

Drug-Based Modulation of Endogenous Stem Cells Promotes Functional Remyelination *In Vivo*

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Introduction

Multiple sclerosis (MS) involves an aberrant autoimmune response and progressive failure of remyelination in the CNS. Prevention of neural degeneration and subsequent disability requires remyelination through the generation of new oligodendrocytes, but current treatments exclusively target the immune system. Oligodendrocyte progenitor cells (OPCs) are stem cells in the CNS and the principal source of myelinating oligodendrocytes (Goldman et al., 2012). These progenitor cells are abundant in demyelinated regions of patients with MS yet fail to differentiate, thereby representing a cellular target for pharmacological intervention (Chang et al., 2002). To discover therapeutic compounds for enhancing myelination from endogenous OPCs, we screened a library of bioactive small molecules on mouse pluripotent epiblast stem cell (EpiSC)-derived OPCs (Brons et al., 2007; Tesar et al., 2007; Najm et al., 2011). Here we discuss seven drugs that function at nanomolar doses to selectively enhance the generation of mature oligodendrocytes from progenitor cells *in vitro*.

Two drugs—miconazole and clobetasol—are effective *in vitro* for promoting precocious myelination in organotypic cerebellar slice cultures, and *in vivo*, in early postnatal mouse pups. Systemic delivery of each of the two drugs significantly increases the number of new oligodendrocytes and enhances remyelination in a lysolecithin-induced mouse model of focal demyelination. Administering each drug at the peak of disease in an experimental autoimmune encephalomyelitis mouse model of chronic progressive MS results in a striking reversal of disease severity. Immune response assays show that miconazole functions directly as a remyelinating drug with no effect on the immune system, whereas clobetasol is a potent immunosuppressant as well as a remyelinating agent. Mechanistic studies show that miconazole and clobetasol function in OPCs through mitogen-activated protein (MAP) kinase and glucocorticoid receptor signaling, respectively. Furthermore, both drugs enhance the generation of human oligodendrocytes from human OPCs *in vitro*. Collectively, our results provide a rationale for testing miconazole and clobetasol (or structurally modified derivatives) to enhance remyelination in patients.

Results

Lead generation

Repair of damaged myelin may provide therapeutic benefit in MS and other demyelinating disorders (Dubois-Dalcq et al., 2005; Franklin et al., 2008;

Mi et al., 2009; Fancy et al., 2010; Bai et al., 2012; Deshmukh et al., 2013; Mei et al., 2014). Therefore, we set out to identify drugs that could be repurposed as remyelinating therapeutics. We selected the US National Institutes of Health (NIH) Clinical Collection I and II libraries comprising 727 drugs with a history of safe use in clinical trials, to test for maturation of OPCs into myelinating oligodendrocytes. Using mouse EpiSC-derived OPCs, we developed an *in vitro* phenotypic screen that accurately quantified differentiation into mature oligodendrocytes using high-content imaging of myelin protein expression (Fig. 1a).

Two batches (>100 million cells each) of pure OPCs were generated from independent mouse pluripotent EpiSC lines of opposite sex. EpiSC-derived OPCs shared virtually all defining molecular and cellular properties, including gene expression profiles, with *in vivo* isolated OPCs but provided the key advantage of being highly scalable (Najm et al., 2011). For *in vitro* screening, the seeding density, endpoint assays, and DMSO (vehicle) tolerance were optimized in pilot studies to ensure accurate and reproducible measurement of OPC differentiation in a 96-well format.

For the primary screen, OPCs were treated with vehicle alone (0.05% [v/v] DMSO) as a negative control, thyroid hormone (a known OPC differentiation inducer) as a positive control (Barres et al., 1994), or drug dissolved in DMSO at a concentration of 5 μ M. After 72 h, cells were fixed and labeled with antibodies to MBP, and the length and intensity of MBP-labeled oligodendrocyte processes were measured (Fig. 1a). These features were reliable indicators of alteration in cellular phenotype, as indicated by consistency and high signal-to-background ratio of positive and vehicle controls across all screening plates. We then normalized the experimental data for the tested drugs against thyroid hormone (set value of 100) on a per-plate basis. On the basis of this analysis, we identified the 22 drugs that enhanced oligodendrocyte formation > 5 SD above DMSO treatment and outperformed thyroid hormone in the measured parameters (Fig. 1b). Notably, one of the top 22 drugs was benztropine, a muscarinic receptor antagonist recently shown to induce OPC differentiation and remyelination (Deshmukh et al., 2013; Mei et al., 2014).

To validate and prioritize the 22 drug hits, the assay was repeated using alternative OPCs, reagents, and parameters to eliminate screen-specific artifacts. Drugs were ranked by their dose-dependent ability

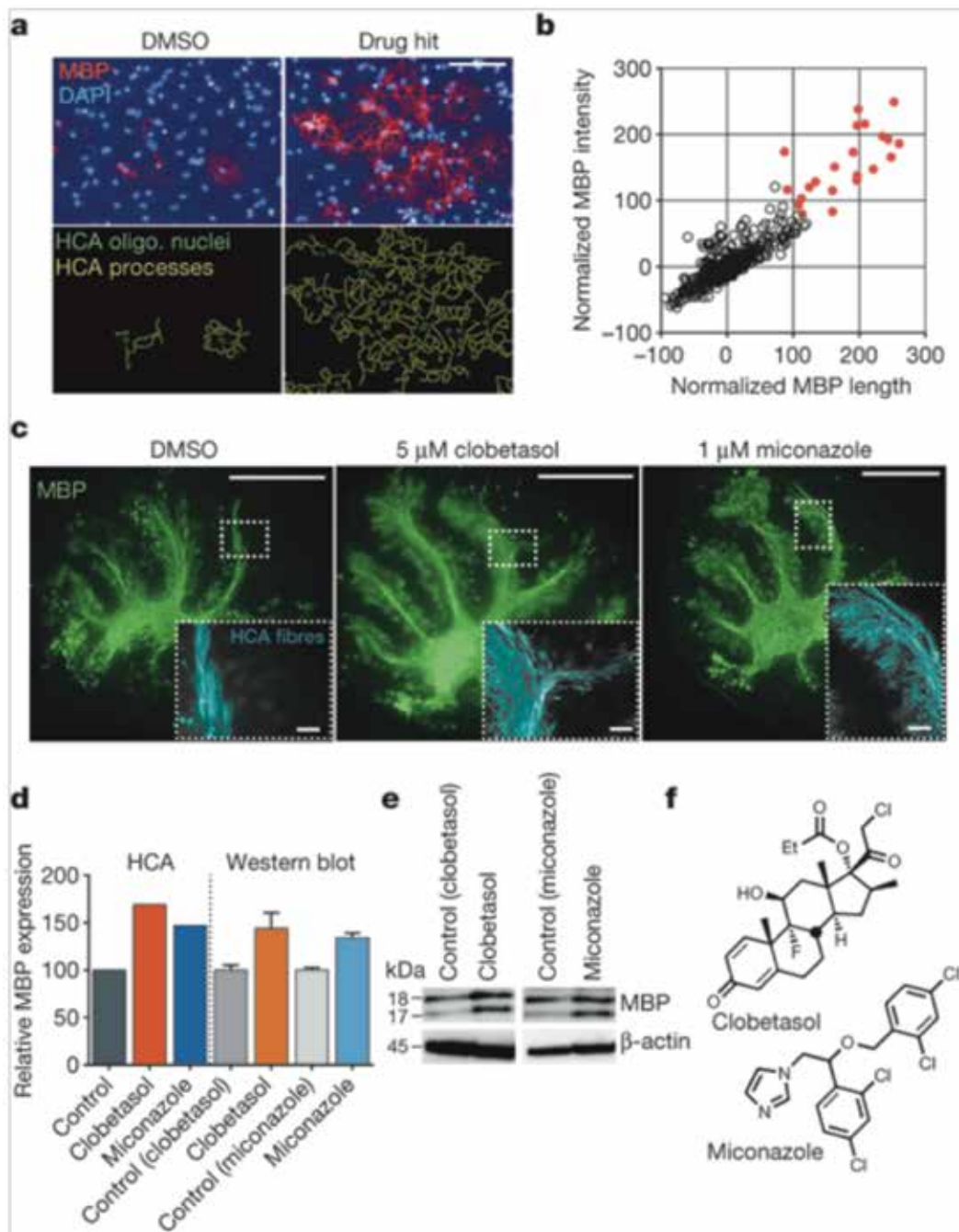


Figure 1. A pluripotent stem cell-based phenotypic screening platform to identify modulators of OPC differentiation and maturation. **a**, Representative images of vehicle-treated and drug hit-treated mouse EpiSC-derived OPCs from the primary screen. Nuclear (DAPI [4',6-diamidino-2-phenylindole], blue) and MBP (red) staining along with HCA to identify oligodendrocyte (oligo.) nuclei (green) and MBP⁺ processes (yellow). Scale bar, 100 μm. **b**, Scatter plot of primary screen results displayed as normalized values of MBP process length and intensity for all 727 drugs, with the 22 hits marked in red. Baseline (vehicle) was set at 0, and thyroid hormone (positive control) was set at 100. **c**, Montaged images of whole postnatal day 7 mouse cerebellar slices treated with drug or vehicle for 5 d and stained for MBP (green). Insets show a representative example of the HCA script used to identify and quantify MBP⁺-aligned fibers (light blue). Scale bars, 1 mm for whole slices and 100 μm for insets. **d**, Relative quantification of HCA and Western blot data from cerebellar slices treated for 5 d. For HCA screen, $n = 1$, with 6–12 slices averaged per group. For Western blot, $n = 3$ independent replicates of 12 slices per group. Values are mean for HCA and mean \pm SEM for Western blot. **e**, Representative Western blot of MBP isoforms and β -actin (loading control) of cerebellar slices treated for 5 d. **f**, Chemical structures of clobetasol and miconazole. Reprinted with permission from Najr et al. (2015), their Fig. 1.

to induce oligodendrocyte generation from OPCs without toxicity. To demonstrate reproducibility, an independent laboratory tested selected drug hits using distinct equipment, plate format (1536-well), personnel, and imaging/analysis scripts. Of the 16 hits tested at the external screening site, 14 were validated as potent inducers of oligodendrocyte differentiation.

Testing drug leads *in vitro*

We next tested whether the drug hits could promote the maturation of native OPCs in CNS tissue. Cerebellar slices were generated from mice at postnatal day 7—a time that precedes widespread myelination—and treated *ex vivo* with drug or DMSO (vehicle) for 5 d and labeled with anti-MBP antibodies (Fig. 1c) (Mi et al., 2009; Bai et al., 2012). We screened 11 of the top drugs and used a high-content analysis (HCA) algorithm developed in house to rank them on the basis of their ability to increase the extent of MBP⁺ aligned fibers in whole cerebellar slices. The so-called high-performing group consisted of four drugs that increased the number of MBP⁺ aligned fibers by approximately 150% or greater (Fig. 1d). We validated the accuracy of our high-content screen using semiquantitative western blotting of MBP protein isoforms in independent slice-culture experiments (Figs. 1d, e) (Woodruff et al., 1998, 1999).

Analysis of structure–activity relationships revealed that the top hits from the primary screen segregated into two specific classes containing either a 1,3-diazole with monosubstitution at the 1-position or a sterane base structure. We selected miconazole and clobetasol, the top overall performing hits in each of the imidazole and sterane classes, respectively, for further mechanistic and functional testing after confirming that both drugs readily cross the blood–brain barrier in mice (Fig. 1f). Miconazole is a topical antifungal agent functioning through cytochrome P450 inhibition, and clobetasol is a potent topical corticosteroid, but their functions in OPCs were unknown.

Testing miconazole and clobetasol *in vivo*

To test whether miconazole or clobetasol enhance remyelination *in vivo*, we used a toxin-induced model whereby focal demyelinated lesions are generated in dorsal white matter of the spinal cord of adult mice by localized injection of lysophosphatidylcholine [LPC]). In lesioned

animals, demyelination is complete within 4 d, after which OPCs are recruited into the lesion. Widespread remyelination does not normally start until 14–21 d postlesion (d.p.l.), which provides a defined window from days 4–14 to test the efficacy of drugs to enhance the extent and rate of remyelination (Jeffery et al., 1995). Both miconazole (10 or 40 mg/kg body weight) and clobetasol (2 mg/kg) treatment induced a marked improvement within the lesions of treated mice compared with vehicle-treated controls. At 8 d.p.l., both drugs induced a significant increase in the number of newly generated CC1⁺ oligodendrocytes in the lesion core (Figs. 2a, b). This effect was coincident with extensive MBP staining in the lesions of miconazole-treated and clobetasol-treated, but not vehicle-treated, animals at both 8 and 12 d.p.l. (Fig. 2a). Electron micrographs and tissue sections stained with toluidine blue demonstrated that miconazole and clobetasol each induced a striking increase in the extent of remyelination (Figs. 2c, d). At 12 d.p.l., lesions of vehicle-treated mice consisted mostly of unmyelinated axons (6% myelinated), while those of miconazole-treated and clobetasol-treated mice contained more than 70% remyelinated axons throughout the extent of the lesion (Fig. 2d). Analysis of myelin thickness relative to axon diameter (g ratio) at 12 d.p.l. revealed that miconazole-induced and clobetasol-induced myelin was thinner than intact myelin, a defining characteristic of remyelination (Fig. 2d).

We also evaluated whether miconazole or clobetasol could promote precocious myelination during development, in the absence of injury or disease. We treated mice at postnatal day 2 (a time point that precedes widespread CNS myelination) daily for 4 d with drug or vehicle. In miconazole-treated and clobetasol-treated mice, we found a significant increase in the number of CC1⁺ oligodendrocytes in the lateral corpus callosum compared with vehicle-treated mice (not shown). Additionally, we found that a significantly larger portion of the corpus callosum was populated by MBP⁺ fiber tracts in miconazole-treated and clobetasol-treated mice. This finding suggests that miconazole and clobetasol enhance myelination in the absence of damage or disease. Collectively, the LPC demyelination and developmental mouse models demonstrated that miconazole and clobetasol each function to induce the differentiation of endogenous OPCs in the CNS and promote enhanced myelination.

To determine whether the drugs were working at a particular stage of the OPC differentiation process,

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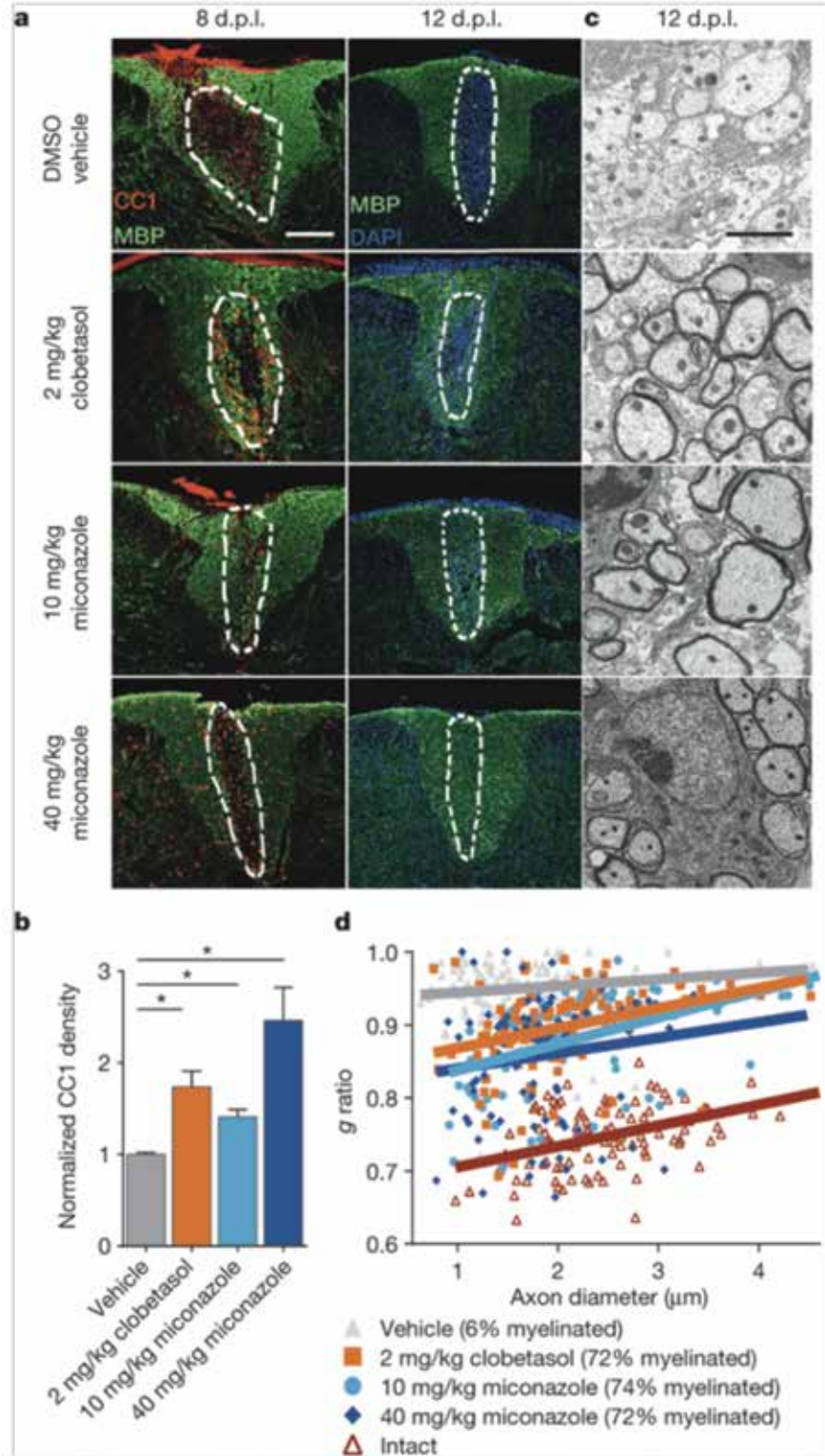


Figure 2. Miconazole and clobetasol each enhance remyelination in the LPC lesion mouse model. **a**, Representative immunohistochemical images of treated mice showing newly generated oligodendrocytes (CC1, red) and MBP (green) within the lesion (approximated by white dashed outline) at 8 and 12 d.p.i. Scale bar, 200 μm . **b**, Quantification of CC1⁺ oligodendrocytes per lesion area at 8 d.p.i. Values are mean \pm SEM; $n = 3$ mice per group. Two-tailed t test; $*p < 0.05$. **c**, Representative electron micrographs showing remyelinated axons within lesions of drug-treated mice at 12 d.p.i. Scale bar, 2 μm . **d**, Scatter plot of g ratios of lesion axons at 12 d.p.i.; $n = 100$ calculated from two mice per group. Percentage of lesion axons myelinated is indicated in the legend. Reprinted with permission from Najr et al. (2015), their Fig. 2.

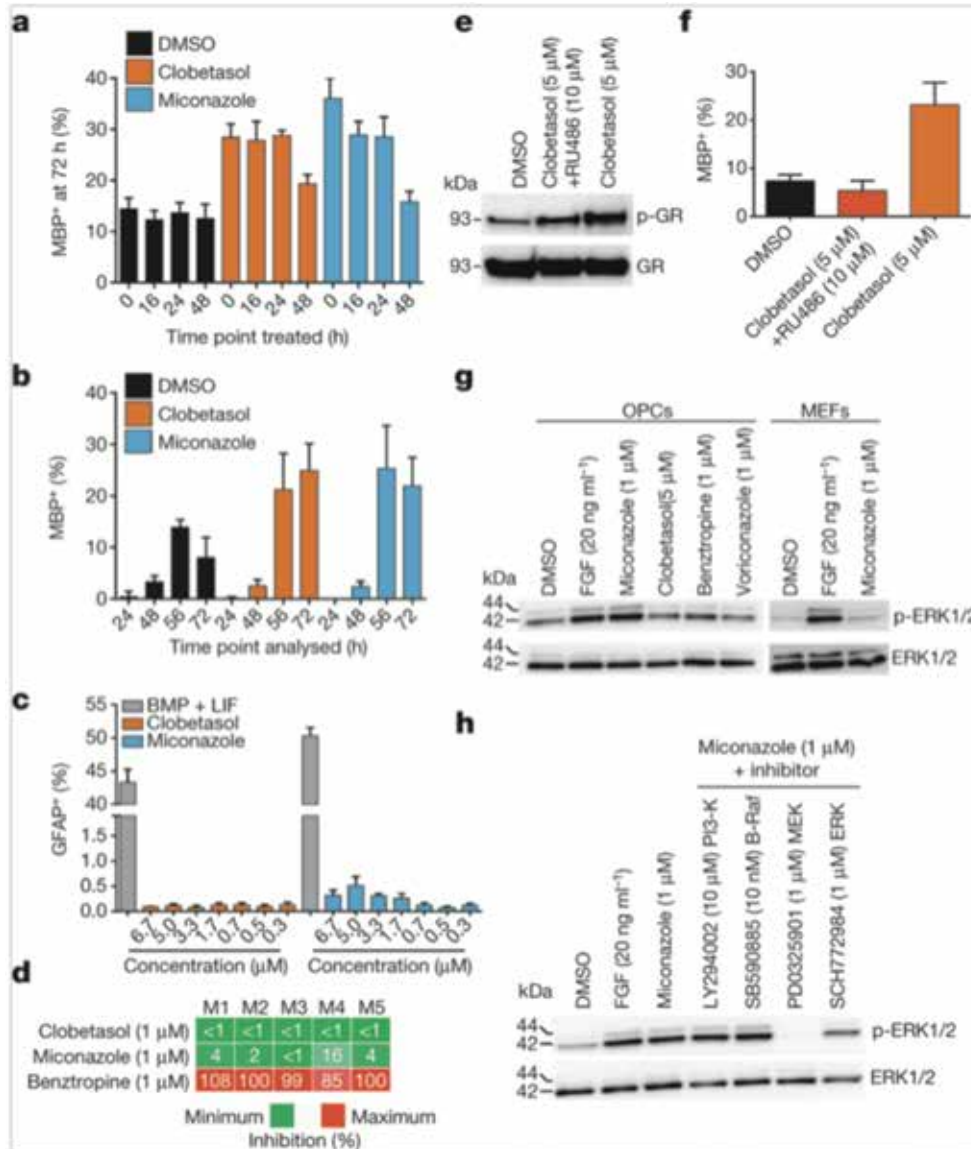


Figure 3. Cellular and molecular effects of miconazole and clobetasol on mouse OPCs. **a**, Percentage MBP⁺ oligodendrocytes generated from OPCs at 72 h with treatments initiated at time points indicated; $n = 6$ wells per condition with > 6000 cells scored per well. **b**, Percentage MBP⁺ oligodendrocytes generated from OPCs treated simultaneously and analyzed at time points indicated; $n = 8$ wells per condition with > 1700 cells scored per well. **c**, Percentage GFAP⁺ astrocytes generated from OPCs at 72 h of treatment; $n = 4$ wells per condition > 2900 cells scored per well. **d**, Heat map depicting biochemical inhibition of muscarinic receptors M1–M5 displayed as percentage inhibition with minimum (green) and maximum (red). **e**, Western blot of total glucocorticoid receptor and its phosphorylation at Ser220 (p-GR) in OPCs treated for 1 h. **f**, Percentage MBP⁺ oligodendrocytes generated from OPCs 72 h after treatment; $n = 6$ wells per condition with > 1400 cells scored per well. **g**, Western blot of total ERK1/2 and their phosphorylation at Thr202/Tyr204 or Thr185/Tyr187 (p-ERK1/2) in cells (OPCs or mouse embryonic fibroblasts [MEFs]) treated for 1 h. Fibroblast growth factor (FGF) served as a positive control for p-ERK1/2 induction. **h**, Western blot of total ERK1/2 and p-ERK1/2 in OPCs treated for 1 h in the presence of the indicated pathway inhibitors. All graphs depict mean \pm SEM. Reprinted with permission from Najr et al. (2015), their Fig. 3.

we seeded OPCs in differentiation conditions and treated them with either miconazole or clobetasol at different time points (0, 16, 24, or 48 h), and assayed MBP expression at 72 h. For both miconazole and clobetasol, the number of MBP⁺ oligodendrocytes present at 72 h was dependent on drug treatment

within the first 24 h of differentiation (Fig. 3a). In agreement with these data, treatment of differentiating OPCs with either drug for different durations (24, 48, 56, and 72 h) induced a progressive, time-dependent increase in the number of MBP⁺ oligodendrocytes (Fig. 3b). These data suggest that

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both drugs function directly on OPCs early in the differentiation process. Additionally, neither drug showed a significant impact on astrocyte formation from OPCs *in vitro*, suggesting they probably function as direct inducers of oligodendrocyte differentiation (Fig. 3c).

Muscarinic receptor antagonists such as benztropine and clemastine have recently been identified as remyelinating agents (Deshmukh et al., 2013; Mei et al., 2014). Therefore, we tested whether miconazole or clobetasol function through the muscarinic acetylcholine pathway using functional cellular reporter assays of all muscarinic receptor subtypes (M1–M5). Neither miconazole nor clobetasol inhibited any of the five muscarinic receptor subtypes (Fig. 3d). We then profiled whether clobetasol or miconazole biochemically inhibited the activity of 414 different kinase isoforms. Neither clobetasol nor miconazole inhibited any of the kinases tested, suggesting their activity is not based on direct inhibition of protein kinases.

Effect on OPC signaling pathways

To explore the signaling pathways in OPCs influenced by these drugs, we performed genome-wide RNA sequencing and phosphoproteomic analyses on mouse OPCs treated with drug or vehicle. Miconazole or clobetasol treatment altered OPC transcript expression and phosphoproteins within hours and influenced the expression of genes in signaling pathways involved in oligodendrocyte maturation and myelination. Clobetasol potentially modulated genes downstream of multiple nuclear hormone receptors, including glucocorticoid receptors, which are known to be important regulators of myelin gene expression (Kumar et al., 1989; Barres et al., 1994). Since glucocorticoid receptor signaling is also known to enhance Schwann cell-mediated myelination in the peripheral nervous system (Morisaki et al., 2010), we tested whether the activity of clobetasol on OPCs was mediated by glucocorticoid receptor signaling. Treatment of OPCs with clobetasol for 1 h increased the phosphorylation of glucocorticoid receptor at Ser220, an activating posttranslational modification (Fig. 3e). RU486, a competitive glucocorticoid receptor antagonist, blocked clobetasol-induced glucocorticoid receptor phosphorylation and oligodendrocyte differentiation (Figs. 3e, f), suggesting that the activity of clobetasol in OPCs is mediated through the glucocorticoid receptor signaling axis.

For miconazole, pathway analyses showed that proteins in the MAP kinase pathway were most strongly affected. Most prominent was the strong

and sustained phosphorylation of both extracellular signal-regulated kinases ERK1 and ERK2 (ERK1/2) at canonical activation sites, which we validated by Western blotting (Fig. 3g). In mice, genetic loss of ERK1/2 in the oligodendrocyte lineage results in normal numbers of OPCs and oligodendrocytes but widespread hypomyelination, while constitutive activation of ERK1/2 results in a profound increase in the extent of remyelination after toxin-induced demyelinating injury (Ishii et al., 2012; Fyffe-Maricich et al., 2013). In contrast to miconazole, treatment of OPCs with clobetasol or benztropine did not induce ERK1/2 phosphorylation (Fig. 3g). Miconazole treatment of a nonneural cell type, mouse fibroblasts, also showed no increase of ERK1/2 phosphorylation, indicating potential cell-type specificity (Fig. 3g). PD0325901, a small-molecule inhibitor of ERK's upstream MAP kinase kinase (MEK), blocked the ability of miconazole to induce ERK1/2 phosphorylation, suggesting that miconazole functions through a MEK-dependent mechanism in OPCs (Fig. 3h). We also treated mouse OPCs with voriconazole, a triazole-containing antifungal cytochrome P450 inhibitor with 80% structural similarity to miconazole, which failed to induce changes in ERK1/2 phosphorylation (Fig. 3g). This result was consistent with the observation that voriconazole did not promote the differentiation of OPCs into oligodendrocytes. Taken together, these results suggest that the effect of miconazole on OPCs is independent of cytochrome P450 inhibition (not shown).

Differentiation of human OPCs into oligodendrocytes

We then assessed whether clobetasol and miconazole treatment would enhance the differentiation of human OPCs into oligodendrocytes. We generated human OPCs from human embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs) (Hu et al., 2009; Wang et al., 2013). We then treated human OPCs with DMSO, clobetasol, or miconazole for 21 d followed by staining for MBP, imaging, and HCA (not shown). Both drugs enhanced human OPC differentiation, with miconazole exhibiting the most reproducible and potent effects.

Therapeutic effect in immune-mediated MS models

To interpret the potential impact of clobetasol or miconazole as therapeutics in immune-mediated MS models, we tested effects on immune cell survival and function. We found that only clobetasol, as expected from its known corticosteroid properties, altered

naive T-cell differentiation and both the proliferation and secretion of cytokines by proteolipid protein (PLP_{139–151})-sensitized or myelin oligodendrocyte glycoprotein (MOG_{35–55})-sensitized lymph node cells (not shown). As such, only clobetasol, but not the solely remyelinating drugs miconazole or benzotroprine, showed efficacy in reducing disease severity in the immune-driven relapsing–remitting PLP_{139–151} experimental autoimmune encephalomyelitis (EAE) model (Fig. 4a). The positive effect of clobetasol in this model resulted from its immunosuppressive effects, as evidenced by the drastic reduction of T cells within the spleen (Fig. 4b).

We also used a second EAE mouse model, MOG_{35–55}-induced, in which the immune response was relatively controlled and disease pathology recapitulated chronic progressive demyelination. We used a therapeutic, rather than prophylactic, treatment regimen to evaluate whether drugs could reverse, rather than prevent, disease. Miconazole-treated and clobetasol-treated animals all exhibited a marked improvement in function, and nearly all animals regained use of one or both hind limbs (Figs. 4c, d). In contrast, vehicle-treated mice exhibited chronic hind limb paralysis during the treatment period. Benzotroprine treatment also resulted in functional

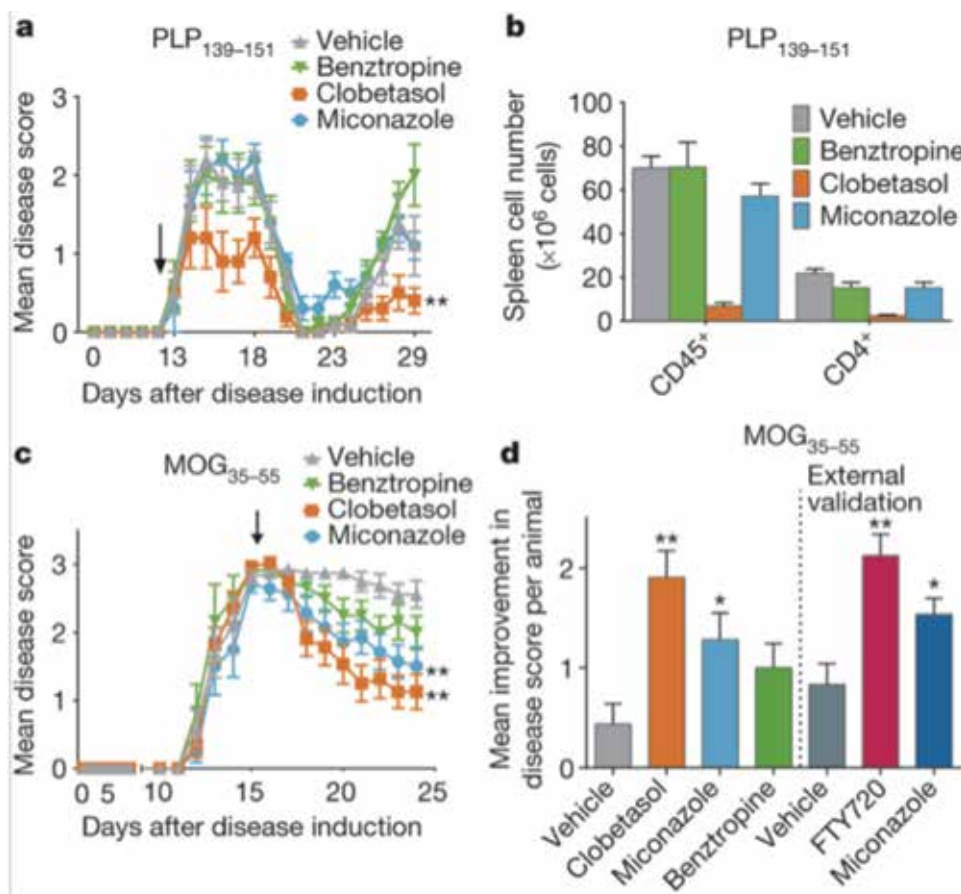


Figure 4. Therapeutic efficacy of miconazole and clobetasol in mouse models of MS. **a**, Scoring of disease severity in relapsing–remitting PLP_{139–151}-induced EAE mice treated beginning on day 13 (black arrow) and ending on day 29; $n = 10$ mice per group. Graph depicts mean daily disease score \pm SEM. **b**, Flow cytometry–based quantification of spleen cell numbers at day 29 from the PLP_{139–151} EAE cohort in **a**. Values are mean \pm SEM; $n = 4$ or 5 mice per group. **c**, Scoring of disease severity in chronic progressive MOG_{35–55}-induced EAE mice treated daily for 10 d beginning at the peak of disease on day 15 (black arrow); $n = 12–16$ mice per group. Graph depicts mean daily disease score \pm SEM. **d**, Mean improvement in disease score (Δ) per animal (peak score minus ending score) of MOG_{35–55} EAE cohort in **c**. Also shown are external validation results in MOG_{35–55} EAE from an independent contract laboratory; $n = 12$ mice per group. For all EAE experiments, drugs were dosed daily by intraperitoneal injection: clobetasol (2 mg/kg), miconazole (10 mg/kg), benzotroprine (10 mg/kg), or FTY720 (1 mg/kg). All EAE disease scoring was as follows: 0, no abnormality; 1, limp tail; 2, limp tail and hind limb weakness; 3, hind limb paralysis; 4, hind limb paralysis and forelimb weakness; and 5, moribund. Two-tailed t test, $*p < 0.05$ and $**p < 0.01$ for drug-treated groups compared with their respective vehicle-treated group. Reprinted with permission from Najr et al. (2015), their Fig. 4.

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improvement, but to a lesser extent than miconazole and clobetasol (Figs. 4c, d). Overt functional recovery of miconazole-treated and clobetasol-treated mice correlated with histological improvements in the spinal cord. Specifically, drug-treated mice showed restoration of MBP expression and a reduction in the extent of demyelination in the spinal cord, whereas vehicle-treated mice showed sustained areas of white-matter disruption (not shown).

Although the immunosuppressive effect of clobetasol makes it challenging to evaluate its remyelinating potential in EAE directly, its consistent and robust induction of OPC differentiation *in vitro*, and enhancement of remyelination in nonimmune-driven *in vivo* assays, suggest that it serves a role in both immunomodulation and promotion of myelination. In contrast, miconazole did not modulate immune cell function, and our data indicate that it acts as a direct remyelinating agent. Given the potential of miconazole as a remyelinating therapeutic, we contracted a separate laboratory to provide independent validation of its efficacy in the MOG_{35–55}-induced EAE preclinical model. The laboratory independently validated the preclinical efficacy of miconazole in MOG_{35–55}-induced EAE for reducing disease severity in treated mice (Fig. 4d).

Conclusions

Since the approval in 1993 of interferon- β -1b for the treatment of MS, therapeutic development has centered on the generation of additional immunomodulatory agents. Despite the effectiveness of many of these drugs to modulate CNS inflammation in patients with MS, none of them prevents chronic progressive disease and disability—largely because of their inability to stop or reverse the failure of remyelination in the CNS. We developed an advanced high-throughput screening platform to discover effective remyelinating therapeutics. This pluripotent stem cell-based system provides unprecedented scalability, purity, and genotypic flexibility to screen for compounds that enhance OPC differentiation and myelination. Using this platform, we identified two drugs approved by the US Food and Drug Administration—miconazole and clobetasol—with newly discovered functions to modulate OPC differentiation directly, enhance remyelination, and significantly reduce disease severity in mouse models of MS. Since miconazole and clobetasol are currently approved only for topical administration in humans, significant optimization of dosing, delivery, and potentially chemical structure will be required to enhance the on-target pharmacology in OPCs while diminishing any potential off-target

side effects. However, the ability of miconazole and clobetasol to cross the blood–brain barrier raises the exciting possibility that these drugs, or their modified derivatives, could advance into clinical trials for the currently untreatable chronic progressive phase of MS.

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