Published in final edited form as:

Neuroscience. 2019 July 15; 411: 255–269. doi:10.1016/j.neuroscience.2019.05.050.

Glucocorticoid-induced reductions of myelination and connexin 43 in mixed central nervous system cell cultures are prevented by mifepristone

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Abstract

Repeated stress induces systemic elevations in glucocorticoid levels. Stress is also associated with alterations in central nervous system astrocytes and oligodendrocytes that involve connexins and myelin proteins. Corticosteroid elevation seems a major factor in stress-induced neuropathology. Changes in astrocyte connexins and myelin components may be important mediators for the neurological effects of corticosteroid elevations. Two primary cell culture models, myelination culture from rat embryonic spinal cord (SC) or cerebral cortex (CC) consisting of neurons and glial cells (oligodendrocytes, microglia and astrocytes), and mixed astrocyte-and-oligodendrocyte culture prepared from postnatal rat CC, were used in this study. Cell cultures were treated with either vehicle, corticosterone (CORT) with or without glucocorticoid receptor antagonist mifepristone, or dexamethasone (DEX) during the period of *in vitro* myelination. Immunoreactivity of astrocyte connexin 43 (Cx43) and oligodendrocyte myelin basic protein (MBP), or the myelination index (co-localization of MBP and phosphorylated neurofilament) were determined by double immunofluorescent labeling. Oligodendrocyte morphology was evaluated by Sholl analysis. Prolonged exposure to CORT or DEX induced dose-dependent reduction of the myelination index, and immunostaining for MBP and Cx43 in SC and CC myelination cultures, which was prevented by mifepristone. In glial cultures single CORT or DEX exposure caused shrinkage and simplification in MBP- or CNPase-positive oligodendrocyte processes. The results support that concurrent effects of glucocorticoids on myelination and astrocyte Cx43 immunoreactivity are mediated by glucocorticoid receptors and may partially account for the involvement of CNS glia in the pathological effects of prolonged stress.

Graphical abstract

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Conflicts of interest statement: The authors have no conflicts of interest to declare.

Keywords

Astrocytes; development; corticosteroids; dexamethasone; myelin; glucocorticoid receptors

INTRODUCTION

Prolonged or repeated exposure to stress is a major risk factor in the development of depression and other psychiatric disorders (Hammen, 2005; Kendler, Gardner, & Prescott, 1999; Mahar, Bambico, Mechawar, & Nobrega, 2014; Swaab, Bao, & Lucassen, 2005). Animals subjected to unpredictable and persistent stress also develop disturbed performance in some cognitive domains as well as depression- and anxiety-like behaviors that respond to psychotropic compounds such as antidepressants (Willner, 2017). Accumulating evidence further shows that oligodendrocyte-derived myelin components and astrocytic gap-junction proteins connexin 43 (Cx43) and connexin 30 (Cx30) (Mahar et al., 2014) are reduced both in rodents under stress and in humans with depression (Ernst et al., 2011; Miguel-Hidalgo et al., 2017; Miguel-Hidalgo et al., 2014; Rajkowska et al., 2015; Sun, Liu, Yuan, Li, & Chen, 2012). Since one of the major hallmarks of stress is both temporary and persistent dramatic elevation of circulating and brain glucocorticoids (mainly cortisol in humans and

corticosterone in rodents) (Barlow, Morrison, & Sullivan, 1975; Cook & Wellman, 2004; Silberman, Wald, & Genaro, 2002; Vogel & Jensh, 1988; Willner, 2017), it is possible that recurrent or sustained plasma glucocorticoid elevations are a causal factor for concurrent neuropathological changes in astrocytes, myelin and the neuronal functions they support. Furthermore, elevation of glucocorticoids and other corticosteroids could receive a substantial contribution from glucocorticoids intrinsic to the CNS (Davies & MacKenzie, 2003; Taves, Gomez-Sanchez, & Soma, 2011).

Exposure to increased glucocorticoid levels could play a role in the neurophysiological and behavioral disturbances of disorders featuring stress as a major risk factor. In fact, *in vitro* studies with isolated, primary cortical astrocytes, as well as *in vivo* research in the cerebral cortex, have found that high levels of corticosterone cause reduction in Cx43 protein (Xia et al., 2017; Xia et al., 2018) or mRNA (Carter, Hamilton, & Thompson, 2013; Samarasinghe, Witchell, & DeFranco, 2012), while in other tissues corticosterone causes Cx43 degradation through autophagy mechanisms (Gao et al., 2016) or the activation of the Akt/mTOR signaling pathway (Shen et al., 2016). Cx43 is the predominant connexin in astrocytes and together with Cx30, which is also significantly produced by astrocytes, plays a major role in myelin maintenance *in vivo*. However, it is unknown whether, during developmental myelin formation or in myelin repair, anomalous activation of glucocorticoid receptors causes changes in the cellular distribution of Cx43 in astrocytes, and whether those changes are paralleled by deficits in myelination-related parameters. It has been shown that some antidepressants, or glucocorticoid receptor antagonists such as mifepristone, mitigate the reduction of Cx43 caused by chronic unpredictable stress in animals (Sun et al., 2012; Xia et al., 2017), but it remains to be ascertained whether during the process of active myelination those antagonists mitigate the adverse effects of exogenous high glucocorticoids on myelin and connexins concurrently.

Several studies have shown that corticosterone treatment downregulates myelin proteins *in vitro* and *in vivo* (Melcangi, Magnaghi, Cavarretta, Riva, & Martini, 1997), although glucocorticoids also appear to upregulate expression of myelin proteins and myelination, greatly depending on developmental stage, specific cellular environment or brain region. For instance, stress and corticosterone result in enhanced oligodendrogenesis in the dentate gyrus of the adult rodent hippocampus (Chetty et al., 2014) and oligodendrocyte precursor differentiation into oligodendrocytes, which could be restored by corticosteroids (Mann et al., 2008). On the other hand, an excess of corticosteroids could cause reduced myelination, which would be in part due to inhibition of the proliferation of NG2 positive oligodendrocyte progenitors (Alonso, 2000). So far, the consequences of prolonged or repeated high glucocorticoids on myelin formation during the process of myelination and expression of myelin markers remain unclear.

Oligodendrocyte development and myelination may also depend on the presence of astrocytes and the gap junctions they form with oligodendrocytes or other astrocytes, although the present research was only directed to determine whether Cx43 immunoreactivity, myelination parameters, and the morphology of oligodendrocytes could be concomitantly reduced by the specific actions of high glucocorticoid levels. Nonetheless, it is worth reminding that differentiation from oligodendrocyte precursors into

oligodendrocytes and the survival of these cells *in vitro* appears to be greatly favored in co-cultures of astrocytes, even in the absence of neurons (Pang et al., 2013; Pang et al., 2012). Likewise, myelin maintenance and formation can also be substantially influenced by the expression of Cx43 and Cx30 in astrocytes, as shown in mice with knock-out of connexins produced by astrocytes and oligodendrocytes (Kiray, Lindsay, Hosseinzadeh, & Barnett, 2016). Furthermore, neuronal activity drives plasticity of oligodendrocyte differentiation and myelin formation (Fields, 2013; Purger, Gibson, & Monje, 2016) as well as the expression of astrocyte connexins (Rouach, Glowinski, & Giaume, 2000), suggesting that myelin maintenance can be both directly and indirectly regulated through gap junction-based interactions between astrocytes and oligodendrocytes.

Previous *in vitro* studies typically used only one or two cell types to investigate the effects of corticosterone exposure on connexin expression or myelination. However, understanding the effects of glucocorticoids on myelination and astrocyte marker expression may require cell cultures where the effects of more complex interactions between different glial cell types and neurons are allowed. Thus, we took advantage of our recently developed neuron-glia co-cultures from the spinal cord (SC) or cerebral cortex (CC) (Pang, Simpson, Miguel-Hidalgo, & Savich, 2018; Pang et al., 2012) to determine the consequences of high glucocorticoid exposure on myelination and Cx43 immunoreactivity. Since previously we observed a more robust, comprehensive, and fast-paced myelination in cultures derived from SC than in those from CC at the same gestational stage (26, 31), in the present study we first treated SC-derived myelination cultures with glucocorticoids and found a significant reduction in Cx43 and myelin basic protein (MBP) immunoreactivities. We next determined whether these effects by corticosteroids also held true during myelination in CC-derived cultures, and whether such effects could be mediated by GC receptors, by treating cells with glucocorticoids in the presence or absence of GC receptor antagonist mifepristone. To gain a first insight into whether the morphological features of differentiating oligodendrocytes are affected by GCs even in the absence of neurons, we conducted additional experiments using mixed glial cell cultures containing only oligodendrocytes and astrocytes.

EXPERIMENTAL PROCEDURES

Cell culture reagents:

all reagents used to prepare N2 medium, corticosterone, dexamethasone, and mifepristone were from Sigma; Dulbecco's modified Eagle Medium (DMEM)/Ham's F12, Neural basal medium (NBM), B27, and fetal bovine serum (FBS) were from Invitrogen. Nerve growth factor (NGF) and neurotrophin-3 (NT-3) were from R&D system (Minneapolis, MN, USA).

Cell cultures and treatments

Myelination cultures—Myelination cultures were established using dissociated neural cells of SC or CC from Wistar rat embryos at gestation day 16 (E16) according to methods previously described (Pang et al., 2018; Pang et al., 2012). The rationale for using two different anatomical origins for our myelination cultures was that generally SC-derived cultures generate faster and more robust myelination, which enabled us to detect putative glucocorticoid-induced changes more readily for an initial feasibility and proof-of-concept

testing, while CC-derived cultures are more relevant to understand mechanisms of astrocyte and myelin involvement in *in vivo* models of stress-related CNS pathology. Regardless of the tissue source, the dissociated embryonic neural cells in these two culture systems differentiate to a similar extent into neurons and macroglial cells (oligodendrocytes and astrocytes), although SC-derived cultures contain significantly more microglia than the CC cultures (10% vs 2%) (Pang et al., 2018; Pang et al., 2012). About 6 to 8 embryos from one pregnant rat were pooled together for each cell culture preparation, and 3 independent primary cell cultures were performed for the entire study. Briefly, dissected tissue was carefully stripped off meninges and cut into small blocks under a stereomicroscope, and digested with trypsin. Dissociated cells were plated on poly-L-lysine coated glass coverslips placed in 12-well plates at a cell density of 1.5×10^5 cells/coverslip, and maintained in a 1:1 mixture (volume:volume) of N2 and NBM+B27 media. To encourage neuronal differentiation, NGF (50 ng/ml) and NT3 (10 ng/ml) were included in the medium during the first week. The medium was switched to a mixed (4:1, volume:volume) insulin-free N2 and NBM+B27 medium starting at 10 days in vitro (DIV) to prevent glial cell overgrowth, which can cause detachment of cells from coverslips (26). At DIV12 cells were treated with 5 μ M corticosterone (CORT), 50 μ M CORT, 50 μ M CORT plus 10 μ M mifepristone, 10 μ M mifepristone alone, 50 μ M dexamethasone (DEX50), or vehicle (DMSO) (mifepristone treatments were applied only to CC cultures). Fresh medium containing treatment reagents was replenished every 3 days. Cells were fixed with 4% paraformaldehyde (PFA) at DIV28, at which time long stretches of myelinated axons are readily detected by immunofluorescence.

Mixed oligodendrocytes-astrocytes cultures—Mixed glial cells were prepared from postnatal day 1 (P1) rat brain cerebral cortex (CC), as previously described (Pang et al., 2010). This type of culture contains astrocytes and oligodendrocytes, but not neurons (Pang et al., 2010). Briefly, dissociated cells were maintained in 10% fetal bovine serum (FBS) until reaching confluency. Culture flasks were shaken at 200 rpm overnight, which causes mainly astrocytes and oligodendrocyte precursors to remain attached to the flask while microglia detach, going into suspension in the medium and are discarded together with the supernatant at the time of medium replacement. The supernatant was replaced with fresh medium and the cells were cultured for an additional week. Cells were then dissociated with trypsin and plated on poly-L-lysine-coated glass coverslips at 1.5×10^5 cells/coverslip. The mixed oligodendrocytes-astrocytes cultures were treated once with CORT5, CORT50 and DEX50 on DIV 10, maintained for 4 days and fixed with 4% PFA for immunocytochemistry.

Immunocytochemistry

At the end of treatment, the culture medium was carefully aspirated and cultures were immediately fixed with 4% PFA for 15 minutes at room temperature (RT) and stored in fresh PBS at 4 °C. From each treatment, three coverslips were subjected to multiple immunolabeling using different combinations of primary antibodies at RT. The antibodies were against the following proteins: myelin basic protein (MBP, for both mature oligodendrocytes and myelin, chicken antibody, catalog #AB9348, Millipore, Temecula, CA, USA, working dilution 1:200), phosphorylated neurofilament (pNF; for mature axons, mouse SMI-31 antibody, catalog #801602, Biolegend, San Diego, CA, USA, 1:1000),

Connexin 43 (Cx43; for astrocyte gap junctions/hemichannels, mouse antibody, catalog # 610062, BD Bioscience, Franklin Lakes, NJ, USA, 1:300), CNPase (for both developing and mature oligodendrocytes, mouse RIP antibody, catalog # MAB1580, Millipore, Temecula, CA, USA, 1:1000), and glial fibrillary acidic protein (GFAP, for astrocyte cytoskeleton, rabbit antibody catalog #AB5804, Millipore, Temecula, CA, USA, 1:1000). CNPase and MBP immunolabeling were also used for morphological analysis of oligodendrocytes. Following washes in PBS, cells were then incubated with secondary antibodies conjugated with Alexa Fluor-488 or Alexa Fluor-555 for 1 h at RT. Coverslips were air-dried and mounted on slides using a DAPI-containing mounting medium.

Morphometric analysis

Area fraction of immunoreactivity: To determine the extent of immunofluorescent structures for each marker or of co-labeling with specific pairs of markers, 20 optic fields (herein called samples) were randomly selected under the 10× or 20× objectives from three coverslip cultures immunostained for a particular marker combination (7 fields from each of 2 slides and 6 fields from the remaining slides). In each slide the microscope objective was aimed at the approximate center of the slide and one field was selected randomly at left or right of the starting point then, and the rest of the fields (samples) were chosen picking one sample every 4 fields until reaching the 7 or six fields per slide. Pictures were taken at a fluorescence Nikon Eclipse E800 microscope with Metamorph software (6. 2r2) using a constant gain setting in all cultures for each wavelength channel of fluorescent light. To determine the extent of neurofilament immunolabeling, fluorescent structures were thresholded at 20 optic density levels over the non-immunoreactive background (dark) with optic density ranging from level 0 (darkest, no immunofluorescence) to level 255 (brightest, maximum immunofluorescence). In each sample the extent of immunolabeling was calculated as an area fraction, which is the ratio (expressed as percentage) of the area taken by immunofluorescent structures to the total 2-dimensional area of the sample. The extent of MBP-, Cx43- and neurofilament-immunofluorescent structures within each sampled optic field was also expressed as an area fraction by the same method.

Myelination index: The extent of myelination along axons was estimated by measuring the length of MBP-immunoreactivity aligned with NF immunoreactive axons (Fig. 1) within doubly-labeled samples (that is, the length of NF+ fiber segments with MPB+ co-labeling in doubly immunostained coverslip cultures) and dividing that length into the total area fraction of neurofilament immunofluorescence in the sample. The resulting quotient was considered the myelination index. To take into account the possibility of variation in the total number of cells among samples, measurements of area fraction or myelination extent were also referred to the number of DAPI-positive nuclei counted in each sample.

Sholl analysis: In CC mixed glial cultures with only two cell types (oligodendrocytes and astrocytes), cells were simultaneously labeled with DAPI (to estimate total cell number) and with MBP or CNPase immunofluorescence to label oligodendrocytes and their processes. Morphology of MBP or CNPase-labeled processes branching out from the cell bodies was assessed by Sholl analysis implemented according to the procedure provided in the image analysis software ImageJ (version 2.0.0), using the same analysis parameters for all cells

detected. This procedure involved overlapping the immunolabeled cell and its processes with a template consisting of a series of concentric circles with increasingly larger radius covering to the farthest reach of the cell's processes (branches). The procedure determines the location and numbers of intersections of the processes with the circles of the template and the ramifications of those processes. Several variables can be derived from the intersection pattern such as: sum of intersections, ending radius, branching from the cell body, ramification index (an indication of how much the branches stemming from the center ramify), and critical value (maximum number of intersections of cell processes with the template at a particular distance from the center of the cell). These variables measure the degree and location of branching and ramifications from the main branches, which indicates the complexity and extent of the processes originating from the cell body. We obtained values for all the variables mentioned above. The distance between adjacent circles in the template used in the analysis of MBP and CNPase immunoreactive cells was 3 μm . Values for the Sholl variables were obtained using the ImageJ Sholl procedure plugin (Ferreira et al., 2014) and averaged for all cells in the samples of a particular treatment (treatments were control (vehicle), CORT5, CORT50 or DEX50). The variable averages in each treatment group were statistically analyzed and compared to other groups as explained below. All immunoreactive cells in each randomly selected sample were included in the analysis.

Statistics:

Measurements of area fraction, myelination index, numbers of DAPI-positive nuclei and variables derived from the Sholl analysis were compared among the treatment groups defined above (each treatment group including about 20 samples (optic fields) from myelination cultures or from astrocyte-oligodendrocyte cultures. For all variables, the distribution of values did not significantly deviate from the normal distribution, so we used ANOVA and if ANOVA was statistically significant, pairwise comparisons were made to the control group with Dunnett's tests to correct for the number of comparisons for data derived from SC cultures, and from CC astrocyte-oligodendrocyte short-term two-cell-type cultures. Alternatively, Sidak tests were used when, in addition to comparing to the controls, comparisons were planned between mifepristone treated samples and CORT50 or controls in data from CC myelination cultures. Group differences in the means of the measured variables were considered significant when the probability of their random occurrence in ANOVA or post-hoc tests was smaller than 0.05 ($p < 0.05$). Each treatment group consisted of 3 to 4 coverslips, and 3 separate experiments (from 3 separate primary cell culture preparations) were conducted.

RESULTS

EFFECTS OF CORT AND DEX ON MYELINATION AND CX43 EXPRESSION IN SC MYELINATION CULTURES

In the control, vehicle-only treated cultures, many pNF+ axons and bundles were closely aligned with MBP-immunoreactive (MBP+) segments on DIV 28 (Fig. 1). In addition, MBP + oligodendrocyte cell bodies, processes and membrane-like expansions were often observed at various locations within the coverslips (Fig. 1, Fig. 2 left panel). In contrast, CORT exposure resulted in a dramatic reduction in the length of myelinated axons identified by co-

localized MBP and pNF immunoreactivity (Fig. 2, middle panel), as reflected in the dose-dependent reduction of the myelinated length per cell (Figs. 3A) or the myelination index (Fig. 3B). CORT treatment also drastically reduced the area fraction of overall MBP immunoreactivity in the examined samples in a dose-dependent manner (Fig. 3C). DEX50 caused a significant reduction of the myelination index (Fig. 3B), but did not result in a reduction of the area fraction of overall MBP immunoreactivity as compared to control samples (Fig. 3C,D). The area fraction of pNF-immunoreactive structures and the number of cells were not significantly different between treatments (Fig. 3E and Fig. 4B, respectively).

Since mixed-cell myelination cultures contain neurons, oligodendrocytes and astrocytes, and astrocytes express Cx43, we also determined punctuated Cx43 immunoreactivity in these cultures. Cx43 immunoreactivity appeared as a multitude of small immunoreactive granules or puncta across the cultured cells (Fig. 4 top middle micrograph). Treatment with CORT or DEX50 also resulted in a strong dose-dependent decrease of the area fraction of Cx43 immunoreactivity relative to cell number (Fig. 4 micrographs, Fig. 4A), although there was no significant change in cell numbers (Fig. 4B) in the glucocorticoid-treated cell cultures.

EFFECTS OF CORT, DEX AND GLUCOCORTICOID RECEPTOR ANTAGONIST MIFEPRISTONE ON CC-DERIVED MYELINATION CULTURES

We next determined whether the adverse effects of glucocorticoids could be replicated in cortical myelination cultures, since alterations of glia cells and/or myelination has been frequently reported in the cerebral cortex, especially in the frontal cortex of depressed patients and rodent models of unpredictable stress. In addition to the control and corticosteroid treatments, some cell cultures were also treated with 50 μ M CORT plus 10 μ M mifepristone, or 10 μ M mifepristone alone and examined at DIV 28. Similar to the SC-derived culture, well-defined linear MBP-immunoreactive fragments were clearly aligned with stretches of pNF+ axons, while the overall MBP immunoreactivity was mostly displayed in cell bodies, processes and membranous expansions (Fig. 5) in CC-derived control cultures. Treatment with CORT resulted in a dramatic dose-dependent decrease in both the myelination index (Fig 5 middle panels, Fig. 6A, B) and the overall area fraction of MBP immunoreactivity (Fig 6C). DEX50 also caused a substantial and significant drop in the myelination index (Fig. 6A, B), but not in the area fraction of the overall MBP immunoreactivity (Fig. 6C). Mifepristone largely prevented the reduction in myelination index and area fraction of MBP immunoreactivity caused by CORT50. In addition, mifepristone applied alone, while not changing significantly the area fraction of MBP immunostaining, produced a significant increase in the myelination index as compared to control CC cultures (Fig. 6A). Cell number and area fraction of NF+ fibers were not changed by treatments (Fig. 6D, E) or when compared to controls, with the exception of cell number in mifepristone-only-treated cultures, which was significantly larger than in CORT50 cultures (Fig. 6D).

To determine Cx43 immunoreactivity changes, coverslips that contained the cells shown in figure 7 were, cultured at the same time and from the same mixtures of cells as those coverslips corresponding to the images in figure 5. That is, the mixture of cells (neurons, oligodendrocytes and astrocytes) in the coverslips for both figures was the same, the only

difference being that the coverslips for figure 5 were processed to detect MBP, neurofilaments and DAPI, and those in figure 7 to detect GFAP, Cx43 and DAPI. In CC-derived myelination cultures, CORT resulted in a dose-dependent decrease in Cx43 area fraction, with the maximal effects observed in cultures treated with 50 μ M CORT, where only a few Cx43 granules were detectable (Figs. 7 and 8). DEX also induced a reduction in Cx43 staining, but it was intermediate between the two doses of CORT (Fig. 8). Treatment with mifepristone in CORT50-exposed cultures largely prevented the reduction of Cx43 immunoreactivity (Figs. 7 and 8). The recovery was best visualized in GFAP-immunostained astrocytes by the reappearance of Cx43 immunoreactive puncta on GFAP immunoreactive astrocytes in CORT50 samples co-treated with mifepristone, since neither CORT50 alone or mifepristone alone noticeably reduced GFAP immunostaining as compared to control cultures (Fig. 7). Mifepristone alone did not significantly alter the area fraction of Cx43 or GFAP immunostaining area fraction in CC cultures (Fig. 8).

EFFECTS OF SINGLE CORT OR DEX EXPOSURE IN MIXED CC GLIAL CELL CULTURES

To determine whether the morphology of differentiating oligodendrocytes co-cultured with astrocytes could be also affected by glucocorticoids in an environment without neurons (and thus lacking myelination), cultures containing only astrocytes and oligodendrocytes were treated with CORT5, CORT50 or DEX50. There were no differences between treatments in the total cell number estimate (DAPI stained cell nuclei) per sample. However, the proportion of identifiable CNPase immunolabeled cells was lower in CORT50 as compared to control samples and there was a tendency for CORT50 samples to also contain less MBP labeled cells than control samples. Overall there was a higher proportion of visible CNPase positive cells as compared to MBP cells in all treatments ($1.262 \pm 0.115\%$ MBP+ cells in the total count vs $1.756 \pm 0.124\%$ CNPase+ cells; $t=2.86$, $p=0.012$). Sholl analysis of MBP immunoreactive cells showed a decrease in the sum of intersections of MBP+ processes in CORT50 and DEX50 treated samples, and lower ramification index in CORT50 samples as compared to controls (Fig. 9A–C). The critical value for intersections was significantly smaller in CORT5, CORT50 and DEX50 treated samples than in controls (Fig. 9D). For CNPase-immunoreactive cells there was significantly lower sum of intersections, ramification index and critical value in CORT5, CORT50 and DEX50 as compared to control samples, but there were no differences in the number of branches directly originating from the cell bodies (Fig. 10).

DISCUSSION

The research reported here shows that exposing primary mixed cell cultures from the rat CNS to high glucocorticoid levels of during the *in vitro* period of myelination resulted in dramatic depletion of the area fraction of Cx43 and MBP immunoreactivities and a strong reduction of a myelination index, while total cell number, immunoreactivity of neurofilament positive axons, and GFAP immunoreactivity were not affected. Furthermore, application of the glucocorticoid receptor antagonist mifepristone in CC cultures prevented the glucocorticoid-induced reduction of both Cx43 immunoreactivity and the myelination index. Interestingly, in the same long-term SC and CC cultures, the effects of glucocorticoid

analog DEX50 on the overall MBP immunoreactivity and Cx43 labeling were noticeably smaller than those caused by CORT50 or CORT5.

INVOLVEMENT OF GLUCOCORTICOID RECEPTORS IN THE EFFECTS OF CORTICOSTEROIDS

The concentrations of CORT and DEX used in this study are much higher than CORT plasma levels induced by stress, and most likely reached maximal receptor occupancy during a significant part of the exposure period. Accordingly, the differences between the effects of CORT and DEX may be due to differences in the nature of the receptors that each molecule activates in the CNS, although it cannot be ruled out that the ability of cells to differentially metabolize CORT and DEX by 11 β -hydroxysteroid dehydrogenase type 1 (11 β HSD1) or 11 β HSD2 enzymes was also involved in the effects. Since we did not determine possible alterations in receptors or in 11 β HSD1 or 11 β HSD2 it is not possible to determine to what extent alterations in the receptors or corticosteroid metabolizing enzymes may have influenced the results obtained. However, as suggested above, at the concentrations used corticosterone would maximally activate glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs), both of which, upon binding to CORT, may translocate to the nucleus to act as transcription factors, causing widespread gene expression changes. In addition, CORT may also act on non-genomic GRs (Groeneweg, Karst, de Kloet, & Joels, 2011), and there is evidence that CORT binding to this type of receptors induces significant effects in the CNS (Groeneweg, Karst, de Kloet, & Joels, 2012; Haller, Mikics, & Makara, 2008), including regulation of Cx43-bearing gap junctions (Samarasinghe et al., 2011). In contrast, DEX would activate GRs, but much less so than MRs, and, due to a significantly lower affinity for some non-genomic GRs, DEX would not activate G-protein-dependent non-genomic receptors (or others) that are activated by CORT (Guo, Chen, Xu, & Fu, 1995), although non-genomic effects of DEX in our cultures cannot be ruled out because such effects have also been demonstrated in other systems (Samarasinghe et al., 2011), and differences with CORT might be rather due to biased agonism on non-genomic receptors and differential affinity at genomic receptors. Only further research will allow discriminating these possibilities. Other studies have found that in the rat brain some receptors are activated by corticosterone and mineralocorticoids but not by DEX (Groeneweg et al., 2012; Guo et al., 1995), suggesting an additional mechanism that would explain differential effects of CORT and DEX on the expression of MBP in oligodendrocytes and its deployment in myelinating membranous expansions along axons. Thus, the suppression of myelination in mixed cultures may be due to the activation of GRs by either CORT or DEX, while the drastic reduction of MBP expression and its labeling in cytoplasmic expansions of oligodendrocytes may be due to actions of CORT on non-genomic receptors, or to a combination of both. Clearly, since we only used one DEX concentration, the possibility that higher DEX concentrations exert effects similar to those of CORT cannot be ruled out. Since mifepristone acts as an antagonist of both genomic and non-genomic effects of glucocorticoids it is reasonable that it largely abrogates both the reduction of myelination index by CORT and the overall MBP immunoreactivity loss, although further experiments would be needed to fully confirm that some of the DEX effects depend on receptor and messenger systems shared with CORT.

INVOLVEMENT OF ASTROCYTES AND OLIGODENDROCYTES IN THE EFFECTS OF CORTICOSTEROIDS

Since we used primary mixed cultures containing neurons and at least 3 glial cell types, effects on myelination may have been a consequence of direct, indirect or a mixture of both kinds of glucocorticoid actions on oligodendrocyte function. Direct effects are certainly possible because genomic and non-genomic GRs are present in oligodendrocytes (in addition to astrocytes and neurons) (Bohn, Howard, Vielkind, & Krozowski, 1991; Cintra et al., 1994; Vielkind, Walencewicz, Levine, & Bohn, 1990), so that oligodendrocyte GR activation would result in impaired myelination or reduced myelin protein expression. Indirect effects on myelination and myelin protein expression, however, may play a major role as well, because both genomic and non-genomic GR activation in neurons and astrocytes would affect the release of neurotransmitters and other factors from those cells (Abraham, Juhasz, Kekesi, & Kovacs, 1998; Berry et al., 2016; Chatterjee & Sikdar, 2013; Cho & Little, 1999; Gubba, Fawcett, & Herbert, 2004; Jacobsson, Persson, Hansson, & Ronnback, 2006; Lindholm et al., 1992; Moghaddam, Bolinao, Stein-Behrens, & Sapolsky, 1994; Treccani et al., 2014). Some of these factors in turn are known to strongly regulate the process of myelination (Fields, 2013; Ishibashi et al., 2006; Simons & Trajkovic, 2006; Stevens, Tanner, & Fields, 1998; Watkins, Emery, Mulinyawe, & Barres, 2008).

In the present study, we did not address whether the corticosterone-induced change in Cx43 was causally connected to myelin alterations. However, the contribution of astrocytes to the regulation of myelination may be important since, even in the obvious presence of neurons, myelination seems to be dependent on, or at least highly influenced by, the action of astrocyte-generated factors (Barnett & Linington, 2013; Ishibashi et al., 2006; Moore, Abdullah, Brown, Arulpragasam, & Crocker, 2011; Pang et al., 2013). Some of these factors, such as BDNF or TIMP1, could be released to the extracellular space (Fulmer et al., 2014; Lundgaard et al., 2013; Moore, Milner, et al., 2011). Astrocytes also produce connexins 43 and 30, which are membrane proteins that assemble into connexons to form gap junctions between astrocytes and oligodendrocytes and are crucial to myelin maintenance *in vivo*. Thus, in the present investigation, we also tested whether exposure to high CORT and DEX produced disturbances in Cx43 immunoreactivity concurrent with myelin alterations and found that both in SC- and CC-derived myelination cultures there was a significant decrease in Cx43 immunoreactivity, raising the possibility that Cx43 deficits contribute to reduced myelination. Clearly, further experiments centered around possible mechanisms linking astrocyte Cx43 to myelination should test whether reduced astrocyte Cx43 plays a causative role in myelin disturbance following elevated corticosteroids. Alternatively, anomalies in the release of soluble factors from astrocytes (for instance BDNF, TIMP) (57, 59) could have a crucial contribution or act additively with reduced Cx43 in alterations of myelin formation caused by sustained high corticosteroid levels.

FUNCTIONAL IMPLICATIONS OF CORTICOSTEROID-INDUCED MYELIN DISTURBANCE

Dose-dependent, adverse effects of high CORT levels on the myelination index are consistent with the delayed remyelination by resident oligodendrocytes caused by glucocorticoids on spinal cord during remyelination after myelin damage. (Chari, Zhao, Kotter, Blakemore, & Franklin, 2006), suggesting that long-term or frequent exposure to

high corticosteroids (at the very least once every three days in our cultures, coinciding with culture medium replacement) may cause disturbances in myelin in proportion to the amount and/or frequency of the CORT elevation. Thus, even CORT elevations over the baseline smaller than the concentrations applied to our cultures may in the long run produce defects in myelination or myelin structure that, without being necessarily catastrophic, may lead to impaired myelin maintenance and disturbed propagation of action potentials. Those elevations could be one of the factors playing in reduced myelin thickness around axons observed in the prefrontal cortex (but not in other brain regions) of mice subjected to social isolation stress (Liu et al., 2012).

Since high plasma corticosterone elevations observed in animals under stress do not appear to reach the levels we used *in vitro*, the argument could be made that CORT or DEX overexposure in our experiments may be supra-physiological, non-specifically activating other receptor systems or producing significant amounts of cell death and thus causing a catastrophic loss of myelin, oligodendrocytes or astrocytes. However, the available data strongly indicate that there was no substantial cell death, loss of neurons or their axons or depletion of astrocytes in our cultures. Furthermore, mifepristone was able to block most of the effects of high CORT, pointing to the specific mediation of glucocorticoid receptors. On the other hand, it appears that stress-induced increases in corticosteroid concentration in the brain parenchyma, although increasing in parallel with plasma levels, persist long after plasma levels revert to baseline, in some cases staying substantially higher than plasma CORT for days (Croft et al., 2008), which could be due to the presence of intrinsic corticosteroid synthesis or conversion within brain tissue (Seckl & Walker, 2001; Taves et al., 2011). If these mechanisms for stress-dependent augmented corticosteroid actions in the extracellular space of oligodendrocytes and astrocytes operate in situations of repeated or prolonged stress, then they might exert effects resembling those observed in our experiments with myelination cultures. A putative ability of primary CNS mixed cultures to synthesize endogenous corticosteroids may exert a regulatory role in myelin formation, which would help to explain that mifepristone alone appeared to have some positive effect on the extent of myelinated axon segments relative to the number of cells, although without significant effect on other MBP or Cx43 immunoreactive structures. Clearly, further studies are needed to test this speculation. Also, additional experimentation with a wider range of corticosteroid concentrations of *in vitro*, in slice cultures and in whole animals should allow for a better understanding of how stress-related glucocorticoid responses in the brain may affect the short-and long-term maintenance of myelin and the expression of gap junction proteins.

MORPHOLOGICAL EFFECTS OF A SINGLE CORTICOSTEROID EXPOSURE

Whether single, short-term substantial CORT elevations may also result in long term consequences for myelin maintenance (or oligodendrocyte morphology) *in vivo* comparable to the effects of repeated exposure on myelination cultures remains to be elucidated. Nevertheless, all glucocorticoid doses (5 μ M CORT, 50 μ M CORT and 50 μ M DEX) tested in the one exposure study with mixed glial cultures (without neurons) appeared to significantly alter the morphology of MBP or CNPase immunoreactive oligodendrocytes by reducing the ramification and reach of their processes, without a comparable change in total cell number as compared to controls, which would be consistent with an effect of corticosteroids on the

differentiation of oligodendrocytes or their morphological maturation, even if the single exposure to glucocorticoids does not appear to dramatically suppress the expression of MBP at oligodendrocyte cell bodies. The morphological change may depend on altered formation of membranous expansions that, if axons were present, would have contributed to myelin formation during the period of myelination, although it cannot be entirely dismissed that deficient trafficking of specific molecules such as MBP and CNPase contributes to the changes in morphological parameters measured on the basis of fluorescence immunolabeling. If future experiments determine that the observed morphological changes correspond to a genuine disruption in the ability of oligodendrocytes to extend membranes in particular circumstances, this could in part explain the paucity of myelin formation along axons in the mixed cultures. Alternatively, in DEX-treated mixed cultures, the coincidence of preserved MBP/CNPase immunoreactivity in oligodendrocyte expansions with decreased myelination of axonal segments could suggest that the ability of extending membranous expansions by oligodendrocytes and the formation of myelin are two mechanistically distinguishable morphogenetic processes.

In summary, the present study provides original evidence that repeated exposure to high levels of corticosteroids during the period of myelination of spinal cord and cerebral cortex primary mixed cell cultures leads to a concurrent loss of myelination, a reduction in membranous expansions immunoreactive for myelin proteins and a dramatic decrease in immunoreactivity for the astrocytic gap junction protein Cx43, effects that are dependent on the activation of glucocorticoid receptors. These results suggest that concurrent astrocyte and oligodendrocyte neurobiological processes known to regulate the production of myelin, and thus the establishment of adequate signal propagation, may be affected by abnormally high exposure to corticosteroids during the myelination process.

Acknowledgments:

The authors acknowledge funding support from NIMH grant R56MH113828 (JJMH), IRSP grants from the University of Mississippi Medical Center (JJMH) and the Imaging Core of NIGMS grant P30GM103328. Funding sources had no role in the preparation of the article, study design, the analysis and interpretation of data or the decision to submit the article for publication.

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Highlights

- High glucocorticoids reduce axon myelination and connexin 43 in primary cultures from spinal cord and cerebral cortex.
- Mifepristone prevents decreases in myelination and connexin 43 immunoreactivity induced by glucocorticoids.
- High glucocorticoids induced shrinkage of oligodendrocyte processes in mixed glial cell cultures devoid of neurons.

Fig. 1: Myelination in spinal cord (SC)-derived cultures based on MBP immunoreactivity. Micrographs taken from a microscopic field containing structures labeled by fluorescence immunolabeling of MBP (A) and pNF (B), in addition to nuclear stain DAPI (C) in a mixed culture from the SC at DIV28. The three stains are merged in D to illustrate MBP+ labeling along pNF+ axons (arrows) and the presence of MBP+ oligodendrocytes (arrowheads). The lightning bolt-shaped arrows point to an MBP-immunoreactive membrane-like expansions that were often found in control cultures at this stage of the myelination process. Calibration bar: 100 μ m.

Fig. 2:

CORT and DEX treatment significantly reduced myelination index and MBP area fraction in the SC cultures. Top panels: Micrographs from microscope fields of CONTROL (left panel), 50 μ M CORT (CORT50) (middle) and 50 μ M DEX (DEX50) (right) cultures at DIV28 with nuclear DAPI staining (blue), and immunofluorescence labeling of MBP (green or yellow) and neurofilament (red). Note that linear stretches of MBP immunostaining are readily identifiable (white arrows) along pNF immunolabeled axons in the CONTROL culture, but are mostly absent from axons in CORT50 or DEX50 cultures. However, both CONTROL and DEX50 cultures contained membrane like MBP-immunoreactive expansions (lightning bolt-shaped arrows), which were very rare in CORT50 cultures. Calibration bar: 100 μ m.

Fig. 3:

Graphs A-E: Quantification of the length of MBP-immunoreactive stretches aligned with pNF+ axons relative to the number of total cells (DAPI stained cell nuclei) (A) or relative to the total area fraction of pNF+ axons (B) (this latter quotient is referred as myelination index in the main text) in control, 5 μ M CORT, 50 μ M CORT, and 50 μ M DEX treated cultures. The total area fraction of MBP immunoreactivity (that includes all positive cell bodies, processes, membrane-like expansions and myelinated portions of axons) relative to total cell numbers is presented in C, and the ratio of myelinated length of axons to the total area fraction of MBP in D. Panel E illustrates the area fraction (%) of pNF immunoreactivity for the same cultures as above. Since all ANOVAS (in panel A, $F(3,68)=9.315$, $p=0.0001$; B, $F(3,68)=7.894$, $p=0.0001$; C, $F(3,68)=5.469$, $p=0.002$; D, $F(3,68)=7.693$, $p=0.0002$), except that of pNF+ area fraction (panel E; $F(3,68)=2.643$, $p=0.0562$) showed a significant

effect of group, stars (*) denote where the pairwise Dunnett's tests for multiple comparisons to the control group showed a statistical difference at $p \leq 0.01$.

Fig. 4:

Exposure to glucocorticoids suppressed Cx43 expression in SC myelination cultures. Micrographs from microscopic fields of Control (top panels), 5 μ M CORT (CORT5) (second row), 50 μ M CORT (CORT50) (third row) and 50 μ M DEX (DEX50) (bottom panels) at DIV28 with immunofluorescence labeling of MBP (green), Cx43 (red), and the merged image (MBP+Cx43+DAPI) illustrating the reduced amount of Cx43 and MBP immunolabeling by corticosteroid treatment. Calibration bar: 150 μ m. Graphs A and B: Quantification of the area fraction of Cx43 immunoreactivity relative to the total number of

cells (A) and average numbers of cells in the same samples (B). Since the ANOVA showed a significant effect of group for Cx43 area fraction ($F(3,35) = 8.336$, $p = 0.0003$), stars denote where the pairwise Dunnett's tests (for multiple pairwise comparisons to the control group) revealed a statistically significant difference at $p = 0.01$.

Fig. 5: Mifepristone significantly prevented CORT-induced myelination deficits in CC-derived cultures. Micrographs from microscope fields of glucocorticoid-untreated Control (top panels), 50 μ M CORT-treated (middle panels) or 50 μ M CORT plus 10 μ M mifepristone-treated (bottom panels) in primary CC-derived cultures at DIV28 with nuclear DAPI staining (blue), and immunofluorescence labeling of MBP (green or yellow in left panel, green in the right panel) and pNF(red). Note that linear stretches of MBP immunostaining are readily identifiable (white arrows) along pNF+ immunolabeled axons in the Control and

CORT50+mifepristone cultures but are mostly absent from axons in cultures treated only with CORT50 (middle panels). Scale bar: 150 μm .

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Fig. 6:

Quantification of the myelination index in CC-derived cultures treated with glucocorticoids. The length of MBP-immunoreactive segments aligned with pNF-positive axons (myelin length) relative to the total area fraction of pNF+ axons (myelination index, in A) or relative to the total number of cells (DAPI stained cell nuclei, in B), and the total area fraction of MBP immunoreactivity relative to cell numbers (C), were quantified in Control, 5 μ M CORT, 50 μ M CORT, 50 μ M DEX, 50 μ M CORT+10 μ M mifepristone, and 10 μ M mifepristone alone treated CC cultures. No significant changes in total cell number (D) or

area fraction of pNF+ immunoreactivity (E) were caused by any treatments as compared to controls. Since most ANOVAS showed a significant effect of group for all variables studied (panel A, $F(4,43) = 8.4$, $p = 0.0001$; B, $F(5,52) = 8.963$, $p = 0.0001$; C, $F(5,52) = 30.15$, $p = 0.0001$) pairwise Sidak's tests for pairwise multiple planned comparisons to control or the CORT50-treated group were performed and revealed a statistically significant difference as compared to controls (stars, *), or significant differences between the CORT50 only treatment and the CORT50+mifp or the mifp only treatments (pound symbol, #) at $p = 0.01$. mifp = mifepristone

Fig. 7:

Glucocorticoid exposure resulted in a dramatic reduction of Cx43 immunoreactive puncta but not GFAP immunoreactivity in CC-derived myelination cultures. Micrographs from microscope fields of controls (left 3 panels), 50 μ M CORT-treated (middle column of panels) or 50 μ M CORT plus 10 μ M mifepristone-treated (right 3 panels) in CC cultures at DIV28 immunofluorescence with labeling for GFAP (red, top row of panels), Cx43 (green, middle row of panels). The bottom 3 panels are the merged images of the overlying two panels plus the nuclear DAPI staining. Note that CORT50 treatment dramatically reduced Cx43 immunoreactive puncta, but co-incubation of CORT50 with mifepristone largely prevented that reduction (middle and bottom rows of panels). Calibration bar: 200 μ m.

Fig. 8:

Bar graph summarizing the quantification of the area fraction of Cx43 immunoreactivity relative to the number of cells in CC-derived cultures untreated with glucocorticoids (Control) or treated with 5 μ M CORT, 50 μ M CORT, 50 μ M DEX, 50 μ M CORT+10 μ M mifepristone (mifp), or 10 μ M mifp only. ANOVA showed a significant effect of treatment group for Cx43 area fraction ($F(5,56)=8.01$, $p=0.0001$). Thus, stars denote where the pairwise Sidak's tests showed a statistical difference as compared to the control cultures at $p=0.01$, and the pound symbol (#) denotes significant differences between the CORT50-only treatment and the CORT50+mifepristone treatment in the same Sidak's test, with significance at $p=0.01$. mifp = mifepristone.

Fig. 9:

Effect of glucocorticoid exposure on MBP-immunoreactive oligodendrocyte morphology in mixed astrocytes-oligodendrocyte cultures from the cerebral cortex. Top and middle panels: Micrographs from microscope fields of mixed glial cultures exposed to vehicle (Control), 5 μM CORT (CORT5), 50 μM CORT (CORT50) and 50 μM DEX (DEX50) for 4 days. Immunofluorescence labeling of MBP (green) (top panels) and the merged image (MBP +DAPI) (middle panels) illustrate the shrunk appearance of MBP immunoreactive cells in corticosteroids-treated cultures as compared to controls. Calibration bar: 200 μm. Bottom graphs: Quantification of Sholl analysis parameters for MBP-immunoreactive cells in mixed glial cultures from the control (CONT), 5 μM CORT (C5), 50 μM CORT (C50) or 50 μM DEX (DEX50) treatments. The parameters determined were: Sum of intersections of MBP immunoreactive cell processes with the concentric circles of the templates (A), Ending radius of the longest processes (B), Ramification index (C) and Critical value (D). Since all ANOVAS but one demonstrated a significant effect of group for all four variables (panel A, $F(3, 136) = 8.057$, $p = 0.0001$; B, $F(3, 136) = 2.493$, $p = 0.0628$; C, $F(3, 136) = 3.495$, $p = 0.02$; D, $F(3, 131) = 12.32$, $p = 0.0001$), stars (*) denote where the pairwise Dunnett's tests showed a statistical difference between a particular treatment and the untreated control cultures at $p < 0.01$.

Fig. 10:

Effect of glucocorticoid exposure on CNPase-immunoreactive oligodendrocyte morphology in mixed astrocytes-oligodendrocyte cultures from the cerebral cortex. Top and middle panels: Micrographs from microscope fields of control, 5 μ M CORT (CORT5), 50 μ M CORT (CORT50) and 50 μ M DEX (DEX50)-treated mixed glial cultures immunostained with myelin proteins CNPase (green in the top panels) and the merged image (CNPase +DAPI) (middle panels) illustrate the shrunk appearance of CNPase immunoreactive oligodendrocytes. Calibration bar: 200 μ m. Bottom panels: Quantification of Sholl analysis parameters for CNPase immunoreactive cells in CC-derived cultures control (CONT) or cultures treated with 5 μ M CORT (C5), 50 μ M CORT (C50) or 50 μ M DEX (DEX50). The parameters determined were: Sum of intersections of CNPase immunoreactive cell processes with the concentric circles of the templates (A), Ending radius of the longest processes (B), Ramification index (C) and Critical value (D). Since all ANOVAS demonstrated a significant effect of group for all four variables (panel A, $F(3, 293) = 12.84$, $p = 0.0001$; B, $F(3, 293) = 8.2$, $p < 0.0001$; C, $F(3, 293) = 3.137$, $p = 0.03$; D, $F(3, 293) = 17.39$, $p = 0.0001$) stars (*) denote where the pairwise Dunnett's tests showed a statistical difference between a particular treatment and the untreated control cultures at $p < 0.01$.