slice cultures obtained from postnatal Day 10 rat pups and maintained in culture for 7 days to allow abundant myelination (Birgbauer \textit{et al.}, 2004; Hussain \textit{et al.}, 2011). Severe myelin loss was observed 4 days after treatment with lysophosphatidyl choline for 17–18 h (0.5 mg/ml of culture medium) (Fig. 6A). However, the lysophosphatidyl choline treatment did not affect the number of axons beside the loss of their myelin sheaths (Fig. 6A and B). When slices were treated for 4 days with 10 \( \mu \)M testosterone after lysophosphatidyl choline removal, most axons were remyelinated.

We then examined the consequences of pharmacological androgen receptor blockade on testosterone-mediated myelin repair in these organotypic cultures. When lysophosphatidyl choline-demyelinated slices in culture were treated with testosterone for 4 days, the immunofluorescence intensity of myelin basic protein-positive fibre networks was increased 3-fold compared with control slices treated with vehicle (Fig. 6C and D). The remyelinating effect of testosterone was completely blocked by simultaneously adding the selective androgen receptor antagonist flutamide (10 \( \mu \)M). Conversely, the stimulation of remyelination could be mimicked by the non-aromatizable testosterone metabolite 5a-DHT (1 \( \mu \)M), a potent androgen receptor ligand (Fig. 6D). These \textit{in vitro} results suggest that cerebellar androgen receptor is required for testosterone-dependent remyelination.

**Genetic evidence for remyelination signalling of testosterone through the androgen receptor**

To assess whether the stimulation of remyelination by testosterone involves the classical intracellular androgen receptor, we examined whether testosterone promotes myelin repair in \( \text{AR}^{\text{Tfm}} \) mice. In these mice, a frame shift mutation in exon 1 of the \text{AR} gene produces a non-functional protein (Gaspar \textit{et al.}, 1991; Merlet \textit{et al.}, 2007). After 12 weeks of cuprizone administration, followed...
by 6 weeks of treatment with an empty Silastic® implant, the number of CA II+ oligodendrocytes and myelin basic protein immunoreactivity were markedly reduced in the corpus callosum of AR Tfm male mice, as previously observed in wild-type mice (Fig. 7A and B). However, in contrast to wild-type mice, 6 weeks of testosterone therapy did not allow restoration of the oligodendrocytes and myelin in AR Tfm mice.

Because in AR Tfm mice the androgen receptor is non-functional in all tissues and males exhibit an abnormal reproductive phenotype with an altered endocrine status (Zuloaga et al., 2008), we examined the specific relevance of brain androgen receptor to testosterone-dependent myelin repair. We used a mouse model that we previously developed with selective deletion of androgen receptor in neural cells including neurons and macroglia but sparing the androgen receptor in microglia [AR nefCre; Tg(NesCre)], designated as AR nefCre mice thereafter (Raskin et al., 2009).

Unlike AR Tfm mice, the AR nefCre mice have a normal development of the urogenital tract, produce offspring and have moderately increased levels of circulating testosterone and oestradiol when compared with wild-types (Raskin et al., 2009).

Castrated AR nefCre male mice were exposed to cuprizone for 12 weeks and were then treated during 6 weeks with empty or testosterone-filled Silastic® implants. After a severe depletion of CA II+ oligodendrocytes and pronounced demyelination by cuprizone, testosterone-treated AR nefCre mice remained severely deficient in oligodendrocytes and myelin (Fig. 7C–F).

**Figure 3** Testosterone therapy promotes the remyelination of corpus callosum. (A) Representative semi-thin plastic-embedded tissue sections stained with toluidine blue (top) and ultra-thin sections examined by electron microscopy (EM) (bottom) from corpus callosum of castrated control male mice (Cont), cuprizone-fed mice for 12 weeks (CUP 12 W), cuprizone-fed mice for 12 weeks followed by a treatment of 6 weeks with empty implants (6 W – T) and cuprizone-fed mice treated testosterone-filled Silastic® implants (6 W + T). Dramatic loss of myelin was observed after 12 weeks of cuprizone treatment (CUP-12 W) and the following weeks (CUP12 + W6 – T). We noticed a significant reduction of axonal diameter as a consequence of myelin loss. Testosterone promoted the remyelination of axons CUP12 W+(6 W + T), and the the axonal diameter of remyelinated axons returned to normal size. (B) The number of myelinated axons counted from electron microscopy micrographs [group differences F(3, 15) = 144, P < 0.001] (n = 4–5 per group). (C) The average of axonal diameters measured from electron microscopy graphs [group differences F(3, 15) = 78, P < 0.001] (n = 4–5 per group). (D) Density of SMI-31+ axons quantified by light microscopy on 50 μm vibratome sections [group differences F(3, 17) = 2.9, P = 0.063] (n = 5–6 per group). Values represent means ± SEM and were analysed by one-way ANOVA followed by Newman-Keuls post hoc tests. Significance: ***P < 0.001 when compared with control or testosterone-treated mice or as indicated.
Figure 4 Testosterone treatment significantly increased the number of Olig2+ cells and NG2+ oligodendrocyte precursor cells in the chronically demyelinated corpus callosum. Following cuprizone-induced chronic demyelination, castrated male mice were treated for 6 weeks with empty (−T) or testosterone-filled (+T) subcutaneous Silastic® implants. (A and C) Following the feeding of cuprizone (CUP), the density of Olig2+ cells was markedly decreased within the corpus callosum, and testosterone (T) treatment stimulated their recruitment [group differences $F(2, 20) = 34.3, P < 0.001$] ($n = 6–9$ per group). (B and D) Testosterone treatment also caused a 5-fold increase in the number of NG2+ oligodendrocyte precursor cells [group differences $F(2, 9) = 32.4, P < 0.001$] ($n = 4$ per group). Values presented in C and D correspond to means ± SEM and were analysed by one-way ANOVA followed by Newman-Keuls post hoc tests. Significance: ***P < 0.001 when compared with controls or testosterone-treated mice (C), or when compared with controls or mice receiving an empty implant (D). **P < 0.01 and *P < 0.05 as indicated. (E–J) The identification of cells induced by testosterone within the cuprizone-demyelinated corpus callosum. (E) Cells double-labelled for Olig2 (yellow-green stained nuclei) and NG2 (black cell processes). (F) Peroxidase immunostaining of Olig2+ nuclei. (G) Double-labeling of the peroxidase immunostained Olig2+ nuclei (black) and CA II+ oligodendrocytes (green) is indicated by arrows. (H) Immunodetection of BrdU+ nuclei of dividing cells. (I) Immunodetection of CA II+ oligodendroglial cells. (J) Co-localization of BrdU and CA II (arrows), visualized by merging H and I.
Those experiments demonstrate that the presence of neural androgen receptor is required for the action of testosterone in myelin repair, and that of microglial androgen receptor alone is not sufficient.

The androgen receptor is a promising therapeutic target in myelin repair

The identification of androgen receptor as a drug target for remyelination strategies opens new perspectives for the therapeutic use of selective androgen receptor modulators. Here, we tested the synthetic testosterone analogue 7α-methyl-19-nortestosterone, which binds with high affinity to androgen receptor but is not a substrate of the 5α-reductase, and hence is not converted to 5α-DHT, the active androgen in the prostate (Prasad et al., 2009). Indeed, 7α-methyl-19-nortestosterone possesses several potential therapeutic benefits over testosterone including improved pharmacological and pharmacokinetic properties allowing longer periods of administration at lower doses.

After 12 weeks of cuprizone demyelination of the corpus callosum, castrated male mice were treated for 6 weeks with subcutaneous Silastic® implants filled either with testosterone, 5α-DHT or...
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7α-methyl-19-nortestosterone. Controls received an empty implant. As reported above, the testosterone implants produced plasma and brain levels of the hormone comparable with those observed in normal males. The 5α-DHT implants produced physiological levels of the hormone in plasma and brain as determined by gas chromatography/mass spectroscopy (0.55 ± 0.12 ng/ml in serum and 0.96 ± 0.14 ng/g in brain). For comparison, brain levels of 5α-DHT measured in intact males or in castrated males receiving a testosterone implant were 0.25 ± 0.06 ng/g and 0.5 ± 0.04 ng/g, respectively. The 7α-methyl-19-nortestosterone

Figure 6 Testosterone promotes the remyelination of cerebellar slice cultures after lysophosphatidyl choline (LPC)-induced demyelination. (A and B) Effect of testosterone (T) and lysophosphatidyl choline treatment on the number of myelinated axons. (A) Antibodies against MBP and calbindin protein were used for the immunostaining of myelin (green) and axons (red). Most axons surrounded by myelin sheaths in control slices were remyelinated after 4 days of treatment with testosterone following lysophosphatidyl choline–induced demyelination. The arrowhead indicates a myelinating oligodendrocyte and the arrow denotes a Purkinje cell. Images were analysed using confocal microscopy. (B) While most axonal myelin was lost, the number of axons was not significantly affected by lysophosphatidyl choline treatment [group differences F(2, 13) = 0.86, P > 0.4], but testosterone restored the number of myelinated axons to normal level [group differences F(2, 12) = 45.0, P ≤ 0.001] (n = 5–6 per group). Values represent means ± SEM and were analysed by one-way ANOVA followed by Newman-Keuls post hoc tests. Significance: ***P < 0.001 when compared with sections treated with vehicle (controls) or testosterone (10 μM) after lysophosphatidyl choline. (C and D) The remyelinating effect of testosterone can be blocked by the selective androgen receptor antagonist flutamide and mimicked by its non-aromatizable metabolite 5α-DHT. (C) Representative immunofluorescence of MBP+ myelin in control slices, after lysophosphatidyl choline–induced demyelination followed by 4 days of vehicle, testosterone or testosterone + flutamide treatment. (D) The corresponding quantification of myelin basic protein immunofluorescence in the cerebellar slices [group differences F(4, 68) = 39.7, P ≤ 0.001] (n = 9–20 per group). Values represent means ± SEM (% of control) and were analysed by one-way ANOVA followed by Newman-Keuls post hoc tests. Significance: ***P < 0.001 when compared with controls or sections treated with testosterone alone or 5α-DHT.
Figure 7  Testosterone fails to stimulate remyelination in the chronically demyelinated corpus callosum of mice with a non-functional androgen receptor (AR<sup>Trm</sup> mice) or after selective neural androgen receptor ablation (AR<sup>Nes<sub>Cre</sub></sup> mice). (A and B) In AR<sup>Trm</sup> mice, the corpus callosum was depleted of CA II<sup>+</sup> oligodendrocytes and MBP<sup>+</sup> myelin after 12 weeks of cuprizone (CUP) treatment followed by the administration of empty implants during 6 weeks (CUP – T). Treatment for 6 weeks with testosterone implants (CUP + T) failed to stimulate the recruitment of new oligodendrocytes and myelin repair [group differences for CA II<sup>+</sup> oligodendrocytes: F(2, 16) = 56.7, P ≤ 0.001, and for myelin basic protein immunofluorescence: F(2, 15) = 21.3, P ≤ 0.001] (n = 6–7 per group). (C–F) As in AR<sup>Trm</sup> mice, testosterone treatment failed to stimulate oligodendrocyte recruitment and remyelination of the cuprizone-demyelinated corpus callosum of AR<sup>Nes<sub>Cre</sub></sup> mice. (C and E) CA II<sup>+</sup> oligodendrocytes in the corpus callosum of control AR<sup>Nes<sub>Cre</sub></sup> mice or after feeding cuprizone for 12 weeks followed by treatment for 6 weeks with empty (CUP – T) or testosterone-filled (CUP + T) Silastic<sup>®</sup> implants [group differences F(2, 12) = 48.6, P ≤ 0.001]. (D and F) Myelin basic protein immunofluorescence staining of the corpus callosum of control, CUP – T and CUP + T AR<sup>Nes<sub>Cre</sub></sup> mice. Results are expressed as % of control [group differences F(2, 13) = 76.3, P ≤ 0.001] (n = 4–7 per group). Values represent means ± SEM and were analysed by one-way ANOVA followed by Newman-Keuls post hoc tests. Significance: ***P ≤ 0.001 when compared with the corresponding controls.
implants produced plasma levels of the androgen in the range required for a full suppression of sperm production in males as determined by a specific radioimmunoassay (2.12 ± 0.31 ng/ml) (von Eckardstein et al., 2003). Under these experimental conditions, testosterone, 5α-DHT and 7α-methyl-19-nortestosterone were equally efficient in stimulating the recovery of oligodendrocytes (Fig. 8A) and strong remyelination (Fig. 8B).

Discussion

The stimulation of endogenous myelin repair remains an important therapeutic challenge in multiple sclerosis. Under conditions of severe and chronic demyelination, we found that testosterone is a potent inducer of remyelination. Despite the previously reported sexual dimorphism affecting the structure of myelin and the number of oligodendrocytes (Cerghet et al., 2006), and differences in the incidence of multiple sclerosis between males and females (Confavreux and Vukusic, 2006; Fromont et al., 2010), a potential role of testosterone and its receptor in myelin repair has been neglected.

A strong remyelinating effect of testosterone was demonstrated in the cuprizone model of persistent demyelination, where no spontaneous recovery of myelin can be detected (Matsushima and Morell, 2001; Harsan et al., 2008). In this model, only the transplantation of oligodendrocyte precursor cells or the administration of thyroid hormone have been found to promote myelin repair (Mason et al., 2004; Harsan et al., 2008). Testosterone treatment for 6 weeks was sufficient to stimulate the recruitment of Olig2+ cells and NG2+ oligodendrocyte precursor cells, leading to efficient remyelination in both males and females. This is a remarkable effect after depletion of ~80% of CA II+ oligodendrocytes and a >50% reduction in the number of NG2+ precursors in response to 12 weeks of cuprizone feeding. Most repopulating Olig2+ cells and the proliferative CA II+ cells as well as EGFP+ cells (not shown) were predominantly detected in the corpus callosum, suggesting a local neo-oligodendrogenesis in the damaged white matter. However, proliferation was also observed in the subventricular zone, although less extensive. The present observation is supported by previously published results about the local induction of oligodendrocytes by thyroid hormone in demyelinated corpus callosum (Harsan et al., 2008) and in myelinated tracts after the ablation of oligodendrocytes during postnatal development (Jalabi et al., 2005). Moreover, oligodendrocyte regeneration was also observed in early multiple sclerosis lesions (for review see Franklin and ffrench-Constant, 2008). It is worthy to note that the atrophy of axons caused by myelin loss observed in the present study is also supported by previous observations in adult and young mice (Jalabi et al., 2005; Harsan et al., 2008), and it can be accurately explained by the important role of oligodendroglia and myelin in the maintenance of healthy axons (Wake et al., 2011; Lee et al., 2012).

The cuprizone-induced demyelination and depletion of oligodendrocytes was also accompanied by a significant increase in the number of astrocytes (S100+ and GFAP+ cells) and microglial cells (Iba1+ cells). Although prolonged and strong inflammation can cause severe damage to neurons and nerve fibres (Howell et al., 2010), a mild inflammatory response may be beneficial for remyelination (Franklin, 2002; Li et al., 2005). Our results show that both astrocytes and microglial cells remain activated during the process of testosterone-dependent myelin repair, when they may be supportive, but that they are downregulated by the hormone at a more advanced stage of remyelination.

Figure 8 After cuprizone-induced chronic demyelination of the corpus callosum, the remyelinating effect of testosterone (T) can be mimicked by its non-aromatizable metabolite 5α-DHT and by the potent androgen receptor ligand 7α-methyl-19-nortestosterone (MENT). (A) Treatment of castrated males after cuprizone withdrawal for 6 weeks with a Silastic® implant filled with testosterone, 5α-DHT or 7α-methyl-19-nortestosterone efficiently replenished the number of CA II+ oligodendrocytes. The number of oligodendrocytes remained low in controls (C) receiving an empty implant [group differences F(3, 24) = 62, P < 0.001] (n = 5–7 per group). (B) Treatment with testosterone, 5α-DHT or 7α-methyl-19-nortestosterone also efficiently restored myelin basic protein immunoreactive myelin, whereas myelin basic protein immunofluorescence remained low in controls. Results are expressed as % of myelin basic protein immunofluorescence in normal males [group differences F(3, 25) = 25.2, P < 0.001] (n = 5–13 per group). Values represent means ± SEM and were analysed by one-way ANOVA followed by Newman-Keuls post hoc tests. Significance: ***P ≤ 0.001 when compared with the corresponding controls.
Such a temporal regulation of neuroinflammatory responses may represent an additional therapeutic benefit of testosterone.

It is important to note that delivery of testosterone from subcutaneous implants, providing normal basal levels observed in male mice (1–5 ng/ml) (Nyby, 2008), was sufficient to stimulate efficient remyelination of axons. In sexually aroused males, circulating testosterone can reach concentrations >20 ng/ml (James et al., 2006; Nyby, 2008). These observations indicate that levels of testosterone may influence the capacity of the brain to regenerate myelin, and cast a new light on previous reports of impaired testicular functions and reduced testosterone levels in patients with multiple sclerosis (Wei and Lightman, 1997; Safarinejad, 2008). Basal levels of testosterone in normal males are ~5 ng/ml, thus similar to the ones induced in our experimental study. They were found to be only slightly reduced in relapsing-remitting multiple sclerosis, but significantly decreased when multiple sclerosis becomes progressive. Moreover, the response of serum testosterone to gonadotropin stimulation is blunted in progressive multiple sclerosis (Safarinejad, 2008). These findings also raise the interesting question of a possible relationship between the age-dependent decline of testosterone levels (Rosario et al., 2004) and the rapid progression of multiple sclerosis in ageing males. In addition, androgens also play a significant role in female health (Chen et al., 2006), and an increased number of multiple sclerosis lesions have been reported in females with reduced levels of testosterone (Tomassini et al., 2005).

The finding that testosterone, at physiological levels, is a potent promoter of myelin formation sheds a new light on the role of steroid hormones in demyelinating diseases and extends our therapeutic possibilities. However, the remyelinating effect of testosterone alone does not explain all the complex aspects of gender differences in multiple sclerosis: why more females than males have the disease, and why it affects males and females differently (Confavreux and Vukusic, 2006; Fromont et al., 2010). First, in this study, we specifically addressed the problem of myelin repair, more related to the recovery from multiple sclerosis attacks and to the progress of the disease than to its incidence, which involves adaptive immune responses. Second, gender differences, whether they affect the immune system or the remyelination process, involve multiple hormonal and genetic factors. Thus, myelin formation can also be influenced by the major female reproductive hormones, oestradiol and progesterone, and by neurosteroids that are locally synthesized within the brain and spinal cord (Gold and Voskuhl, 2009; Hussain et al., 2011). Moreover, hormone-dependent structural differences in myelin between males and females may affect demyelination and remyelination (Cerghet et al., 2006). Recent studies suggest that intrinsic genetic sex differences in neural cells may also play a significant role (Smith-Bouvier et al., 2008). Male gender has been associated with an earlier assignment of irreversible multiple sclerosis disability landmarks (Confavreux and Vukusic, 2006). Interestingly, this observation is consistent with experimental studies showing that older female rats more efficiently remyelinate axons than males after toxin-induced demyelination (Li et al., 2006). This type of experimental model should allow us to further explore specific aspects of gender differences in multiple sclerosis progression.

Using pharmacological and genetic tools, we demonstrate in vitro and in vivo that the androgen receptor mediates the remyelination action of testosterone. A functional androgen receptor is indeed required, as testosterone failed to stimulate the replenishment of oligodendrocytes and remyelination in the corpus callosum of AR<sup>TM</sup> mice, expressing a non-functional androgen receptor. Importantly, the brain androgen receptor represents a valuable target for myelin repair because testosterone failed to stimulate remyelination after its neural cell-specific deletion in AR<sup>NecCre</sup> mice. In these mice, the androgen receptor is selectively inactivated in neurons and macroglial cells, but continues to be expressed in microglial cells. Thus, the presence of androgen receptor in microglia alone (Garcia-Ovejero et al., 2002) is not sufficient to induce testosterone-dependent myelin repair.

Given that, in the nervous system, the androgen receptor is predominantly expressed in neurons and is particularly abundant in axons and dendrites of the cerebral cortex, subcortical structures including the corpus callosum and less abundant in astrocytes, the remyelinating effects of testosterone might be indirect and mediated by these cells (DonCarlos et al., 2003; Lorenz et al., 2005). Both cell types indeed play key roles in myelin formation by producing trophic factors (Talbott et al., 2005; Trapp and Nave, 2008).

Whatever the cellular targets of testosterone, the brain androgen receptor is a particularly attractive drug target for long-term remyelination-based therapies, as the receptor is upregulated by androgens (Lu et al., 1998). Further investigation, including the generation of different transgenic mouse lines for cell-type specific ablation of androgen receptor, may be required for long-term studies in the field.

We also aimed to reduce the peripheral effects of testosterone, attributed mainly to its natural metabolite 5α-DHT. Our findings provide support for the therapeutic use of androgens to demyelinating diseases. We show that 7α-methyl-19-nortestosterone, at a contraceptive dose, is as efficient as testosterone or 5α-DHT in stimulating myelin repair. This synthetic androgen, which has been developed for long-term male contraception and androgen replacement therapy in hypogonadal men, has several potential therapeutic benefits over testosterone: (i) it is ~10 times more potent than testosterone, allowing the administration of low doses through sustained-release delivery systems; (ii) it is resistant to 5α reduction and therefore, its action is not amplified in the prostate and other peripheral targets of testosterone; and (iii) it has improved pharmacological and pharmacokinetic properties (Prasad et al., 2009).

In summary, the present study is the first to demonstrate a strong remyelinating effect of testosterone mediated by its receptor. We identify the brain androgen receptor as a promising therapeutic target for myelin repair. Selective synthetic drug ligands of the androgen receptor, such as the prostate sparing 7α-methyl-19-nortestosterone, are promising therapeutic agents for promoting remyelination. These observations thus provide the preclinical basis for new clinical trials aimed to test the therapeutic efficacy of androgens in males with multiple sclerosis.
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