Glucocorticoid pulsatility : implications for brain functioning

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Chapter 3

Subregion-specific differences in translocation patterns of mineralocorticoid and glucocorticoid receptors in rat hippocampus

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Brain Res. 2009 Jan 16; 1249:43-53

Abstract

Corticosteroids exert important effects on brain function via glucocorticoid (GRs) and mineralocorticoid receptors (MRs) by inducing receptor translocation to the nucleus, where the receptor-ligand complexes modulate transcription of target genes. Based on studies describing uneven receptor expression patterns, regionally different corticosterone action, and the importance of timing of corticosteroid effects, we hypothesised that differential patterns of MR and GR translocation exist in the rat hippocampus in response to a single glucocorticoid stimulus.

Temporal patterns of receptor translocation were investigated in both intact and adrenalectomised (ADX) male Sprague-Dawley rats. Animals were sacrificed at different time points after intraperitoneal administration of 3 mg/kg corticosterone. Specific MR and GR primary antibodies were used for immunohistochemistry and confocal microscopy to visualise and quantify receptor subcellular localisation in the different subregions of the hippocampus.

Results indicate (1) significant subregion-specific differences in translocation patterns for both MR and GR, with respect to the extent and timing of nuclear translocation and (2) specific differences between ADX and intact animals that are most prominent in the dentate gyrus: while corticosterone given to intact animals induced a marked nuclear increase in both MR and GR, in ADX animals nuclear MR signal was already elevated in the absence of hormone and did not change after steroid treatment.

We conclude that in response to a single stress-like increase in corticosterone distinct region-specific MR- and GR-dependent translocation patterns exist in the rat hippocampus, which may underlie region-specific effects relevant to homeostatic control.

Introduction

Adrenal corticosteroid hormones are important for the adequate onset and termination of the stress response, and as such, are essential factors in the maintenance of homeostasis. Their actions include numerous regulatory effects on brain function such as modulation of neurogenesis and memory formation (Herbert et al. 2006), behavioural and neuroendocrine responses (Oitzl et al. 1997), but also neuronal excitability and neurotransmitter release (Joels 2008). Therefore, excessive corticosteroid levels or insufficient adaptation to stress may impair homeostasis and brain function severely and are thus considered major risk factors for the onset of stress-related disorders like depression and post-traumatic stress disorder (de Kloet et al. 2005).

One essential issue concerning the functioning of the stress system is to understand how the effects of corticosteroids are differently mediated by the two types of corticosteroid receptors, the mineralocorticoid and glucocorticoid receptor [MR and GR; (Reul & de Kloet 1985)]. MR and GR are members of the nuclear receptor superfamily known to act as ligand-activated transcription factors (Mangelsdorf et al. 1995) and are widely present in brain. Both receptors are colocalised in high abundance in the hippocampus (Van Eekelen & De Kloet 1992, Han et al. 2005), a brain structure important for learning and memory and particularly susceptible to the effects of corticosteroids. Currently, it is widely accepted that in absence of the ligand, the receptors are part of a large protein heterocomplex containing a number of chaperone proteins (i.e. Hsp90 and p23), stabilising the complex in the cytoplasm. Activation of MR and GR by ligand binding results in dynamic conformational changes thereby rapidly remodelling the chaperone complex and exposing the nuclear localisation signal. As a consequence, the receptor-ligand complex is actively translocated to the nucleus using the microtubule cytoskeleton as a guiding scaffold. In the nucleus, it associates with glucocorticoid response elements (GREs) in the promoter regions of corticosteroid target genes in order to modulate their expression (Pratt et al. 2004, Nishi & Kawata 2006, Picard 2006). Importantly, corticosteroids differentially affect different neuronal cell populations underscoring the relevance of cell specific mechanisms of signalling (Joels 2006, Fitzsimons et al. 2008).

GRE occupancy by GR is a primary determinant of glucocorticoid responsiveness in cultured cells (So et al. 2007), and is also essential in spatial memory formation (Oitzl et al. 2001). Since the import and export of the receptor from the nucleus are obviously necessary steps in the signalling mechanism, receptor translocation could potentially be a more upstream rate limiting step in the transduction of the steroid signal to nerve cells in the brain. However, most knowledge concerning the dynamics of the corticosteroid receptors is based on cell culture models (Nishi & Kawata 2006) and the exact mechanism through which these

receptors act in brain is still unclear emphasising the need for more in vivo studies. For instance, upon hormone withdrawal in cell lines, GR reassociates rapidly into a cochaperone complex, but only slowly redistributes to the cytoplasm over periods that can extend over 12–24 h (Hache et al. 1999), while a recent study using rat hippocampal homogenate demonstrated more rapid GR translocation to and from the nucleus after administration of hormone pulses (Conway-Campbell et al. 2007).

Previous in vivo studies have focused more on the distribution of MR and GR in the rat hippocampus at limited time points (Ahima & Harlan 1990, Ahima et al. 1991, Cintra et al. 1994, van Steensel et al. 1996, Han et al. 2005), providing more time-restricted information. Others have used hippocampal homogenate to study receptor translocation (Kitchener et al. 2004, Conway-Campbell et al. 2007), thereby losing detailed information of receptor kinetics in different hippocampal subregions. The contribution of the different hippocampal subregions on specific processes like neuronal excitability (Joels 2006), but also on learning and memory formation (Kubik et al. 2007), has been described previously emphasising their individual importance. In line with this concept, it has been suggested that hippocampal cells may have unique transporting mechanisms for translocating corticosteroid receptors to the nucleus (Nishi & Kawata 2006). Indeed, differential expression of components of the chaperone complex in the hippocampus like cytoskeletal proteins (Arnold & Trojanowski 1996) and hsp90 (Patchev et al. 1994) may suggest region-specific translocation patterns of the receptors and consequently may explain region-specific regulatory effects of glucocorticoids.

Therefore, we assessed the dynamic response of MR and GR in different subregions of the rat hippocampus after corticosteroid treatment providing high resolution and detailed information with the use of immunohistochemistry and confocal imaging. We quantified MR and GR translocation patterns in intact and adrenalectomised rats after glucocorticoid treatment at different time points after injection and found that subcellular (changes in) localisation patterns differ between hippocampal regions.

Experimental procedures

Animals

Adult male Sprague-Dawley rats (mean weight ± 250 grams) were purchased from Harlan (Leiden, The Netherlands) and group housed in rooms on a 12h/12h light/dark cycle (lights on at 07:00 AM). Food pellets and drinking water were available ad libitum. Animals were handled daily for a week before the start of the experiment. Animals that underwent bilateral adrenalectomy, performed under isoflurane anaesthesia, were let to recover for

three days while their drinking water was supplemented with 0.9% saline. Experiments were approved by the Local Committee for Animal Health, Ethics and Research of the University of Leiden (DEC nr. 05080). Animal care was conducted in accordance with the EC Council Directive of November 1986 (86/609/EEC).

Experimental design

In order to study MR and GR translocation patterns in the rat hippocampus, both intact (n = 4 per group) and adrenalectomised rats (ADX; n = 3 per group) were injected intraperitoneally (i.p.) with 3 mg/kg corticosterone (CORT-HBC; Sigma, St. Louis, USA) and sacrificed at different time points thereafter. Previous studies have shown that this concentration is sufficient to induce hippocampal GR translocation to the nucleus as detected by Western blot (Kitchener et al. 2004, Conway-Campbell et al. 2007) and immunofluorescence (van Steensel et al. 1996). ADX animals were sacrificed respectively 0, 30, 60 and 120 min after injection while intact animals were sacrificed at 0, 60, 120 and 180 minutes. As a control, intact animals were injected with vehicle (0.9% NaCl) and sacrificed at 0 and 60 min after injection. Though recently it was demonstrated that there is no difference in nuclear AM and PM hippocampal protein levels, at least for GR (Furay et al. 2006), the study was performed in the morning between 08.00-12.00 AM. All animals were sacrificed before the circadian rise in corticosterone levels to prevent interference of endogenously circulating corticosterone levels. In all groups, plasma corticosterone concentrations were measured to verify that the adrenal glands were removed completely and to monitor corticosterone levels in blood at multiple time points after injection. This was done using a corticosterone radioimmunoassay kit (MP Biomedicals Inc., CA., USA) according to the manufacturer's instructions.

Tissue preparation and processing

Transcardial perfusion-fixation was performed to process the brains for tissue sectioning and immunohistochemistry as described previously (Heine et al. 2004). Briefly, animals were deeply anaesthetised in the morning by i.p. injection of pentobarbital sodium salt (Nembutal 1 ml/kg bodyweight; A.U.V., Cuijk, The Netherlands) and then perfused intracardially with ice-cold 0.9% saline, pH 7.4 followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4. Afterwards, brains were removed and cryoprotected by placing them in a solution of 30% sucrose in 4% PFA, pH 7.4 until complete saturation. Brains were snap-frozen and using a Leica 1900 cryostat sectioned coronally at 30 μm and stored in an antifreeze solution at -20 ˚C until further use.

Free-floating immunohistochemistry

To study changes in subcellular distribution pattern of MR and GR immunoreactivity (IR) in the rat hippocampus as a consequence of corticosterone administration, free-floating immunohistochemistry was performed on brain slices. First, sections were rinsed thoroughly with 0.1M PBS, pH 7.4. To block non-specific binding, slices were incubated with 0.1M PB, pH 7.4 containing 2% bovine serum albumine (BSA) and 0.3% TX-100 for two hours. After rinsing, sections were incubated with rabbit polyclonal GR (1:500, H300, Santa Cruz Biotechnology, USA) and mouse monoclonal MR antibodies [1:500, MR1-18 1D5; (Gomez-Sanchez et al. 2006)] diluted in PBS containing 0.3% TX-100 for 72 hours at 4 °C. After washing, sections were incubated with AlexaFluor-488 labelled goat-anti-rabbit IgG and AlexaFluor-A594 goat anti-mouse IgG (1:1000; Molecular Probes, USA) in PBS containing 0.3% TX-100 and 2% BSA for two hours. Finally, sections were washed and nuclei were visualised with Hoechst 33242 (Molecular Probes, USA) in PBS after which sections were mounted with Aqua Polymount (Polysciences, Inc) and stored in the dark until further analysis. Controls sections were incubated with equal amounts of normal rabbit and mouse IgG (Santa Cruz Biotechnology, USA) which were used as substitute for the primary antibody. Additionally, secondary fluorescent labels were swapped to check cross-reactivity and sections were incubated without any primary antibodies to check for any aspecific binding of the secondary antibodies.

Confocal microscopy and image analysis

To examine the subcellular distribution pattern of MR and GR IR in the different subfields of the rat hippocampus, a Leica Q550IW confocal microscope was used. Images (630x magnification, 155 x 155 μm, 1 μm focal plane) were acquired from the pyramidal cell fields CA1, CA2 and CA3 and dentate gyrus (DG). To relate changes in nuclear IR due to experimental conditions and not due to microscopical parameters, all settings for filters, lasers and images were left unchanged during imaging. Per animal, two frames per hippocampal subfield were imaged with 30-40 cells on average.

To assess the percentage of IR cells in each hippocampal subfield, MR and GR positive cells with a signal above background were included as indicated by aspecific antibodies (normal IgGs). Background levels were indicated by sections that were incubated with equal amounts of normal IgG. Per animal (ADX n = 12, intact n = 14), all cells from two confocal frames for each hippocampal subregion were counted. The multiple sections that were imaged per animal all had a different plane of focus. The amount of positive cells as percentage of the total amount of cells as indicated by the nuclear Hoechst stain is expressed as mean percentage (± SEM). No statistical difference was found between the percentages in ADX and intact animals.

To quantify differences in MR and GR subcellular distribution patterns, changes in fluorescence intensity values of nuclear immunoreactivity were measured using ImageJ 1.32j analysis software (NIH, USA; http://rsb.info.nih.gov/ij/) similarly as described previously (van der Laan et al. 2005, Morsink et al. 2006). Briefly, Hoechst staining was used to identify the boundaries between the nuclear surface and cytoplasm of individual cells and was circled with the analysis software. These circles served as a template and were pasted onto the corresponding MR and GR images to measure the optical density (mean grey levels) within the nucleus. Non-specific binding (normal mouse and rabbit IgG) and background staining of the sections were also measured and subtracted from the total signal to obtain the specific signal. Only cells that had a clear ovally-shaped nucleus with a diameter of approximately 5-7 μm and showed IR clearly above background were included for analysis thereby excluding cells that were not in the plane of focus.

Statistical analysis

Data are presented as mean ± SEM. Differences in mean optical density were examined by a one-way ANOVA. Tukey's post-hoc testing was applied to compare individual groups where applicable. Statistical significance was accepted at p-value < 0.05.

Results

Distribution of MR and GR immunoreactivity in rat hippocampus

Dual labelling immunohistochemistry and confocal imaging using specific MR and GR primary antibodies were used to study subcellular distribution patterns in the rat hippocampus. The specificity of these antibodies for MR and GR was shown earlier by others (Ko et al. 2002, Wochnik et al. 2005, Gomez-Sanchez et al. 2006), and was here again verified for immunohistochemistry by comparing the distribution pattern of the antibodies to previous studies. Results demonstrate a classic distribution pattern for both MR and GR expression in the rat hippocampus (Fig. 1A). As expected, high immunoreactivity (IR) for both receptor types was found in CA1, CA2 and DG while in CA3, very low levels of GR and high levels of MR IR were detected. On basis of these results, it was concluded that the antibodies were suitable for detection of MR and GR and therefore appropriate for detecting changes in subcellular distribution patterns in the rat hippocampus.

Before measuring translocation patterns of MR and GR in all subregions of the hippocampus, the fraction of cells that contributed to the observed effect was assessed. Therefore, we quantified the amount of MR and GR IR cells as a percentage of the total cell population

in each of the individual subregions labelled by the nuclear Hoechst staining (Fig. 1B). No significant differences were found between ADX and intact animals. On average, the fraction of cells that expressed MR and GR were approximately 95% in CA1, CA2 and DG. As expected, in CA3 the percentage of GR IR cells was much lower (44 ± 4 %; average ± SEM)) while MR was expressed in more cells (83 ± 2 %). The degree of colocalisation was also assessed in CA1 cells and in percentages were found to be 92.1 ± 2.4 and 91.9 ± 2.0 for GR+/MR+ and MR+/GR+ positive cells respectively (data not shown; average ± SEM). These number are highly similar to those reported in a recent study that thoroughly described the degree of colocalisation between MR and GR IR cells (Han et al. 2005).

Confocal imaging of MR & GR translocation patterns in hippocampal subregions

To study changes in subcellular localisation of MR and GR as a consequence of corticosteroid treatment, we injected rats with a high dose of corticosteroids (3 mg/kg i.p.) and sacrificed them at different time points thereafter. As a control, intact animals were injected with vehicle and sacrificed either at 0 or 60 min after injection. Confocal imaging and quantification of MR and GR IR in CA1 cells showed no change in nuclear intensity as a consequence of vehicle injection (Fig. 2A).

Initially, to avoid interference of endogenous corticosteroids, adrenalectomised animals were used as a model system to investigate MR and GR translocation as a direct consequence of corticosteroid treatment. In this first study, using ADX rats, predominant nuclear localisation of the signal was still observed 120 minutes after the injection. To also get information on the termination of the response to a single injection, in the second experiment with intact animals, we adjusted the time course by omitting an earlier time point (30 min) and included a later time point (180 min after injection). Corticosterone levels before and after injection were monitored in vehicle injected intact rats and corticosterone injected ADX and intact rats (Fig. 2B). As expected, no significant increase in corticosterone level was observed in vehicle injected intact rats. Before injection in ADX rats (0 min), no endogenously circulating corticosterone was detectable, while in intact animals basal levels were 36 ± 9 ng/ml. A bolus injection of 3 mg/kg corticosterone resulted in similar peak levels of 492 ± 61 ng/ml in ADX and 410 ± 56 ng/ml corticosterone 30 min after injection which then gradually returned to baseline.

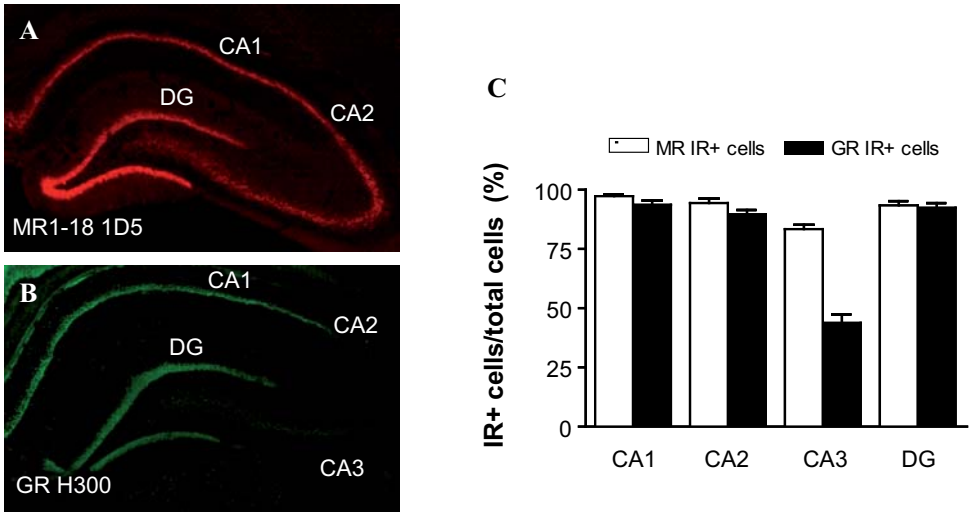

Figure 1 | Distribution and quantification of MR and GR immunoreactive (IR) cells in the rat hippocampus. A and B| Confocal imaging of MR and GR IR using specific MR (upper panel) and GR (lower panel) antibodies in the rat hippocampus demonstrate a classic staining pattern. Typically, MR is distributed in the cells of pyramidal cell layers CA1-3 and dentate gyrus. GR is also expressed in these hippocampal subfields but at very low levels in CA3 subfield. C | Quantification of the percentage of MR (open bars) and GR (filled bars) IR cells in different subfields of the rat hippocampus. IR cells that were clearly above background were included in the analysis. Background levels were indicated by expression levels of non-specific antibodies. In CA1, CA2 and CA3 almost all cells expressed MR and GR. In the CA3 field, GR is expressed at low levels in approximately 44 ± 4 % of the cell population. Bars indicate mean ± SEM (n=26).

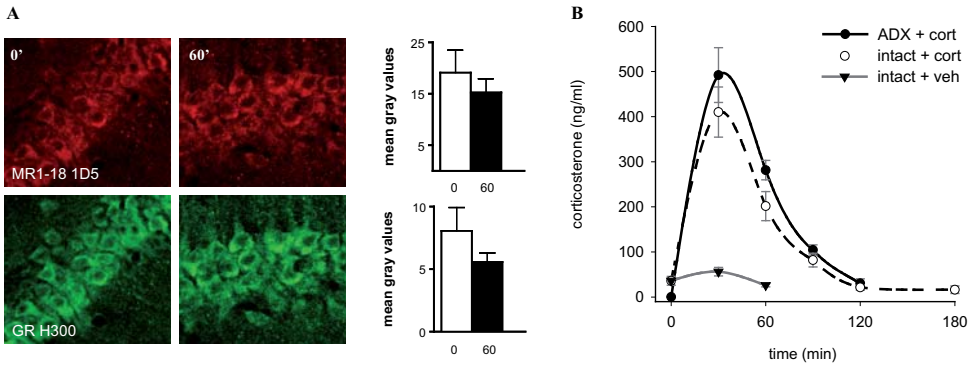

Figure 2 | Representative confocal imaging and quantification of MR and GR subcellular distribution in CA1 cells of vehicle injected intact animals and plasma corticosterone levels. A | As demonstrated by confocal imaging and quantification, no translocation of either MR nor GR is observed 60 min after vehicle injection in intact animals in CA1 cells. B | Plasma corticosterone levels in vehicle injected intact rats and corticosterone injected vehicle and ADX rats at multiple time points after an i.p. injection of 3 mg/kg corticosterone. Values represent mean ± SEM.

Confocal imaging of MR and GR IR at different time points after corticosteroid treatment in hippocampus of ADX rats demonstrated subregion-specific differences in MR and GR translocation patterns as illustrated for CA1 and DG in Figure 3. In CA1 (Fig. 3A), we observed rather uniform translocation patterns for MR and GR. For both receptors, IR was found to be localised predominantly in the cytoplasmic compartment at time point 0 min. Localisation relative to the nuclear compartment was verified using Hoechst as a nuclear marker. After corticosterone injection, increasing levels of nuclear IR for both receptors were observed with a maximum at 60 min after injection, demonstrating a classic translocation pattern from the cytoplasmic to the nuclear compartment. Surprisingly, in DG (Fig. 3B), and to a lesser extent in CA2 and CA3 (data not shown), a considerable amount of MR and GR IR was already present in the nucleus at time point 0 min, when no endogenously circulating hormone was present. After injection with corticosterone, an additional increase in nuclear MR and GR IR was observed which was more evident for GR than MR. Quantification of the translocation patterns of all the subregions is shown in Figure 4-7.

Quantification of MR and GR translocation patterns in hippocampal subregions

Changes in MR and GR subcellular distribution were quantified by measuring fluorescence intensity values in the nucleus of MR and GR positive cells in each of the hippocampal subfields of ADX and intact rats (Fig. 4-7). A significant increase in nuclear IR for both MR and GR was observed in CA1 in ADX rats with maximal levels at 60 minutes after corticosterone injection (Fig. 4A). In intact animals, a similar response was observed and at 180 min nuclear levels of MR and GR IR were back to basal again (Fig. 4B). A notable difference between intact and ADX rats in MR and GR translocation was found in the DG (Fig. 5). While the pattern of translocation of MR and GR in DG of intact rats highly resembled CA1 with increased levels at 60 and 120 min and basal levels again at 180 min after injection (Fig. 5B), the pattern in ADX rats was quite different (Fig. 5A). Already at 0 min, nuclear localisation of MR and GR was observed in the DG. Corticosterone injection increased nuclear levels of GR significantly 120 min after injection whilst surprisingly, no additional increase in nuclear MR IR was observed.

In CA2 (Fig. 6), similar translocation patterns for both intact and ADX rats were observed with increased nuclear levels for both MR and GR after 60 min. Though a slight drop in nuclear receptor levels was observed at 180 min, levels remained significantly higher than the control (0 min) for all the time points measured for GR but not for MR. In CA3 in both intact and ADX animals (Fig. 7), a slight but statistically not significant increase in nuclear GR levels was observed while MR nuclear signal did increase significantly after 60 min in ADX and 120 min in intact animals.

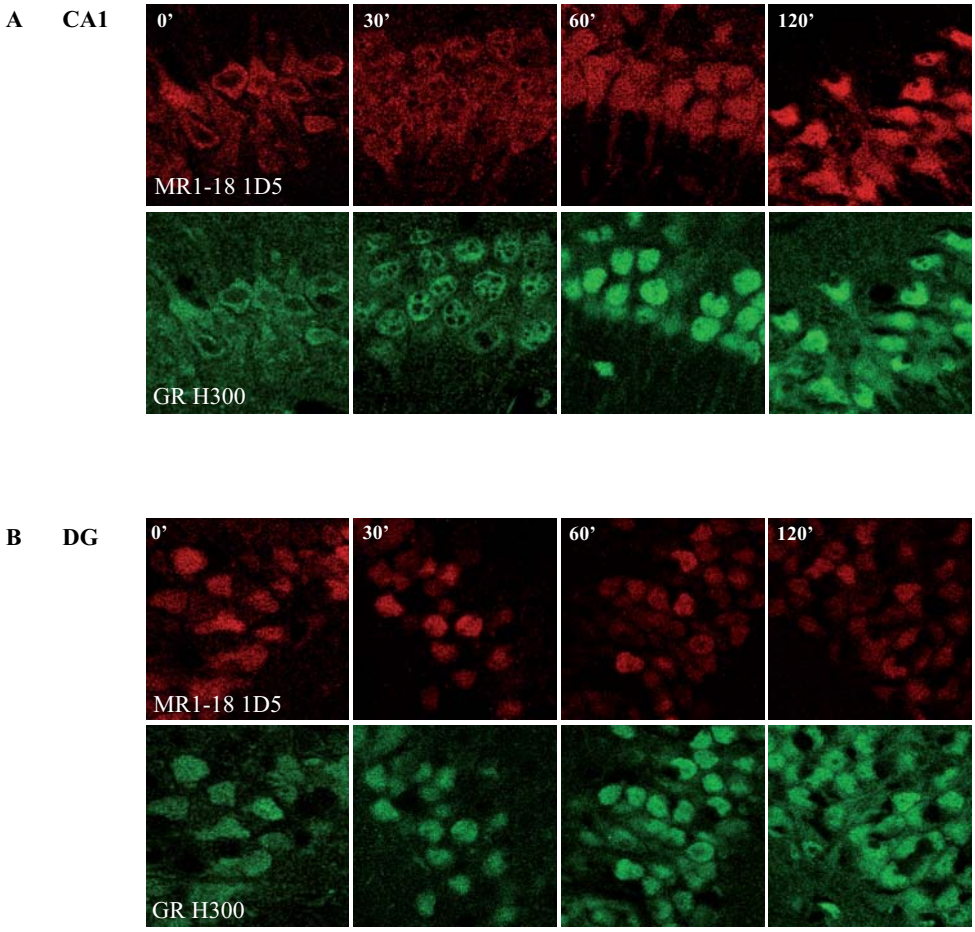

Figure 3 | Representative confocal imaging of MR (red) and GR (green) translocation patterns in hippocampus of ADX rats after corticosterone treatment. A | Classic translocation patterns for both MR and GR are observed in CA1. At t=0 min, MR and GR IR is localised mainly in the cytoplasm. After injection, MR and GR IR starts to accumulate in the nucleus and is completely nuclear at t =60 min. B | In the DG, nuclear IR of MR and GR is already present at t=0. After injection, an additional increase in nuclear IR for both receptors is observed, though to a lesser extent than in CA1.

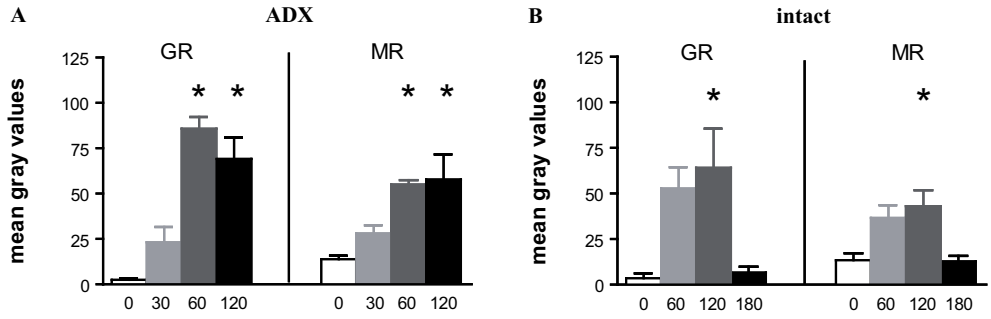

Figure 4 | Quantification of MR and GR nuclear translocation in CA1 of ADX and intact rats at different time points after corticosterone treatment. Nuclear localisation of MR and GR in CA1 at different time points after corticosterone injection is rather similar between A | ADX and B | intact animals for the time points measured. Bars represent corrected mean grey levels (mean ± SEM). Asterisks indicate significant differences at a p-value smaller than 0.05 compared to control (0 min).

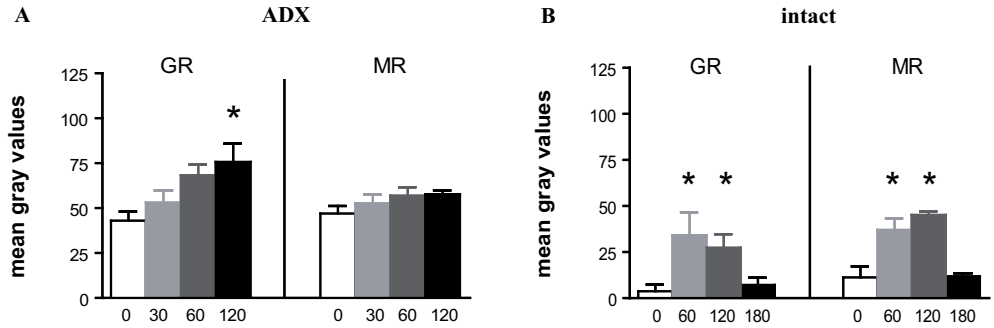

Figure 5 | Quantification of MR and GR nuclear translocation in DG of ADX and intact rats at different time points after corticosterone treatment. Nuclear translocation of MR and GR in B | intact animals resembles translocation patterns in CA1. A | ADX animals show higher nuclear localisation of MR and GR in controls (0 min) compared to intact animals but no significant increase in MR translocation is observed. Bars represent corrected mean grey levels (mean ± SEM). Asterisks indicate significant differences at a p-value smaller than 0.05 compared to control (0 min).

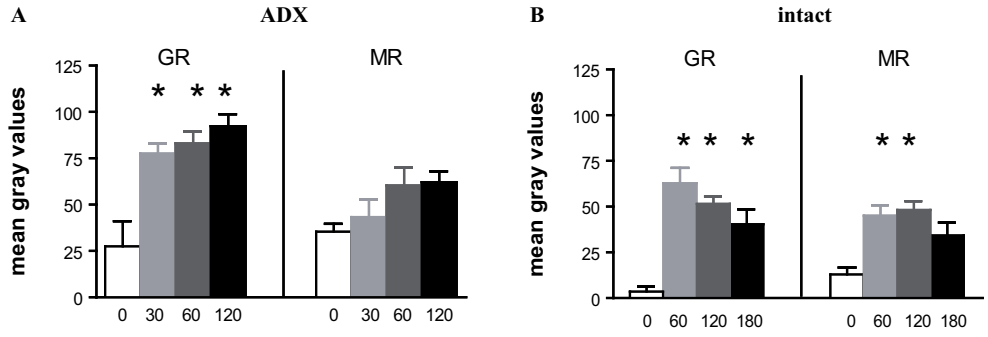

Figure 6 | Quantification of MR and GR nuclear translocation in CA2 of ADX and intact rats at different time points after corticosterone treatment. Nuclear localisation of MR and GR in CA2 cells demonstrate similar translocation patterns between A | ADX and B | intact animals at different time points after corticosterone injection. Bars represent corrected mean grey levels (mean ± SEM). Asterisks indicate significant differences at a p-value smaller than 0.05 compared to control (0 min).

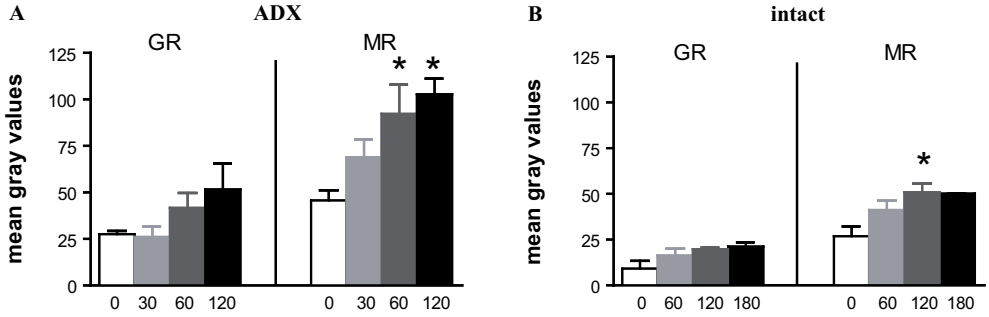

Figure 7 | Quantification of MR and GR nuclear translocation in CA3 of ADX and intact rats at different time points after corticosterone treatment. Though the extent of MR nuclear translocation is larger in A | ADX compared to B | intact animals, the patterns are quite similar. No significant increase in nuclear translocation of GR is observed in this area. Bars represent corrected mean grey levels (mean ± SEM). Asterisks indicate significant differences at a p-value smaller than 0.05 compared to control (0 min).

Discussion

Over the last decades, the distribution patterns of MR and GR in rodent brain have been investigated intensively (Ahima & Harlan 1990, Ahima et al. 1991, Van Eekelen & De Kloet 1992, Cintra et al. 1994, Han et al. 2005). While these studies have proven to be fundamental for the current knowledge of MR and GR localisation in the brain, they did not investigate the temporal aspects of MR and GR dynamics as a consequence of acute corticosteroid treatment in such detail. In this study we investigated MR and GR translocation at different time points after acute treatment using GR rabbit polyclonal antibody (H300; Santa Cruz Biotechnology) and MR mouse monoclonal antibody (1D5 1-18) both of which have been described and used in literature previously (Ko et al. 2002, Wochnik et al. 2005, Gomez-Sanchez et al. 2006, Conway-Campbell et al. 2007).

The expression patterns we obtained for MR and GR are in agreement with previously published papers as mentioned above. As expected, both antibodies demonstrated a typical distribution pattern with clear immunoreactivity in hippocampal subregions CA1, CA2, CA3 and DG while GR was expressed at very low expression levels in a subset of the CA3 cell population. Combined with information from previously published papers (Ko et al. 2002, Wochnik et al. 2005, Gomez-Sanchez et al. 2006), we therefore assumed that these antibodies indeed were highly capable in specifically recognising MR and GR protein in the rat hippocampus and thus can contribute to investigating tissue distribution and the detailed changes in subcellular localisation of MR and GR which can be detected by immunohistochemistry and confocal imaging.

Earlier studies reported on the effects of a single dose of corticosterone or aldosterone on MR and GR translocation in different regions of the hippocampus 5 min and 2 hours after injection in both ADX and intact rats (Ahima & Harlan 1991, Ahima et al. 1991). Using the high spatial resolution that fluorescent immunohistochemistry provides combined with the temporal aspects of the experimental design, the present study provides new and more detailed information simultaneously describing the translocation patterns of MR and GR in the different subfields of the rat hippocampus at multiple time points between 0 and 180 min in both ADX and intact animals. We show that differences exist in translocation speed and extent between various subfields after corticosteroid treatment with CA1 and DG as most interesting examples.

The phenomenon of hippocampal subregion-specific differences has been described previously in the literature, though not yet with respect to MR and GR dynamics. It is known that specific hippocampal subregions contribute uniquely to specific behavioural processes like learning and memory formation (Gilbert et al. 2001, Kubik et al. 2007). In relation to

corticosteroids, differential effects on different cell populations have been described (Joels 2006, Kavushansky et al. 2006), implying region-specific signalling mechanism. For instance, differences in corticosterone-induced effects on voltage-dependent calcium currents and the expression of calcium channel subunits between CA1 and DG have been observed (Joels et al. 2008b) but also subregion-specific effects on serotonin-1A receptor mRNA expression down regulation under control of MR and GR (Meijer & de Kloet 1994, Meijer & de Kloet 1995). Furthermore, in CA1, a U-shaped dose-dependency of corticosterone on certain responses like serotonin-1A receptor hyperpolarisation is observed but not in other regions like DG (Joels 2006). The differences in translocation and thus participation of both MR and GR between the respective hippocampal subregions may therefore contribute to this phenomenon.

The differential regulatory effects of glucocorticoids on the subregions of the hippocampus could therefore already start at the level of receptor translocation and thus be a necessary step in the proper transduction of the steroid signal in nerve cells, converging into higher functional levels.

An intriguing question left to be answered is what could be the underlying cause for these differences in translocation between hippocampal subregions and conditions. Still very little is known about region-specific factors that determine steroid receptor translocation rate and extent. Differential expression of genes in the hippocampus that are influencing nuclear translocation provide more insight. Unequal distribution patterns of microtubule-associated proteins [MAPs; (Arnold & Trojanowski 1996)] and chaperone proteins (Patchev et al. 1994) in the hippocampus have been described previously but large-scale expression profiling studies on laser-microdissected hippocampal subregions in combination with GeneChip technology revealed more detail in specific differences in cellular context between hippocampal subregions (Datson et al. 2004). Interestingly, Datson and colleagues found many genes to be differentially expressed between CA3 and DG that belong to the receptor transport complex including several MAPs, chaperone proteins like FKbp1a and parts of the dynein transport complex. Though the exact functional contribution of many proteins of the translocation mechanism have yet to be fully elucidated, research concentrating largely on GR dynamics have produced promising models in which MAPs are heavily involved in the transport of GR in a cell type-specific manner (Fitzsimons et al. 2008).

The local concentration of corticosterone, or hormonal bioavailability, is another factor that could contribute to the subregion-specific differences in translocation patterns, determining the exact amount of corticosterone that is sensed by the cells of the different subregions. One factor could be the energy-dependent efflux transporter P-glycoprotein (Pgp) mdr1b, capable of transporting corticosterone. Specific mdr1b mRNA expression was found in all divisions of the hippocampus although part of the signal seems to be present outside

the principal layers (Volk et al. 2004). Dentate granule cells express relatively high levels of mdr1b mRNA as detected by in situ hybridisation (Karssen et al. 2004) which could contribute to the lesser extent of nuclear translocation we observed in this area. Another factor is the local enzymatic activity of 11β-HSD1 that catalyses the accumulation of active corticosteroids, thereby controlling the concentration of corticosterone available for corticosteroid receptor binding in brain (Seckl 1997). However, studies on regional differences in the tissue distribution of 11β-HSD1 in the hippocampus have not been conclusive. Jamieson and colleagues described a more homogenous distribution with higher expression levels in CA3 (Jamieson et al. 1999), while a more recent paper described regional differences with high expression levels in CA1 and DG (Buren et al. 2007). To what extent 11β-HSD1 and Pgp mdr1b contribute to the subregional specificity in translocation patterns remains to be established.

Though we observed rather similar translocation patterns of MR and GR between intact and ADX rats, the DG provided an interesting exception. In DG, and to a lesser extent in CA2 and CA3, even before corticosteroid treatment, a substantial amount of MR and GR is located in the nucleus of ADX animals. The receptors therefore seem to be retained in the nucleus specifically in these regions after removal of the adrenals, or lack of glucocorticoids. Such retention of unliganded receptor has been noted before (Brink et al. 1992, Pekki et al. 1992), but remains unexplained. At least for MR it is known that the receptor can act promiscuous. For instance, not only corticosterone and aldosterone are naturally occurring ligands but cross-talk with other steroids, like progesterone, also occurs and could therefore influence MR localisation in absence of glucocorticoids (Rupprecht et al. 1993). These results raise the question whether corticosteroid receptors dynamics may also be regulated in a cell-specific manner by other ligands which in vivo only become evident after ADX and may also apply for GR. Additionally, nuclear localisation of MR and GR after ADX is also known to depend on the time after adrenalectomy (Ahima & Harlan 1991, Ahima et al. 1991). Subregional expression profiling of glucocorticoid effects has not yet been done and the possible changes in the local cellular context due to changes in glucocorticoid levels have yet to be described and could provide more insight in these DG-specific effects.

MR and GR differ in their affinity for several ligands. While GR is also selective for naturally occurring and synthetic glucocorticoids; MR binds corticosterone and aldosterone with a very high affinity, i.e. about 10-fold higher than GR (de Kloet et al. 2005). Given that endogenously circulating levels of glucocorticoids should be enough to extensively occupy MR (Reul & de Kloet 1985) and the lack of MR nuclear localisation in CA1 of basal adrenally intact animals is striking (Fig. 4B). Possibly, the differences in strain of rats, experimental design, fixation of the tissue and the choice of different methodological approaches (i.e. the use of antibodies) could explain these discrepancies with earlier studies. A partial explanation may involve the very low levels of corticosterone secretion in the morning observed

in Sprague-Dawley rats, which may preclude extensive MR occupation (Spiga et al. 2007). Based on the higher affinity of MR for corticosterone, one mightalso expect to see differences in translocation kinetics of MR and GR, e.g. a more rapid translocation of MR. However, we observed rather similar translocation rates of MR and GR to the nucleus, with the exception of the DG after ADX. This uniform behaviour is most likely explained by the dose of corticosterone that was used in this study. Peak levels of approximately 410-490 ng/ml (30 min after injection; Fig. 2B) saturate both MR and GR thereby masking the outcome of differences in receptor affinities for the extent of receptor translocation to the nucleus. The expected differences in translocation rate and onset may probably only be evident when lower, more physiological doses of corticosterone levels are administered. Additional information about the relationship between hormone plasma concentrations and receptor translocation could come from correlations in individual animals. Unfortunately, the statistical power of such correlations in the present experiment is very weak, and no meaningful relationships resulted from such an analysis (data not shown).

In summary, the results of this study suggest a revision of the view that MR and GR translocate uniformly in the rat hippocampus since we have demonstrated that they are transported in a subregion-specific manner. Even though these data indicate the need for further studies on the underlying mechanism of this differential translocation in the hippocampus, they could help to explain the cell type-specific effects of corticosteroids in the hippocampus. Also, the data show that in CA1, the translocation of MR to the nucleus occurs in response to a rise in ligand concentrations; hence the ligand concentration is rate limiting for at least nuclear localisation. In other subfields, the nuclear localisation in absence of ligand is not further increased when ligand concentrations rise, hence the receptor seems to be the rate limiting factor. Furthermore, our results illustrate that immunohistochemical studies can provide detailed information about the localisation of the receptors in vivo, which can assist in the evaluation of receptor activation during pharmacological treatments and physiological conditions.

Acknowledgements

This research was supported by NWO Mozaïek grant 017.002.021. We gratefully acknowledge Dr. Gomez-Sanchez for kindly providing the MR1-18 1D5 antibody, Gerda Lamers for technical assistance with confocal microscopy and Dr. Carlos Fitzsimons for critically reading the manuscript.