Methods in Neurosciences

Volume 22

Neurobiology of Steroids

Edited by
E. Ronald de Kloet
Win Sutanto
Division of Medical Pharmacology
Center for Bio-Pharmaceutical Sciences
Leiden/Amsterdam Center for Drug Research
University of Leiden
Sylvius Laboratories
2300 RA Leiden
The Netherlands

ACADEMIC PRESS
San Diego New York Boston London Sydney Tokyo Toronto

Front cover photograph: Light micrograph of the rat MBH which shows the result of a double immunostaining experiment for NPT and ß-endorphin using the NiDAB/DAB double immunostaining technique. Immunoreactivity for NPY was labeled by the dark-blue to black NiDAB reaction, while ß-endorphin-immunoreactive neurons were labeled with the brown DAB chromogen. For more details, see Chapter 25, Figure 6. Photograph courtesy of Drs. Csaba Leranth, Frederick Naftolin, Marya Shanabrough, and Tamas L. Horvath, Yale University School of Medicine, New Haven, Connecticut.

This book is printed on acid-free paper. ♾

Academic Press, Inc.
A Division of Harcourt Brace & Company
525 B Street, Suite 1900, San Diego, California 92101-4495

United Kingdom Edition published by
Academic Press Limited
24-28 Oval Road, London NW1 7DX

International Standard Serial Number: 1043-9471

International Standard Book Number: 0-12-185292-X

PRINTED IN THE UNITED STATES OF AMERICA
94 95 96 97 98 99 EB 9 8 7 6 5 4 3 2 1

[9] Immunocytochemical Studies on Glucocorticoid Receptor

Antonio Cintra, Gunnar Akner, Rafael Coveñas, Mercedes de León, Ann-Charlotte Wikström, Luigi F. Agnati, Jan-Åke Gustafsson, and Kjell Fuxe

Monoclonal Antibodies Demonstrate Glucocorticoid Receptor in Rat Central Nervous System

Glucocorticoid Receptors: Neuronal Localization

The localization of the glucocorticoid receptor (GR)-immunoreactive neurons in the rat central nervous system (CNS) has been studied using an anti-GR monoclonal mouse antibody, MAb 7 (28), directed against an epitope in the N-terminal domain of the rat GR (16–18, 23). Double-immunoperoxidase or immunofluorescence techniques using different chromogens or fluorophores (Table I) (13) allowed the identification of the neuronal populations containing GRs. A strong nuclear GR immunoreactivity has been demonstrated *inter alia* in the stress-sensitive monoaminergic neurons of the brain stem (24) and in several hypothalamic neurons containing hypophysiotropic hormones, but not in the luteinizing hormone-releasing hormone (LHRH) system of the male rat (12, 13). Also, GR was not demonstrated in cholinergic neurons (37). Another monoclonal antibody, BUGR-2, which identifies an epitope adjacent to the DNA-binding domain of the rat GR, has demonstrated a widespread distribution of neuronal and glial GRs in the central nervous system of the rat (1, 7). Using this antibody glucocorticoid receptor immunoreactivity has been shown in catecholaminergic neurons (31). Glucocorticoid receptor immunoreactivity has even been demonstrated in a small population of LHRH-immunoreactive neurons of the preoptic region (3).

There is a great similarity between the pattern of distribution of GR immunoreactivity shown by MAb 7 and BUGR-2 monoclonal antibodies. Table II summarizes the regions where there are some discrepancies. MAb 7 shows strongly the GR-immunoreactive neurons, for example, within layers II/III of the cerebral cortex (Fig. 1), the hippocampal formation (Fig. 2), and the arcuate, periventricular, and paraventricular hypothalamic nuclei. Also some thalamic nuclei, for example, the parafascicular nucleus, revealed a strong GR immunoreactivity. In addition, BUGR-2 revealed strong GR immunoreactivity in the CA3 pyramidal cells as well as within the mitral cells of the

Methods in Neurosciences, Volume 22

TABLE I *Staining of Glucocorticoid Receptor Using Avidin–Biotin–Peroxidase Technique*

Step	*Comments*[a]
Fixation	4% (v/v) Paraformaldehyde, 0.2% (w/v) picric acid, 0.1 *M* phosphate buffer
Cryoprotection	30% (w/v) sucrose, 0.1 *M* phosphate-buffered saline (PBS)
Sectioning	20-μm-thick cryostat sections, collected free floating
Mouse monoclonal antibody against rat liver GR	Diluted in 0.1 *M* PBS containing 0.3% (v/v) Triton X-100 (Sigma) 1% (v/v) goat whole serum (Cappel). Incubated at room temperature, overnight
Biotinylated horse anti-mouse antibody	Diluted in 0.3 *M* PBS. Incubated at room temperature, 2 hr
Avidin–biotin complex	Diluted in 0.1 *M* PBS. Incubated at room temperature, 1 hr
Color-generating substrate	0.03–0.05% (w/v) 3,3'-diaminobenzidine tetrahydrochloride (Sigma), 0.015% (v/v) H_2O_2, 0.03% (w/v) ammonium nickel sulfate (BDH Chemicals, Ltd.), 50 m*M* Tris-HCl (pH 7.4)
Mounting	Rinsed in Tris-HCl; mounted on gelatin-coated slides; dehydrated in alcohols/xylene; coverslipped with Enthelan (Merck)

[a] Supplier locations: Sigma (St. Louis, MO); Cappel (West Chester, PA); BDH Chemicals, Ltd. (Poole, England); Merck (Rahway, NJ).

olfactory bulb, the substantia nigra, and the Purkinje cells of the cerebellar cortex (1, 26), where MAb 7 showed slight or no GR immunoreactivity. Important contributions to the central GR localization and regulation were also made by the use of another monoclonal antibody (35).

Semiquantitative Evaluations of Glucocorticoid Receptor Immunoreactivity

Subjective analyses reporting various degrees of intensity/density of GR immunoreactivity can be improved by computer-assisted image analysis to provide semiquantitative microdensitometric evaluations. We have used the IBAS system (Zeiss Kontron image analysis; Kontron Elektronik, Germany). Glucocorticoid receptor immunoreactivity in different areas of the rat CNS can be expressed in mean gray values (MGVs). Figure 2 contains measurements of MGVs in different areas of the hippocampal formation. Another parameter provided by computer-assisted image analysis is the density of GR-immunoreactive neurons or glial cells present in different CNS areas (Fig. 1).

TABLE II Glucocorticoid Receptor Immunoreactivities Using MAb 7 and BUGR-2 Monoclonal Antibodies against Rat Liver Glucocorticoid Receptor

Brain region	Antibody	
	MAb 7[a]	BUGR-2[b]
Telencephalon		
Internal granular layer of olfactory bulb	+	+++
Mitral cell layer of olfactory bulb	0	++
Islands of Calleja	+	++
Nucleus of horizontal diagonal band	0/+	+/++
Nucleus of vertical diagonal band	0/+	++
CA3 hippocampal area	+	+++
Subfornical organ	+++	nd
Diencephalon		
Median preoptic nucleus	+++	+
Anterior hypothalamic area	+	++
Suprachiasmatic nucleus	0/+	+
Paraventricular hypothalamic nucleus, parvocellular part	+++	++
Periventricular nucleus	+++	+
Arcuate nucleus	+++	+
Tuber cinereum	+++	+/++
Dorsomedial thalamic nucleus	+++	++
Laterodorsal thalamic nucleus	+	+++
Parafascicular thalamic nucleus	+++	nd
Zona incerta	0/+	+/++
Mesencephalon		
Substantia nigra, pars compacta	+	++/+++
Substantia nigra, pars lateralis	+	++
Central gray, dorsal	++	+++
Central gray, medial	++	0/+
Central gray, lateral	+/++	+++
Oculomotor nucleus	0/+	+++
Darschewitsch nucleus	0/+	+++
Pons		
Nucleus ambiquus	+	++/+++
Dorsal nucleus of the lateral lemniscus	+/++	nd
Parabraquial nucleus, medial	+	+++
C1 adrenaline cells	++	+++
C2 adrenaline cells	++	+++
C3 adrenaline cells	++	+++
Cerebellum		
Purkinje cell layer	0	++
Granular cell layer	++	+++

(*continued*)

TABLE II (*continued*)

Brain region	Antibody	
	MAb 7[a]	BUGR-2[a]
Spinal cord		
Layer I	0/+	nd
Layer II	++	+++
Layer 8, 9	+	+/++
Layer 10	+	nd
White matter	+/++	nd
Intermediolateral cell column	+	++

[a] From Okret *et al.* (28); Fuxe *et al.* (17); Fuxe *et al.* (18).
[b] From Antakly and Eisen (7); Ahima and Harlan (1).

Glucocorticoid Receptors: Glial Localization

Fuxe *et al.* (17) and Ahima and Harlan (1) referred to the nuclear localization of GR in glial cells, assuming that it is present within small nuclear profiles. Within the white matter of the rat CNS, especially within the white matter of the lower brain stem and the spinal cord, low-intensity GR immunoreactivity was observed in glial profiles organized in rows. It seems likely that these glial profiles of the white matter represent both oligodendrocytes (mainly telencephalon) as well as fibrillary astrocytes (mainly lower brain stem and spinal cord). On the basis of the scattered distribution without relationship to nerve cell bodies, the GR-immunoreactive glial profiles within the gray matter might represent protoplasmatic astrocytes. Nevertheless, in some cranial nerve cell nuclei of the brain stem, such as the motor nucleus of the facial and trigeminal nerve, a perineuronal location could be found, suggesting that some of these glial profiles also represent perineuronal oligodendrocytes. In line with these results glucocorticoids have been demonstrated to influence the metabolism of glia cells and the degree of myelinization (21). On the basis of the low to moderate intensity of GR immunoreactivity and relatively high density of GR-immunoreactive glial profiles within restricted regions of the gray matter, it seems likely that several glial cell populations found especially in the brain stem and spinal cord represent a major target for the action of the glucocorticoids.

An *in vitro* study using BUGR-2 (33) revealed the presence of GR immunoreactivity in primary cultures of mixed glial cells and within glial cell lines. This receptor was found in astrocytes, oligodendrocytes, and Schwannoma cells (33).

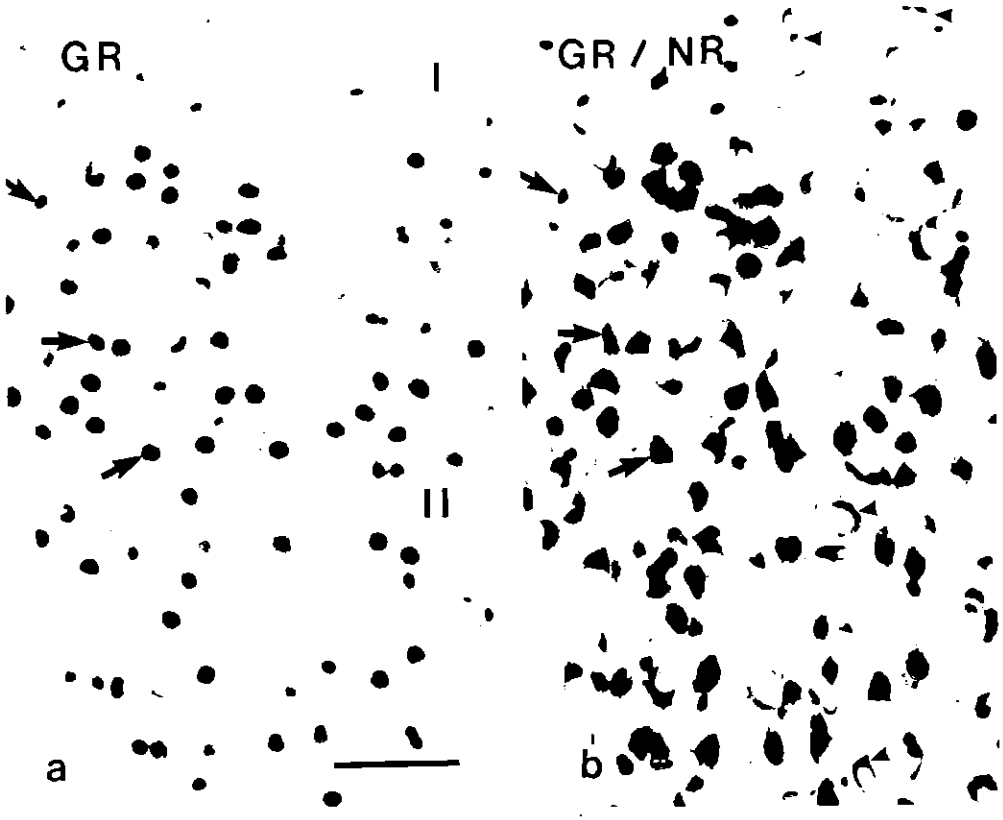
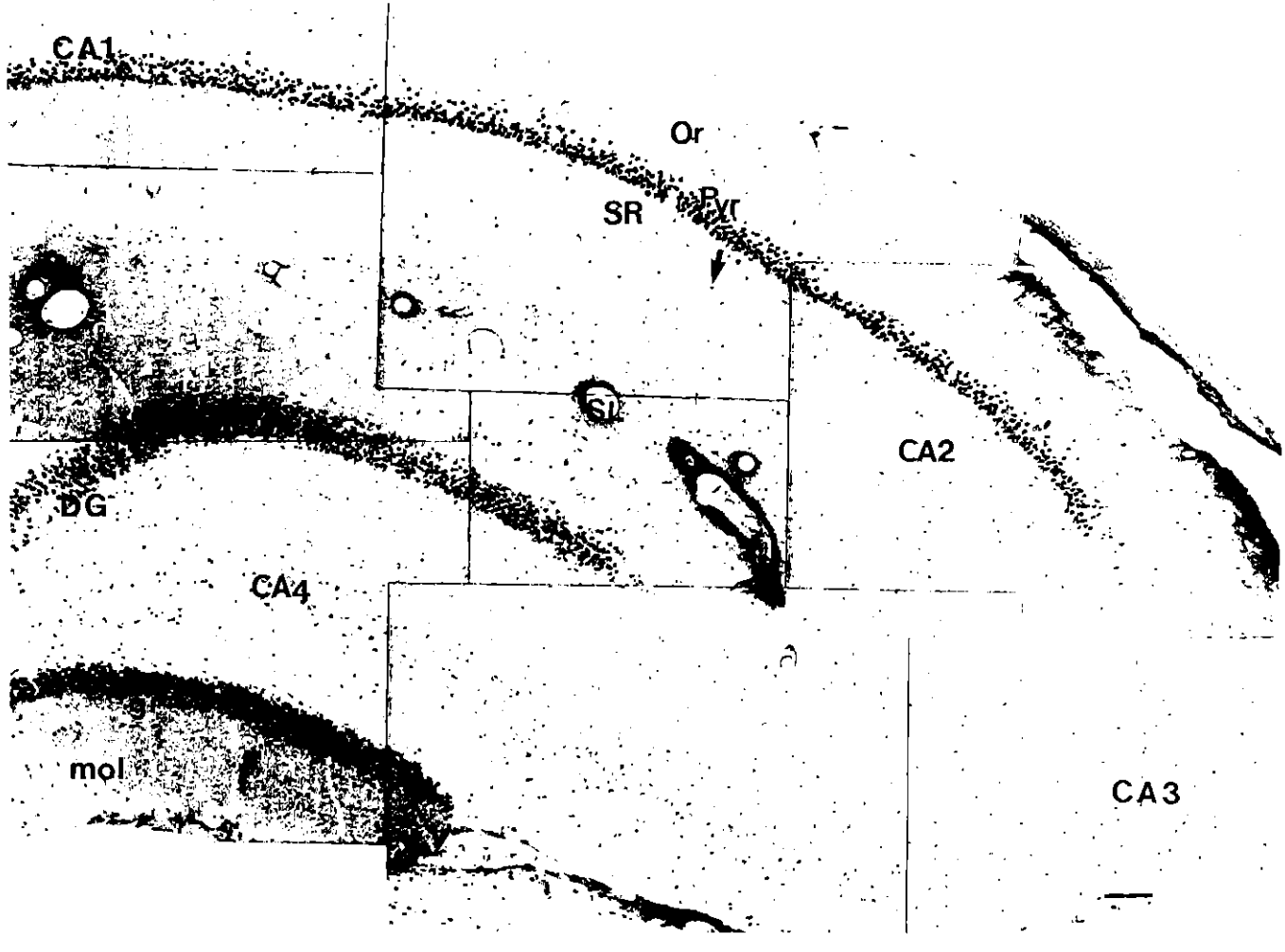


FIG. 1 Glucocorticoid receptor immunoreactivity is shown in a coronal section of the rat parietal cortex stained by the GR (MAb 7) antibody in combination with the immunoperoxidase technique with diaminobenzidine as the chromogen. (a) GR immunoreactivity in the molecular (I) and the granular cell (II) layers; (b) same field after counterstaining with neutral red (NR). The majority of the granular cells have nuclear GR (arrows). Arrowheads point to cells devoid of GR immunoreactivity in the molecular layer or to endothelial cells in the granular cell layer. Bar: 100 μm. Using computer-assisted image analysis (IBAS system), the GR nuclear profiles within layers II/III were found to be distributed in two classes: profiles with a mean diameter of 13 ± 4.5 μm with a density of $1.3/10^4$ μm^2 (glial profiles) and profiles with a mean diameter of 32 ± 13 μm with a density of $11.7/10^4$ μm^2 (neuronal profiles). Sample area: 70,750 μm^2.


CA1
Or
SR
Pyr
SL
CA2
DG
CA4
mol
CA3

Subcellular Distribution of Glucocorticoid Receptors

The intracellular distribution of GR is still a matter of controversy. An accurate knowledge of the subcellular localization of GR would be of major importance in the elucidation of signal transduction by glucocorticoid hormones. A large number of biochemical cell fractionation reports have shown unanimously that treatment with glucocorticoid hormones induced a shift in GR distribution from the high-speed supernatant (designated "cytosol") to the pellet (designated "nuclear fraction"). The concept of "cytosol" was (erroneously) regarded as representing the cytoplasm and thus, the "two-step model" of GR action was founded: glucocorticoid hormones induce a translocation of the hormone–GR complex from the cytoplasm to the nucleus followed by regulation of target gene transcription (20). However, the result of immunolocalization studies of individual cells, using mono- or polyclonal antibodies, has been inconsistent and has formed the basis for several notions regarding GR localization with or without the presence of receptor agonist: (a) GR is a nuclear protein both in the absence and presence of glucocorticoid hormones (9, 29); (b) GR is present both in the cytoplasm and nucleus in the absence or presence of ligand. Glucocorticoid treatment does not change the relative intracellular GR distribution (19, 25); and (c) GR is present both in the cytoplasm and nucleus in the absence or presence of ligand. Glucocorticoid treatment induces a nuclear translocation of a part of or the whole cytoplasmic GR pool (11, 30) Autoradiographic studies using radiolabeled GR agonists have also given various results, but suffer from the potential problem of ligand binding affecting the intracellular receptor localization.

Some of the discrepancies may be explained by technical circumstances such as different species, cell types, anti-GR antibodies, fixation/permeabilization procedures, and detection techniques. Other problems may have in-

---

FIG. 2 Glucocorticoid receptor immunoreactivity is shown in a coronal section of the rat brain at bregma level −3.3 mm. The MAb 7 antibody was employed and the labeling was performed by the immunoperoxidase technique, using diaminobenzidine as chromogen. The strongest GR immunoreactivity is found in the pyramidal cell layer (Pyr) of the CA1–CA2 area. The following microdensitometric values for the intensity of the immunostaining (expressed as mean gray values) were obtained by computer-assisted image analysis: stratum oriens (Or), 79; pyramidal cell layer of the CA1 area, 108; stratum radiatum (SR), 74; stratum lacunosum (SL) moleculare, 61; pyramidal cell layer of the CA2 area, 111; pyramidal cell layer of the CA3 area, 44; dentate gyrus (DG) molecular layer (mol), 64; and dentate gyrus granular cell layer (gr), 90. Bar: 200 μm.

volved (a) direct comparison between cytology and histology, because these methods imply different means of cell or tissue sample processing prior to immunostaining; (b) comparison between the distribution of endogenous GR in target cells and exogenous, transfected, often heterologous, GR in nontarget cells; and (c) selection bias when choosing fields of view for microphotography.

Nuclear versus Cytoplasmic Location of Glucocorticoid Receptor

In histological sections of the rat brain and spinal cord, GR was mainly restricted to the nuclei of neuronal and glial cells. This preferential localization of the GR into nuclear profiles can have several explanations; one is that cytoplasmic but not nuclear GR immunoreactivity is abolished by the detergent Triton X-100 used in the immunocytochemical experiments (36). Indeed, a weak cytoplasmic GR immunoreactivity is observed only in some large nerve cells such as the pyramidal nerve cells of the cerebral cortex. The monoclonal antibodies may also preferentially recognize the liganded form of the GR. Conformational changes of the GR after binding the ligand may expose antigenic determinants on the GR. The disappearance of nuclear GR immunoreactivity in the absence of glucocorticoids, for example, following adrenalectomy (ADX) (1, 17), would be in line with this explanation. The effect of long-term ADX was studied in detail (2, 32). Parallel to the disappearance of nuclear GR immunoreactivity after ADX and reappearance after corticosterone administration some groups of neurons also displayed a cytoplasmic GR immunoreactivity. Most GRs recognized by antibodies in histological sections are activated GRs. Thus, GR immunoreactivity in a brain area will be dependent not only on the number of receptors found in a cell population but also on the affinity of these receptors to corticosterone and on the relative amount of hormone that can reach the receptors in that brain area. The degree of labeling of the various GR-immunoreactive neuronal and glial cell populations as well as the GR distribution pattern will therefore vary with the physiological state of the animal. Indeed, a circadian variation of neuronal GR immunoreactivity has been reported (32).

Cytological Studies of Glucocorticoid Receptor in Nonneuronal Cell Cultures

We have studied the intracellular immunocytological distribution of GR in a number of different mammalian cell types, both primary cultures and established cell lines, originating from the entodermic and mesodermic em-

TABLE III Immunocytological Staining of Glucocorticoid Receptor Using Indirect Immunofluorescence Technique

Step	Comments[a]
Fixation/permeabilization	Techniques compared *Cross linking* F/T: 4% (v/v) formaldehyde in 0.14 *M* (PBS), 4°C, 10–15 min followed by 0.05% (v/v) Triton X-100, room temperature, 30 min *Precipitating* M: methanol (100%), −20°C, 10 min
Mouse monoclonal antibody against rat liver GR	Diluted in 0.14 *M* PBS, yielding a final protein concentration of 10–20 μg/ml. Incubated at room temperature, 1–2 hr
Secondary antibody	Goat anti-mouse IgG, FITC labeled, diluted in 0.14 *M* PBS, yielding a final protein concentration of 25 μg/ml. Incubated at room temperature, 1 hr
Mounting	Washed in 0.14 *M* PBS. Mounted upside down on glass slides in 50% (v/v) glycerol–0.14 *M* PBS

[a] PBS, phosphate-buffered saline; IgG, immunoglobulin G; FITC, fluorescein isothiocyanate.

bryonal germinal layers, focusing on human primary culture of gingival fibroblasts. In these experiments we have used several different monoclonal mouse anti-rat liver GR antibodies that recognize different epitopes in the N-terminal *trans*-activation domain of the GR (28), a number of different fixation/permeabilization techniques, and several detection systems. The antibodies cross-react well with human GR (10). Of major interest was the comparison between various fixation techniques employing different chemical principles, such as cross-linking (aldehyde/detergent) and precipitating (organic solvents) methods in various combinations and concentrations.

Emphasis was placed on comparison between a standard fixation technique previously used in our laboratory (Table III), that is, *cross-linking* followed by 0.05% (v/v) Triton X-100 (36), and a *precipitation technique* (4, 5). As an indirect immunodetection system we have used fluorescence, peroxidase, and gold techniques, and have obtained similar results. We have concentrated on indirect immunofluorescence because of the sharp contrast in the images, the potential for double-staining of individual cells using two different fluorochromes with nonoverlapping excitation and emission spectra, and the use of confocal microscopy (see below).

Besides conventional transmission light microscopy, we have also used confocal laser scanning microscopy (CLSM) in collaboration with the Department of Physics IV (Royal Institute of Technology, Stockholm, Sweden).

The stained cell monolayer is excited one point at a time by a focused laser beam and the emitted light is detected by a photomultiplier tube (PM tube). This CLSM technique has several advantages over conventional transmission light microscopy: (a) CLSM provides thin optical sections of the cells by reducing the out-of-focus contributions of the emitted light. The "thickness" of each optical section in these studies ranges from 1 to 2 μm and depends on the numerical aperture (NA) of the objective, the refractive index of the immersion medium, and the wavelength of the light. Optical sectioning reduces the risk of projection artifacts; (b) CLSM provides better resolution than conventional microscopy; (c) CLSM presents data in a digitized form that allows various kinds of direct image analysis such as subtraction of one image from another and quantitation of relative fluorescence intensities; and (d) CLSM allows serial sectioning of individual cells followed by two- or three-dimensional reconstruction. (For a more detailed description of the CLSM technique see Refs. 27 and 38.)

The data recorded by the microscope and the PM tube are stored as digital images, for which the value of each image element, lying between 0 and 255, is proportional to the fluorescent light emitted from corresponding points in the specimen. To calculate the relative intensity distribution in the nucleus and cytoplasm in the same transverse optical section through the central part of a particular cell, the image is shown on a TV monitor and the whole cell, the cell nucleus, and a part of the background are circled. The intensity of GR immunoreactivity is measured by the PM tube and expressed in relative units. The cytoplasmic intensity is defined by subtracting the signal due to the nucleus from that of the whole cell. The mean photometric immunointensity of GR in the nuclear, cytoplasmic, and whole cell compartments, respectively, is quantified in 40–60 cells for each variable, followed by statistical analysis.

Cytoplasmic versus Nuclear Localization

Without the addition of any exogenous glucocorticoid, GR was found to be localized in both the nucleus (n) and the cytoplasm (c). On average, the relative $c:n$ ratio $\approx 9:1$ with a significant heterogeneity of GR intensity in both compartments. This heterogeneity was more easily recognized visually after methanol (M) rather than formaldehyde/Triton (F/T) fixation, but was photometrically detected in both instances. F/T yielded a two to three times higher intracellular GR concentration, indicating that M caused a much higher extraction of GR from all cellular compartments compared to F/T.

The subcellular distribution of GR was observed to be similar using both F/T and M fixations (Fig. 3). During interphase, cytoplasmic GR was observed in three distinct distributions: (a) along cytoplasmic microtubules,

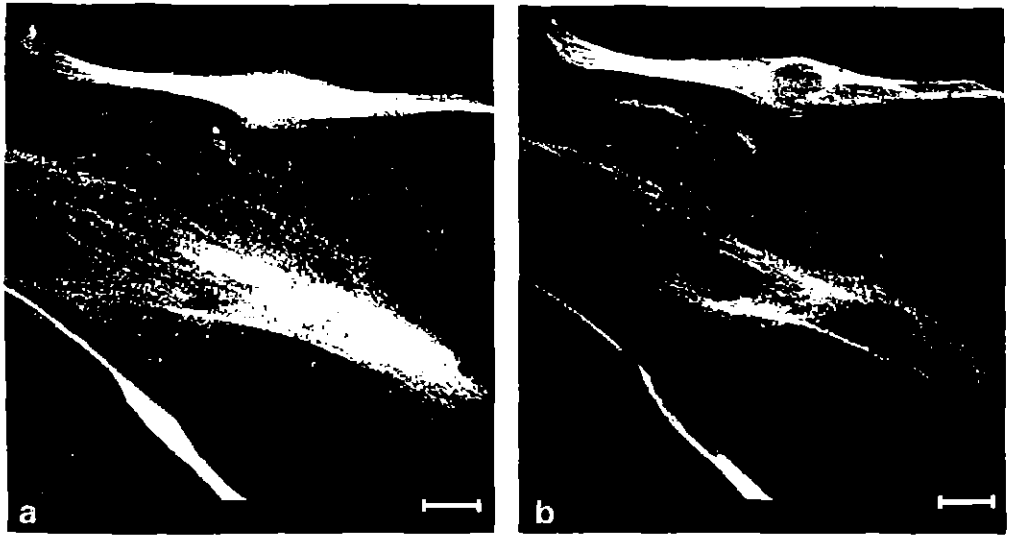


FIG. 3 An interphase human fibroblast primary culture was fixed with methanol and stained for glucocorticoid receptor (a) and tubulin (b) by means of the double-immunofluorescence technique. Confocal laser scanning microscopy provided a 1-μm-thick optical, transversal section through the central part of the cell. Glucocorticoid receptor immunoreactivity exhibits a cytoplasmic fibrillar staining pattern that colocalizes well with cytoplasmic microtubules. In the nucleus there is both a diffuse and granular GR signal, but little, if any, tubulin immunoreactivity. Bar: 20 μm.

(b) in individual centrioles, and (c) in the plasma membrane. The nuclear GR was found to be diffuse in all, and in addition granular in ~30% of the nuclei in a cell monolayer, leaving the nucleoli unstained. During mitosis, GR was concentrated in the centriolar regions and in the mitotic spindle apparatus during all stages of mitosis, together with a diffuse staining pattern in the surrounding cytoplasm. (For a more detailed description of the intracellular GR distribution see Refs. 4–6.)

Treatment with dexamethasone (10^{-6} *M* for 1 hr) caused a visually observable increase in nuclear GR staining in ~30% of the nuclei; however, this was found only after M, not F/T, fixation (see below). Photometry of GR on thin optical CLSM sections revealed a significant hormone-induced increase in the average GR intensity in both the nucleus and the cytoplasm, again detectable only after M but not F/T fixation. The increment in GR intensity was ~60% in the nucleus and ~35% in the cytoplasm. Also, when expressing the average GR intensity per picture element (pixel), we found ligand-induced increments in both compartments (~60% and ~13% in the nucleus and cytoplasm, respec-

tively), thereby eliminating the impact of differences in compartment size. The qualitative subcellular distribution of GR in the cytoplasm and nucleus remained unchanged after hormone treatment (see above).

Gasc *et al.* (19) studied the distribution of GR in rat liver by immunohistology, using two different monoclonal anti-GR antibodies, I GR 49/4 (35) and MAb 7 (28), and various fixation techniques. Their observation of an increase in nuclear GR after dexamethasone treatment was interpreted as indicating less loss of hormone–receptor complexes during the fixation procedure as compared to hormone-free GR, rather than demonstrating nuclear translocation of GR. These results are in agreement with ours.

On the basis of these results, we have formulated an "extraction hypothesis" regarding GR localization: during basal conditions, GR is present both in the cytoplasmic and nuclear compartments, exhibiting a strong heterogeneity between the individual cells. Treatment with glucocorticoid hormones does not induce any detectable compartment shift, but changes the affinity of GR for its intracellular docking sites, both in the nucleus and in the cytoplasm. This affinity change can be detected (visually or photometrically) only after precipitating fixatives such as methanol, which extract less GR from hormone-treated than nontreated cells, but not after cross-linking fixatives such as aldehyde, which locks both nonliganded and liganded GR to its docking sites and does not allow any detergent-induced extraction. Because there is less methanol-induced extraction of GR from the nucleus than the cytoplasm, this may give rise to a false visual or photometric impression of a hormone-induced apparent nuclear translocation of GR.

In summary, our results illustrate the fundamental role of the cell or tissue sample processing prior to the immunostaining procedure. The diffuse and partly granular nucleus GR population probably participates in nuclear gene transcriptional regulation. The extranuclear GR pool does not seem to shift toward the nucleus following hormone administration. This cytoplasmic GR may constitute a storage form of GR, but may also reflect the potential of GR to exert effect(s) on sites in the cytoplasm without involving nuclear genomic transcription. One may speculate that the observed distribution of GR along cytoplasmic and mitotic microtubules as well as in cytoplasmic and mitotic centrioles may reflect direct sites of GR action.

Evidence has been presented in support of an association between another member of the steroid hormone receptor superfamily, that is, the vitamin D_3 receptor, and microtubules (8).

Glucocorticoid Receptors and Pain Mechanisms

Glucocorticoid receptor-immunoreactive neurons are particularly abundant in brain and spinal cord areas related to the nociceptive pathway and are

suggested to be under glucocorticoid regulation. High densities of moderately to strongly GR-immunoreactive neurons exist within the substantia gelatinosa of the dorsal horn, the central gray, the midline thalamic nuclei, as well as within the posterior nuclear group and the ventral posterior nuclei of the thalamus. Pain pathways are known to project into these areas. High densities of GR-immunoreactive neurons are found all over the cerebral cortex, with the exception of layer IV and the outer part of layer V. Thus, pain, which may be globally perceived at the cortical level, may be under glucocorticoid control. Furthermore, the antinociceptive systems projecting into the substantia gelatinosa from the lower brain stem, such as the 5-hydroxytryptamine and noradrenaline descending systems, exhibit strong GR immunostaining, showing that systems mediating or reducing pain may be modulated by glucocorticoids in an integrated way. This view is supported by the phenomenon of stress-induced analgesia, which may involve both the nociceptive and the antinociceptive systems.

We designed some experiments to understand how glucocorticoids are implicated in pain mechanisms (see ref. 39 and 40).

Glucocorticoid Receptor and Neuropeptide Contents of Primary Afferent Neurons

In the spinal and sensitive cranial nerves, several neuropeptides are implicated in pain mechanisms: substance P (SP), calcitonin gene-related peptide (CGRP), somatostatin (SOM), galanin (GAL), and neuropeptide Y (NPY). We performed a study on the presence of GR immunoreactivity in the ganglionic neuronal populations containing one or more of these peptides. Most of these cells are small neurons giving rise to amyelinic fibers. The double-immunofluorescence technique was employed (14). One-third of both the CGRP- and SP-containing neurons of the spinal and trigeminal ganglia demonstrated nuclear GR (Fig. 4), whereas 50% of the GAL immunoreactive neurons of spinal ganglion, but only occasionally in the trigeminal ganglion, showed GR. Neither SOM- nor NPY-immunoreactive neurons demonstrated GR. The results suggest that glucocorticoids may regulate the synthesis of peptides via GR in some chemically identified neuronal populations of the spinal and trigeminal ganglia.

Regulation of Neuropeptide Contents of Spinal Ganglion by Glucocorticoids

The hypothesis of glucocorticoid regulation of peptide-containing nerve cell populations within the spinal ganglia was tested by performing adrenalectomy

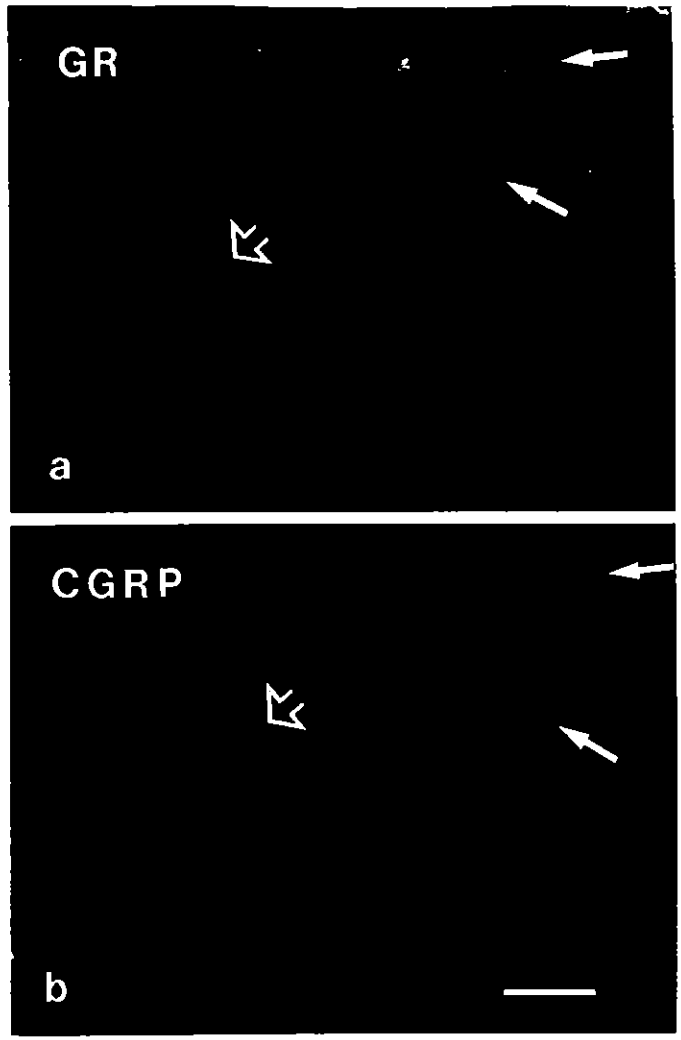


FIG. 4 Coronal section of the rat lumbar dorsal root ganglion double labeled for (a) GR and (b) calcitonin gene-related peptide (CGRP). After an incubation with both GR (MAb 7) and CGRP (Milab, Malmö, Sweden) antibodies at 4°C overnight, the secondary antibodies were linked to fluorescein-conjugated donkey anti-mouse and Texas Red-conjugated donkey anti-rabbit immunoglobulins, respectively (Jackson, West Grove, PA) at 37°C for 2 hr. Arrows point to large ganglion cells containing both nuclear GR and cytoplasmic CGRP. An open arrow shows a neuron with GR but lacking CGRP. Bar: 50 μm.

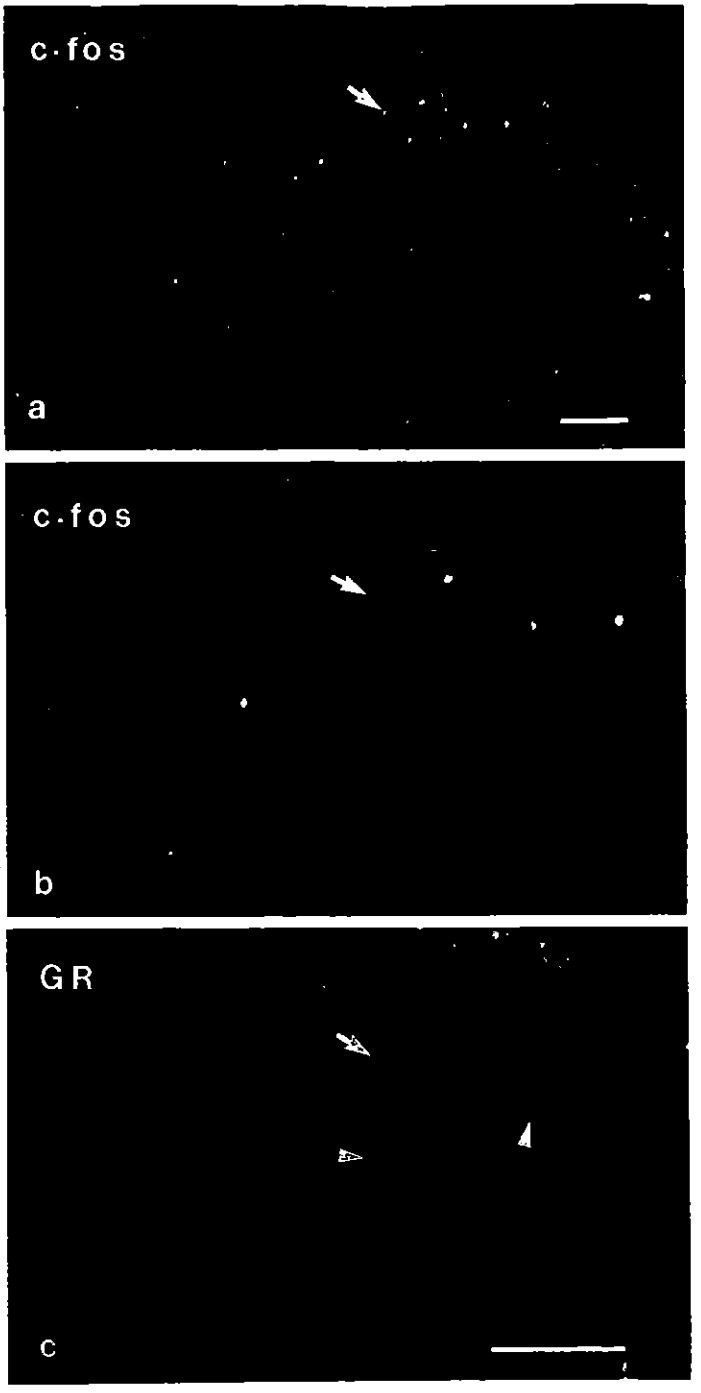

c-fos
a
c-fos
b
GR
c

FIG. 5 Immunoreactivities of c-Fos and GR are shown in a coronal section of the rat spinal cord by means of a double-immunofluorescence procedure. The immunostaining was performed in two steps: first, binding of the GR antibody (MAb 7), at 4°C overnight, and labeling with fluorescein-conjugated donkey anti-mouse antibody (Jackson); second, binding of the c-Fos antibody (Affiniti, Derbyshire, UK), at 4°C overnight, and labeling with Texas Red-conjugated donkey anti-goat immunoglobulins (Jackson). Electric stimulation of the sciatic nerve 2 hr before sacrifice elicited c-Fos. (a) shown in large magnification in (b). Few (less than 10%) of the c-Fos-induced neurons of the superficial layers (I–II) of the dorsal horn exhibit GR (arrows). Arrowheads in (c) point to GR in neurons without c-Fos. Bars: 100 μm.

populations in the CNS. The results are compatible with the existence of both a cytoplasmic and nuclear pool of GR but a translocation of cytoplasmic GR to the nucleus still remains to be clearly demonstrated. Glucocorticoid receptors may directly modulate pain pathways at various levels (primary sensory neurons, periaqueductal gray matter, thalamus, and cerebral cortex) but not importantly at the level of the dorsal horn. The results emphasize the role played by the glucocorticoids in nuclear genomic transcription.

Acknowledgments

This work has been supported by Grants 04X-715 and 13X-2819 from the Swedish Medical Research Council, from the Swedish Medical Society, and from the Lars Hierta Minne Foundation.

References

1. R. S. Ahima and R. E. Harlan, *Neuroscience* **39,** 579 (1990).
2. R. S. Ahima and R. E. Harlan, *Endocrinology (Baltimore)* **129,** 226 (1991).
3. R. S. Ahima and R. E. Harlan, *Neuroendocrinology* **56,** 845 (1992).
4. G. Akner, K. G. Sundqvist, M. Denis, A. Wikström, and J.-Å. Gustafsson, *Eur. J. Cell Biol.* **53,** 390 (1990).
5. G. Akner, K. Mossberg, A.-C. Wikström, K. Sundqvist, and J.-Å. Gustafsson, *J. Steroid Biochem. Mol. Biol.* **39,** 419 (1991).
6. G. Akner, A.-C. Wikström, K. Mossberg, K. Sundqvist, and J.-Å. Gustafsson, *J. Histochem. Cytochem.* **42,** 645 (1994).
7. T. Antakly and H. J. Eisen, *Endocrinology* **115,** 1984 (1984).
8. J. Barsony and W. McKoy, *J. Biol. Chem.* **267,** 24457 (1992).
9. M. Brink, B. M. Humbel, E. R. de Kloet, and R. Van Driel, *Endocrinology* **130,** 3575 (1992).

35. H. Westphal, G. Moldenhauer, and M. Beato, *EMBO J.* **1,** 1467 (1982).
36. A.-C. Wikström, O. Bakke, S. Okret, M. Brönnegård, and J.-Å. Gustafsson, *Endocrinology (Baltimore)* **120,** 1232 (1987).
37. M. Zoli, A. Cintra, I. Zini, L. Hersh, J.-Å. Gustafsson, K. Fuxe, and L. Agnati, *J. Chem. Neuroanat.* **3,** 355 (1990).
38. N. Åslund, K. Carlsson, A. Liljeborg, and L. Majlöf, *Proc. Scand. Conf. Image Anal., 3rd* pp. 338–343. Studentlitteratur, Lund, 1983.
39. R. Coveñas, M. de León, G. Chadi, A. Cintra, J.-Å. Gustafsson, J. A. Narvaez, and K. Fuxe, *Brain Res.* **640,** 352 (1994).
40. M. de León, R. Coveñas, G. Chadi, J. A. Narvaez, K. Fuxe, and A. Cintra, *Brain Res.* **636,** 338 (1994).