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Immunocytochemical study on the intracellular localization of the type 2 glucocorticoid receptor in the rat brain

J.A.M. van Eekelen, J.Z. Kiss*, H.M. Westphal** and E.R. de Kloet

Rudolf Magnus Institute for Pharmacology, Medical Faculty, University of Utrecht, Utrecht (The Netherlands)

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The localization of the glucocorticoid receptor (GR) (type 2) in the rat brain was studied with immunocytochemistry using a monoclonal antibody against the rat liver GR. Strong GR immunoreactivity (GR-ir) was observed in neurons of limbic and brainstem structures known to be associated with the stress-activated circuitry, which suggest that these sites are responsive to glucocorticoid feedback. The intracellular localization of GR-ir was examined in CA_1 and CA_2 pyramidal neurons of the hippocampus. In intact rats GR-ir is predominantly present in the cell nucleus. Adrenalectomy (ADX) caused a slow depletion of the GR-ir signal from the cell nucleus until near detection limits at two weeks postsurgery. At that time, 1 h after administration to longterm ADX rats the synthetic glucocorticoid (type 2) agonist RU 28362 as well as a moderate and high dose of corticosterone (CORT) markedly enhanced the cell nuclear GR-ir. The type 2 antagonist RU 38486 also caused an increase of GR immunostaining in cell nuclei upon acute administration to ADX rats. The mineralocorticoid aldosterone (ALDO) and a low dose of CORT, which bind almost exclusively to type 1 corticosteroid receptors, were ineffective. In conclusion, our data suggest that in the hippocampal CA_{1-2} neurons type 1 and type 2 corticosteroid receptors may coexist. The steroid-induced changes in cell nuclear immunoreactive GR staining intensity suggest possible cytoplasmic-cell nuclear translocation of GR and/or exposure of immunogenic GR domains.

INTRODUCTION

Biochemical and autoradiographic studies have revealed the presence of two types of corticosteroid receptors in the rat brain[7,15,27,32,33]. This resolution into two types was achieved when novel synthetic glucocorticoids became available that exhibited exclusive glucocorticoid properties[22,24]. New methodology involving an in vitro autoradiographical procedure and image analysis permitted to visualize these two receptors[9,28,31]. The type 1 corticosteroid receptor resembles the kidney mineralocorticoid receptor[18,39]. However, a subtype of these type 1 receptors, i.e. the type corticosterone preferring sites (CR) display predominant localization in neurons of the hippocampus and septum[15,19,28]. The latter type 1 receptors bind with high-affinity corticosterone (CORT) as well as aldosterone (ALDO), but functional studies have shown that these sites respond to CORT as agonist, while ALDO has antagonistic properties[8,9,20]. Type 2 receptors, which represent the classical glucocorticoid receptor (GR) are similar to the liver GR[27]. Type 2 has a widespread localization in the brain and binds potent synthetic glucocorticoids with highest affinity. Type 2 receptors have a 10-fold lower affinity to CORT than displayed by type 1 (ref. 27).

Only recently immunocytochemical research on corticosteroid receptors in the brain was feasible when poly- and monoclonal antibodies became available that were raised against the rat liver GR. Fuxe et al.[1,12,13] reported a detailed neuroanatomical distribution of GR-immunoreactive (GR-ir) neurons and glial cells in the central nervous system of the rat. These observations appeared largely in agreement

* Present address: Institute of Histology and Embryology, University of Geneva, Medical School, 1211 Geneva 4, Switzerland.
** Present address: Institut für Molekularbiologie und Tumorforschung, Emil-Mannkopfstrasse 1, D-3350 Marburg/Lahn, F.R.G.
Correspondence: J.A.M. van Eekelen, Rudolf Magnus Institute for Pharmacology, Medical Faculty, University of Utrecht, Vondellaan 6, 3521 GD Utrecht, The Netherlands.

with the type 2 distribution observed after autoradiography of in vitro labelled tissue sections. Type 2 and GR-ir localization were demonstrated in the CA_1 and CA_2 pyramidal neurons of the hippocampus, the cell field that also contains type 1 receptors as shown by high-resolution autoradiography after administration of tracer doses [^{3}H]CORT and with the in vitro autoradiographical procedures[7,15,26,28,30,34].

In the present study we have further examined the immunocytochemical localization of type 2 receptors in the rat brain using a monoclonal antibody to the rat liver GR. We show that GR-ir is localized in abundance in key sites of the stress-activated brain circuitry and may coexist with type 1 in the hippocampus. The GR-ir signal in cell nuclei is attenuated at longer time intervals after adrenalectomy (ADX), and can be enhanced upon type 2 glucocorticoid agonist and antagonist administration, but not by type 1 ligands.

MATERIALS AND METHODS

Male Wistar rats (150–180 g b. wt.) were used in this study. The animals were maintained under standard light (lights on between 06.00 and 20.00 h) and temperature (23 °C) conditions. Food and water were available ad libitum. In general, bilateral adrenalectomy (ADX) was performed in the afternoon under ether anesthesia via the dorsal approach. The rats were allowed to recover for 7–10 days, while having access to food and 0.9% NaCl solution.

The following experiments were performed: (1) regional distribution of GR-ir in the rat brain. (2) A time course of changes in GR-ir after ADX. The rats were sacrificed at 24 h, 1 week and 2 weeks after surgery, respectively. (3) Administration of steroids to ADX rats. Aldosterone (ALDO, 30 μg/100 g b. wt. s.c., postinjection survival time 30–60 min), CORT (low dose 1 μg/100 g b. wt., s.c., moderate dose 10 μg/100 g b. wt., s.c., high dose 300 μg/100 g b. wt., s.c., survival time for all doses 1 h) and the synthetic glucocorticoid RU 28362 (100 μg/ 100 g b. wt., s.c., survival time 2 h). (4) The effect of GR-antagonist RU 38486 on the intracellular localization of GR in the hippocampus. In one experiment an injection of RU 38486 (1 mg/100 g b. wt., s.c., suvival time 90 min) was given. Another experiment involved administration of the same dose of RU 38486, followed by an injection of the GR agonist, RU 28362 (100 μg/100 g b. wt., s.c., survival time 60 min) 30 min later. In Exp. 4 and 5 all drugs were dissolved in 2% ethanol and polyethylene glycol (PEG). Sham-operated rats as well as ADX rats that were given an injection of PEG s.c. served as controls.

Tissue preparation and immunocytochemistry

The animals were anesthetized with pentobarbital (0.25 ml/100 g b. wt., i.p.). Just prior to perfusion blood samples were taken from the heart and collected in chilled EDTA-coated tubes for plasma CORT by radioimmunoassay. Fixation of the brain was performed by transaortic perfusion and the choice of fixative was dependent on the area studied. In the case of the hippocampus, the brain was fixed with 4% paraformaldehyde, 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4 (4 °C, method A). For the study on the supraoptic nucleus 4% paraformaldehyde, 0.2% picric acid in 0.1 M phosphate buffer, pH 6.0 (4 °C), followed by 4% paraformaldehyde in 0.1 M borate buffer, pH 9.0 (4 °C) was used for perfusion (method B). The brains were placed overnight in the fixative of method A and the second fixative of method B, respectively, at 4 °C and then washed in several changes of 0.1 M sodium phosphate buffer, pH 7.2. Coronal sections (30–50 μm) were cut with a vibratome and processed for immunocytochemistry. At first the buffer was replaced by normal horse serum (5% in sodium phosphate buffer, containing 0.3% Triton X-100) for 1 h at room temperature. Subsequently the floating sections were incubated with GR antiserum (diluted 1:1000 in sodium phosphate buffer) for 48 h at 4 °C. Further incubation with biotinylated antimouse IgG (diluted 1:500 in 0.05 M Tris buffer, pH 7.6, containing 0.3% Triton X-100) and avidin–biotin–peroxidase complex (ABC, 40 μl avidin + 40 μl biotinylated peroxidase in 10 ml Tris buffer) was performed for 1 h each at room temperature. The ABC components were obtained in kit form from Vector Labs. (Burlingame, CA). Between all incubation steps the sections were rinsed twice with Tris buffer at room temperature. 3,3-Aminobenzidine tetrahydrochloride (Polysciences) was applied as the chromogen. Finally the sections were mounted on gelatin-coated slides, dehydrated and coverslipped.

The monoclonal antibody 1 GR 49/4 was generated against rat liver glucocorticoid receptor[37] and

was kindly provided by H.M.W.

In the control studies, no immunoreactivity was observed when sections were incubated with ascites fluid nor when the primary antibody was omitted. The immunoperoxidase reaction was markedly reduced after pre-absorption of the antiserum with a purified GR preparation of rat liver.

RESULTS

Optimal visualization of GR immunoreactivity (GR-ir) seemed to be dependent on the nature of the fixative. In the current study we used two perfusion methods A and B, in which the fixatives differed mainly in pH. To obtain satisfactory detection of GR-ir in the hippocampus, method A with almost neutral pH 7.4 gave the best results, However, GR-ir in cell nuclei of the supraoptic nucleus (SON) was substantially reduced after application of this perfusion procedure, but could be preserved with fixation method B (Kiss et al., in preparation). The latter perfusion is characterized by a rapid change of pH 6.0 to pH 9.0 and forms a slight modification on the method as described by Berod et al.[3].

Our neuroanatomical distribution study showed a widespread pattern of GR localization in the brain. In the hippocampus, mainly the pyramidal nerve cells in the CA_1 and CA_2 region and the granular cells of the dentate gyrus (DG) expressed immunostaining for GR, while no GR-ir was observed in the CA_3 and CA_4 region of the hippocampus (Fig. 1). In the hypothalamus, the parvocellular neurons of the paraventricular nucleus (PVN) and the arcuate nucleus (AN) showed GR-ir and the SON revealed distinct GR immunostaining in the magnocellular neurons (not shown). A scan of the brainstem showed immunocytochemical detection of GR mainly in the nucleus of the solitary tract (NTS, Fig. 2A,B), the area postrema (AP, Fig. 2A,B) and the locus coeruleus (LC, Fig. 2C,D). In all the targets mentioned immunoreactivity was localized in the cell nuclei. Cytoplasmic GR-ir was faint, thereby difficult to distinguish from background immunostaining.

The effect of steroid administration on GR-ir was examined in the rat hippocampus at 1–2 weeks after ADX (Fig. 3). In the hippocampus, such rats had, at that time interval, almost no GR-immunopositive staining in the cell nucleus (Fig. 3B). After ALDO injection (30 μg/100 g b. wt., s.c., Fig. 3F) or a low dose of CORT (1 μg/100 g b. wt., s.c., not shown), GR-ir was almost nil. A moderate dose of CORT (10 μg/100 g b. wt., Fig. 3C) effectuated an intermediate

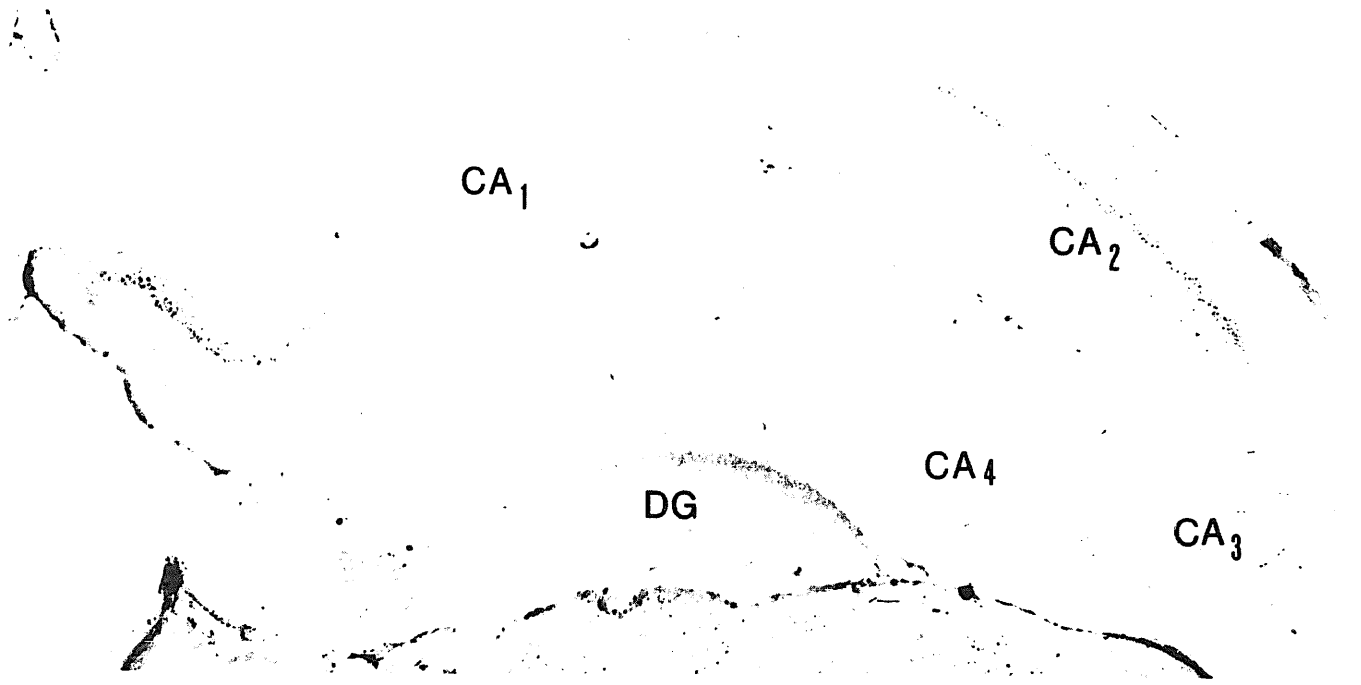

Fig. 1. Distribution of GR-ir in the hippocampus of the rat. Photomicrographs show a coronal section of the hippocampus with the CA_{1-4} pyramidal cell fields and the dentate gyrus (DG), ×39.

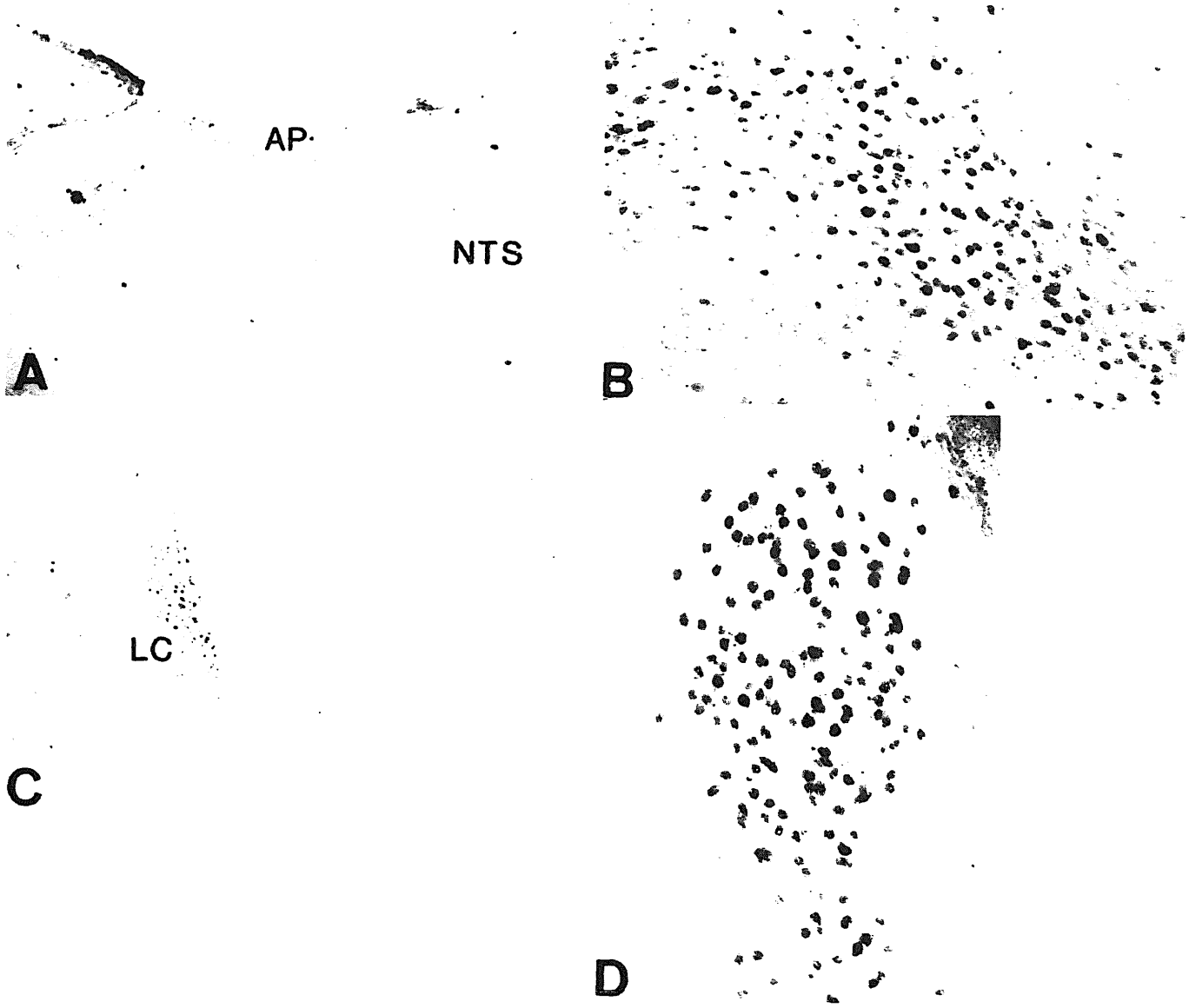

Fig. 2. GR-ir in the brainstem of the rat brain. A: GR-immunopositive neurons in the nucleus of the solitary tract (NTS) and the area postrema (AP). ×39. B: high-power photomicrograph of the NTS. ×137. C: GR immunostaining in the locus coeruleus (LC). ×39. D: high-power photomicrograph of the LC. ×139. Note GR-ir in the cell nuclei of the neurons.

intensity of GR-ir in the cell nuclei, which was comparable to GR-ir in a sham-operated animal (Fig. 3A vs C). Strong GR-ir appeared when the synthetic glucocorticoid RU 28362 was administered. The immunoreaction was very distinct and well-outlined darkly GR-immunostained cell nuclei became visible (Fig. 3E). Although a high dose of CORT induced substantial GR-ir, the cell nuclear intensity appeared to be expressed to a somewhat lesser extent in comparison to application of the pure glucocorticoid analogue (Fig. 3D vs E).

The use of type 2 GR-antagonist, RU 38486, provided striking results concerning the intracellular localization of GR (Fig. 4). GR-ir was predominantly found in the cell nucleus after administration of RU 38486 (1 mg/100 g b. wt., s.c., Fig. 4D). A similarity in nuclear GR-ir could be observed after either GR agonist administration (RU 28362, 100 μg/100 g b. wt., s.c., Fig. 4C), or GR antagonist administration (RU 38486, Fig. 4D) or application of GR antagonist succeeded by GR agonist 30 min later (Fig. 4E). In the 3 situations mentioned, cell-nuclear GR-ir was stronger in intensity in comparison with the sham-operated animal (Fig. 4C–E vs A). With respect to the ADX-rat, GR-ir was close to detection limits in this experiment (Fig. 4B).

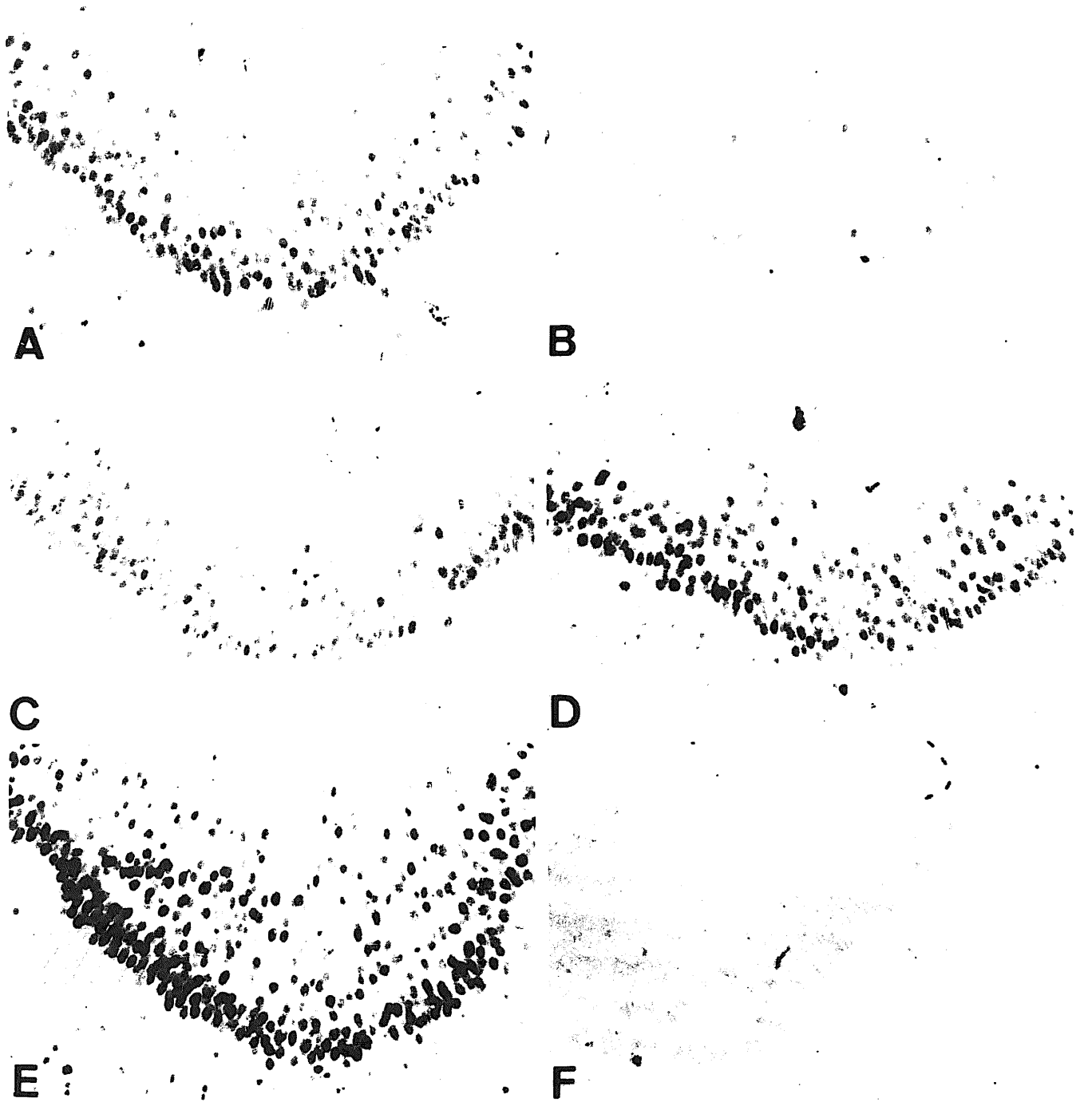

Fig. 3. The effect of s.c. administration of glucocorticoids on GR-ir in the hippocampus. Photomicrographs show cell-nuclear GR-ir in the CA_1 pyramidal neurons of the dorsal subiculum in a sham-operated rat (A) and after corticosteroid injection (CORT, 10 μg/100 g b. wt. (C); 300 μg/100 g b. wt. (D)) or after RU 28362 injection (100 μg/100 g b. wt. (E)) to an ADX rat. No GR-ir is shown after administration of aldosterone (ALDO, 30 μg/100 g b. wt. (F)) and in an ADX rat (B). Note the well-defined darkly GR-immunostained cell nuclei after RU 28362 treatment. ×133.

DISCUSSION

The present study shows a widespread, but uneven regional localization pattern of GR in the rat brain using immunocytochemistry with a monoclonal antibody against the rat liver GR. In the hippocampus, strong cell nuclear GR-ir was found in the CA_1 and CA_2 pyramidal cell fields, moderate GR-ir was detected in the granular cells of the dentate gyrus, while the cell nuclei of CA_3 and CA_4 lacked detectable GR

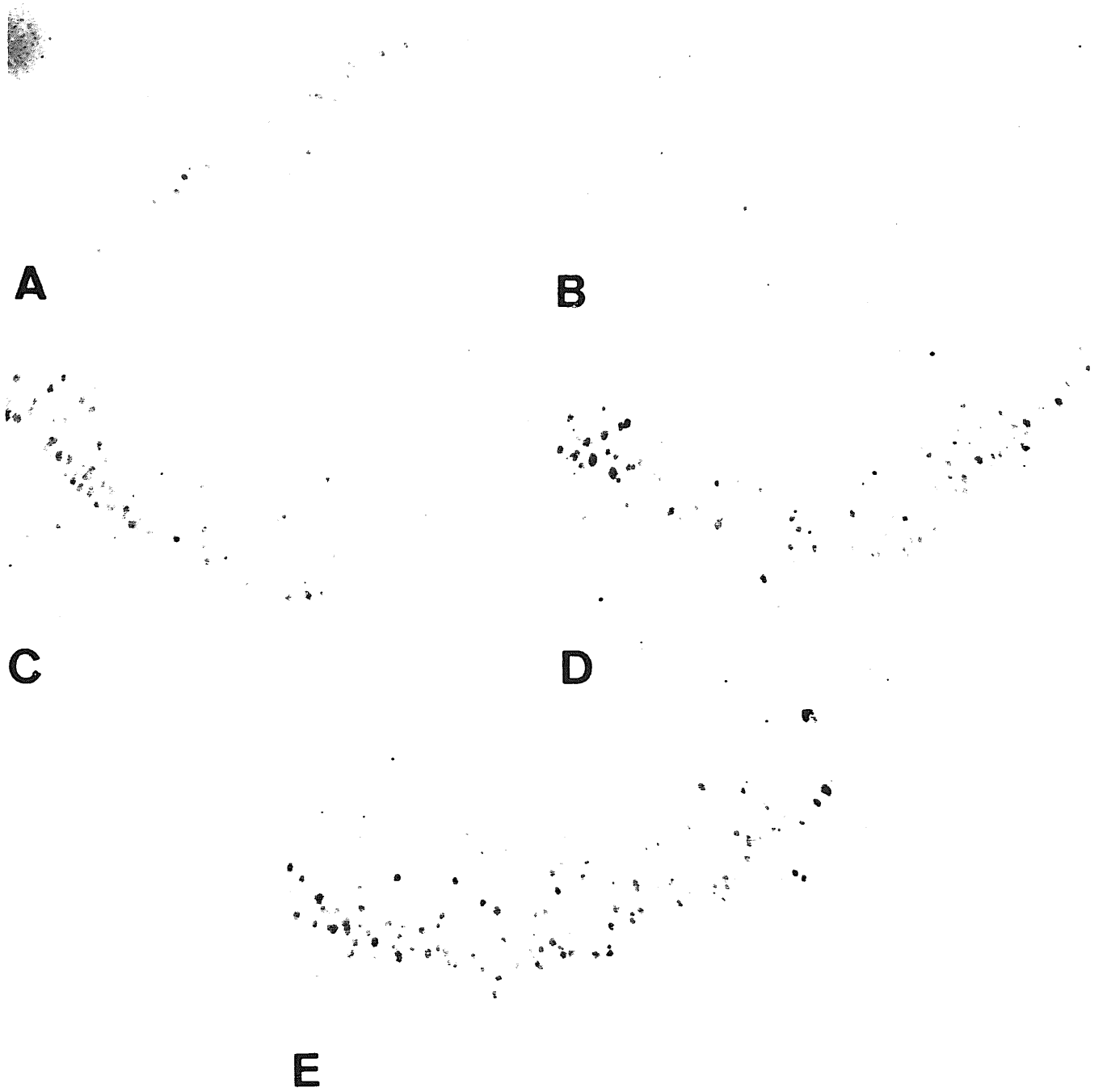

Fig. 4. The effect of antiglucocorticoid administration s.c. on GR-ir in the hippocampus. Photomicrographs show cell-nuclear GR-ir in the pyramidal nerve cells of the dorsal subiculum in a sham-operated rat (A), after injection of GR-agonist RU 28362 (100 μg 100 g b. wt. (C)), after injection of GR antagonist RU 38486 (1 mg/100 g b. wt. (D)) and after administration of GR-antagonist RU 38486 followed by GR agonist RU 28362 30 min later (E). No GR-ir can be detected in the ADX-rat (B). ×136.

immunostaining. In the hypothalamus, in particular in the parvocellular neurons of the PVN and in the AN, GR-ir was found. The brainstem exhibited pronounced GR-ir in particular in the catecholaminergic cell groups of the NTS and the LC, and the AP. These findings are consistent with a recent detailed immunocytochemical report of Fuxe et al.[1,12,13] as well as with cytosol binding and autoradiographical

results of Reul and de Kloet[27,28]. Additionally we have observed strong GR-immunopositive staining in the cell nuclei of the magnocellular neurons of the supraoptic nucleus (Kiss et al., in preparation). Taken together, these data show that type 2 GR are located in abundance in cell groups implicated in the organization of neuroendocrine, autonomous and behavioral responses to stressful stimuli, and suggest that glucocorticoids feedback in these loci on stress-activated circuitry and metabolism[9,17].

The time course of GR-ir after ADX revealed changes in GR immunostaining of the cell nuclei. Parallel with time, in comparison with GR-ir in the sham-operated rat, cell-nuclear GR-ir reduced in intensity up to undetectable levels at 2 weeks after ADX. If the endogenous corticosteroid is required for GR-immunostaining, the slow disappearance from the cell nuclei could be explained by steroid depletion following ADX. However, previous studies involving labelled steroids suggest that such depletion is already completed within approximately 4 h[29]. The ADX-dependent loss of cell nuclear GR-ir could imply translocation of the steroid-devoid GR to the cytoplasmic compartment or be indicative for a different receptor state[21,38]. A similar shift in intracellular GR-ir following ADX was also reported by Fuxe et al[12]. The issue of the intracellular localization of the unoccupied receptor remains controversial[2,21,38]. Unliganded estrogen and progesterone receptors are localized in the nuclear compartment as judged from immunocytochemical and enucleation studies[11,16,35,36]. The histochemical studies were mostly unable to detect extranuclear immunostaining distinct from background staining.

We have taken advantage of the time-dependent loss of GR-ir from the cell nucleus in chosing 7–10 days as the most suitable time point to study the effect of acute administration to ADX rats on the amount of GR-ir in the cell nucleus. Strong cell-nuclear GR-ir was evoked by administration of the potent synthetic glucocorticoid RU 28362 (100 μg/100 g b. wt.,) that binds exclusively to type 2 GR with high affinity[22]. A moderate dose of CORT (10 μg/100 g b. wt.) revealed a cell nuclear GR-ir in an intensity that was comparable to GR-ir in hippocampal cell nuclei of a sham-operated rat, while a high dose of CORT (300 μg/100 g b. wt.) induced a more intensified cell nuclear GR-ir, as has been reported elsewhere[12]. The doses applied resulted in CORT blood levels of 1.2 and 23.3 μg/dl resp., which are normally observed at basal and at diurnal peak or stressful conditions. Circadian differences in GR-ir were observed (not shown), which is in correspondence with the finding that concurrent with a progressive rise in CORT-blood levels during the day, type 2 GR corticoid occupation was up to 50%[27]. A low dose of CORT (1 μg/100 g b. wt., s.c.) was shown to be insufficient to occupy type 2 GR[27], and neither did ALDO, which is known to have a very low affinity for type 2 GR (K_d = ± 30 nM[33]). This may explain why injection of the low dose of CORT or the administration of ALDO did not induce appearance of cell nuclear GR-ir. So far high-resolution autoradiography after administration of tracer doses (± 1 μg) of [^{3}H]ALDO and [^{3}H]CORT to ADX rats, revealed nuclear retention in the CA_1, CA_2 and CA_3 pyramidal neurons and the dentate gyrus neurons of the hippocampus[7,15,26–28,30,34]. These receptors represent the type 1 CR[9]. Accordingly, the present immunocytochemical data could imply a co-localization of type 1 CR and type 2 GR in these target cells. If indeed type 1 and type 2 coexist in the same cell then it is of interest that a moderate dose of CORT (10 μg/100 g b. wt.) gave a substantial GR-ir signal. Frequently, a slightly higher dose of CORT (30 μg/100g b. wt.) has been used in functional studies on behavior and neurotransmission. The specificity of the CORT-effect measured with these parameters suggested binding to type 1 to be a pre-requisite[5,10]. The present study suggests that this dose also produced binding of CORT to type 2.

That also the anti-glucocorticoid RU 38486 evoked a strong GR-ir in the cell nucleus upon acute administration to ADX rats may shed some light on the mode of action of the antagonist. RU 38486 displays a very high affinity to the glucocorticoid receptor[14,23,25]. Our results show that in vivo the RU 38486–GR complex can be detected within the cell nuclear compartment, which is in agreement with previous in vitro studies suggesting that nuclear transfer of the antagonist–receptor complex may occur[4,6,23]. Whatever the mode of action of the antiglucocorticoid is, subsequent administration of the agonist RU 28362 did not alter the GR-ir signal evoked by RU 38486.

In conclusion, our immunocytochemical study shows that GR-ir in the cell nuclear compartment re-

sponds to administration of glucocorticoid agonist and antagonist with an increase in staining intensity, reflecting a high specificity of the antibody for the brain type 2 GR. With regard to the hippocampus, high-resolution autoradiography has demonstrated localization of type 1 CR in the CA_1 and CA_2 pyramidal neurons, shown to contain type 2 GR in the present study. While the type 1 receptor in the hippocampus is thought to be involved in synchronization and activation of daily activities (exploration, food-seeking behavior) and sleep-related events[9,10], the current study opens up the possibility that the very same cells contain type 2 GR sites, which mediate in a time-dependent fashion feedback action of CORT on the stress- and circadian-activated hippocampal circuitry.

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