Identifying the cellular targets of drug action in the central nervous system following corticosteroid therapy

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Abstract

Corticosteroid (CS) therapy is used widely in the treatment of a range of pathologies, but can delay

production of myelin, the insulating sheath around central nervous system nerve fibers. The cellular targets

of CS action are not fully understood, *i.e.* 'direct' action on cells involved in myelin genesis

[oligodendrocytes and their progenitors the oligodendrocyte precursor cells (OPCs)] versus 'indirect' action

on other neural cells. We evaluated the effects of the widely used CS dexamethasone (DEX) on purified

OPCs and oligodendrocytes, employing complementary histological and transcriptional analyses.

Histological assessments showed no DEX effects on OPC proliferation or oligodendrocyte

genesis/maturation (key processes underpinning myelin genesis). Immunostaining and RT-PCR analyses

show that both cell types express GR (the target for DEX action), ruling out receptor expression as a causal

factor in the lack of DEX-responsiveness. GRs function as ligand-activated transcription factors, so we

simultaneously analyzed DEX-induced transcriptional responses using microarray analyses; these

substantiated the histological findings, with limited gene expression changes in DEX-treated OPCs and

oligodendrocytes. With identical treatment, microglial cells showed profound and global changes *post*-DEX

addition; an unexpected finding was the identification of the transcription factor *Olig1*, a master regulator of

myelination, as a DEX responsive gene in microglia. Our data indicate that CS-induced myelination delays

are unlikely to be due to direct drug action on OPCs or oligodendrocytes, and may occur secondary to

alterations in other neural cells, such as the immune component. To the best of our knowledge, this is the

first comparative molecular and cellular analysis of CS effects in glial cells, to investigate the targets of this

major class of anti-inflammatory drugs as a basis for myelination deficits.

Key words: oligodendrocyte; Olig1; corticosteroid; glucocorticoid receptor; microglia; microarray

Introduction

Corticosteroid (CS) therapy is currently used widely for the treatment of a range of pathologies, but can

delay myelin genesis during development and repair (remyelination) in major white matter tracts of the

central nervous system (CNS) including the optic nerve and corpus callosum.[1–7] Notably, high and multiple

CS doses are frequently used during periods of developmental myelination,[8,9] and to treat conditions

involving myelin injury/repair such as Multiple Sclerosis and spinal cord injury.[10,11] The cellular

mechanisms underpinning the reported CS induced defects in myelin genesis are largely unknown, but

observations that myelination delays occur without accompanying axon loss[2,5] indicate that such effects may

be primarily underpinned by glial responses to CS.

Myelination involves a sequential process: initially, proliferative and migratory precursors called

oligodendrocyte precursor cells (OPCs) populate the CNS[12] and receive signals to generate

oligodendrocytes. Astrocytes are important mediators of myelin genesis, by influencing OPC migration,

proliferation and differentiation into oligodendrocytes;[13] the latter engage in complex, intercellular cross-talk

with axons to generate functional myelin.[14] Oligodendrocytes continue to generate myelin sheaths well into

adulthood (about the fifth decade) in the temporal and parietal lobes, with perturbations to this process being

linked to cognitive decline in Alzheimer's disease.[15] Following myelin injury, remyelination broadly

recapitulates developmental myelination, and critically depends on a timed orchestration of cellular and

molecular events in lesions.[16] Myelin genesis in the normal and diseased CNS is therefore a complex

multifactorial process, and studies aiming to establish the cellular targets of CS action during

myelinogenesis yield contradictory information.[17] Evidence for direct effects on OPCs includes CS

treatment of CG4 cells (an OPC line)[18] and adrenalectomized rats,[19] where OPC proliferation was inhibited.

Further, CS have pro-differentiation effects on myelinogenic cells,[20,21] but in demyelination models CS can

delay[22] or enhance[23] remyelination. These confounding responses may relate to the immunomodulatory

effects of CS on microglia; the latter remove myelin debris (inhibitory to myelin genesis) and secrete

stimulatory/inhibitory cytokines that can impact oligodendrocyte development.[24] However, CS treatment of

astrocytes can also down-regulate oligodendroglial differentiation factors.[25] While neurons engage in cross-

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talk with oligodendroglial cells during myelination, one study has reported limited CS effects in striatal neurons, suggesting that at least some neurons are not direct targets of CS action.[26]

Such observations suggest that CS may impact myelination through *indirect* effects mediated by non-myelinogenic glial cell intermediaries, but rigorous evaluation of *direct* effects of CS on oligodendroglia is lacking. There is heavy reliance in the field on histological observations in cultures, but the constituent cell types are often poorly characterized.[27,28] In terms of drug action, CS are lipid-soluble, readily entering cells where they bind to appropriate cytosolic ligand-activated receptors, with nuclear translocation of complexes and action on hormone response elements to regulate the associated genes.[29] In this context, the critical question of whether oligodendrocyte lineage cells express the glucocorticoid receptor (GR) is also not fully resolved, with two studies yielding contradictory results.[19,30]

The widespread clinical use of CS in conjunction with the critical roles of myelin in neuroprotection and regulation of electrical conductivity[16] highlight the need to resolve such conflicting information by defining the mechanisms underpinning aberrant myelinogenesis. To address this issue, and specifically, to evaluate if CS exert direct effects on oligodendroglial cells, we have employed a two-pronged methodological approach comprising histological analyses complemented with parallel transcriptional analyses (as CS effects are mediated by transcriptional regulation). We have employed isolated purified cultures, and short time frames for the studies, to evaluate direct cellular actions of CS and the potential primary/early targets of drug action respectively. We consider this approach necessary to provide comprehensive insights into the mechanisms by which CS therapy may impact myelination.

Results and Discussion

To the best of our knowledge, this is the first study to employ a dual methodological approach (that provides independent and corroborative readouts of CS effects at the morphological and molecular levels) in order to investigate the mechanisms by which this major class of anti-inflammatory drugs may impact myelin genesis. The inclusion of independent transcriptional analysis of DEX treated cultures, in conjunction with histological assays, provides an unbiased approach unhindered by prior expectation of CS effects on neural cells. Further, the use of isolated, purified glial cultures in these experiments allows for examination of *direct* actions of CS which cannot be evaluated within the context of the multicellular lesion environment *in vivo*.[3] High purity OPC, astrocyte and microglial cultures were generated as judged by staining for cell specific markers; values were: $96.4 \pm 0.5\%$ A2B5[+] ($n = 6$) and $96.9 \pm 1.2\%$ NG2^{+} for OPCs ($n = 3$); $97.8 \pm 0.8\%$ OX42^{+} for microglia ($n = 6$); and $94.4 \pm 1.7\%$ glial fibrillary acidic protein positive (GFAP[+]) for astrocytes ($n = 3$). Highly enriched oligodendrocyte cultures were obtained: $81.2 \pm 5.2\%$ myelin basic protein positive (MBP[+]; $n = 6$), with the majority of the remaining cells showing morphological features of earlier stages of the oligodendrocyte lineage under phase contrast microscopy; microglial cells (<5%) were readily identifiable by their morphologies, and astrocytic morphologies were never observed in these cultures.

Histological analyses indicate that oligodendrocyte lineage cells are unaffected by CS treatment

In control (untreated and vehicle) and DEX treated cultures, OPCs displayed typical bipolar phenotypes, with similar morphologies of A2B5[+] and NG2^{+} cells observed between treatment groups (Figure 1a-b). No DEX effects on OPC proliferation/survival were observed in terms of total number of nuclei per microscopic field or percentage of pyknotic nuclei (Figure 1c-d). No differences were found between the groups in terms of numbers or percentages of A2B5[+] or NG2^{+} cells per microscopic field (Figure 1e-h).

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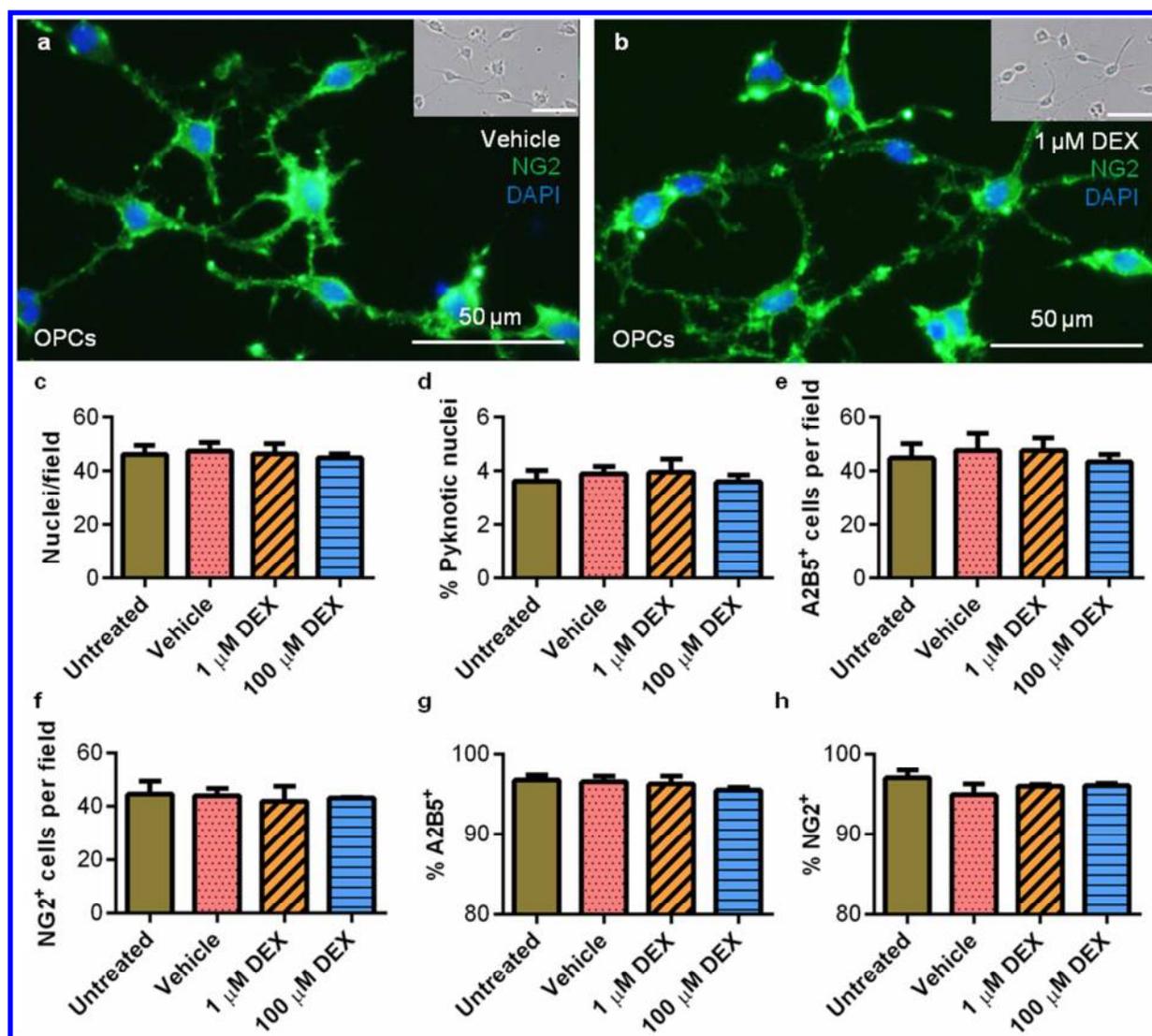
Figure 1 DEX does not affect the survival, proliferation or antigenic profiles of OPCs. *Typical*

fluorescence micrographs (with phase contrast counterparts, inset) showing NG2$^+$ OPCs with similar

staining profiles and morphologies in vehicle control (a) and treated cultures (DEX; 72 h; 1 μM) (b). For

all treatment conditions, bar graphs illustrate the number of healthy nuclei per microscopic field (c),

percentage of pyknotic nuclei (d), number of A2B5$^+$ (e) and NG2$^+$ (f) cells per microscopic field, and the

percentage of A2B5$^+$ (g) and NG2$^+$ (h) cells in OPC cultures [72 h DEX; for (c) and (d) n = 6; *for (e-h)* n =

3; (a-b) insets, scale bar = 50 μm].

To monitor the effects of DEX on oligodendrocyte *differentiation*, OPC cultures (propagated in OPC-MM

for 24 h) were switched to Sato differentiation medium and allowed to differentiate +/- DEX for up to 7

days. Complex process-bearing oligodendrocytes were found in both control and DEX treated

oligodendrocyte cultures (Figure 2a-b). No differences were observed between groups in the total number of

nuclei or the percentage of pyknotic nuclei per microscopic field (Figure 2c-d). Further, using our

morphological scoring criteria, no CS effects were discernible on oligodendrocyte maturation in terms of the

extent of process/membrane elaboration (Figure 2g). Therefore, DEX treatment did not affect the survival,

proliferation, antigenic profiles or morphologies (including extent of differentiation) of oligodendrocyte

lineage cells.
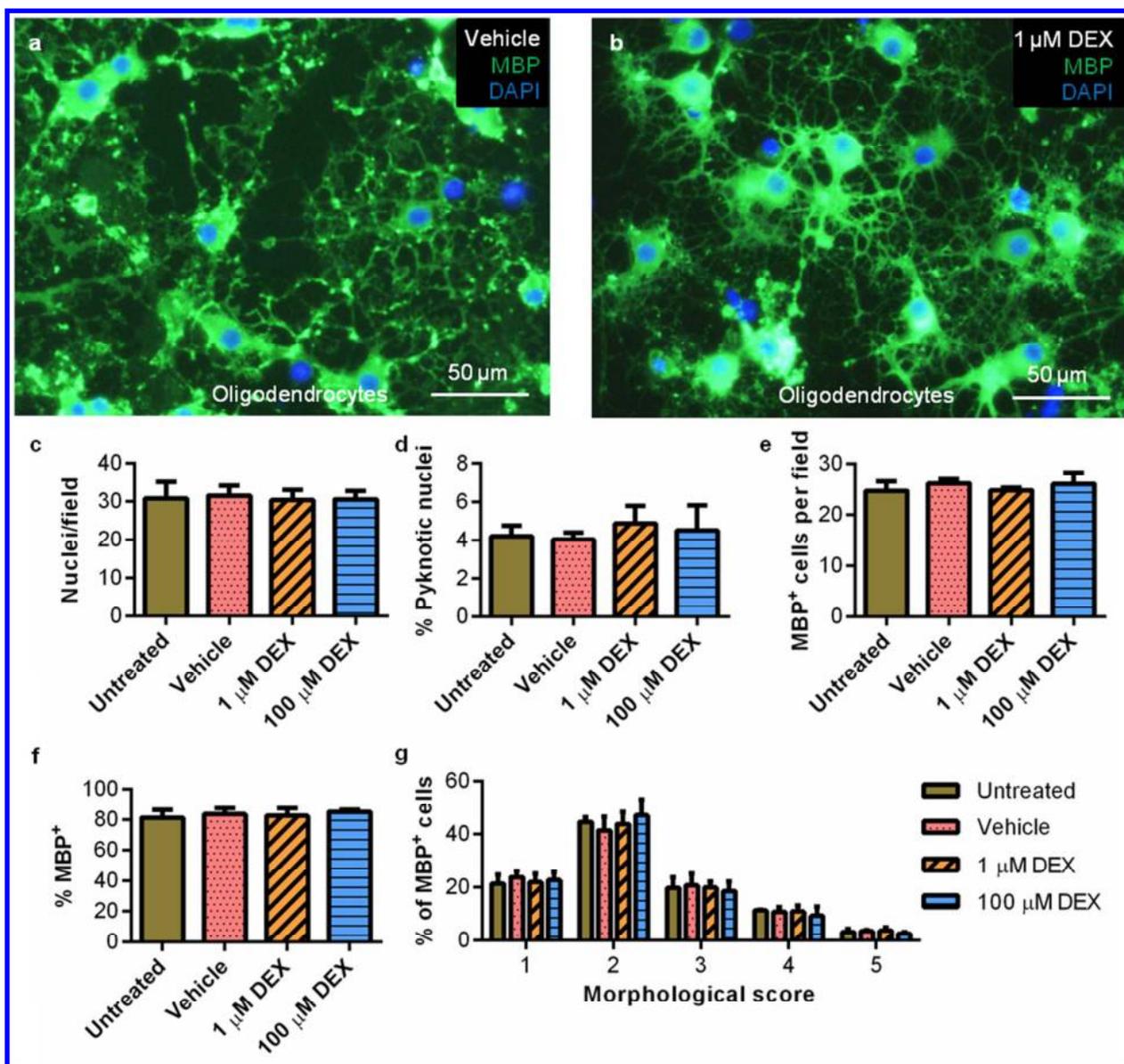
Figure 2 DEX does not affect the survival or maturation of oligodendrocytes. *Typical fluorescence*

micrographs showing MBP⁺ oligodendrocytes with similar morphologies and staining profiles at different

stages of differentiation in vehicle control (a) and treated cultures (DEX; 7 d; 1 µM) (b). Note the extensive and complex branching of processes in both cultures. For all treatment conditions, bar graphs illustrate the number of healthy nuclei per microscopic field (c), percentage of pyknotic nuclei (d), number of MBP+ cells per microscopic field (e), the percentage of MBP+ cells (f) and morphological scoring of MBP+ oligodendrocytes indicating extent of differentiation/maturation (g) in oligodendrocyte cultures (7 d DEX; n = 3 for all graphs).

Positive control cultures: Untreated microglial cultures and those treated with vehicle showed cells with a branching ramified morphology (Figure 3a). DEX treatment dramatically reduced cell densities with many cells showing a rounded appearance (Figure 3b-c) confirming that the CS preparations used were physiologically active, and the spectrophotometrically confirmed doses (see Methods) were appropriate for eliciting cellular responses. No significant differences were noted in microglial response to 1 and 100 µM DEX, presumably due to saturating concentrations of DEX employed. In this context, the dissociation constant (Kd) of GR with DEX is unknown for microglia, but is reported to be 3.5 – 6.0 nM in multicellular cultures derived from rat brain.[31-32] This is greatly exceeded by the lowest DEX dose tested (1 µM), and as DEX readily diffuses across the cell membrane,[33] this supports the concept of receptor saturation. Further, it should be noted that biological responses to DEX in microglia[34] and other cell types[35-37] have been shown to display similar plateau effects.
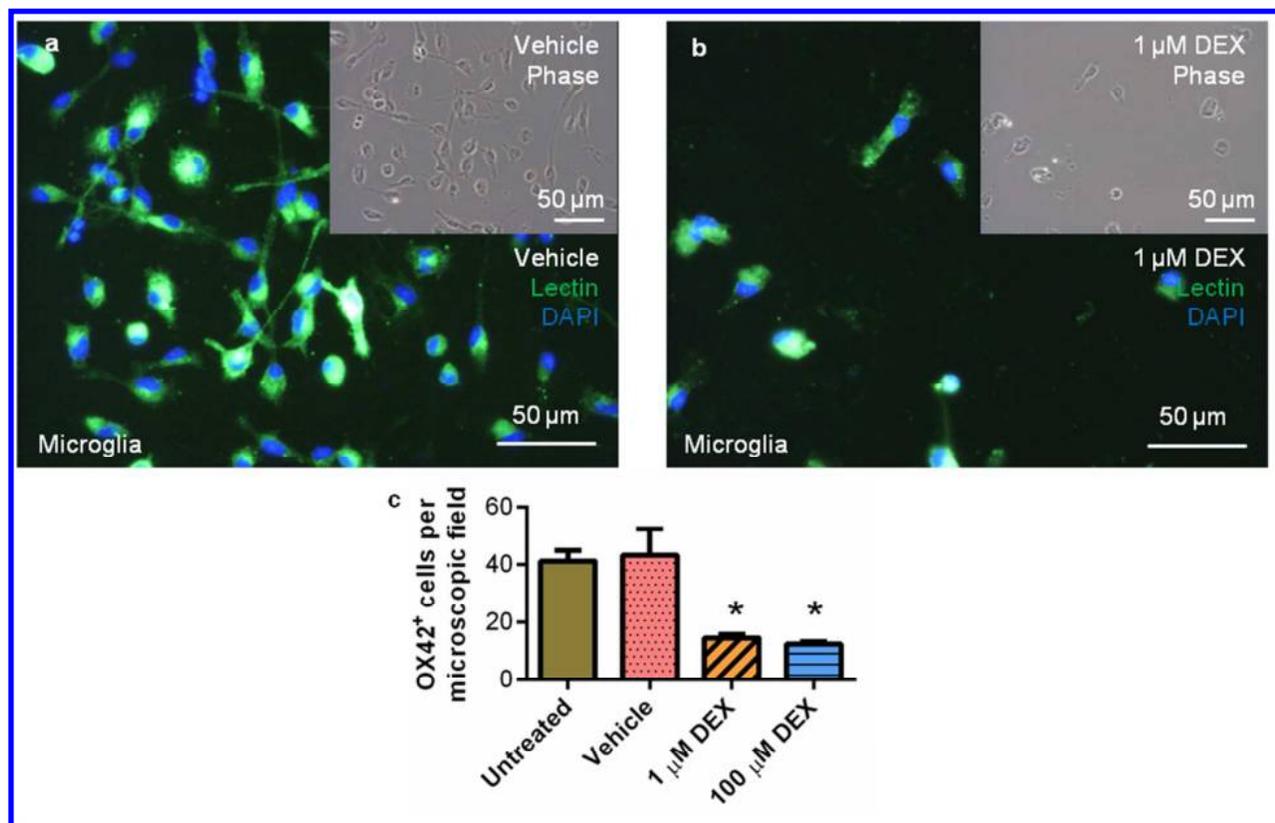
Figure 3 DEX dramatically alters numbers and morphologies of microglia. *Typical fluorescence*

micrographs (with phase contrast counterparts, inset) showing many process bearing OX42$^+$ microglia in a

vehicle control culture (a) compared with a dramatic reduction in cell numbers and alterations to

morphology following treatment (DEX; 72 h; 1 µM) (b). (c) Bar graph illustrating the number of OX42$^+$

cells per microscopic field in microglial cultures for all treatment conditions (72 h DEX; **p < 0.05 versus*

untreated control; one-way ANOVA, Bonferroni's post-test; n *= 3).*

The absence of CS effects on oligodendrocyte lineage cells cannot be attributed to the absence of GR expression

The lack of an overt oligodendroglial response to CS treatment could be explained by an absence of the

necessary receptor (GR) expression. Earlier studies report that oligodendroglial lineage cells express GR

mRNA but evidence for GR expression in rat OPCs/oligodendrocytes has not been definitive. Although

several studies report GR (mRNA or protein) expression in 'glial cells', often poorly characterized cultures

are employed in the experiments, leaving doubt regarding cell specific receptor expression and related CS effects. For example, Vielkind et al.[28] show GR expression in "*tentatively identified*" OPCs in mixed glial cultures, without OPC-specific counterstaining (some of these cells were GFAP$^+$, suggesting that they are astrocytes), while Jung-Testas and Baulieu[27] employed uncharacterized mixed glial cultures, identifying only GFAP$^+$ astrocytes and CNP$^+$ or MBP$^+$ oligodendrocytes, with neither study immunostaining for microglia. The unresolved question of oligodendroglial expression of GR was addressed here using immunostaining for GR protein and RT-PCR for GR mRNA detection.

Microglia and astrocytes (the positive controls) expressed GR mRNA (Figure 4a) and immunostained for GR protein (Figure 4b-c). Both cytoplasmic and nuclear localization of GR were observed in both cell types. Similarly, both detection methods confirmed GR mRNA and protein expression in OPCs and oligodendrocytes (Figure 4a, d-e) with a comparable pattern of intracellular distribution of receptor. Therefore, we have shown here that all the major glial cell types studied (using well characterized glial culture systems) including the oligodendrocyte lineage cells, express both GR mRNA and protein, independently validated by PCR and immunocytochemical analyses, confirming their potential to respond to CS treatment.
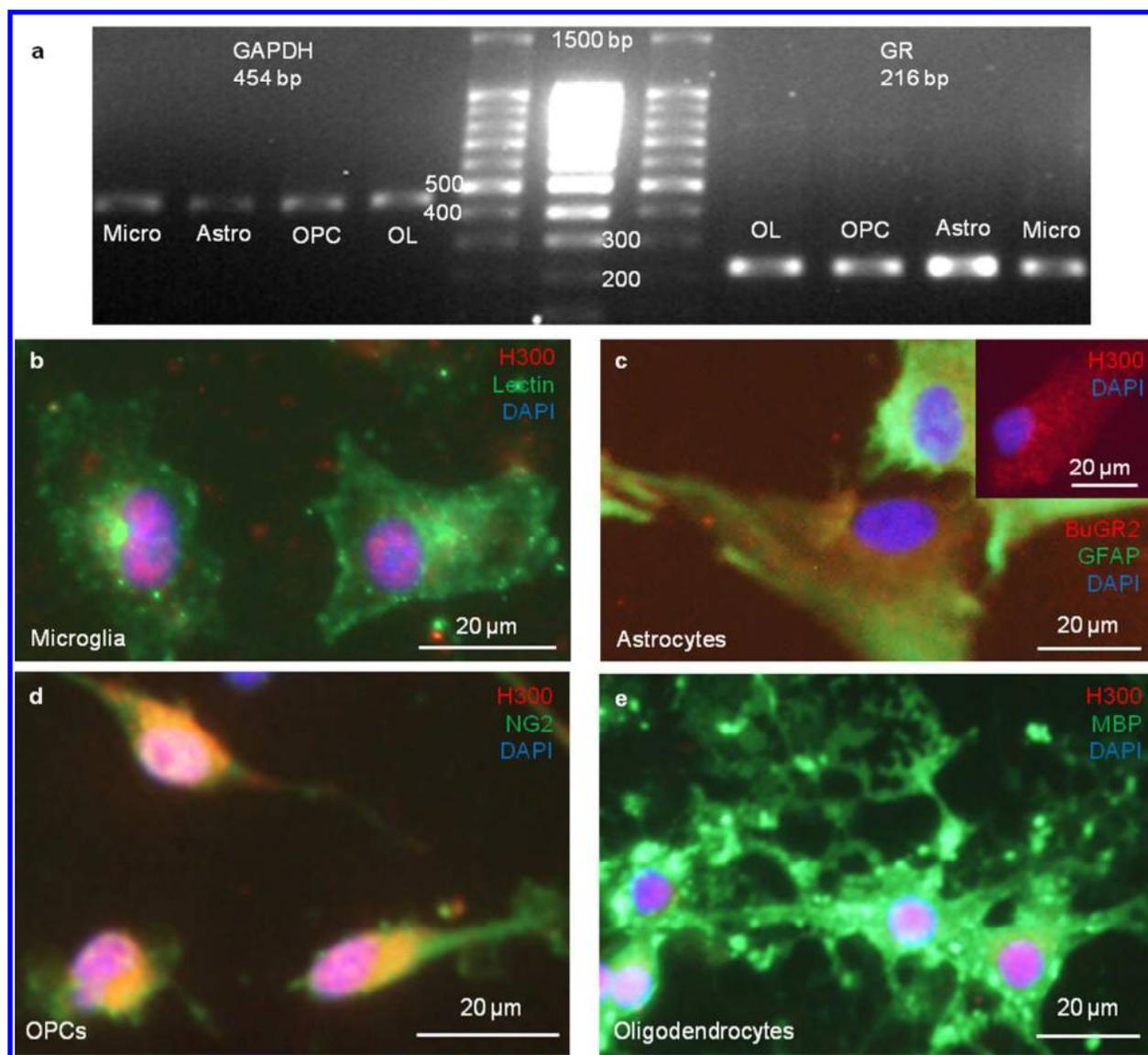
Figure 4 All glial cell types express GR mRNA and protein. *(a) RT-PCR analyses showing expression by all glial cell types of GAPDH mRNA (452 bp product; housekeeping gene) and GR mRNA (216 bp product). Central numbers indicate bp values of ladders. Fluorescence micrographs showing GR expression (H300 and BuGR2 antibodies) in lectin$^+$ microglia (b), GFAP$^+$ (c) and morphologically-identified astrocytes (c, inset), NG2$^+$ OPCs (d) and MBP$^+$ oligodendrocytes (e). OL = oligodendrocyte.*

Transcriptional analyses support the concept that oligodendrocyte lineage cells are not primary, direct targets of CS action

Supporting the histological observations, the transcriptional effects following DEX addition were most pronounced in microglia and astrocytes (positive controls for CS induced gene expression changes; Figure 5a-b), where 257 and 38 genes were differentially expressed, respectively. In contrast, the oligodendrocyte lineage cells showed modest responses with 5 and 10 differentially expressed genes in OPCs and oligodendrocytes, respectively (Figure 5c-d).
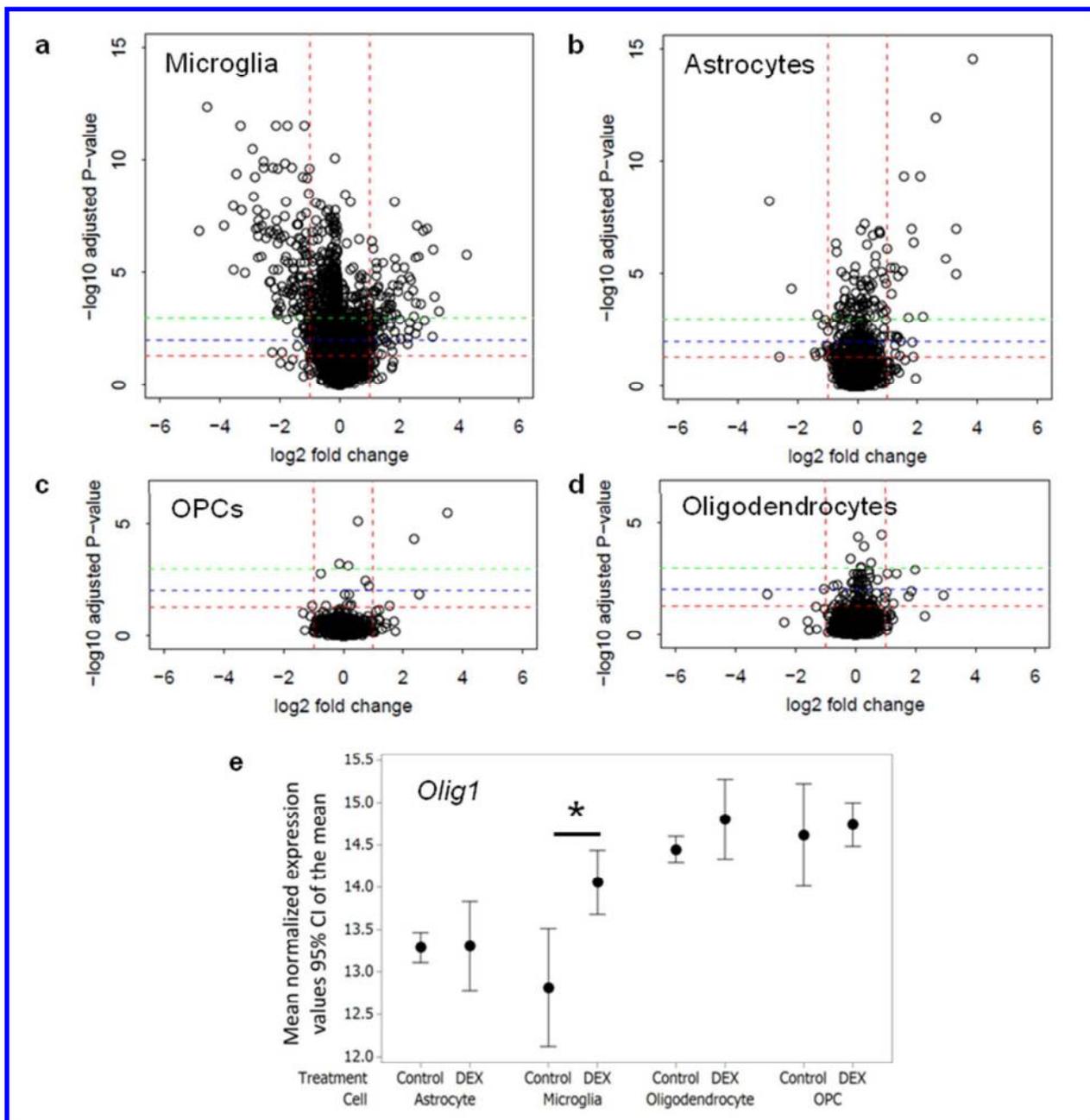
Figure 5 DEX induced transcriptional changes are extensive in microglia and astrocytes, but limited in

oligodendroglia. Volcano plots illustrate the transcriptional response to dexamethasone treatment (DEX; 48

h; 1 μM) detected by microarray analysis of purified microglial (a), astrocyte (b), OPC (c) and

oligodendrocyte cultures (d). Positive fold change represents upregulation, negative fold change represents

*downregulation (versus vehicle controls; x axis). Dashed horizontal lines represent* $p < 0.05$ *(red),* $p < 0.01$

(blue) and $p < 0.001$ *(green). Note extensive changes in microglia and astrocytes, but limited changes in*

OPCs and oligodendrocytes. (e) Mean normalized expression values (error bars represent 95% confidence

intervals) for Olig1 showing elevated expression in DEX treated microglia (unpaired t-*test;* $p < 0.05$*; n =*

3).

The majority of differentially expressed microglial genes are related to immune function but some individual

genes of interest were identified. Two such key genes are *Olig1* (Figure 5e) a basic helix-loop-helix

transcription factor involved in the maturation of oligodendrocytes[38] and oligodendrocyte-myelin

glycoprotein (*Omg* ; human homolog known as *OMG* and *OMGP*), both upregulated in microglia following

DEX treatment. The former finding was confirmed using immunostaining to detect Olig1 protein; both

OPCs and oligodendrocytes (positive controls; Figure 6a-b) expressed Olig1, as did microglial cells (Figure

6c). In addition, a significant increase in Olig1 expression was evident in DEX treated microglial cultures

compared to controls (Figure 6d-e). Within each culture, DEX treated microglia consistently demonstrated

significantly higher optical density values than controls (DEX minus vehicle for each culture; Figure 6f).

Differentially expressed genes identified in astrocytes treated with DEX were significantly enriched in a

number of gene ontologies including "response to corticosteroid stimulus" (see Supporting Information,

Astrocyte.Enrichment_Analysis).
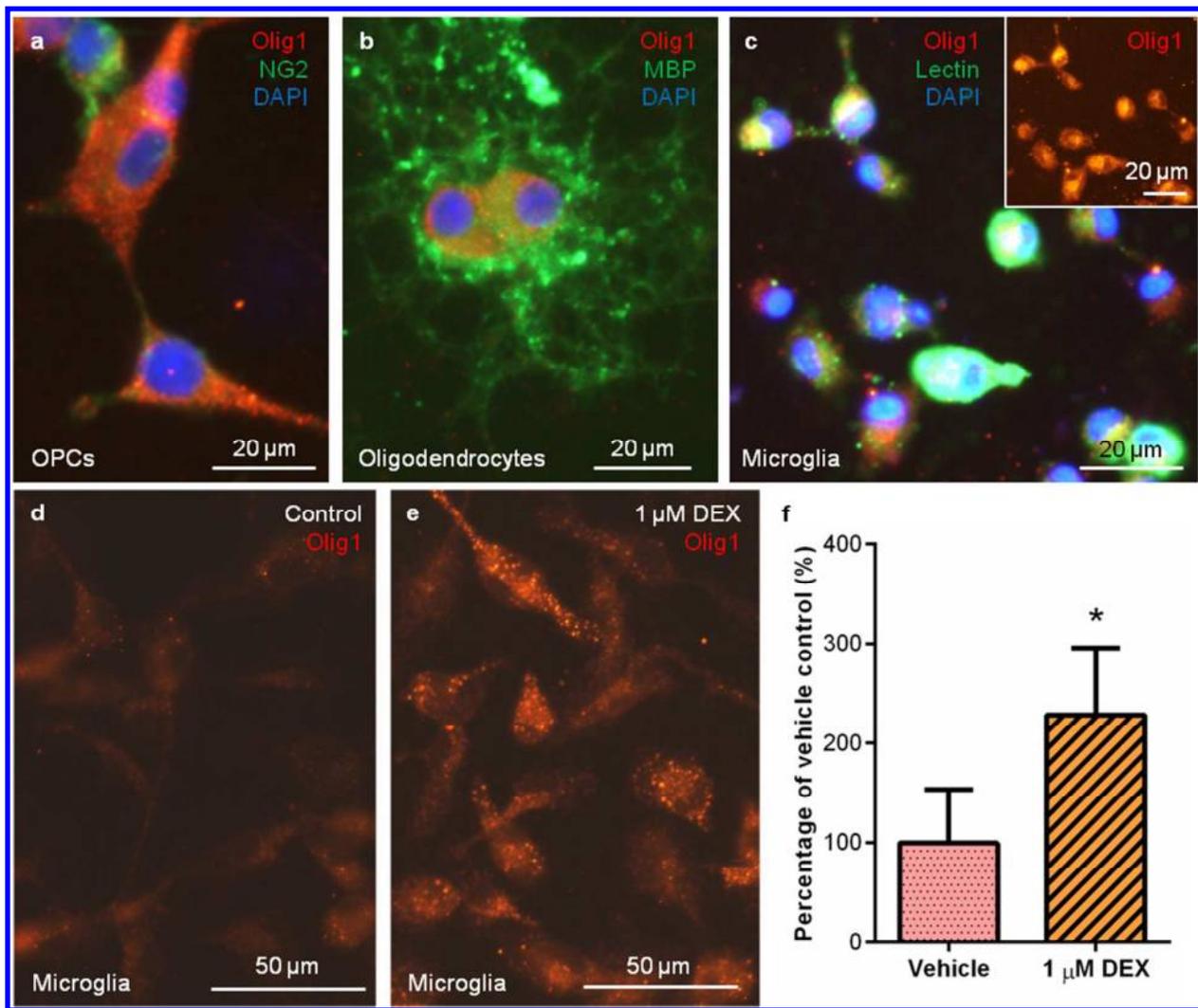
Figure 6 Microglia express Olig1 protein, and upregulate Olig1 expression following DEX treatment.

Fluorescence micrographs showing Olig1 expression in NG2$^+$ OPCs (a), MBP$^+$ oligodendrocytes (b) and lectin$^+$ microglia (c; inset shows Olig1counterpart image). (d) Olig1expression in a vehicle control microglial culture. (e) Marked increase in microglial Olig1expression following DEX addition (DEX; 48 h; 1 µM). (f) Bar graph showing normalized mean optical density measurements of Olig1 expression, with elevated values in DEX treated microglia over vehicle controls (48 h; 1 µM; paired t-test; p < 0.05; n = 3).

By contrast, for OPCs no enriched terms were identified which passed our inclusion criteria. For oligodendrocytes, a single pathway map "Role of ZNF202 in regulation of expression of genes involved in Atherosclerosis" was highlighted by pathway analysis (ZNF202 is a transcription factor; genes in this pathway are those encoding HDL proteins and APOE). Multiple gene ontology terms were identified, with

the most enriched being "regulation of growth" (genes encoding: HTRA1, HDL proteins, Alpha crystallin B, Neuromodulin and APOE) and "response to reactive oxygen species" (genes encoding: Cystatin C, HDL proteins, Alpha crystallin B and APOE). Additional terms were also identified for astrocytes and microglia, with the full results provided as Supporting Information. Whilst the gene expression changes in oligodendrocytes are limited, there were a few changes of note. Both *Apoe* and *Gap43* were downregulated in oligodendrocytes on DEX addition. APOE has a major role in lipid metabolism,[39] but its role in the genesis of lipid rich myelin sheaths in the CNS is currently unclear. Gap43 is expressed in immature oligodendrocytes but down regulated during maturation[40] – although we did not detect morphological alterations in CS treated oligodendrocytes, we cannot currently rule out alterations in their engagement with axons.

In contrast to OPCs and oligodendrocytes, both astrocytes and microglia showed more global and pronounced effects following identical CS treatment. For astrocytes, the affected genes included well known glucocorticoid-responsive genes, including *Sgk*,[41] *Fkbp5*[42] and *Tsc22d3* (aka *Gilz*).[43] Further, five of the affected genes (*Sult1a1*, *Sgk*, *Ddit4*, *Klf9* and *Tsc22d3*) were recently shown to be up-regulated by short-term (4 h) glucocorticoid treatment of striatal astrocytes,[26] whereas the most down-regulated gene (*Ednrb*) was also reported to be negatively regulated by glucocorticoids in neural progenitor cells.[44] Together, these observations underscore the validity/robustness of the microarray procedures utilized here. With respect to the microglial arrays, an unexpected finding was the identification of the transcription factor *Olig1* as a DEX responsive gene, given its role as a master regulator of myelination, with expression reported in oligodendroglia and radial glia.[45,46] Nevertheless, immunocytochemical approaches unequivocally demonstrated (i) Olig1 expression in cultured microglia, and (ii) up-regulation of protein expression by DEX treatment, further validating the sensitivity of the microarray system used here. The implications of this finding remain to be established, but do question the prevailing view that Olig1 expression in the CNS is restricted to oligodendrocyte lineage cells.

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It is well established that different tissues/cell types exhibit markedly differing responses to glucocorticoids; indeed, glucocorticoid sensitivity can vary throughout the cell cycle within a single cell type.[47] Consequently, the finding that the various cell types exhibit differing transcriptional responses to DEX (as shown here for the first time with CNS glia) is not without precedent. However, elucidation of the factors accounting for the inter-cellular differences — and limited glucocorticoid sensitivity of oligodendroglia, in particular — will require extensive further study. For example, whilst all glial cell types express the GR, information is lacking regarding the complement of GR isoforms expressed in each cell type. This is important because (i) diverse GR subtypes can be generated from the Nr3c1 gene locus (by alternative splicing coupled with the usage of alternative translation initiation sites), each subtype being further subject to multiple post-translational modifications (including phosphorylation, sumoylation, acetylation and ubiquitination), and; (ii) different protein isoforms can exhibit distinct transactivation/transrepression patterns of gene regulation.[47-49] Other factors influencing glucocorticoid action and which may differ between glial cell types include: GR chaperones/co-chaperones, transcriptional co-regulators and chromatin remodelling complexes;[47] transcription factors such as AP-1, NF-KB and STATs that participate in protein-protein interaction with – and are modulated by – the GR;[47-49] the lncRNA GAS5 which acts as a decoy GRE to riborepress the GR;[50] and chromatin architecture.[51]

CS are undoubtedly highly effective immunosuppressive agents that assist recovery in a range of pathologies. However, we consider that elucidation of the mechanisms of adverse effects of CS based immunotherapies is essential given the intimate relationship between myelination and axonal function/survival. For example, axons produce aberrant branches without the inhibitory effects of myelin.[52] The ordered arrangement of ion channels at the nodes of Ranvier (and therefore conductive properties of axons) also critically depends on correct myelination.[53] Oligodendrocytes are considered to be essential to long-term axonal integrity, potentially through trophic support mechanisms and axon-glia metabolic coupling mechanisms[54,55] leading to a widely held view that axon-myelin interactions have an important neuroprotective role (and by extension impact disease progression when perturbed).[16] Consequently, CS-induced delays in myelination are of significant clinical importance when considering treatment of a range

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of paediatric and adult pathologies. Our findings demonstrate that OL lineage cells show limited responses to CS and are therefore not likely to constitute the direct cellular targets of CS action in the CNS. This is a somewhat unexpected finding, given that both OPCs and oligodendrocytes are key players in the process of myelin genesis.

However, the exact mechanisms by which microglia impact myelin production *in vivo* are not fully resolved. Our pathway analyses do not indicate specific mechanisms by which microglial gene expression changes may directly impact myelin genesis, however more complex secondary effects may account for CS-induced myelin perturbations *in vivo*. During regeneration, impaired remyelination following microglial suppression is likely due to impaired myelin debris clearance.[56] Microglial roles in *developmental* myelination are less clear, however at least one study suggests that myelin synthesis can be stimulated by microglial cells,[57] through unknown secretory mechanisms. Further, it is established that microglia secrete several soluble mediators including those with key roles in the development of the oligodendroglial lineage, such as IGF-1.[58,59] The release of such mediators is the critical first step towards successful myelin regeneration, and it is feasible that similar mechanisms may be operational during developmental myelination,[56] and therefore perturbed by immunosuppressive therapies. We also cannot exclude the possibility that CS effects on myelin production may simply be related to a decrease in microglial numbers, as reported previously. *In vivo* DEX treatment has been shown to elicit a decrease in microglial numbers, this effect being dependent on both the dose employed[60] and the duration of treatment.[61] However, systematic correlative analyses of CS-induced microglial depletion and the progression of developmental myelination have not been conducted so far. Microglia remove supernumerary neurons during development, but it is not clear whether they perform the same role for supernumerary oligodendroglial cells (which are known to be reduced by up to 50% during development). Depleted numbers of microglia, and/or microglial dysfunction could also impair removal of dead cells and regulation of glial population numbers, essential to establishing normal neuron:glia ratios and interactions during development.[62]

In light of these points, the following transcriptional changes in our study may be of relevance. Analysis of the combination of all transcriptional changes on cellular function using the Ingenuity Pathway analysis tool

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showed (i) DEX-treatment resulted in altered expression of genes relating to 'cell movement' (including

'migration of microglia'), therefore CS-treated microglia may demonstrate an impaired ability to migrate

towards and phagocytose cells/synapses and myelin debris. (ii) Microglial cytokine secretion can affect

neural/glial specification[59] and cytokines such as interleukin-2 (IL-2), whose gene expression was shown to

be altered in our analyses, can exert toxic effects on oligodendrocytes and myelin. The consequences of such

changes on global myelin genesis are as yet unknown. Future work will need to establish whether our *in*

vitro findings can be extrapolated to lesions *in vivo*, where the complex, intercellular crosstalk in sites of

pathology is maintained, and where the CS doses encountered by cells may be different to those employed in

our study. Nevertheless, our findings can provide a basis for future investigations into the precise

relationship between microglia, immune suppression and myelination.

Summary

A detailed understanding of the cellular and molecular mechanisms underpinning adverse effects of

immunosuppressive drugs in the nervous system is critical to the development of better classes of

therapeutic agents. Our findings indicate that CS effects on myelin genesis are not primarily mediated by

oligodendrocyte lineage cells. Instead, the primary mediators of such effects could be the microglial or

astrocytic cells. The role of cellular cross-talk between astrocytes and other neural cells during development

and remyelination is well established.[14] Further, while microglial interactions can impact regenerative

processes,[24] their role in neural development remains elusive. Evidence is gathering, however, that microglia

play critical roles in the regulation of precursor cell numbers[63] and development of cortical neurons;[64] the

impact that immunotherapies have in the context of repair and development is therefore an important issue

and will require elucidation. The current experiments were conducted within a short timeframe in purified

cultures to attempt to identify the primary/early targets of drug action in the response cascade. However, we

cannot rule out the possibility that cellular response profiles may be different following prolonged CS

exposure. Therefore, further investigations to identify downstream effectors/target cells will require

proteomic/secretomic analyses of CS treated glial populations, at later time points and with longer

exposures, all within experimental systems that allow for examination of neural cell cross-talk. Such work

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can potentially offer evidence for an indirect mechanism of CS effects on myelin genesis via a

microglial/astrocytic cell intermediary. This detailed understanding can provide information relevant to the

development of novel therapeutic agents/immunotherapies that limit adverse effects of CS, along with

refinements to the timing and dose of existing CS therapy.

Materials and methods

The care and use of animals was in accordance with the Animals (Scientific Procedures) Act of 1986

(United Kingdom) with approval by the local ethics committee.

Reagents: Tissue culture materials were from Fisher Scientific (UK). Recombinant human platelet-derived

growth factor (PDGF-AA) and basic fibroblast growth factor (FGF2) were from Peprotech (UK).

Dexamethasone (DEX) 21-phosphate disodium (D4902; ≥97% pure), culture media and anti-biotin

secondary antibodies [Cy3- or fluorescein isothiocyanate (FITC)-conjugated] were from Sigma-Aldrich

(UK). All other secondary antibodies were from Jackson ImmunoResearch Laboratories Inc. (USA).

Mounting medium [with 4',6-diamidino-2-phenylindole (DAPI)] was from Vector Laboratories (UK). RQ1

RNase-free DNase and DNA ladders were from Promega (UK), Immolase DNA polymerase was from

Bioline (UK), specific primers were from MWG Operon (Germany), random hexamer primers, Superscript

II reverse transcriptase and RNase OUT were from Invitrogen (UK).

Cell cultures: Primary mixed glial cultures were prepared from dissociated cerebral cortices of Sprague-

Dawley rats at postnatal day 1 – 3 and glial populations were isolated by sequential rotary shaking

procedures using well-established protocols.[65,66] Briefly, microglia were derived first (200 rpm, 1 h) and

plated at 6×10^4 cells/cm^2 in D10 medium [Dulbecco's modified Eagle's medium (DMEM) supplemented

with 10% fetal bovine serum (FBS), 2 mM glutaMAX-I, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50

µg/ml streptomycin]. OPCs were derived next (200 rpm, 18 h) and plated at 4.5×10^4 cells/cm^2 in OPC

maintenance medium (OPC-MM: DMEM supplemented with 2 mM glutaMAX-I, 1 mM sodium pyruvate,

10 nM biotin, 10 nM hydrocortisone, 30 nM sodium selenite, 50 µg/ml transferrin, 5 µg/ml insulin, 0.1%

bovine serum albumin, 50 U/ml penicillin, 50 µg/ml streptomycin, 10 ng/ml PDGF-AA, and 10 ng/ml

FGF2). Finally, after an additional shake (200 rpm, 18 h) to deplete residual OPCs, adherent astrocytes were

trypsinized and plated at 4×10^4 cells/cm^2 in D10. To deplete microglia from OPC and astrocyte fractions,

cells were transferred to non-tissue-culture grade petri dishes (to which microglia readily attach) and after 30

min the unattached cells were collected. To establish oligodendrocyte cultures, OPC cultures were switched

to Sato differentiation medium [DMEM supplemented with 2 mM glutaMAX-I, 1 mM sodium pyruvate, 1x

N2 supplement (5 µg/ml insulin; 20 nM progesterone; 100 µM putrescine; 30 nM selenium; 100 µg/ml

transferrin), 30 nM thyroxine, 30 nM triiodothyronine, 50 U/ml penicillin, and 50 µg/ml streptomycin]. All

cultures were incubated at 37°C in 5% CO_2/95% humidified air. Cells were plated on poly-D-lysine (PDL)

coated 6-well plates for microarray/RNA studies, or PDL-coated chamber slides or coverslips in 24-well

plates for histological studies.

DEX treatment of cultures: DEX is frequently employed in clinical practice,[8,11] particularly for the

treatment of respiratory distress syndrome,[9] reduction of cerebral oedema associated with intracranial

neoplasms,[67] and following acute spinal cord injury.[10] This drug was therefore selected for use in the

experimental studies, and is reported to act almost exclusively on the GR.[68] DEX was prepared in ethanol

with the concentration verified spectrophotometrically (Genesys 10S UV-Vis spectrophotometer,

ThermoScientific, USA) then diluted in appropriate culture medium to the indicated concentration; the final

concentration of ethanol was 0.27% (v/v) at all doses and for vehicle controls. The drug doses used here

have been frequently used with neural cells including for microarray analysis,[36,69] validating their use in the

current study. Further, CS treatment of microglia promotes apoptosis,[70] providing a simple and effective

measure of CS effects, making microglia suitable positive controls to validate the biological efficacy of the

DEX doses selected. Table 1 summarizes the protocols and timings of DEX treatment for individual assays.

Table 1 Protocol timings for DEX treatment of glial cells							
Experiment	**Cell type**	**Culture medium[1]**	**[DEX]**	**DEX application *post*-plating**	**Period of DEX treatment**	**Total culture period**	**Processing at end of DEX treatment; immunostaining for cell markers[2]**
Transcriptional effects of CS (microarray)	Astrocyte	D10	1 µM	24 h	48 h	3 d	RNA extraction
	Microglia	D10	1 µM	24 h	48 h	3 d	RNA extraction
	OPC	OPC-MM	1 µM	24 h	48 h	3 d	RNA extraction
	OL	24 h OPC-MM, then Sato	1 µM	4 d	48 h	6 d	RNA extraction
CS effects on proliferation/ differentiation/ morphology	Microglia	D10	1 µM, 100 µM	24 h	3 d	4 d	Histology; OX42
	OPC	OPC-MM	1 µM, 100 µM	24 h	3 d	4 d	Histology; A2B5, NG2
	OPC/OL	24 h OPC-MM, then Sato	1 µM, 100 µM	24 h (applied in Sato)	7 d; refreshed every 2-3 d	8 d	Histology; MBP
GR detection	Astrocyte	D10	1 µM	3 d	30 min	3 d	Histology; H300, BuGR2/GFAP
	Microglia	D10	1 µM	3 d	30 min	3 d	Histology; H300/Lectin
	OPC	OPC-MM	1 µM	3 d	30 min	3 d	Histology; H300/NG2
	OL	24 h OPC-MM, then Sato	1 µM	9 d	30 min	9 d	Histology; H300/MBP
Olig1 detection	Microglia	D10	1 µM	24 h	48 h	3 d	Histology; Olig1/Lectin
	OPC	OPC-MM	1 µM	24 h	48 h	3 d	Histology; Olig1/NG2
	OL	24 h OPC-MM, then Sato	1 µM	7 d	48 h	9 d	Histology; Olig1/MBP

DEX = dexamethasone; GR = glucocorticoid receptor; OL = oligodendrocyte; OPC = oligodendrocyte precursor cell; [1]see 'methods, cell cultures' for medium details; [2]see Table 2 for antibody/marker details

Histological analyses

Immunocytochemistry: Cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) [room temperature (RT); 30 min] and washed in PBS. Table 2 summarizes the antibodies used to detect specific cell types and the immunostaining protocols. Cells were blocked (RT; 30 min), incubated overnight

with primary antibody in blocking solution (4°C; simultaneous antibodies for double-staining), washed,

blocked, incubated with the appropriate FITC- or Cy3-conjugated secondary antibody in blocking solution

(1:200; RT; 2 h; simultaneous antibodies for double-staining), washed and mounted with the nuclear stain

DAPI. As both H300 and GFAP require anti-rabbit secondary antibody, BuGR2 was used with GFAP to

double-stain astrocytes.

Table 2 Antibodies and immunostaining protocols

Antibody	Supplier	Blocking solution in PBS	Antibody concentration
Mouse anti-A2B5, OPC marker	Sigma-Aldrich, UK	5% serum	1:200
BuGR2, mouse anti-GR	Abcam, UK	5% serum, 0.3% Triton	1:100
Rabbit anti-GFAP, astrocyte marker	DakoCytomation, UK	5% serum, 0.3% Triton	1:500
H300, rabbit anti-GR	Santa Cruz Biotech, USA	5% serum, 0.3% Triton	1:100
Lectin (*Lycopersicon esculentum*, biotin-conjugated), microglial marker	Sigma-Aldrich, UK	5% serum	1:150
Rat anti-MBP, oligodendrocyte marker	Serotech Ltd., UK	5% serum, 0.3% Triton	1:200
Rabbit anti-NG2, OPC marker	Millipore, UK	5% serum	1:150
Mouse anti-Olig1	Millipore, UK	5% serum, 0.1% Triton	1:200
OX42 (mouse anti-Cd11b), microglial marker	Serotech Ltd., UK	5% serum[1]	1:500

GR = glucocorticoid receptor; OPC = oligodendrocyte precursor cell; PBS = phosphate buffered saline; Triton = Triton X-100; [1]pre-permeabilised cells with 1% Triton in PBS, 20 min, room temperature

Image analysis: Immunostained samples were imaged using fixed exposure settings on an Axio Scope A1

fluorescence microscope (Carl Zeiss MicroImaging GmbH, Germany), and the images merged using

Photoshop CS3 (version 10.0.1; Adobe, USA). Culture purity was assessed by scoring the coincidence of

DAPI-stained nuclei with immunostaining for the appropriate cell marker, for a minimum of three

micrographs and 100 nuclei per treatment condition. Simultaneously, nuclei were scored for pyknosis,

indicated by fragmented, intensely stained nuclei, as a measure of toxicity. For oligodendrocyte cultures, MBP^{+} cells were assigned a morphological score to semi-quantitatively assess extent of differentiation/maturation as follows: (1) few or no processes, (2) processed but displaying some bipolarity, (3) multiple processes with loss of bipolarity (displaying radial symmetry), (4) highly-processed and (5) dense and elaborate/flattened processes.

To validate microarray findings regarding *Olig1* expression (see Microarray analysis section), fluorescence micrographs of control and CS-treated microglia were converted to grayscale (Photoshop) and calibrated as a batch (optical density step-tablet, Rodbard equation; ImageJ, National Institutes of Health, USA). The relative expression of Olig1 protein was quantified using optical density measurements of individual cells (minimum of 30 lectin[+] microglia and 3 images per condition per culture), with background readings subtracted.

RNA analyses

Total RNA extraction: For these experiments, microglia and astrocytes (from the same parent primary cultures as OPCs/oligodendrocytes) were used as positive controls. Both express the GR[71,72] (providing RT-PCR validation) and are CS responsive,[26,73,74] so can be predicted to show significant alterations in gene expression, making both cell types suitable positive controls for microarray analysis. All cell types were washed with nuclease-free PBS then RNA was extracted using an RNeasy Mini Kit (Qiagen, UK), as per manufacturer's instructions. Concentrations were determined by Nanodrop spectrophotometry (Labtech, UK) and samples stored at -80°C.

RT-PCR: Residual genomic DNA from vehicle only samples was removed using RNase-free DNase then RNA was reverse transcribed using random hexamer primers and reverse transcriptase, all according to the manufacturer's protocols. cDNA was amplified by hot-start RT-PCR with specific primers for GR[75] (36 cycles) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH;[76] 34 cycles). Products were electrophoresed on 2% agarose gels with 100 bp DNA ladders. Primer sequences were:

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GR: fwd: 5'GAG CAG AGA ATG TCT CTA CCC; rev: 5'GAC GAT GGC TTT TCC TAG CTC.

GAPDH: fwd: 5'ACC ACA GTC CAT GCC ATC AC; rev: 5'TCC ACC ACC CTG TTG CTG TA.

Microarray analysis: For each cell type, RNA samples from four cultures were dispatched to Source Bioscience UK Ltd. (Nottingham, UK) for processing and hybridisation. RNA integrity was determined using the Bioanalyzer (Agilent, USA) and the three pairs of RNA samples (DEX treated and vehicle only control samples from the same culture) with the highest quality per condition were selected for microarray analysis. 750 ng of processed cRNA was hybridized to Illumina RatRef-12 bead chips. Differentially expressed genes were identified using limma package of R.[77] Data was normalized using the neqc function of the limma package found to be robust for Beadarray analysis.[78] Beads with quality scores (detection probability) < 95% in any sample were removed, resulting in 15,498 genes analyzed. Significantly differentially-expressed genes were identified using a modified t-test with Benjamini-Hochberg test to control false discovery rate (FDR) for multiple testing. Genes were considered as differentially-expressed if corrected p values were < 0.05 and log2 fold change values were $\geq |1|$. Enrichment and Pathway analysis was conducted using the MetaCore™ platform, with all genes reported here having an enrichment FDR corrected p value < 0.01 and a minimum of two genes in an enriched category. One transcriptionally-altered gene (*Olig1*) was selected in order to validate the microarray findings by double-immunostaining microglia for both lectin and Olig1, using OPCs (NG2 staining) and oligodendrocytes (MBP staining) as positive controls as Olig1 expression is considered to be restricted to oligodendrocyte lineage cells.[38]

Statistical analysis: Other than for the array analysis described above, data were analyzed using Prism statistical analysis software (GraphPad, USA). All data are expressed as mean ± SEM. The number of experiments (*n*) refers to the number of mixed glial cultures from which isolated cultures were derived, with each primary culture being established from a different rat litter. For CS effects on oligodendroglia and microglia, data were analyzed by one-way ANOVA with Bonferroni's multiple comparison *post*-tests for *post*-hoc analysis. Optical density measurements of Olig1 expression were averaged for each culture/condition, normalized then analyzed by a paired *t*-test.

Supporting information available: Microarray analyses are provided as Excel files.

Gene.lists.with.annotations; Astrocyte.Enrichment_Analysis; Mglia.Enrichment_Analysis;

Oligo.Enrichment_Analysis; OPC.Enrichment_Analysis. The complete microarray dataset has been

deposited at ArrayExpress (Experiment name: Corticosteroid treatment of glial cell types; ArrayExpress

accession: E-MTAB-1695). This material is available free of charge via the Internet at http://pubs.acs.org.

Abbreviations: CNS, central nervous system; CS, corticosteroid; DEX, dexamethasone; GFAP, glial

fibrillary acidic protein; GR, glucocorticoid receptor; MBP, myelin basic protein; OPC, oligodendrocyte

precursor cell.

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