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The brain mineralocorticoid receptor: greedy for ligand, mysterious in function

Johannes M.H.M. Reul *, Angela Gesing, Susanne Droste, Ingemar S.M. Stec, Anja Weber, Cornelius Bachmann, Alicia Bilang-Bleuel, Florian Holsboer, Astrid C.E. Linthorst

Section of Neuropsychopharmacology, Max Planck Institute of Psychiatry, Kraepelinstrasse 2, 80804 Munich, Germany

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Abstract

Glucocorticoids exert their regulatory effects on the hypothalamic–pituitary–adrenocortical axis via two types of corticosteroid receptors: the glucocorticoid receptor and the mineralocorticoid receptor. Whereas the glucocorticoid receptor has a broad distribution in the brain, highest levels of mineralocorticoid receptor are found in the hippocampus. Based on the differential occupancy profile by endogenous glucocorticoids, glucocorticoid receptors are thought to mediate negative feedback signals of elevated glucocorticoid levels, whereas mineralocorticoid receptors control the inhibitory tone of the hippocampus on hypothalamic–pituitary–adrenocortical axis activity. Dysfunction of mineralocorticoid receptors and glucocorticoid receptors are thought to be implicated in stress-related psychiatric diseases such as major depression. Because of its intriguing features, we focus in this review on the mineralocorticoid receptor and provide data which reveal novel aspects of the pharmacology and physiology of mineralocorticoid receptors. Newly obtained results are presented, which help to solve the paradox of why dexamethasone binds with high affinity to mineralocorticoid receptors in vitro, yet binds poorly in vivo. Until recently, mineralocorticoid receptor protein and mRNA levels could only be routinely studied with in vitro cytosol binding assays, in vitro and in vivo receptor autoradiography, Northern blot analysis, and in situ hybridization. These methods are unfortunately hampered by several flaws, such as the necessity of adrenalectomy, no or poor neuroanatomical resolution, the fact that mRNA does not provide the same information as protein, or combinations of these factors. We present immunohistochemical data on mineralocorticoid receptors in the brain obtained by using commercially available antibodies, which alleviate many of these shortcomings. Furthermore, an in vivo microdialysis method is presented which allows the assessment of free corticosterone levels in the brain, which is critical for the study of the pharmacological basis of mineralocorticoid receptor (and glucocorticoid receptor) function. Finally, a novel aspect of the regulation of mineralocorticoid receptors is described which provides evidence that this receptor system is dynamically regulated. In conjunction with previously reported effects of antidepressants, these results have initiated a new concept on the cause of the hypothalamic–pituitary–adrenocortical axis disturbances often seen in stress-related psychiatric disorders such as major depression.

Keywords: Mineralocorticoid receptor; Glucocorticoid receptor; Hypothalamic–pituitary–adrenocortical axis; Stress; Antidepressants; Major depression

1. Introduction

During almost 40 years of research on glucocorticoid hormone binding receptors in the central nervous system, it has become clear that two types of such receptors exist: the glucocorticoid receptor and the mineralocorticoid receptor (for review, see De Kloet et al., 1998). Differentiation of these receptor sites was accomplished with pharmacological, neuroanatomical and molecular cloning techniques. In concert, as ligand-dependent transcription factors (Trapp et al., 1994), mineralocorticoid receptors and glucocorticoid receptors convey signals of a broad range of glucocorticoid hormone concentrations to the genome. Given that the concentration of circulating glucocorticoid hormone varies over the diurnal cycle, mineralocorticoid receptors and glucocorticoid receptors regulate both ongoing processes as well as mechanisms during and after stress (Reul and De Kloet, 1985; De Kloet and Reul, 1987; De Kloet et al., 1998).

* Corresponding author. Tel.: +49-89-30622-443; fax: +49-89-30622-371.

E-mail address: reul@mpipsykl.mpg.de (J.M.H.M. Reul).

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Owing to its particular properties ("greedy for ligand") and its still not clarified functions ("mysterious in function"), the mineralocorticoid receptor is an intriguing receptor and is of sufficient interest to be highlighted here.

2. Discovery of mineralocorticoid receptor in the brain

The presence of mineralocorticoid receptors in the brain was discovered at the end of the 1960s (McEwen et al., 1968; Gerlach and McEwen, 1972). Bruce McEwen and colleagues injected adrenalectomized rats with a tracer dose of [^{3}H]corticosterone and observed the uptake and retention of the radioactive label mainly in pyramidal and granular neurons of the hippocampus, the dorsolateral septum and brain stem motor nuclei. To a much lesser extent, the label was taken up and retained in the central nucleus of the amygdala and the cerebral cortex (McEwen et al., 1968). As the injected tracer was a glucocorticoid hormone, it was thought at the time that the receptor binding [^{3}H]corticosterone was the glucocorticoid receptor. However, during the following decades, data were collected which indicated that this was not the complete story. De Kloet et al. (1975) discovered that [^{3}H]dexamethasone, a synthetic glucocorticoid hormone, when injected in adrenalectomized rats was not taken up by pyramidal and granular neurons in the hippocampus but instead showed a diffuse uptake all over the brain and high retention in the pituitary. This observation was the first indication that [^{3}H]corticosterone and [^{3}H]dexamethasone might bind to different receptor sites. Confusing at the time was also the observation that injection of [^{3}H]aldosterone, a natural mineralocorticoid, resulted in a similar uptake and retention pattern as [^{3}H]corticosterone (Birmingham et al., 1979; De Nicola et al., 1981). Furthermore, the newly developed cell-free "cytosolic" binding assays using [^{3}H]corticosterone and [^{3}H]dexamethasone as ligands revealed a much broader presence of glucocorticoid-binding sites in the brain than was expected from the in vivo uptake studies (De Kloet et al., 1975).

The field made a major leap forward in the early 1980s when the selective glucocorticoid receptor ligands, RU 26988 (11β,17β-dihydroxy-17α-(1-propynyl)-androsta-1,4,6-triene-3-one) and RU 28362 (11β,17β-dihydroxy-6-methyl-17α-(1-propynyl)-androsta-1,4,6-triene-3-one), became available (Philibert and Moguilewsky, 1983). In contrast to natural glucocorticoids, these ligands have no affinity for binding to mineralocorticoid receptors. The use of these ligands made it possible to distinguish between binding of [^{3}H]corticosterone to two different receptor types, the mineralocorticoid receptor (also called corticosterone-preferring or type I receptor) and the glucocorticoid receptor (also called type II receptor) (Moguilewsky and Raynaud, 1980; Veldhuis et al., 1982; Krozowski and Funder, 1983). Studies conducted to gain insight into the putative receptor heterogeneity made use of physicochemical methods such as isoelectric focusing (Wrange and Yu, 1983) and density gradient centrifugation (Coirini et al., 1983), and (competition) binding assays with selective ligands (Anderson and Fanestil, 1976; Moguilewsky and Raynaud, 1980; De Nicola et al., 1981; Veldhuis et al., 1982; Krozowski and Funder, 1983; Coirini et al., 1983; Beaumont and Fanestil, 1983; Wrange and Yu, 1983; Reul and De Kloet, 1985). Apart from these studies in rats, the RU ligands were used to distinguish between mineralocorticoid receptor and glucocorticoid receptor populations in mice (Luttge et al., 1989; Reul, unpublished) and dogs (Reul et al., 1990a).

Importantly, radioligand binding assays revealed a marked difference between the binding affinity of [^{3}H]corticosterone at mineralocorticoid receptors and at glucocorticoid receptors. Reul and de Kloet (1985) determined the apparent binding affinity (K_d) of [^{3}H]corticosterone at mineralocorticoid receptors and glucocorticoid receptors to be ≈ 0.5 and 2–5 nM, respectively. Nowadays, due to longer incubation times (overnight instead of 3–6 h; thus, better approaching complete binding equilibrium) than in the older publications, K_d values of [^{3}H]corticosterone and [^{3}H]aldosterone range between 0.1 and 0.3 nM (Reul et al., 1993, 1994a,b). This more than 10-fold difference between the affinity values (mineralocorticoid receptor vs. glucocorticoid receptor for binding corticosterone) proved to have tremendous consequences for the occupancy of mineralocorticoid receptors and glucocorticoid receptors during baseline and stress conditions and, thus, for the conceptual understanding of glucocorticoid hormone action in the brain (Reul and De Kloet, 1985; De Kloet and Reul, 1987; Reul et al., 1987; De Kloet et al., 1998). Reul and De Kloet observed in rats killed early in the morning (i.e., at the trough of the hypothalamic–pituitary–adrenocortical axis diurnal rhythm; plasma corticosterone level < 1.5 μg/100 ml) that mineralocorticoid receptors were already occupied for more than 80% by endogenous ligands (presumably corticosterone), whereas glucocorticoid receptors were only about 10% occupied under these conditions (Reul and De Kloet, 1985). In contrast, glucocorticoid receptors became substantially occupied only under conditions of higher plasma corticosterone levels (> 5–10 μg/100 ml) such as occurring at the circadian peak and during stress (Reul and De Kloet, 1985; Reul et al., 1987). They also showed that after injection of minute amounts of corticosterone (i.e., 1 μg/100 g body weight) in adrenalectomized rats, already more than 80% of hippocampal mineralocorticoid receptors were occupied, whereas glucocorticoid receptors remained virtually unoccupied (Reul and De Kloet, 1985). Given that this dose is equivalent to the tracer dosages administered to rats for the uptake and retention studies, it is clear that the receptor type labeled in those studies (McEwen et al., 1968; Gerlach and McEwen, 1972; Warembourg, 1975) is the mineralocorticoid receptor and not the glucocorticoid receptor.

3. Visualization or mineralocorticoid receptor in the brain

The newly developed glucocorticoid analogs RU 26988 and RU 28362 made it possible to investigate the topography of mineralocorticoid receptor and glucocorticoid receptor in more detail. Using a cytosol binding assay on micropunched brain material, Reul and De Kloet (1985) showed that highest mineralocorticoid receptor levels were present in the dorsal subiculum and CA1 area of the hippocampus, and in the dentate gyrus. The CA3 area expressed a lower receptor density. A further step in neuroanatomical resolution was made possible by using a method called in vitro autoradiography, in which cryosections of brain are incubated with radioligands. In this manner, mineralocorticoid receptors and glucocorticoid receptors could be visualized by autoradiography in brain sections. This method confirmed that mineralocorticoid receptors are mainly present in the hippocampus and dorsolateral septum, and that within the hippocampus these receptor sites are heterogeneously localized. Highest levels of mineralocorticoid receptors were observed in the pyramidal layer of the CA1 and CA2 and in the granular layer of the dentate gyrus (Sarrieau et al., 1984; Reul and De Kloet, 1986). Glucocorticoid receptors were found to be ubiquitously distributed in the brain with high levels in the cerebral cortex, hippocampus, thalamus and paraventricular and supraoptic nucleus of the hypothalamus (Reul and De Kloet, 1986; Kiss et al., 1988).

The cloning of the cDNA's for mineralocorticoid receptors (Arriza et al., 1987) and glucocorticoid receptors (Hollenberg et al., 1985) in the 1980s opened the possibility to perform in situ hybridization studies to visualize the mRNA (and hnRNA) species for these corticosteroid receptors in the brain (Van Eekelen et al., 1988; Herman et al., 1989a). The pattern of mineralocorticoid receptor mRNA expression in the hippocampus is similar to that shown for in vitro autoradiography of mineralocorticoid receptor binding, with highest levels in the CA1–CA2 pyramidal layer and moderate levels in the pyramidal layer of CA3 and granular layer of the dentate gyrus. Glucocorticoid receptor mRNA expression is present in both neurons and glia and, in the hippocampus, it is highest in CA1–CA2, moderate in the dentate gyrus and low in the CA3 area (Van Eekelen et al., 1988; Herman et al., 1989a).

When studying receptor binding properties, mRNA expression and/or neuroanatomical topography of corticosteroid receptors, the pros and cons of the applied methodology should be borne in mind. The great advantage of cytosol binding assays is the highly quantitative output, allowing the assessment of maximal binding capacity (B_{max}), affinity constants (K_d) and association (K_1)/dissociation (K_{-1}) kinetics (f.i. Reul et al., 1990a and see below). Nevertheless, a major drawback is the necessity of using adrenalectomized animals because endogenous corticosteroids severely hamper the determination of binding parameters. It cannot be excluded, however, that adrenalectomy might be a confounding factor for the assessment of mineralocorticoid receptor and glucocorticoid receptor levels. These considerations do not apply for the determination of corticosteroid receptor mRNA levels as endogenous hormones are in this case irrelevant for the measurement. Although it has often been reasoned that mRNA levels would vary in parallel with protein or binding levels, there is clear-cut evidence that this is not the case. Study of the changes in mineralocorticoid receptor mRNA and mineralocorticoid receptor binding over time after adrenalectomy and treatment with placebo or dexamethasone revealed that these variables followed disparate time courses as a result of either treatment (Reul et al., 1989). Thus, detection of mRNA by in situ hybridization can provide data on the topographical distribution of transcriptional activity of corticosteroid receptor genes, but cannot replace the information obtained by measuring binding capacity or protein levels. Moreover, in vitro autoradiography and in situ hybridization share the drawback that only the signal of cell groups can be detected, and not, or limitedly, the signal of individual cells. An exception to the latter remark may be the counting of silver grains using the cover slip method of autoradiography, which is quite tedious and often has poor resolution.

Due to the recent commercial availability of specific antibodies raised against mineralocorticoid receptors (Santa Cruz Biotechnology, Santa Cruz, CA), it is now possible to study with immunohistochemistry and Western blot analysis this receptor type in rats (Gesing et al., submitted for publication), mice (see below) and possibly other species including humans (as yet unknown). In this way, adrenalectomy is no longer required for the determination of receptor binding capacity. Importantly, since the mineralocorticoid receptor antibody recognizes both the occupied and the unoccupied receptor (Gesing et al., submitted for publication), it is possible to estimate the total mineralocorticoid receptor population. Fig. 1 shows the distribution of mineralocorticoid receptor immunoreactivity in the dorsal hippocampus of the mouse. It is clear that, overall, highest mineralocorticoid receptor concentrations are present in the pyramidal neurons of the medial portion of CA1 ("CA1a") and CA2 cell field. Moderate levels of mineralocorticoid receptors are found in the pyramidal neurons of the lateral portion of CA1 ("CA1b") and in the granular neurons of the dentate gyrus, whereas a lower staining intensity is observed in the pyramidal neurons of the CA3 cell field. Furthermore, neurons expressing considerable levels of mineralocorticoid receptors were observed in the dorsolateral septum, the central nucleus of the amygdala, the amygdalo–hippocampal nucleus, induseum griseum and tenia tecta (not shown). Generally, this staining pattern is similar to the mineralocorticoid receptor labeling results obtained with in vitro autoradiography (Reul and De Kloet, 1986, rat; Reul, unpublished, mouse).

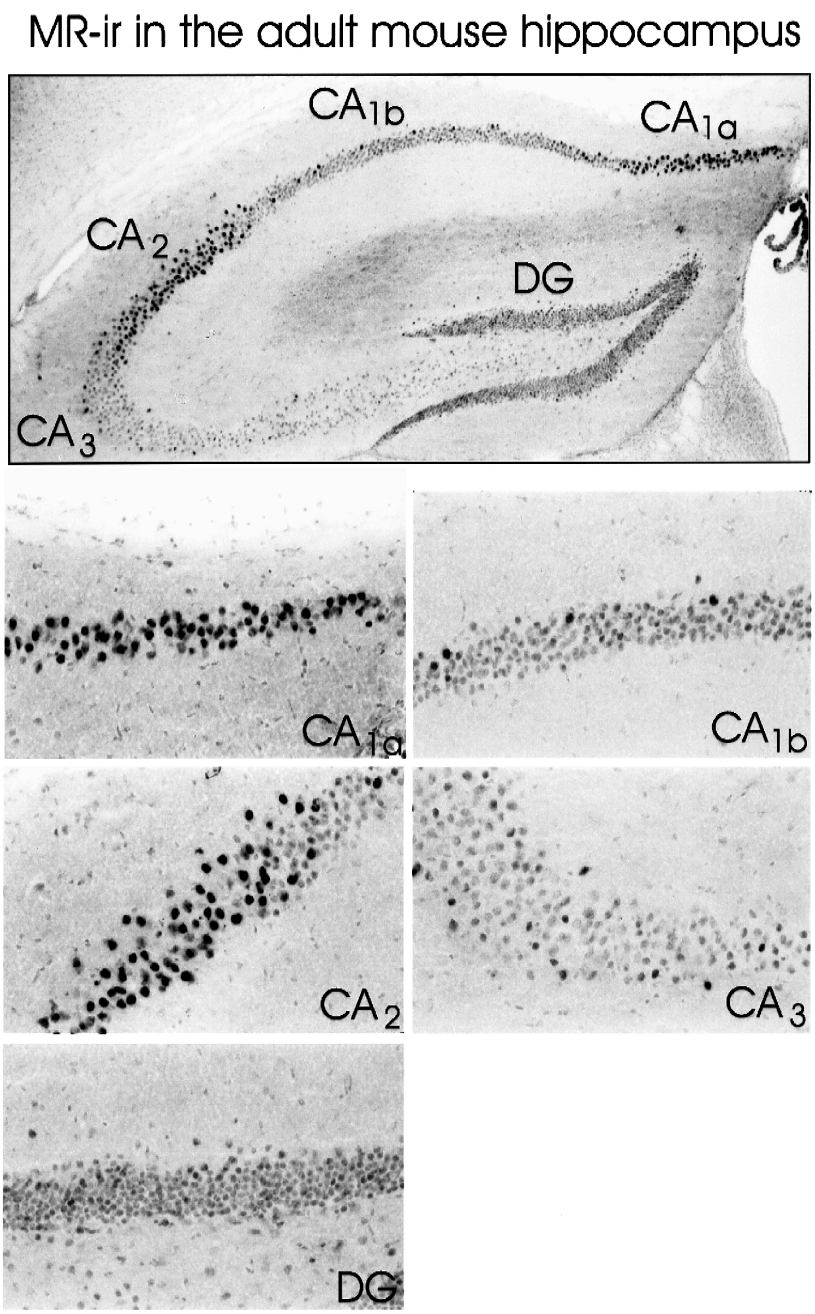


Fig. 1. Immunohistochemistry of mineralocorticoid receptors in the dorsal hippocampus of a male adult mouse (C57BL6). Cryosections (20 μm) of mouse brain were post-fixed with 4% paraformaldehyde and incubated overnight with a goat anti-mineralocorticoid receptor antibody (dilution 1:800; Santa Cruz). Using standard protocols, mineralocorticoid receptor immunoreactivity was visualized by diaminobenzidine with Ni^{+} enhancement (Gesing et al., submitted for publication). CA_{1-3}, pyramidal layers of the hippocampus; dentate gyrus (DG). The CA_1 subfield is distinguished in (a) and (b) area because of differences in morphology and mineralocorticoid receptor staining pattern. An overview image (top) of the whole dorsal hippocampus is shown (magnification 20 ×) as well as detailed images of CA_{1-3} and DG (magnification 320 ×). Western blot analysis of hippocampal extracts revealed one band of mineralocorticoid receptor immunoreactivity with an apparent molecular weight of 116 kDa (not shown). Preabsorption of anti-mineralocorticoid receptor antibody with blocking peptide completely blocked immunoreactivity in brain sections and Western blots (not shown).

However, the pictures taken at higher magnification (320 ×) illustrate the advantage of immunolabeling mineralocorticoid receptors: the labeling of individual neurons is easily discernable (Fig. 1). Also, the mineralocorticoid receptor signal is localized exclusively in the nucleus, irrespective of whether the animals were killed under baseline or stress conditions. Thus, the ability to detect mineralocorticoid receptors in individual neuronal nuclei makes it possible to determine the spectrum of mineralocorticoid receptor density among individual neurons within a given subfield or region. It is clear from Fig. 1 that there are marked variations in the mineralocorticoid receptor

content among neurons in the different hippocampal subfields and in the dentate gyrus granular layer, i.e., there are high and low mineralocorticoid receptor expressing neurons. The density of mineralocorticoid receptor immunoreactivity in individual neuronal nuclei can be determined with a video image analysis program that provides data on the Gaussian distribution of mineralocorticoid receptor density in a neuronal population (data not shown) and on the mean mineralocorticoid receptor density in selected subfield or areas (Gesing et al., submitted for publication). The reason for the heterogeneous neuronal expression of mineralocorticoid receptors in the subfields is currently unknown, but the availability of these antibodies allows detailed studies using double/triple immunofluorescence, eventually combined with tracing methods to further assess the phenotype and the neuroanatomical context of these neurons. Using confocal scanning laser microscopy and double labeling immunocytochemistry, Van Steensel et al. (1996) showed that mineralocorticoid receptors and glucocorticoid receptors are colocalized in many clusters in nuclei of rat pyramidal CA1 neurons.

4. Ligand-binding preferences: the dexamethasone paradox

Numerous in vitro competition binding studies with a variety of species including rat, mouse, hamster, dog and human have shown that the mineralocorticoid receptor prefers to bind natural glucocorticoid (i.e., corticosterone, cortisol) and mineralocorticoid hormones (i.e., aldosterone, deoxycorticosterone) (Veldhuis et al., 1982; Krozowski and Funder, 1983; Coirini et al., 1983; Sutanto and De Kloet, 1987; Luttge et al., 1989; Reul et al., 1990a; Rupprecht et al., 1993). In contrast, glucocorticoid receptors preferentially bind synthetic glucocorticoids such as dexamethasone and RU 28362, moderately bind natural glucocorticoids, and hardly bind mineralocorticoid hormones (Veldhuis et al., 1982; Arriza et al., 1987; Sutanto and De Kloet, 1987; Reul et al., 1990a; Rupprecht et al., 1993). If one considers the literature on the dexamethasone–mineralocorticoid receptor interaction during the course of the last 25 years, strikingly paradoxical observations have been made. The paradox essentially boils down to the different in vivo vs. in vitro binding properties of dexamethasone at mineralocorticoid receptors. De Kloet et al. (1975) reported that [^{3}H]dexamethasone, in contrast to [^{3}H]corticosterone was not taken up and retained by mineralocorticoid receptors (see above) in hippocampal neurons of adrenalectomized rats. However, in vitro, dexamethasone has been shown in several species (rat (Davies and Watkins, 1982), mouse (Luttge et al., 1989), dog (Reul et al., 1990a), human (Arriza et al., 1987; Rupprecht et al., 1993), but not hamster (Sutanto and De Kloet, 1987)) to bind with high affinity (K_d 0.5–1 nM) to hippocampal mineralocorticoid receptors. For this reason, and because dexamethasone does not bind to corticosterone-binding globulin (CBG, transcortin), [^{3}H]dexamethasone has been used by some investigators as a ligand for determining the mineralocorticoid receptor binding capacity of brain cytosol preparations (Brinton and McEwen, 1988; Chao et al., 1989; Spencer et al., 1990; Dhabhar et al., 1995). In vivo, dexamethasone exerts hardly any agonistic effects via kidney or brain mineralocorticoid receptors (Funder and Sheppard, 1987; De Kloet, 1991; De Kloet et al., 1998), and might even act as an mineralocorticoid receptor antagonist in the brain (Bohus and De Kloet, 1981). Bohus and De Kloet showed that dexamethasone antagonizes the effect of corticosterone on forced extinction of a passive avoidance response. The question is how to explain the incapacity of dexamethasone to be bound and retained in mineralocorticoid receptor-containing tissue and to produce mineralocorticoid receptor-mediated effects in the face of its high in vitro binding affinity for this receptor.

One factor that limits the access of dexamethasone to mineralocorticoid receptors is the mdr1a P-glycoprotein, which is expressed in the apical membranes of endothelial cells of the blood–brain barrier (Cordon-Cardo et al., 1989). This protein acts as an energy-dependent pump, removing xenobiotic substances including synthetic steroids from the brain parenchyma. This notion was further substantiated in mutant mice with a defunct mdr1a gene which showed an increased uptake of tracer amounts of [^{3}H]dexamethasone in the brain as compared to wild-type mice (Schinkel et al., 1995). De Kloet (1997) and Meijer et al. (1998) expanded these observations by showing that adrenalectomized mdr1a mutant mice have a higher uptake of [^{3}H]dexamethasone in brain than control mice, with cell nuclear uptake in the hippocampus and paraventricular nucleus being up to 10-fold higher. However, despite the importance of these observations, they do not explain why dexamethasone does not bind to renal mineralocorticoid receptors in vivo (Funder et al., 1973; Funder and Sheppard, 1987). The presence of the enzyme 11β-hydroxysteroid dehydrogenase (which physiologically metabolizes natural glucocorticoids, thereby allowing the "selective" access of mineralocorticoid hormones to mineralocorticoid receptors) in this tissue is irrelevant as dexamethasone is not a substrate for this enzyme (Funder et al., 1988; Edwards et al., 1988; Monder, 1991). In addition, the mdr1a P-glycoprotein, although present in the kidney (see references in Schinkel et al., 1995), does not seem to remove dexamethasone from this organ (Schinkel et al., 1995). Thus, a limited access of dexamethasone to the brain cannot be the whole reason why this synthetic glucocorticoid does not bind and activate mineralocorticoid receptors in the brain.

Further evidence that mineralocorticoid receptors in brain resist binding of dexamethasone in vivo comes from receptor occupancy experiments. Administration of dexamethasone (25 μg/100 g body weight) to rats resulted in a rise in the occupancy of glucocorticoid receptors, whereas

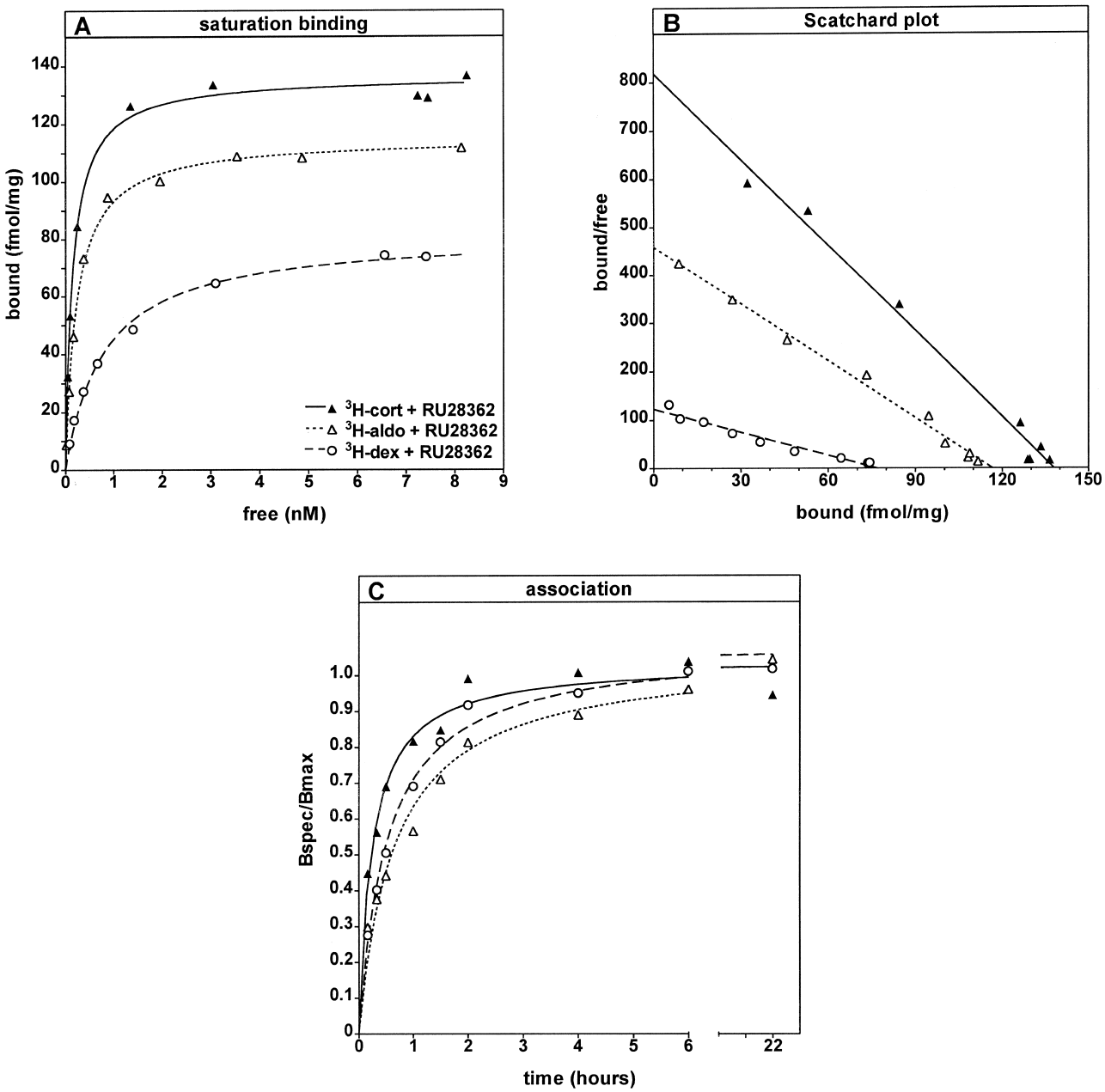


Fig. 2. In vitro binding properties of [^{3}H]corticosterone, [^{3}H]aldosterone and [^{3}H]dexamethasone regarding the rat hippocampal mineralocorticoid receptor. Cytosol was prepared from hippocampus of adrenalectomized adult male rats and binding assays were performed basically as described previously (Reul et al., 1993, 1994a,b). To prevent [^{3}H]steroids from binding to glucocorticoid receptor, a 100-fold excess of RU 28362 was included. (A) Saturation binding (only the specific binding is shown) was determined by incubation of cytosol with [^{3}H]steroids at a concentration range of 0.05–9 nM for 20–24 h. (B) Scatchard plot analysis based on saturation binding shown in (A). (C) For association binding (specific binding only), cytosol was incubated with [^{3}H]steroids (15 nM) + RU 28362 and, after the start of incubation, aliquots were taken at various time points (10 min–22 h). (D) For dissociation binding (specific binding), after 22 h of incubation with [^{3}H]steroids, a 1000-fold excess of the corresponding nonradioactive steroid was added and aliquots were taken after various time intervals (0–48 h). The observed association (K_{obs}) and dissociation (K_{-1}) rates were analyzed with a curve fitting program (Bylund and Yamamura, 1990). The association constant K_1 and the derived affinity constant K_{Dkin} were calculated from the equations $K_1 = (K_{obs} - K_{-1})/[[^3H]steroid]$ and $K_{Dkin} = K_{-1}/K_1$. For calculated binding parameters, see Table 1. (E) For determination of stability of [^{3}H]steroid–receptor complexes, cytosol (without the receptor complex stabilizer sodium molybdate) was incubated with [^{3}H]steroid + RU 28362 or with [^{3}H]dexamethasone alone for 18 h, after which, either 0.1 M (final concentration) KSCN was added for 30 min, or cytosol mixtures were brought to 25°C for 30 min, or mixtures were left at 0–4°C (= control). Thereafter, free and bound [^{3}H]steroid were separated (for details, see above-mentioned references) and specific binding (expressed as fmol/200 μl, mean ± S.E.M.) was determined. [^{3}H]dexamethasone binding to glucocorticoid receptor was determined by subtracting the specific binding of [^{3}H]dexamethasone + RU 28362 from that of [^{3}H]dexamethasone alone. In all binding assays, also nonspecific binding of [^{3}H]steroids was determined (not shown). Here, data for one representative experiment out of three independent experiments are shown, except for (E): $n = 3$ independent experiments.

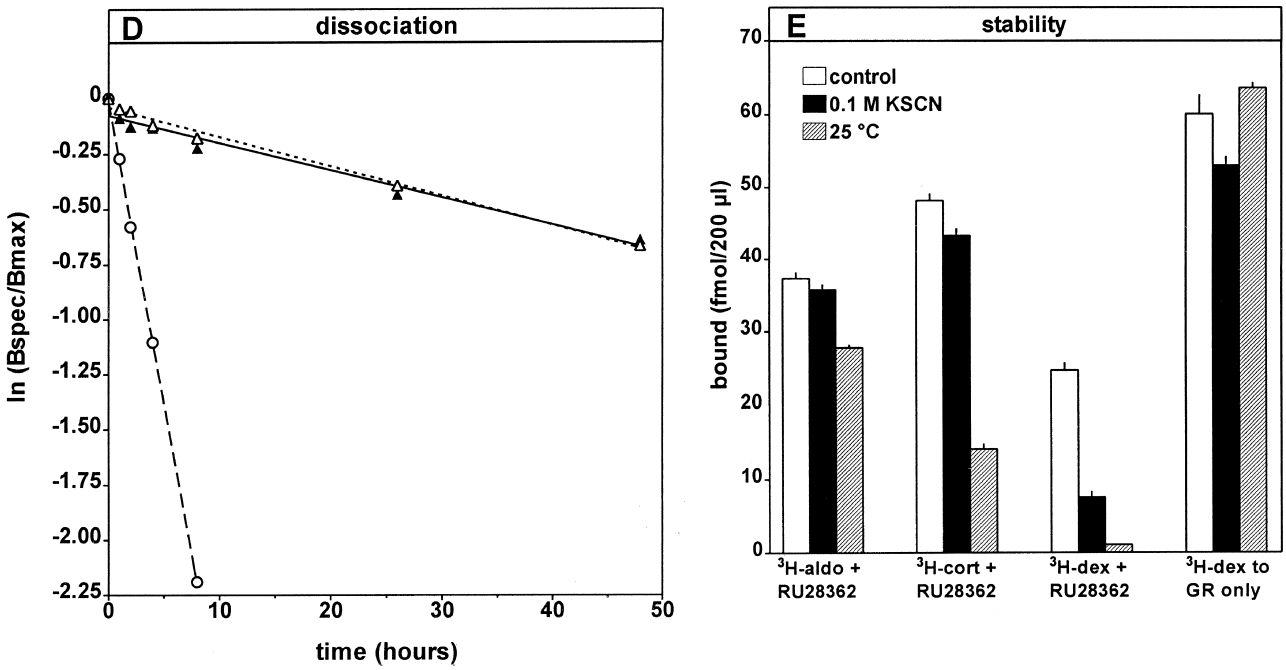


Fig. 2 (*continued*).

mineralocorticoid receptor occupancy was not affected (Reul et al., 1987). Thus, although a considerable dose of dexamethasone was injected and although mineralocorticoid receptors and glucocorticoid receptors display comparable affinity constants for binding of this steroid in vitro, the resultant occupancy changes were markedly different. In addition, the observation that glucocorticoid receptors indeed became occupied by dexamethasone proves that the steroid is able to penetrate the blood–brain barrier and enter brain cells.

To further elucidate the discrepancy between mineralocorticoid receptor binding of dexamethasone in vitro vs. in vivo, we conducted a series of binding experiments in which we compared the saturation binding, association and dissociation kinetics, and stability of [^{3}H]ligand-receptor complexes after activation of [^{3}H]corticosterone, [^{3}H]aldosterone and [^{3}H]dexamethasone at rat hippocampal mineralocorticoid receptors (Fig. 2; Stec and Reul, unpublished). To exclude binding of these ligands to glucocorticoid receptors, a 100-fold excess of the selective glucocorticoid RU 28362 was included in the assays. Table 1 depicts a summary of the binding parameters of the three ligands for their interaction with mineralocorticoid receptors. Saturation binding (Fig. 2A) and Scatchard analyses (Fig. 2B) showed that [^{3}H]dexamethasone bound to mineralocorticoid receptors with an approximately three- to four-fold lower affinity ("K_{Dsat}" in Table 1) and a 30% lower maximal binding capacity ("B_{max}" in Table 1) than [^{3}H]corticosterone and [^{3}H]aldosterone. Thus, it seems as if [^{3}H]dexamethasone can detect only about 70% of the total number of mineralocorticoid receptors in the cytosol preparation. The apparent discrepancy between [^{3}H]corticosterone and [^{3}H]aldosterone is due to contamination of the cytosol by CBG (of approximately 20 fmol/mg protein), to which [^{3}H]corticosterone binds whereas [^{3}H]aldosterone and [^{3}H]dexamethasone do not. An explanation for the aberrant B_{max} value determined with [^{3}H]dexamethasone can be derived from the kinetic

Table 1
In vitro binding parameters of ^{3}H-labeled corticosterone, aldosterone and dexamethasone in hippocampal cytosol of adrenalectomized rats

^{3}H-steroid	K_1 (M^{-1} min^{-1})	K_{-1} (min^{-1})	K_{Dkin} (nM)	K_{Dsat} (nM)	B_{max} (fmol/mg)
Corticosterone	1.5×10^6	1.8×10^{-4}	0.12	0.17	138
Aldosterone	6.1×10^5	2.0×10^{-4}	0.33	0.25	117
Dexamethasone	1.3×10^6	4.4×10^{-3}	3.42	0.67	81

K_1, association rate; K_{-1}, dissociation rate; K_{Dkin}, affinity binding constant derived from K_1 and K_{-1} ($K_{Dkin} = K_{-1}/K_1$); K_{Dsat}, affinity binding constant derived from saturation binding and Scatchard analysis. Calculation of parameters is based on data presented in Fig. 2.

experiments. Whereas the association binding rate of [^{3}H]dexamethasone is almost as high as that of [^{3}H]corticosterone, the dissociation rate is markedly (i.e., more than 20-fold) higher than that of the two other [^{3}H]steroids (Fig. 2C and D, Table 1). These differences in association and dissociation kinetics result in different K_{Dkin} constants (i.e., affinity binding constants derived from the kinetic binding parameters K_1 (i.e., association rate constant) and K_{-1} (i.e., dissociation rate constant); $K_{Dkin} = K_{-1}/K_1$; Table 1). However, the affinity binding constants derived from saturation (K_{Dsat}) and kinetic (K_{Dkin}) analysis should be comparable, which was indeed the case for [^{3}H]corticosterone and [^{3}H]aldosterone but not for [^{3}H]dexamethasone. Regarding the latter ligand, there was a large discrepancy in affinity binding constant values (0.67 vs. 3.42 nM), the reason for which is unclear, but may involve the extremely high off-rate of [^{3}H]dexamethasone from mineralocorticoid receptors (Table 1). Based on these novel (cf. [^{3}H]dexamethasone) binding data, it may be concluded that [^{3}H]dexamethasone binds to mineralocorticoid receptors in an unstable fashion.

This was further substantiated in an experiment in which the stability of the three [^{3}H]steroid–mineralocorticoid receptor complexes was tested by activating the complexes with either potassium thiocyanate (KSCN) or heat (25°C) (Fig. 2E). Fig. 2E shows that [^{3}H]dexamethasone–mineralocorticoid receptor complexes are much less stable than complexes with the other two ligands, thus underscoring the relatively fragile interaction of dexamethasone with the mineralocorticoid receptor. In contrast to the labile ^{3}H-dexamethasone–mineralocorticoid receptor complexes, ^{3}H-dexamethasone–glucocorticoid receptor complexes are quite stable (Fig. 2E). We propose that these particular characteristics of how dexamethasone interacts with mineralocorticoid receptors contribute substantially to the inability of this ligand to activate mineralocorticoid receptors in vivo. Moreover, dexamethasone might even have antagonistic properties toward the mineralocorticoid receptor (Bohus and De Kloet, 1981).

5. Role of mineralocorticoid receptors in hypothalamic–pituitary–adrenocortical axis regulation

5.1. Differential occupancy of mineralocorticoid receptors and glucocorticoid receptors

Both corticosteroid receptor types, mineralocorticoid receptor and glucocorticoid receptor, are primary regulators of the hypothalamic–pituitary–adrenocortical axis. It was recognized early that the glucocorticoid receptor is the receptor which mediates negative feedback signals of naturally occurring or synthetic glucocorticoid hormones (Keller-Wood and Dallman, 1984; Antoni, 1986; Dallman et al., 1987). The site of these negative feedback effects is mainly the PVN and the anterior pituitary (reviewed by De Kloet et al., 1998), although synthetic glucocorticoids such as dexamethasone can act more potently at the pituitary level because these steroids are not impeded by the intracellular CBG-like molecules present in pituitary cells (De Kloet et al., 1977; Hammond, 1990). On the basis of lesion and electrical stimulation studies, it is thought that the hippocampus, i.e., the site where mineralocorticoid receptors are mainly localized, has an overall inhibitory influence on the activity of the hypothalamic–pituitary–adrenocortical axis (reviewed by Jacobson and Sapolsky, 1991). Hence, ablation of the inhibitory influence of the hippocampus by dorsal hippocampectomy or fornix transection results in a rise in baseline hypothalamic–pituitary–adrenocortical axis activity and increased corticotropin-releasing hormone (CRH) mRNA and vasopressin mRNA expression in the paraventricular during the morning hours (i.e., at the trough of the diurnal hypothalamic–pituitary–adrenocortical activity cycle (Herman et al., 1989b). The role of hippocampal mineralocorticoid receptors in hypothalamic–pituitary–adrenocortical regulation was, however, unclear for some time. A step forward was made when occupancy studies showed that, due to the high affinity for endogenous glucocorticoids, hippocampal mineralocorticoid receptors are more than 80% occupied at the trough of the diurnal hypothalamic–pituitary–adrenocortical cycle (Reul and De Kloet, 1985; Reul et al., 1987, 1990b). On the basis of the different occupancy of mineralocorticoid receptors (i.e., always $\approx$ 80%) and glucocorticoid receptors (i.e., high only at circadian peak or after stress), it was proposed that mineralocorticoid receptors mediate the tonic inhibitory control of the hippocampus on hypothalamic–pituitary–adrenocortical activity, whereas glucocorticoid receptors mediate the negative feedback of elevated glucocorticoid levels to restrain hypothalamic–pituitary–adrenocortical drive (De Kloet and Reul, 1987). In the following years, this concept was further substantiated by studies using selective mineralocorticoid receptor antagonists and animal models with decreased mineralocorticoid receptor levels. Intracerebroventricular (i.c.v.) and intrahippocampal injection of the synthetic mineralocorticoid receptor antagonist RU 28318 results in an elevation of baseline corticosterone levels (Ratka et al., 1989; Oitzl et al., 1995; Van Haarst et al., 1997, Gesing et al., submitted for publication). Rats treated with RU 28318 or with antisense oligodeoxynucleotides against mineralocorticoid receptor mRNA show increased hypothalamic–pituitary–adrenocortical responses to psychological stressors (Ratka et al., 1989; Reul et al., 1997). Implantation of corticosterone-, but not dexamethasone-releasing, pellets in the dorsal hippocampus results in a suppression of adrenalectomy-induced increases in plasma adrenocorticotropin (ACTH) (Kovacs and Makara, 1988). Except for one report (Michelson et al., 1994), studies in humans show that treatment with the mineralocorticoid receptor antagonists, spironolactone and canrenoate, results in an elevation of baseline hypothalamic–pituitary–adrenocorti-

cal axis activity (Born et al., 1991; Dodt et al., 1993; Deuschle et al., 1998). Aged rats, which generally have reduced hippocampal levels of mineralocorticoid receptors (and also often of glucocorticoid receptors), show elevated baseline levels of hypothalamic–pituitary–adrenocortical hormones and extended stress-induced responses (Reul et al., 1988; Van Eekelen et al., 1991; Cizza et al., 1994; Morano et al., 1994; Van Eekelen et al., 1995). Furthermore, aged dogs show decreased septal and hippocampal mineralocorticoid receptor levels (and unaltered glucocorticoid receptor levels) which are accompanied by elevations in basal plasma ACTH and cortisol levels and an enhanced responsiveness to stress (Reul et al., 1991a,b; Rothuizen et al., 1993). The dexamethasone suppression test result was unaltered in aged dogs, which is consistent with the unaltered glucocorticoid receptor system in these animals (Rothuizen et al., 1993). Thus, hippocampal mineralocorticoid receptors control the inhibitory tone of this limbic structure on the hypothalamic–pituitary–adrenocortical axis in terms of both basal activity and stress-evoked reactivity.

5.2. In vivo microdialysis of free corticosterone in brain

The rat hippocampal mineralocorticoid receptors have, as mentioned, a very high affinity for corticosterone, determined at 0–4°C to be about 0.1–0.2 nM. One may predict that at 37°C, i.e., in vivo, the binding affinity is approximately 0.5–1 nM. The consequence of this receptor property is that it will become occupied by corticosterone in concentrations in the low nanomolar range. This is indeed the range of free (i.e., not bound to CBG) corticosterone levels (0.5–1 nM) circulating at the diurnal trough of hypothalamic–pituitary–adrenocortical activity and thus, the reason why mineralocorticoid receptors are already extensively occupied under these conditions. However, the plasma total corticosterone concentration under such baseline conditions is 5–30 nM (i.e., about 0.15–1.0 μg/100 ml). It is clear, therefore, that plasma total corticosterone concentrations can hardly be used to relate to mineralocorticoid receptor physiology and pharmacology. Moreover, two additional confounding factors can be discerned: (1) the CBG levels in blood are not constant but are subject to regulation by, amongst other, glucocorticoids (Siiteri et al., 1982; Smith and Hammond, 1992); (2) the plasma-free corticosterone concentration may not reflect the free steroid concentration in the central nervous system, because physiological glucocorticoids such as corticosterone may be ligands for the mdr1a P-glycoprotein extrusion pump (Ueda et al., 1992). Thus, corticosterone levels in cerebrospinal fluid and extracellular fluid of the brain may be lower than those in blood. In view of these considerations and our aim to relate free corticosterone levels to mineralocorticoid receptor (and glucocorticoid receptor) function, we decided several years ago to develop a method which would enable us to measure free corticosterone levels in the extracellular fluid (which is devoid of CBG) of the brain.

The method of choice was in vivo microdialysis in combination with a high sensitivity radioimmunoassay (Fig. 3A). This method makes it possible to monitor corticosterone levels in a brain region of choice in freely moving animals (we routinely use rats and mice, but the method should be also applicable in other animals such hamsters, guinea pigs, tupaias, etc. and even humans during neurosurgery) at 15-min or longer intervals over the entire diurnal cycle (for example, see Fig. 3B; free corticosterone levels in the rat hippocampus) for at least 8 days (Linthorst et al., unpublished). It can be seen from Fig. 3B that the free glucocorticoid levels in the hippocampus start to rise between 13:00 and 14:00 h, peak around the lights-off time and decrease again during the dark phase.

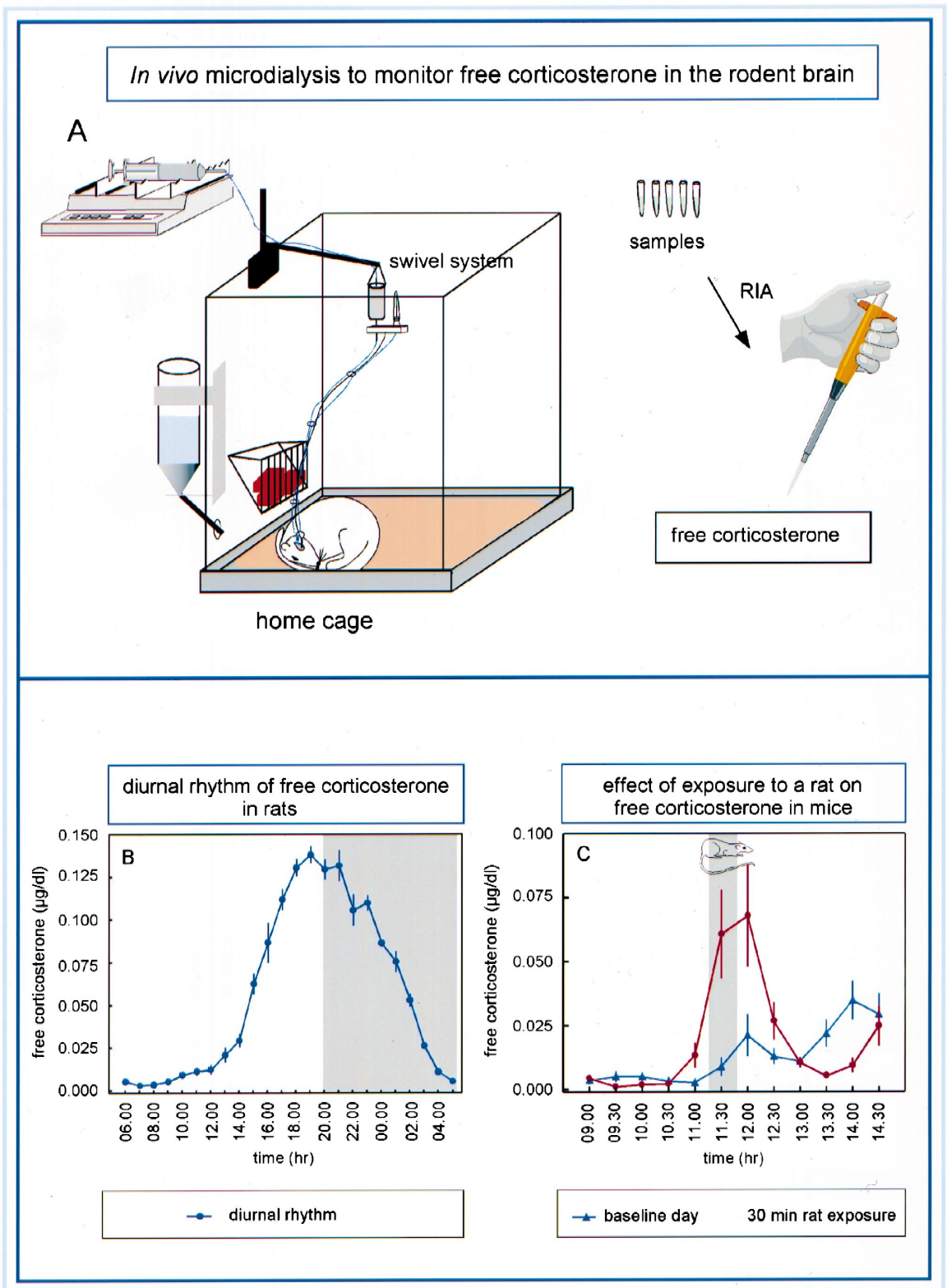
In vivo microdialysis of free corticosterone also makes it possible to monitor changes in glucocorticoid levels in the brain as a result of physiological or pharmacological challenges. Fig. 3C shows the effect of exposure of a mouse to a rat on hippocampal free corticosterone levels in the mouse (taken from Linthorst et al., 2000). As expected, since rats are natural predators of mice, the mouse shows a clear-cut stress response in terms of hypothalamic–pituitary–adrenocortical hormones, as revealed by the rise in free hippocampal corticosterone levels. In this manner, it is possible to determine exactly the concentrations of corticosterone to which neurons in selected brain regions are exposed during defined physiological conditions.

Thus, microdialysis of brain free corticosterone levels is a robust method that provides highly reproducible data (Linthorst et al., 1994, 1995a, 1997; Pauli et al., 1998; Linthorst et al., 2000). Another advantage of this method that should be mentioned is that it can be used to monitor, in parallel, levels of neurotransmitters, such as serotonin and noradrenaline as well (Linthorst et al., 1995a,b, 1997, 2000). Therefore, the method will continue to be used in our laboratory for studies of the hypothalamic–pituitary–adrenocortical axis.

5.3. The mineralocorticoid receptor: a dynamically regulated receptor

The situation of a receptor (i.e., mineralocorticoid receptor) which is always to a large extent occupied by hormone prompts the question whether we are dealing with a static or a dynamic receptor system. A static system would be merely playing a cofactor function, whereas, in contrast, a dynamic receptor system would be responding, in terms of its capacity and function, rapidly to changing requirements. For glucocorticoid receptors, this is much less relevant because this receptor becomes occupied in a graded manner by glucocorticoids during different physiological conditions (cf. circadian trough and peak, stress). Recently, we discovered that hippocampal mineralocorticoid receptors were relatively quickly upregulated by acute

psychological stressors, an action which is mediated by CRH in the brain (Gesing et al., 1999; submitted for publication). Importantly, the rise in mineralocorticoid receptor levels was associated with a stronger inhibitory tonus on hypothalamic–pituitary–adrenocortical axis activity (Gesing et al., 1999; submitted for publication). These data show for the first time that the hippocampal mineralocorticoid receptor is a dynamically regulated receptor which participates in adaptive mechanisms in the brain evoked by stressful events.



These findings on mineralocorticoid receptors may be of relevance for understanding the pathophysiology of stress-related disorders such as major depression. In this psychiatric disease, the overall hyperactivity of the hypothalamic–pituitary–adrenocortical axis appears to be at least in part the result of impaired glucocorticoid receptor (Modell et al., 1997) and mineralocorticoid receptor (Lopez et al., 1998) function, and hyperactivity of CRH (Nemeroff et al., 1984; Owens and Nemeroff, 1993) and vasopressin (Von Bardeleben and Holsboer, 1989; Raadsheer et al., 1994, 1995); (for review, see Holsboer and Barden, 1996). It is currently still unclear whether the state of hypothalamic–pituitary–adrenocortical hyperactivity is initially caused by an increased CRH drive resulting in mineralocorticoid receptor/glucocorticoid receptor dysfunction or by a primary defect in mineralocorticoid receptors and/or glucocorticoid receptors resulting in CRH hypersecretion. The above-described novel mechanism of interaction between CRH and mineralocorticoid receptor expression during stress might provide clues on this chicken and egg problem. By extrapolation, chronic stress results, via CRH hypersecretion and desensitization of central CRH receptors, in an incapacitation of hippocampal mineralocorticoid receptors leading to a progressively deteriorating hypothalamic–pituitary–adrenocortical axis function, resulting in hypothalamic–pituitary–adrenocortical hormone hypersecretion, impaired glucocorticoid receptor function, and changes in neurotransmitter metabolism. In genetically vulnerable persons (Holsboer et al., 1995; Modell et al., 1998), this sequence of events may occur and precipitate major depression or another stress-related (psychiatric) disorder.

5.4. Mineralocorticoid receptor dynamics, hypothalamic–pituitary–adrenocortical regulation and antidepressant drug action

Beside this hypothesis on the role of CRH and mineralocorticoid receptor in major depression, there are other data that strongly indicate that mineralocorticoid receptors have a role in this disease. Several groups have shown that treatment of rats with antidepressant drugs increases the binding capacity and gene expression of mineralocorticoid receptors in the hippocampus and other limbic and cortical brain areas (Brady et al., 1991; Seckl and Fink, 1992; Reul et al., 1993, 1994a; Yau et al., 1995, Gesing et al., unpublished). Time course analyses revealed that hippocampal mineralocorticoid receptor levels increased markedly already after 1–2 weeks, whereas levels of glucocorticoid receptors increased, only moderately, after at least 3–5 weeks of treatment (Brady et al., 1991; Reul et al., 1993, 1994a; Gesing et al., unpublished). The antidepressant-induced increases in mineralocorticoid receptors also preceded the decrease in CRH mRNA expression in the hypothalamic paraventricular nucleus (Brady et al., 1991) and the decline in baseline and stress-induced hypothalamic–pituitary–adrenocortical activity (Reul et al., 1993, 1994a; Gesing et al., unpublished). Thus, the effects of antidepressants on mineralocorticoid receptors may be a key phenomenon in the readjustment of hypothalamic–pituitary–adrenocortical regulation in major depression. This readjustment has indeed been observed to be a prerequisite for stable remission of the disease (Zobel et al., 1999). The effects of antidepressants on the rat hypothalamic–pituitary–adrenocortical axis were independent of their primary pharmacological profile. Accordingly, irrespective of whether rats were treated with tricyclic, pharmacologically unspecific antidepressants (amitriptyline, imipramine, trimipramine), with monoamine oxidase A inhibitor (moclobemide), with selective serotonin reuptake inhibitors (e.g., paroxetine) or with serotonin uptake enhancers (tianeptine), elevated levels of hippocampal mineralocorticoid receptors and an attenuation of hypothalamic–pituitary–adrenocortical activity were observed (Reul et al., 1993, 1994a; Gesing et al., unpublished). The specificity of the effects of antidepressants on the hypothalamic–pituitary–adrenocortical axis is underlined by the observation that chronic treatment with anxiolytics such as librium does not affect mineralocorticoid receptor levels or stress-induced hypothalamic–pituitary–adrenocortical activity (Reul, unpublished). Therefore, we have postulated that the readjustments in hypothalamic–pituitary–adrenocortical axis regulation may be a common denominator for clinically efficacious antidepressant drugs (Reul et al., 1993, 1994a; Gesing et al., in preparation a reviewed by Holsboer and Barden, 1996).

A link between antidepressant drug action, mineralocorticoid receptor function and major depression was observed in a recent clinical study (Hundt et al., unpublished). Here, patients suffering from major depression were treated

Fig. 3. In vivo microdialysis is a powerful method to monitor free corticosterone levels in the brain of freely moving rodents (A) under baseline conditions and during stressful situations. Free corticosterone levels are measured by a sensitive radioimmunoassay (for references, see text); (B) shows the diurnal rhythm of free corticosterone levels (μg/dl) in male young adult Wistar rats (average of diurnal rhythms collected over 5 days in five rats; 60-min samples). Rats were equipped with a microdialysis probe in the hippocampus (CMA/12, CMA/Microdialysis, Stockholm, Sweden; membrane: polycarbonate, molecular cut-off 20,000 Da, length 4 mm, outer diameter 0.5 mm). The shaded area indicates the dark period of the diurnal cycle; (C) shows the effects of exposure to a rat (between 11:30–12:00 h) on free corticosterone levels (μg/dl; 30-min samples) in male $B_6C_3F_1$ mice ($n = 6–8$) (data taken from Linthorst et al., 2000 with permission of the European Neuroscience Association). For details, see Linthorst et al. (2000). Mice were equipped with a microdialysis probe in the hippocampus (CMA/11, CMA/Microdialysis; membrane: cuprophane, molecular cut-off 6000 Da, length 3 mm, outer diameter 0.24 mm). The shaded area highlights the sample collected while the mice were exposed to a rat. For (B) and (C), time points on the *x*-axis represent the time of day at which collection of the respective sample was started. Data are presented as means ± S.E.M.. Please note that corticosterone levels are not corrected for dialysis efficiency (≈ 40% for rat probe; ≈ 12.5% for mouse probe; both determined in vitro at 37°C).

with placebo or the mineralocorticoid receptor antagonist spironolactone during the first 10 days of a course of treatment with amitriptyline. It was shown that the mineralocorticoid receptor antagonist caused a clear-cut impediment of the clinical response, indicating that a fully functional mineralocorticoid receptor is essential for the clinical efficacy of antidepressant drugs. Thus, this clinical study strengthens our concept of the involvement of mineralocorticoid receptors in the etiology of stress-related psychiatric disorders such as major depression. In view of the intricate involvement of CRH in the regulation of mineralocorticoid receptor expression in the limbic system (Gesing et al., 1999; submitted for publication), it can be envisaged that the development of selective nonpeptidergic CRH receptor antagonists will be a promising lead for a novel treatment for major depression (Holsboer, 1999; Zobel et al., 2000).

6. Other functions of the mineralocorticoid receptor

Beside its prominent role in the regulation of the hypothalamic–pituitary–adrenocortical axis and being a primary target for antidepressant drug action in the brain, the hippocampal mineralocorticoid receptor is involved in a variety of processes including serotonergic neurotransmission (Joëls et al., 1991; Meijer and De Kloet, 1998), electrophysiological events such as neuronal excitability (Joëls and De Kloet, 1989, 1990) and long-term potentiation (Pavlides et al., 1994), and behavioral responses (Oitzl et al., 1994; Smythe et al., 1997; Bitran et al., 1998). Furthermore, mineralocorticoid receptors in granular neurons of the dentate gyrus have anti-apoptotic properties (Sloviter et al., 1989; Hassan et al., 1997; Almeida et al., 2000). A full coverage of these functions of mineralocorticoid receptors, however, exceeds the scope of this review. Therefore, we wish to refer to other comprehensive reviews (De Kloet, 1991; Joëls and De Kloet, 1992; De Kloet et al., 1998). Nevertheless, it should be noted that the effects of acute psychological stress, CRH and antidepressants on mineralocorticoid receptors and the significance of these changes in mineralocorticoid receptors for hypothalamic–pituitary–adrenocortical regulation, as described in this review, have hardly been investigated in relation to the above-mentioned mineralocorticoid receptor-regulated processes.

7. Concluding remarks

The brain mineralocorticoid receptor is a remarkably interesting receptor not only because it is mainly localized in pyramidal and granular neurons of the hippocampus but even more because at any physiological level of pituitary–adrenal activity it is almost completely occupied by endogenous glucocorticoid hormone. Two concepts were formulated on the basis of these observations: (1) mineralocorticoid receptor is the glucocorticoid-binding receptor which mediates the tonic inhibitory influence of the hippocampus on the hypothalamic–pituitary–adrenocortical axis, and (2) if the mineralocorticoid receptor-mediated glucocorticoid signal were to change in response to changing physiological needs and conditions, then the capacity of this receptor should be rapidly adjustable. Much evidence has been collected during the last 15 years that supports point (1) and, recently, it has been discovered that acute psychological stressors, via an action of CRH, induce a surge in hippocampal mineralocorticoid receptor capacity (i.e., point (2)), which precipitates an organizational change in hypothalamic–pituitary–adrenocortical axis regulation. Thus, CRH is not only a principal mediator of the stress response but contributes to dynamic changes in mineralocorticoid receptor levels, presumably as part of adaptive mechanisms to cope with the stressful challenge. We have introduced here two methods which may be beneficial for the study of mineralocorticoid receptors in the brain. One method allows the study of mineralocorticoid receptor density at the individual cell level with immunohistochemistry and, the other, the investigation of free levels of corticosterone in brain with in vivo microdialysis. In addition, we have presented data on the labile interaction of dexamethasone with mineralocorticoid receptors, possibly implying that this steroid might act as an mineralocorticoid receptor antagonist in vivo.

For future research, it will be a challenge to further elucidate the significance of mineralocorticoid receptor plasticity not only for hypothalamic–pituitary–adrenocortical regulation but also with regard to its role in neurochemical, electrophysiological, autonomic and behavioral events, in order to gain further insight into stress coping mechanisms and, ultimately, into stress-related psychiatric disorders. Therefore, an investment should be made in the development of biochemical and molecular methods and tools to study mineralocorticoid receptor- (and glucocorticoid receptor-) mediated actions at a cellular and subcellular level in the brain in situ. Until then, the mechanisms and functions regulated by mineralocorticoid receptors will remain mysterious.

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