Glucocorticoid pulsatility : implications for brain functioning

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Chapter 2

Specificity of glucocorticoid receptor primary antibodies for analysis of receptor localisation patterns in cultured cells and rat hippocampus

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Abstract

After glucocorticoid stimulation, glucocorticoid receptors (GRs) are translocated to the nucleus to modulate transcription of glucocorticoid target genes. The subcellular distribution and trafficking of GR in cultured cells has been studied quite intensively using several techniques. However, the intracellular localisation of nuclear receptors in ligand-free and stimulated conditions in vivo is still controversial, in part because of inconsistent results with different antibodies. Knowledge of trafficking of GR in vivo could greatly contribute to understanding nuclear receptor signalling.

Therefore, in this study we systematically compared a panel of different primary GR antibodies using immunohistochemistry and confocal imaging. Nuclear translocation patterns at different time points after glucocorticoid stimulation were compared in cultured AtT20 cells and rat hippocampal CA1 and dentate gyrus cells. The BuGR2 antibody consistently detected GR nuclear translocation patterns between in vivo and in vitro settings, but the other GR primary antibodies provided contradictory results. While GR H300 and P20 strongly detected nuclear GR immunoreactivity after glucocorticoid stimulation in both CA1 and dentate gyrus cells, the same antibodies provided poor results in cultured cells. The opposite was found for the primary GR M20 antibody.

These data indicate that with a particular glucocorticoid receptor antibody the findings in cell culture studies cannot always be extrapolated to in vivo situations. Moreover, different antibodies disclose different features of the glucocorticoid receptor translocation process.

Introduction

Glucocorticoids play a pivotal role in the homeostasis of many biological systems including the stress response, energy metabolism and the immune and inflammatory response (de Kloet et al. 2005). Their effects are mediated by two members of the nuclear receptor family, the mineralocorticoid and glucocorticoid receptor [MR and GR; (Reul & de Kloet 1985, Mangelsdorf et al. 1995)]. MR has a high affinity for naturally occurring glucocorticoids resulting in extensive receptor occupancy, whereas GR has a tenfold lower affinity for corticosterone and is therefore only activated during stress and the circadian peak (Reul & de Kloet 1985, Spencer et al. 1993, Kitchener et al. 2004, Furay et al. 2008).

The most common view on GR activation is that the unliganded receptor is part of a chaperone complex consisting of several proteins such as heat shock protein 90 and immunophilins which stabilise the receptor in the cytosol (DeFranco et al. 1998, Wochnik et al. 2005, Nishi & Kawata 2006, Picard 2006). Upon glucocorticoid binding, the ligand binding domain (LBD) of GR undergoes a major conformational change and dissociates from the chaperone proteins. Exposure of the nuclear localisation signal triggers the recruitment of the transport machinery (i.e. importins) which actively translocate the entire complex to the nucleus via a microtubule network (Harrell et al. 2004, Pratt et al. 2004, Fitzsimons et al. 2008). In the nucleus, GR functions as a ligand-activated transcription factor and mediates genomic events by modulating the transcription of glucocorticoid target genes via transactivation and transrepression (Datson et al. 2008, van der Laan & Meijer 2008).

The subcellular distribution and trafficking of GR in cultured cells has been studied quite intensively using several techniques such as green fluorescent protein (GFP)-tagged receptors, fluorescence after photobleaching (FRAP) and immunohistochemistry which have proven to be powerful tools for mechanistic assessment of nuclear receptor signalling (Htun et al. 1996, Nishi & Kawata 2006, Kawata et al. 2008, Stavreva et al. 2009). In such studies, MR and the progesterone receptor are found in both cellular compartments, while estrogen receptor-α and -β are thought to be localised predominantly in the nucleus (Fejes-Toth et al. 1998, Htun et al. 1999, Lim et al. 1999). On the other hand, GR and the androgen receptor are considered to reside in the cytoplasm (Htun et al. 1996, Georget et al. 1997), but also this has been disputed (Brink et al. 1992). However, there is much controversy about the subcellular distribution patterns of nuclear receptors in ligand-free conditions in vivo, as practical limitations still restrict the use of GFP-tagged receptors and FRAP in most animal models and thus favour the use of antibodies.

Knowledge of trafficking of GR in vivo could greatly contribute to understanding nuclear receptor signalling in the context of target tissues. During the past decades, several

GR-specific primary antibodies have become available for immunohistochemical studies. However, inconsistency in expression patterns among antibodies is not uncommon and has led to controversial results. Only rarely have different primary antibodies been tested in single experiments without any other variable parameters. These studies altogether indicate the need for more systematic comparison of different primary GR antibodies.

In this study we used immunohistochemistry and confocal imaging to screen a panel of different GR-specific primary antibodies in cultured cells and in rat hippocampus to compare nuclear translocation patterns. We found contradictory translocation pattern between the in vitro culture and the living hippocampus. The results indicate that findings from cell culture studies cannot always be extrapolated to in vivo situations and that the outcome of a study may depend on the choice of the antibody.

Experimental procedures

Cell culture

AtT-20/D-16V mouse tumour cells were grown and maintained in Dulbecco's modified Eagle's medium (4500 mg/L glucose; Invitrogen, USA) supplemented with 0.5% penicillin/streptomycin, 10% horse serum, and 10% fetal bovine serum (Invitrogen Life Technologies, The Netherlands) in a humidified atmosphere of 5% CO2 at 37 ºC as described previously (van der Laan et al. 2008a). A day before stimulation, 1 x 105 cells per chamber were plated and maintained in Lab-TekTM Permanox Chamber Slides (Nalge Nunc International, Belgium) in steroid-free medium. This medium is devoid of lipophilic components such as hormones and consists of 4500 mg/L glucose (Invitrogen, USA) supplemented with 0.5% penicillin/streptomycin and 1% stripped foetal bovine serum. The foetal bovine serum was stripped by incubating overnight at 4 ˚C with 2% of dextran-coated charcoal (Sigma-Aldrich, The Netherlands), spinning down for 5 min at 3000 rpm and subsequent filtering with autoclaved filter tops. The entire procedure was repeated after which the serum was aliquoted and stored at -20 ˚C until use.

Animals

Adult male Sprague-Dawley rats (± 250 grams) were purchased from Harlan (Leiden, The Netherlands) and group housed (4 animals/cage) in rooms on a 12h/12h light/dark cycle (lights on at 07:00 h). Food pellets and drinking water were available ad libitum. 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).

Surgery

To exclude interference from endogenously circulating corticosteroids, animals underwent bilateral adrenalectomy (ADX) performed under isoflurane anaesthesia. Animals were let to recover for three days while their drinking water was supplemented with 0.9% saline. In order to study GR translocation patterns in CA1 and dentate gyrus cells of the hippocampus, rats (n = 3-4) were injected intraperitoneally (i.p.) with 3 mg/kg corticosterone (CORT-HBC; Sigma-Aldrich, USA) as described previously (van Steensel et al. 1996, Conway-Campbell et al. 2007). Animals were sacrificed at different time points after injection, respectively 0, 30, 60, or 120 minutes. Blood plasma was collected to verify that the adrenal glands were completely removed and to monitor corticosterone levels in blood after injection (Fig. 4E). Samples were processed using a corticosterone radioimmunoassay kit according to the manufacturer's instructions (MP Biomedicals Inc., CA., USA).

Tissue 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 followed by 4% PFA in 0.1 M phosphate buffer, pH 7.4. Following perfusion the brains were removed, cryoprotected by complete saturation in a solution of 30% sucrose in 4% PFA, pH 7.4, and snap-frozen, Coronal section were cut at 30 μm using a Leica 1900 cryostat, and stored in an antifreeze solution (30% ethylene glycol, 20% glycerol, 0.02 M Na2HPO4, 6.6 mM NaH2PO4) at -20 °C until further use.

GR immunohistochemistry in cultured cells

To study receptor translocation patterns after glucocorticoid treatment, cells were stimulated with either vehicle or 10-7 M dexamethasone (Sigma-Aldrich, The Netherlands) known to induce significant GR translocation in cultured cells (Nishi et al. 1999, Fitzsimons et al. 2008). Respectively 0, 5, 15 and 30 min after stimulation, cells were processed for immunofluorescence to visualise subcellular distribution patterns of GR as described previously (Morsink et al. 2006). Briefly, cells were fixed with 4% paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS), pH 7.4. After blocking in PBS supplemented with 5% normal goat serum and 0.3% TX-100, a panel of different GR-specific primary antibodies was used to detect GR immunoreactivity (IR). The subsequent primary antibodies were used: rabbit polyclonals raised against human GR, M-20 (aa 5-20), H300 (aa 121-420) and P20 (aa 750-769) from Santa Cruz Biotechnology Inc, Germany, or mouse monoclonal BuGR2 (aa 407-423 of rat GR) from Abcam, UK and rabbit polyclonal GR-Kawata (N-terminal domain of rat GR) kindly provided by prof. dr. M. Kawata (Kyoto, Japan). Antibodies were

diluted (1:500 or 1:2000 for GR-Kawata) in PBS containing 1 % BSA and 0.1 % TX-100 for 60 min at room temperature. As a control, the primary antibody was omitted or substituted with equal amounts of normal rabbit or mouse IgG. After washing, cells were incubated with AlexaFluor-488 labelled goat-anti-rabbit IgG or AlexaFluor-A488 goat anti-mouse IgG (1:1000, Molecular Probes, USA) in PBS containing 0.1 % TX-100 and 1 % BSA for 60 min at room temperature. Finally, cells were washed and nuclei were visualised with Hoechst 33258 (1:10000, Molecular Probes, USA). Slides were mounted with Aqua Polymount (Polysciences Inc, USA) and stored in the dark until further analysis.

GR immunohistochemistry in rat brain slices

To study changes in subcellular distribution pattern of GR-IR in the rat hippocampus as a consequence of glucocorticoid treatment, free-floating immunohistochemistry was performed on brain slices as described previously (Sarabdjitsingh et al. 2009). Briefly, non-specific binding was blocked by incubating the sections in 2% BSA, 0.3% TX-100 in 0.1M PBS. After rinsing, sections were incubated with different GR-specific primary antibodies as described above [P20, M20 and H300 (1: 500), Santa Cruz Biotechnology, Germany and BuGR2 (1:500) from Abcam, UK] in 0.3% TX-100 in PBS for 72 hours at 4 ˚C. Serial sections of the same animals were used for the different antibodies. At the time of the current experiment the GR antibody from Kawata was no longer available and therefore not included in this experiment. After washing, sections were incubated with AlexaFluor-488 labelled goat-anti-rabbit IgG or goat-anti-mouse IgG (1:1000; Molecular Probes, USA) in 0.3% TX-100, 2% BSA in PBS for 2 hours at room temperature. Finally, nuclei were visualised with Hoechst 33258 (1:10000, Molecular Probes, USA) after which sections were mounted with Aqua Polymount (Polysciences, Inc), and stored in the dark until further analysis.

Control sections were incubated with equal amounts of normal rabbit IgG (Santa Cruz Biotechnology, USA), which were used as substitute for the primary antibody. Additionally, sections were incubated without any primary antibodies to check for any aspecific binding of the secondary antibodies.

Confocal imaging and quantification

Subcellular distribution patterns of GR-IR in both cultured cells and brain slices was examined by a Leica Q550IW confocal microscope. Images were acquired at 630x magnification (155 x 155 μm, 1 μm focal plane). All settings for filters, lasers and images were left unchanged during imaging in order to relate changes in nuclear and cytoplasmic GR-IR due to experimental conditions and not due to microscope parameters. For the AtT20 cells, a group size of approximately 30 cells per time points was examined, taken from three individual wells. Per animal, two frames of the CA1 pyramidal cell layer or dentate gyrus were imaged with 30-40 cells on average.

Fluorescence intensity values of nuclear and cytoplasmic GR-IR were quantified by measuring optical density (ImageJ 1.32j analysis software; NIH, USA; http://rsb.info.nih.gov/ij/) similarly as described before (van der Laan et al. 2005, Morsink et al. 2006, Sarabdjitsingh et al. 2009). 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 GR images to measure the optical density within and outside the nucleus. Background levels, indicated by sections and cells that were incubated with equal amounts of normal IgG, were also measured and subtracted from the total signal to obtain the specific signal.

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

Visualisation of GR nuclear translocation patterns in AtT20 cells

GR subcellular distribution patterns in AtT20 cells visualised with different GR-specific antibodies at different time points after dexamethasone (dex) treatment are illustrated in Fig. 1. Before stimulation (0 min), GR immunoreactivity (IR) was evenly distributed over the nuclear and cytoplasmic compartment with all five antibodies (Fig. 1 A1-E1). Stimulation with dex resulted in a significant accumulation in nuclear GR-IR observed with the BuGR2 (Fig. 1 A1-A4), M20 (Fig. 1 C1-C4) and GR-Kawata antibody (Fig. 1 E1-E4). This effect was readily observed 5 min after treatment (Fig. 1 A2, C2 and E2) while complete nuclear translocation was reached after 30 min (Fig. 1 A4, C4 and E4). In comparison, dex stimulation resulted in very moderate to no nuclear translocation when visualised with the H300 (Fig.1 B1-B4) and P20 antibody (Fig. 1 D1-D4).

Quantification of GR nuclear translocation patterns in AtT20 cells

Fluorescence intensity values were measured in both the nuclear and cytoplasmic compartment of cells stimulated with dex. Fig. 2 depicts the fold increase in those compartments as visualised with the different GR primary antibodies. Nuclear intensity increased approximately 3-fold after 30 min of dex stimulation observed with the BuGR2, M20 antibody and GR-Kawata (Fig. 2A, C, E, $p < 0.001$). A more modest increase was observed with the H300

(Fig. 2B, $p < 0.001$), while GR-IR with P20 remained unchanged after dex treatment (Fig. 2D, $p = 0.60$). A small decrease in cytoplasmic fluorescent intensity was observed with the M20 antibody, P20 and GR-Kawata (Fig. 2C-E, $p < 0.01$).

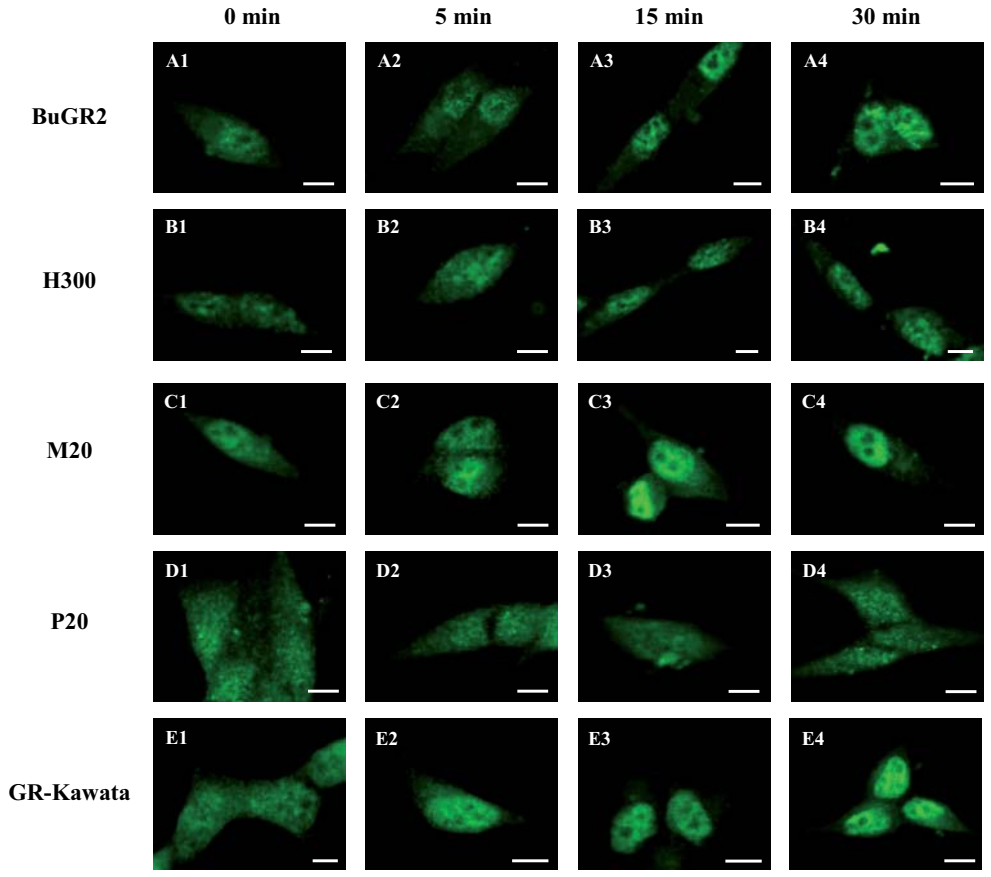


Figure 1 | GR nuclear translocation patterns in cultured Att20 cells. Time curve analysis of subcellular distribution of GR IR in AtT20 cells 0, 5, 15 and 30 min after 10-7 M dexamethasone stimulation using confocal imaging. A1-E1 | No differences in distribution pattern before stimulation (0 min) between the different antibodies M20, GR-Kawata, BuGR2, H300 and P20 were observed. Clear nuclear GR-IR was observed in cells visualised with the A1-A4 | BuGR2, C1-C4 | M20 and E1-E4 | GR-Kawata antibody already 5 min after stimulation, while moderate to no nuclear localisation was observed with the B1-B4 | H300 and D1-D4 | P20 antibody. Scale bar = 10 μm.

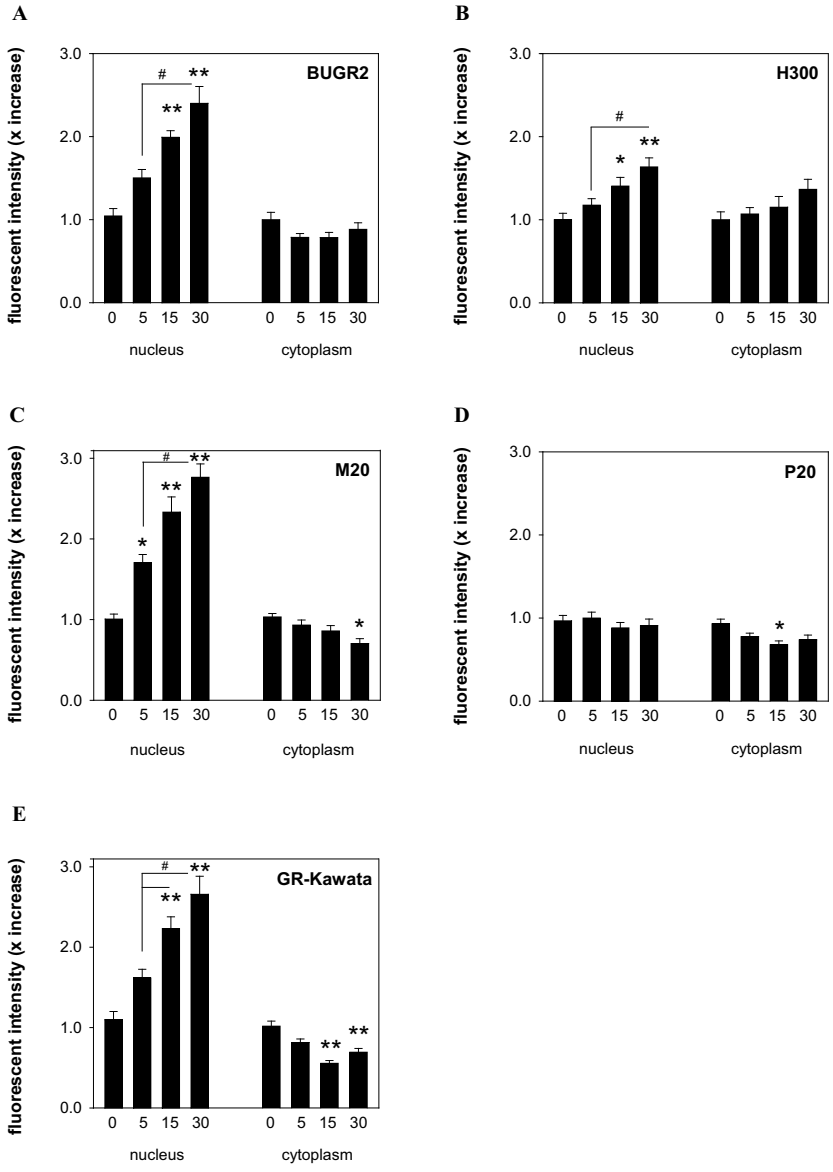


Figure 2 | Quantification of GR nuclear translocation patterns in cultured Att20 cells. Fluorescent intensity values of GR-IR in the nuclear and cytoplasmic compartment are shown at 0, 5, 15 and 30 min after 10-7 M dex stimulation visualised with different GR primary antibodies. Dex stimulation resulted in approximately 3-fold increase in fluorescent intensity in the nuclear compartment visualised with A | BuGR2 ($F(3, 108) = 18.20$, $p < 0.001$), C | M20 ($F(3, 99) = p < 0.001$) and E | GR-Kawata ($F(3, 105) = 22.01$, $p < 0.001$). Cells visualised with B | H300 ($F(3, 112) = 10.37$, $p < 0.001$) and D | P20 ($F(3,99)$, $p = 0.63$) showed modest to no increase in the nuclear compartment after stimulation. Interestingly, a small but significant decrease in cytoplasmic IR was observed with C | M20 ($F(3, 101) = 4.54$, $p < 0.01$), D | P20 ($F(3, 99) = 4.08$, $p < 0.01$ and E | GR-Kawata ($F(3, 120) = 16.05$, $p < 0.001$). Data is expressed as mean ± SEM. One-way ANOVA and Tukey's post-hoc test, ** $p < 0.001$ and * $p < 0.01$ vs veh, # $p < 0.05$ 5 vs 15 and 30 min, n = ± 30 cells per time point.

Visualisation of GR nuclear translocation patterns in rat hippocampal CA1 cells

Before stimulation (0 min), clear GR-IR was detected in the cytoplasmic compartment of CA1 cells with the primary GR BuGR2, H300, M20 and the P20 antibodies (Fig. 3 A1-D1). No nuclear GR IR was observed at this time point with any of the antibodies. After glucocorticoid stimulation strong accumulation of nuclear GR-IR was observed with the BuGR2 antibody, similar to the observations in cultured cells (Fig. 3 A1-A4). However with the other GR primary antibodies striking differences were observed in nuclear translocation patterns compared to cultured cells. While in AtT20 cells the M20 antibody nicely visualised nuclear localisation of GR, in CA1 cells the same antibody was not capable in recognising nuclear GR-IR molecules at any of the time points after injection (Fig. 3 C1-C4). Only an increase in cytoplasmic GR-IR was detected with this antibody. In contrast, while the H300 and P20 antibodies poorly detected nuclear GR in the AtT20 cells, in CA1 cells strong nuclear GR accumulation, especially with the H300 antibody, was observed already from 60 minutes after glucocorticoid treatment (Fig. 3 B1-B4 and D1-D4).

Quantification of GR nuclear translocation patterns in rat hippocampal CA1 cells

Fluorescence intensity values were measured in both the nuclear and cytoplasmic compartment of CA1 cells after corticosterone injection. Fig. 4 depicts the fold increase in those compartments as visualised with the different GR primary antibodies. In agreement with the confocal images, we observed increased GR-IR in the nuclear compartment after glucocorticoid treatment with some of the antibodies. Nuclear intensity increased (more than) approximately 4-fold 120 min after corticosterone injection observed with the BuGR2, H300 (Fig. 4A and B, $p < 0.001$) and P20 antibody (Fig. 4D, $p < 0.01$) while no increase was observed with the M20 antibody (Fig. 4C, $p = 0.42$). In contrast, GR-IR in the cytoplasmic compartment as visualised with the M20 antibody increased significantly to approximately 2-fold (Fig. 4C, $p < 0.01$).

Quantification of GR nuclear translocation patterns in rat dentate gyrus cells

To generalise the in vivo findings in CA1 cells, another hippocampal cell population, granule dentate gyrus cells was analysed (Fig. 5). For this purpose, we compared the H300 and M20 primary antibodies which provided distinctly different results in CA1 cells. In agreement with the results from CA1 cells, we observed increased GR-IR in the nuclear compartment after glucocorticoid treatment visualised with H300 (Fig. 5A, $p < 0.01$), but not with M20 (Fig. 5B, $p = 0.62$). Similarly, an approximately 2-fold increase was observed in cytoplasmic GR-IR intensity with the latter antibody (Fig. 5B, $p = 0.04$).

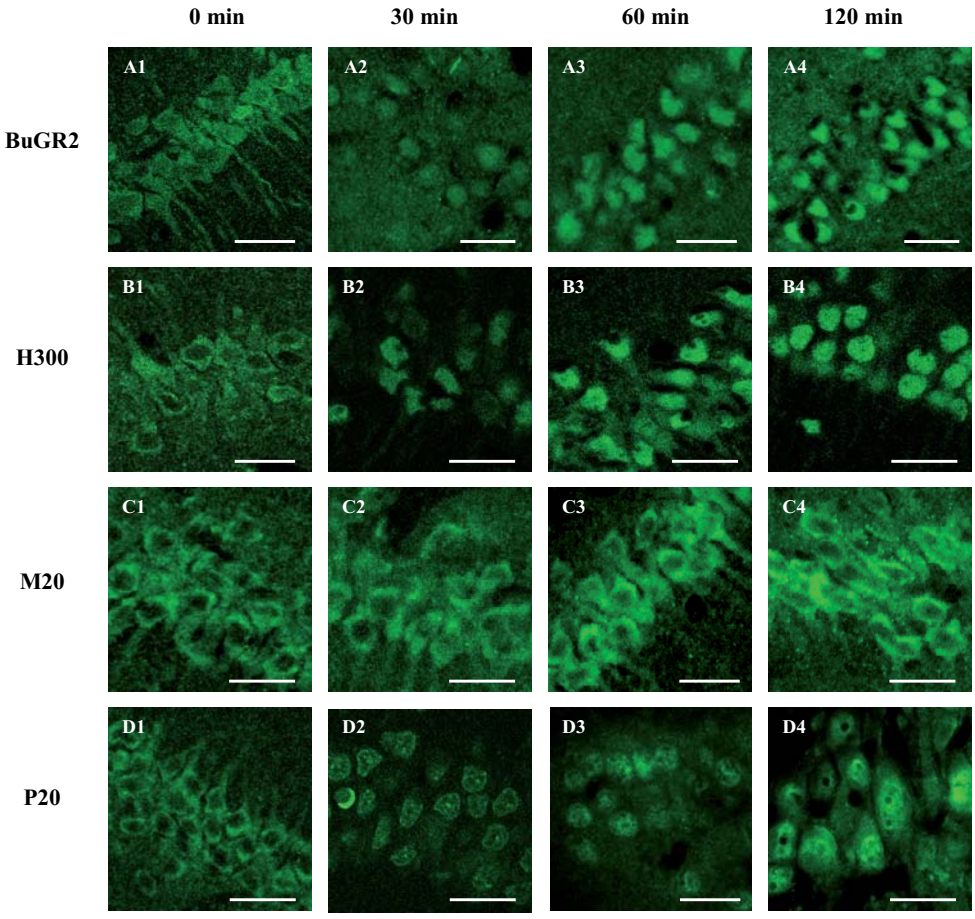
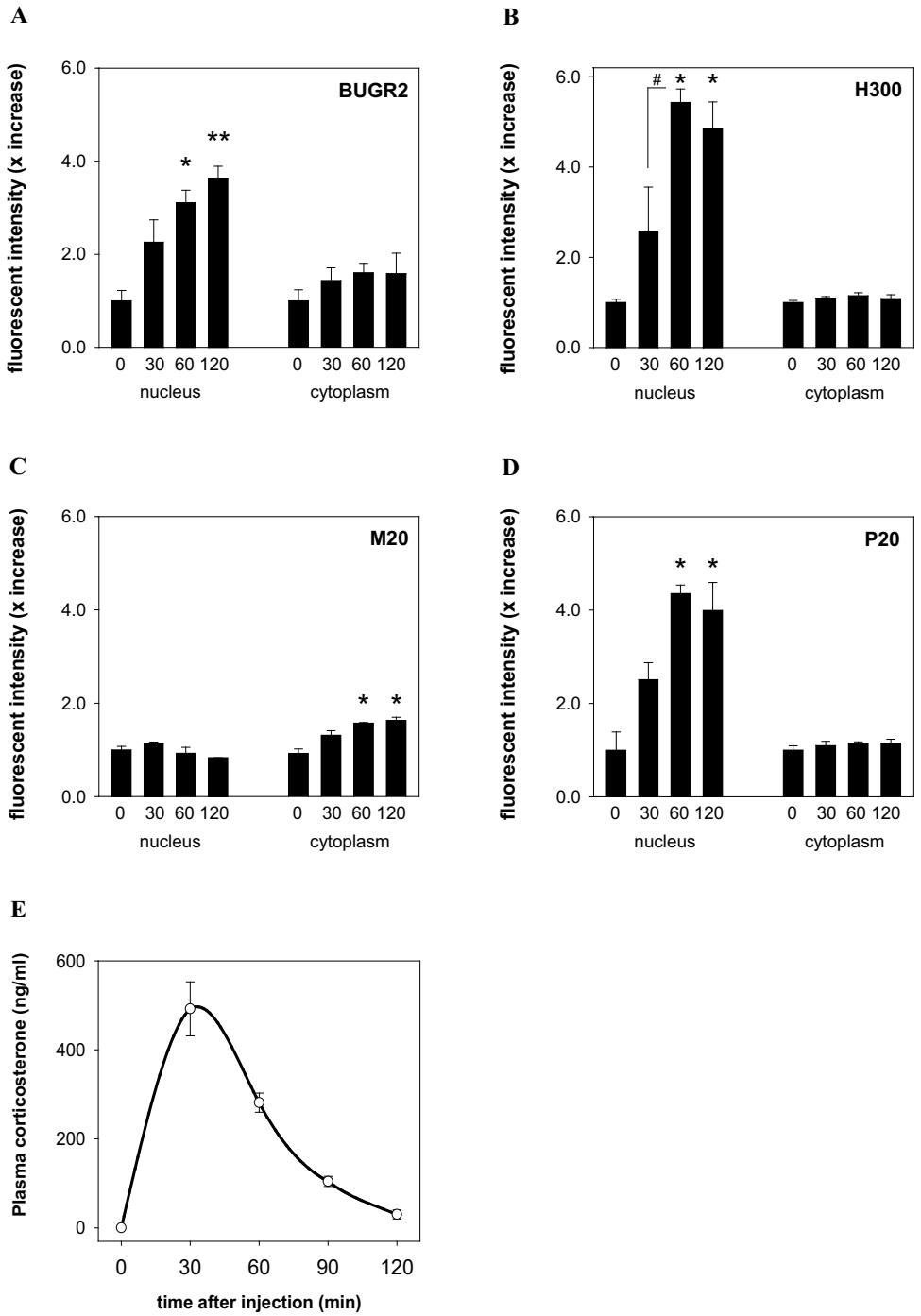


Figure 3 | GR nuclear translocation patterns in rat CA1 cells. Time curve analysis of subcellular distribution of GR-IR in CA1 cells of ADX rat hippocampus at 0, 30, 60 and 120 min after 3 mg/kg corticosterone injection. A1-D1 | Before stimulation (0 min), GR-IR visualised with all GR antibodies was mostly localised in the cytoplasmic compartment. In contrast to the AtT20 cells, no increase in nuclear GR-IR was visible after glucocorticoid stimulation with the A1-A4 | BuGR2 and C1-C4 | M20 antibody while the B1-B4 | H300 and D1-D4 | P20 antibodies clearly visualised nuclear GR distribution. Scale bar = 20 μm.


A
B
C
D
E
fluorescent intensity (x increase)
BUGR2
H300
M20
P20
6.0
4.0
2.0
0.0
0 30 60 120
nucleus
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*
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Plasma corticosterone (ng/ml)
600
400
200
0
0 30 60 90 120
time after injection (min)

< Figure 4 | Quantification of GR nuclear translocation patterns in rat CA1 cells. Fluorescent intensity values of GR-IR in the nuclear and cytoplasmic compartment of ADX rat CA1 cells are shown at 0, 30, 60 and 120 min after 3 mg/kg ip corticosterone injection visualised with different GR primary antibodies. Corticosterone injection resulted in increased fluorescent intensity in the nuclear compartment visualized with A | BuGR2 ($F(3,10) = 14.77$, $p < 0.001$), B | H300 ($F(3, 9) = 14.13$, $p < 0.001$) and D | P20 ($F(3, 10) = p < 0.01$). Interestingly, cells visualised with C | M20 ($F(3, 8) = 1.06$, $p = 0.42$) showed no increase in the nuclear compartment after glucocorticoid stimulation. The opposite was found for the cytoplasmic compartment in which corticosterone injection resulted in increased GR-IR visualised with C | M20 ($F(3, 6) = 12.85$, $p < 0.01$ but not with A | BuGR2 ($F(3, 10) = 1.11$, $p = 0.39$), B | H300 ($F(3, 10) = 1.24$, $p = 0.34$) and D | P20 ($F(3, 11) = 0.78$, $p = 0.53$). E | plasma corticosterone levels were monitored before and at different time points after injection in ADX rats. Before injection (t = 0 min), corticosterone levels were indeed very low or non detectable. Data is expressed as mean ± SEM. One-way ANOVA and Tukey's post-hoc test, ** $p < 0.001$ and * $p < 0.01$ vs veh, # $p < 0.05$ 5 vs 15 and 30 min, n = 3-4 rats per time point.

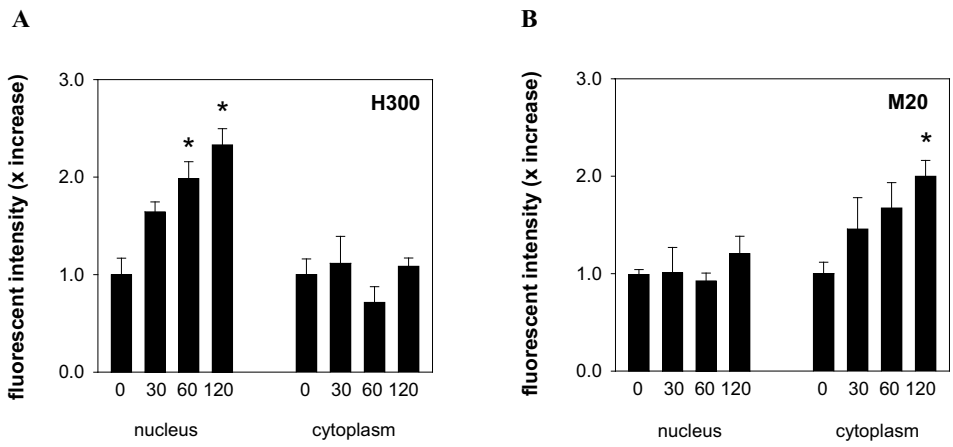

Figure 5 | Quantification of GR nuclear translocation patterns in rat dentate gyrus cells. Fluorescent intensity values of GR-IR in the nuclear and cytoplasmic compartment of ADX rat dentate gyrus granule cells are shown at 0, 30, 60 and 120 min after 3 mg/kg ip corticosterone injection visualised with different GR primary antibodies. Corticosterone injection resulted in increased fluorescent intensity in the nuclear compartment visualised with A | H300 ($F(3,10) = 5.88$, $p = 0.014$) but not with B | M20 ($F(3, 9) = 0.62$, $p < 0.62$). However, cytoplasmic GR-IR increased significantly after glucocorticoid stimulation ($F(3, 7) = 3.60$, $p = 0.04$). Data is expressed as mean ± SEM. One-way ANOVA and Tukey's post-hoc test, * $p < 0.01$ vs veh, n = 3-4 rats per time point.

Discussion

In this study we have investigated the differences between antibodies in determination of GR-IR subcellular distribution patterns after glucocorticoid stimulation in cultured cells and in hippocampus. To prevent confounding differential dose-dependent steroid effects on GR nuclear translocation (Nishi et al. 1999, Spiga & Lightman 2009), and to only study the consequences of the different GR-specific primary antibodies, we choose to expose both cultured cells and rats to very high concentrations of steroid thereby saturating most GR molecules. While some of the antibodies provided consistent cell specific results in GR nuclear translocation patterns between cultured cells and tissue, the data showed for others distinctly different results. The findings demonstrate that the outcome of ligand-induced GR nuclear translocation depends on the particular antibody and cannot always be extrapolated between experimental conditions.

We observed contradictory results in GR nuclear translocation patterns between cultured AtT20 cells and rat CA1 cells with the same primary antibodies (i.e. M20, H300, P20). This may reflect either cell type-specificity or procedural differences, and both may be related to in vitro versus in vivo conditions. While we have no in vivo data for pituitary cells, another cell population in the brain, the granule dentate gyrus cells, showed very similar results as were found in CA1. However, we did find two studies where hippocampal GR immunoreactivity was successfully detected using the M20 antibody (Sheng et al. 2003, Furay et al. 2008). Yet these studies used slightly different protocols optimised for DAB staining. There are also known biological differences in GR between cultured cells and actual tissue. For example, complete redistribution of nuclear GR over the cytoplasm after steroid withdrawal may last more than 24 hours (Hache et al. 1999), while in vivo GR is rapidly shuttled in and out of the nucleus on an hourly time-scale (Conway-Campbell et al. 2007, Sarabdjitsingh et al. 2010). We favour the interpretation that the pattern of nuclear interactions of GR differs in a systematic way between cell lines and tissues, depending on the conditions.

The various stages in the receptor life cycle may also provide differences in the availability of specific epitopes (i.e. steric hindrance due to conformation changes). The antibodies used in this study are all raised against the N-terminal domain of the GR, except the P20 antibody. This antibody is raised against the end of the C-terminus (aa 750-769) that harbours the ligand binding domain. In AtT20 cells, the latter antibody did not detect nuclear GR after glucocorticoid stimulation suggesting that this part of the C-terminus is hidden from the antibody. In rat CA1 cells, the N-terminal raised M20 antibody (aa 5-20) failed to detect changes in nuclear GR-IR. This suggests that in this condition, the immunogenicity of the N-terminal domain rather than the C-terminus is affected by ligand-induced conformational changes. Similarly, it is possible that the counter intuitive increase in cytoplasmic

immunoreactivity after glucocorticoid treatment, in an antibody-specific manner, is due to increased epitope accessibility after stimulation with ligand. Typically, this would not be observed with other antibody based techniques that use denaturing methods (e.g. Western Blot).

It is also possible that the observed differences between antibodies could be explained by the use of different ligands in the two conditions, dexamethasone and corticosterone. The ligand induced conformational change of the receptor is crucial for the distinction between agonists and antagonists, and may also differ to some extent for these two agonists. Functionally, differences between full agonists have been observed for intranuclear mobility, but not for translocation at saturating concentrations (Schaaf & Cidlowski 2003, Schaaf et al. 2005). This in itself does not exclude the agonist dependence of the immunogenicity of GR. Although not all GR antibodies were included then, we have previously used corticosterone in cultured cells and indeed have reported similar GR nuclear translocation with the M20 antibody as described here for dexamethasone (Morsink et al. 2006). Because changes in conformation induced by different agonists primarily take place in the LBD, they probably have little effect on the accessibility of antibodies raised against the N-terminal domain (e.g. H300, M20, BuGR2 and GR-Kawata). The consequences for C-terminally raised antibodies such as the P20, are however unknown, and we have to be aware that particular ligands may cause antibody-dependent outcomes in translocation studies.

Epitope accessibility is also determined by the local cellular context. Conformation changes of GR are partly determined by dissociation of chaperones upon ligand binding, binding for active transport to the nucleus or to other nuclear proteins and is therefore considered a key determinant in receptor function (Pratt et al. 2004, Meijer et al. 2006, Nishi & Kawata 2006, Picard 2006). Differential expression and regulation of molecules that make up GR complexes have not been systematically compared between cultured cells and tissue yet and could therefore contribute to the differences between experimental conditions.

Furthermore, the intracellular localisation of GR may also depend on the composition of the fixative. For instance, addition of gluteraldehyde minimises potential redistribution of the receptor during the fixation procedure (Brink et al. 1992). Taking these methodological aspects into account, some have described a relative absence of GR in the primate hippocampus (Sanchez et al. 2000), while others have shown prominent expression levels of GR in the human hippocampus (Wang 2009).

Although cross-linking fixation methods are regarded as effective means to preserve protein in tissue, it is possible that the different methods of fixative delivery in our study (i.e. direct incubation or transcardial perfusion fixation) result in different fixation strengths [albeit with the same fixative (4% paraformaldehyde, pH 7.4)], and contribute to differences in

epitope accessibility of the antibody. In that respect, antigen retrieval techniques such as heating by microwave or phosphatase pretreatment which aim to reverse deleterious effects of fixation (Shi et al. 1996, Pileri et al. 1997, MacIntyre 2001, Shi et al. 2001, Fritschy 2008), could be used to study the degree of epitope masking in settings like these. However, antigen retrieval techniques which allow renaturation of the structure of fixed protein have been proven beneficial only in particular circumstances and in some cases tend to increase aspecific binding (Lucassen et al. 1995). Though antigen retrieval methods are being widely addressed, more detailed studies with different types and durations of fixatives with ranging pH values on receptor distribution are suggested to elaborate on the consequences on receptor distribution.

In the current study we chose to investigate GR nuclear translocation patterns under steroid-free conditions (e.g. adrenalectomy and stripped serum media). Previously we have shown that GR, but also MR, nuclear translocation indeed depends on the physiological state of an animal but may also interfere with the basal conditions as low levels of both receptors may be retained in the nucleus before glucocorticoid treatment (Sarabdjitsingh et al. 2009, Sarabdjitsingh et al. 2010). However, our approach prevented interference of endogenously circulating glucocorticoids and differences in basal steroid conditions between the in vivo and in vitro situation.

Even though the recent generation of transgenic GR-GFP knock-in mice provide promising animal models for future research (Brewer et al. 2002, Usuku et al. 2005, Nishi et al. 2007), the current ongoing work still heavily depends on the use of primary antibodies.
Besides immunohistochemistry, other antibody-based techniques that study GR in its 'natural' folded conformation such as chromatin immunoprecipitation (as opposed to denaturation with i.e. Western Blotting) may very well deal with similar epitope specificities as describe here. Our data make the point that inconsistency in (subcellular) protein expression patterns and controversy in published results could be attributed to a combination of the type of antibody, epitope accessibility and fixation procedure / composition as well as the local cellular context.

In conclusion, we report that the choice of the primary antibody can have important implications for the interpretation of glucocorticoid action on the identification of receptor subcellular distribution patterns and cannot be extrapolated between different conditions. We propose the use of different GR primary antibodies for different purposes. For direct comparisons between cultured cells and tissue, the BuGR2 and H300 primary antibodies are recommended for visualisation of GR localisation. For comparisons within cultured cells, we recommend the use of BuGR2, M20 and/or GR-Kawata antibody, while we suggest that BuGR2, H300 and P20 are more suitable for brain tissue.

Acknowledgements

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