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CNS Effects of Steroids

CORTICOSTEROIDS AND THE BRAIN

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Summary—Mineralocorticoid (MR) and glucocorticoid receptors (GR) are expressed in the central nervous system. Radioligand binding studies, autoradiography, immunocytochemistry and *in situ* hybridization have shown that MR and GR are found in abundance in neurons of the limbic system (hippocampus), a structure involved in mood, affect and subtle control of the hypothalamic–pituitary–adrenal (HPA) axis. In the hippocampus MR binds corticosterone (CORT) as well as aldosterone (ALDO) with high affinity. MR seems mainly occupied by CORT in the face of its 2–3 order higher circulating concentration. GR binds CORT with a 6–10-fold lower affinity.

MR and GR gene expression, as well as the native receptor proteins, seem to be controlled in a coordinative manner. When GR is down-regulated by excess homologous steroid, MR appears to be increased. Down regulation of MR reduces GR as well. MR and GR display a differential ontogenetic pattern. Ontogeny, particularly that of GR, can be permanently influcnced when animals are exposed during the first post-natal week to maternal deprivation, handling, CORT or $ACTH_{1-24}$ injections. These MR and GR changes persist into senescence and have been proposed to result in altered CORT responsiveness, stress regulation, behavioural adaptation and brain aging.

INTRODUCTION

Since our previous contribution [1] to the Meeting of the International Study Group for Steroid Hormones considerable progress has been made in the research on corticosteroid action in the brain. First of all, this progress was due to the cloning of the steroid receptor genes [2]. Using RNA hybridization procedures, the genes encoding the mineralocoeticoid receptor (MR or Type 1) and the glucocorticoid receptor (GR or Type 2) were found expressed in the central nervous system [3–5]. Second, adrenal steroids and their metabolites showed binding to the membrane-associated $GABA_A$ benzodiazepine–chloride receptor complex and could alter chloride conductances [6]. Third, the enzyme 11β-hydroxysteroiddehydrogenase (11β-OHSD) was found to play a key role in conferring target specificity to mineralocorticoids [7, 8].

We have previously postulated the concept of "tonic and feedback" action of corticosteroids on brain function exerted via MR and GR, respectively [9, 10]. In this concept central (hippocampal) MR and GR colocalized in the same cells are considered to respond in a coordinative manner to a single signal, corticosterone (rat) or cortisol (other mammals), and are critical in maintenance of homeostatic control. The "tonic" influence via MR is constitutive, while GR mediates "feedback action" of CORT aimed to restore disturbances induced by stress. Here we will report recent studies designed to examine the properties and regulation of MR and GR in the central nervous system.

DIVERSITY OF BRAIN CORTICOSTEROID RECEPTORS

The steroid hormone receptors have been cloned and their primary structure is known [2]. MRs and GRs have a 94% sequence homology in the DNA-binding domain, and a 57% homology in the steroid-binding domain. The striking similarity between the MR and GR DNA-binding domains suggests that both receptors may interact with the same or closely related hormone-responsive elements in the genome. Thus it has been proposed that MR and GR may control in a coordinative manner certain gene networks [12].

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The discovery of the MR and GR primary structure and the fact that the chemical structure of these receptors is identical in each corticosteroid target tissue has led to a redefinition of the nomenclature, MR and GR were previously termed Type 1 and Type 2 respectively [13]. The Type 1 was further defined [9–11] as subtypes MR (ALDO-selective) and CR (CORT-preferring) depending on its pharmacology. At present, there is no need to complicate the issue anymore with Type 1 and 2 nomenclature but simply refer to these receptors as MR and GR with the former having aldosterone-selective and CORT-selective pharmacology.

Recently, immunocytochemical and RNA hybridization procedures have shown a widespread distribution of GR in neurons and glial cells throughout the brain. High GR concentrations are found in the limbic system (hippocampus, septum) and in the parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN). These neurons synthesize vasopressin and corticotrophin-releasing factor (CRF), enkephalin (ENK) amongst other neuropeptides. GRs are also present in relatively high concentrations in the ascending monoaminergic neurons in the brain stem. Peptides from the tachykinin (neuropeptide Y) and opioid (POMC, ENK and dynorphin) families are found in these same neurons, and may be under CORT control [14]. Moderate levels are also found in many thalamic nuclei, the striatal areas and in the central amygdaloid nucleus, as well as throughout the cortical hemispheres. The localization of GR mRNA, as detected by *in situ* hybridization, is the same as that of GR protein [3, 4].

In contrast, MR mRNA is restricted to the neurons of the hippocampal formation, lateral septum, amygdala, olfactory nucleus, layer II of the cortex and in brain stem sensory and motor neurons, where it is found in high density [3, 15]. As has been shown by *in situ* hybridization with cRNA probes [3] hippocampal neurons of the CA1 and CA2 subfields and the dentate gyrus express both MR- and GR-mRNA. CA3 neurons express relatively more MR than GR mRNA [3].

A critical difference between the two receptor types is their affinity for both natural and synthetic ligands. GRs show highest affinity to potent glucocorticoids such as dexamethasone (DEX), RU 26988 and RU 28362, a lesser affinity to the natural GCs i.e. CORT (rat) and cortisol (hamster), and a much lower affinity for ALDO [11, 16–19]. In contrast, *in vitro*, MR affinity for both CORT and ALDO (K_d 0.5 nM) is about one order of magnitude higher than that shown by GRs for these ligands. MRs show negligible affinity for the above RU compounds, but still appreciable affinity for DEX [20–22].

High resolution autoradiography of *in vivo* ALDO- and CORT-labelled brain sections showed the characteristic neuroanatomical distribution of MR sites [23]. A substantial fraction of [^{3}H]CORT–receptor complex retained in cell nuclei is bound to sites located at the nuclear matrix (van Steensel, unpublished results). Autoradiographical data show that the uptake and retention mechanism of central MR is responding to very low circulating levels of CORT and ALDO. Similarly low levels of exogenous [^{3}H]DEX are poorly retained in the brain where the steroid is retained in both MR- and GR-containing cells [23]. Note that the uptake of DEX by the pituitary corticotrophs is very high, indicating that *in vivo* at least, the uptake kinetics in brain and pituitary may vary considerably among the steroids [24–27]. The highly specific glucocorticoid agonist, RU 28362, labels brain sections in a pattern similar to the distribution of GR protein and GR mRNA [28, 29]. Quantitative analyses of receptor occupancy revealed that half maximal occupation of MR is achieved by a dose of 0.9 μg CORT/100 g body wt, while the dose has to be increased to 60 μg to reach the ED_{50} of GR [11, 17].

The limbic MR does not discriminate the retention of ALDO and CORT. Circumventricular organs (CVO) and the anterior hypothalamic area retain ALDO better than CORT [30, 31], although with the present technique, MR mRNA in these areas is below detection limits. If indeed MR is present in the CVO, these sites are reminiscent to the aldosterone specific MRs in the kidney. ALDO Specificity of mineralocorticoid target tissues is probably conferred by the enzyme 11β-hydroxysteroid dehydrogenase (11β-OHSD), although the role corticosteroid binding globulin (CBG) cannot be excluded [32, 33]. The enzyme converts CORT to its 11-oxo metabolite, which binds with low affinity to MR or GR and displays strongly reduced biological activity [7, 8, 34]. 11β-OHSD has been found in abundance in the classical mineralocorticoid target tissues such as the kidney and parotid glands. The enzyme and CBG may explain

the preference of the renal MR for ALDO in the face of up to 100–1000-fold excess of circulating CORT. In the hippocampus, low amounts of 11β-OHSD have been detected with immunocytochemical staining [35]. Accordingly, hippocampal (and other central) neurons (which also lack extravascular CBG) will be exposed predominantly to CORT. However, it is possible that the enzyme may be expressed (and become active) under certain physiological conditions in the hippocampus.

Recent evidence indicates that adrenal steroids may also interact with membrane-associated receptor proteins [6, 36–38]. Via membrane receptors the steroids have a fast (milliseconds) influence on synaptic transmission as opposed to the slow acting (minutes to hours) but longer-lasting receptor-mediated genomic actions. The $GABA_A$ benzodiazepine–chloride ionophore complex has been recognized as an important target for membrane action of steroids. Most of the inhibitory neurons are GABAergic. Endogenous steroids such as 3α,5α-tetrahydrodeoxycorticosterone (THDOC) and 3α-hydroxy-5α-dihydroprogesterone (respectively, the metabolites of deoxycorticosterone and progesterone) are potent barbiturate-like ligands of the GABA–receptor complex. *In vitro* these steroids are at least 1000-fold more potent than pentobarbitone in displacing the binding of [^{35}S]*t*-butylbicyclophosphorothionate (TBPS, the "cage convulsant") to the GABA–receptor complex [6, 38]. The steroids potentiate GABA-induced chloride conductances and this action is believed to underly their anesthetic, anticonvulsive, sedative and probably also anxiolytic activity. Interestingly, *in vitro*, steroids such as cortisol and corticosterone [as well as mineralocorticoids (ALDO, deoxycorticosterone) and anti-mineralocorticoids (spironolactone, RU26752)] at nanomolar concentrations enhance the binding of [^{35}S]TBPS to the GABA—receptor complex.

The fact that the abovementioned anticonvulsive steroid metabolites are also present endogenously has opened the possibility that they influence, under physiological and pathophysiological conditions, the GABA–receptor complex and thus, the activity of the central nervous system. Since the brain contains the biosynthetic enzymes required for generation of these active steroids from the cholesterol precursor it cannot be excluded that these steroids are produced *in situ* in the brain (henceforth, neurosteroids), in addition to metabolic conversion from circulating parent steroids [39].

In summary, the binding studies suggest that CORT, perhaps in concert with its metabolites, may interact with several receptor systems localized at the same target, namely neurons in the hippocampus. The action of CORT, exerted via these receptors, differs in mode, onset and duration, but are all aimed to control cellular homeostasis. The $GABA_A$ receptor complex mediates adaptive responses taking place via GABAergic circuits resulting in changes in chloride conductances of the pyramidal neurons.

COORDINATION OF MR AND GR PLASTICITY IN LIMBIC BRAIN

Studies in recent years on the regulation of corticosteroid receptors in brain and pituitary have revealed strong evidence for the existence of a coordinate adjustment of limbic brain MR and GR concentrations which depends on the hormonal state of the animal. Following ADX, hippocampal GR levels are upregulated, whereas, in contrast, MR levels remain relatively constant [40]. Implantation of CORT down-regulates GR leaving MR unaffected. DEX also down-regulates GR, but unlike CORT, it up-regulates MR [40]. Moreover, the DEX-induced upregulation of hippocampal MR is preceded by transient increments in MR mRNA, underscoring the notion that the steroid is affecting MR at the gene expression level, presumably via GR-mediated mechanism [40].

McEwen and coworkers [41, 42] have infused ADX rats intravenously for 3 days with 1 μg/h of either CORT, ALDO or DEX. By the end of the 3-day treatment period, CORT and ALDO administration, as compared to saline-treated controls, had elicited a substantial decrease in measurable MR levels in hippocampus, amygdala and hypothalamus-preoptic area, cortex and cerebellum, and in the pituitary only ALDO was effective. These steroid effects could be explained by receptor occupancy and/or receptor down-regulation of MR. Interestingly, CORT and ALDO infusion also attenuated the post-ADX rise in GR levels in hippocampus, amygdala and hypothalamus-preoptic area, but not in cortex, cerebellum and pituitary. Since the attained CORT and ALDO plasma levels were very low (below detection in RIA), the observed decrease in GR levels was most likely not due to GR occupancy, but rather induced by occupancy of MR by CORT or ALDO.

Infusion of DEX (1 μg/h) resulted in a decrease of GR in all tissues measured (i.e. hippocampus, amygdala, hypothalamic-preoptic area). However, MR levels in these tissues showed a tendency to be up-regulated due to GR occupancy by DEX [42], which is consistent with results from our laboratory [5, 40].

A recent study [43] found that 24 h after a single sc administration (0.5 μg/g body wt) of ALDO to ADX-ovariectomized female mice a profound decrease MR sites in hippocampal, cerebral cortex, hypothalamic, brain stem, cerebellum tissues was observed, while decrements in GR were only noted in hippocampus and cerebral cortex. In addition, the ALDO effect on both MR and GR levels was antagonized by the selective MR-blocker RU 26752, emphasizing that the steroid action was solely mediated through MR ([43], Reul and de Kloet, unpublished observations). Hence, these findings were largely similar to those of McEwen [42]. Following a single s.c. injection (0.5 μg/g body wt) of DEX, a strong binder to both MR and GR in murine brain, both MR and GR were down-regulated. Coinjection of RU 26752 antagonized the DEX effect on MR, but not on GR, indicating that the formation of a DEX–MR complex was required for the DEX-evoked down-regulation of MR [22].

Recent studies have put forward the notion that the homologous regulation of GR is a multi-level regulated process. DEX has been shown to decrease levels of GR mRNA in rat liver [44, 45] and in hippocampus [5], which was due to a reduced transcription rate of the GR gene [44]. The half-life of GR mRNA was not affected by DEX [44]. In addition to the transcriptional regulation of GR gene expression, DEX affected the rate of GR protein turn-over. Hence, in the presence of the steroid, the half-life of the GR protein decreased from 25 to 11 h [44, 45]. Thus, the homologous regulation of GR is regulated at both the transcriptional and post-translational level [5, 44, 45].

Taken together these studies point to a coordinate plasticity of MR and GR sites in limbic brain regions. Down-regulation of MR appeared to be accompanied by a reduction in GR capacity, whereby colocalization of the two receptor types in the same neurons seemed to be a prerequisite. Down-regulation of GR resulted in an increase of MR capacity, although in this case the treatment had to last for at least 3 days. Here again, the effect on MR was only notable when MR and GR were likely to be colocalized. Finally, although the corticosteroid receptor types appear to be regulated in a coordinative manner, large differences were notable in the temporal aspects of MR- and GR-mediated regulation, emphasizing the different modes of steroid action via MR and GR.

ONTOGENY AND AGING OF CORTICOSTEROID RECEPTORS

Presently three methods have been used to analyse the ontogenetic pattern of MR and GR [46–50]. These methods include (i) the *in vitro* binding of radiolabelled steroids to MR and GR extracted from tissue, notably the hippocampus; (ii) the *in vivo* uptake and retention of tracer amounts of [^{3}H]CORT analysed with autoradiography; and (iii) immunocytochemistry of GR proteins. The former two methods have to be performed with ADX rats; the latter does not require ADX. Preliminary data on receptor gene expression have been obtained [51]. These procedures have provided complementary information in that MR and GR have a clearly different development in the rat brain.

In vitro hippocampal cytosol assays show that GRs are present around birth in low amounts and slowly rise reaching adult levels around 4 weeks of age [46, 52, 53]. The affinity of GR for RU 28362 is high at an early age and decreases to adult levels over the same period [46]. Binding constants are not available from other laboratories, where data were derived from single point determinations with saturating concentrations of steroid. Immunocytochemistry of GR protein revealed profound ontogenetic changes both in intensity and localization. From a dense staining early post-natally the topography changed with greater restriction to cells which also express GR at adulthood. Staining intensity displayed an inverted U-shaped curve with minimal intensity around day 12. In some brain areas such as the hippocampal CA3 cell field and the suprachiasmatic nucleus GR staining remained low suggesting that the initial postnatal GR expression becomes suppressed during development [47, 50]. The immunocytochemistry performed in intact animals showed only GR localized in cell nuclei. ADX eliminated staining. Essentially the same ontogenetic pattern was observed in ADX rats treated with RU 28362, suggesting that factors such as receptor activation and cell nuclear binding may be

subject to developmental changes as well. GR gene expression measured with *in situ* hybridization matched the distribution of GR protein [51]. Our previous studies have indicated a pronounced, but transient, increase in GR number following denervation, suggesting that certain neural inputs (dopamine, serotonin) control GR gene expression [54–56]. It would be of interest to examine the role of nerve innervation during development on GR ontogeny.

MR sites in hippocampal cytosol of ADX rats under low ionic strength conditions are detected only around day 8. At that time adult levels in receptor number are reached. However, when extracted with high ionic strength buffer (0.4 M KCl), MR was detected at day 2 [48]. Similar data were obtained by others [53] who showed that the levels increased slowly until day 14. The binding of [^{3}H]ALDO (which also detects GR) is measured at day 3 and increases to adult levels slowly [52]. In the *in vivo* autoradiography using [^{3}H]CORT administered to ADX rats, the radioactivity was visualized in the characteristic pattern for MR in adult rats i.e. in hippocampus, septum, amygdaloid nuclei and the cortical layer II. That these were indeed MR sites was proven, since pretreatment with the selective GR antagonist RU 486 did not displace the labelling. Moreover, MR mRNA shows essentially the same neuro-anatomical distribution [51]. The intensity of [^{3}H]CORT uptake was highest during the second week of life. Note that the uptake and retention of radioactive steroid do not necessarily reflect the receptor number. Metabolism and CBG level, the blood–brain barrier and conformational state of the receptor show pronounced developmental changes and thus all account for changed uptake kinetics [48].

There is a considerable plasticity of MR and GR throughout life as a consequence of brief manipulations during development. In the rat, from approximately post-natal day 4 until day 14, the HPA system is profoundly suppressed. This is the stress hyporesponsive period (SHRP) characterized by the fact that plasma CORT is low; stressors that otherwise in adult animals exert a large HPA response are not (or only minimally) effective in the pup [57, 58]. It is now found that depriving the pups for 24 h from the mother leads to de-repression (or priming) of the HPA axis and a vigorous adrenocortical response to exogenous $ACTH_{1-24}$. This injection into neonates at day 4 results in reduced number of GR at day 60. Hypercorticism due to corticosteroid injection early in life was previously reported to result in reduced CORT binding in the hippocampus, presumably that to GR [59, 60]. Deprivation of the pup for 24 h from the mother had the opposite effect and increased GR was observed at adulthood. A permanent increased GR number also occurred following handling of the neonates for the first week of life [52]. This "handling" effect on CORT binding extended into senescence, although the receptor type was not specified in that study [61]. The effects on MR were smaller and more variable and possibly required additional factors for receptor control (Rosenfeld *et al.* unpublished data). In all cases data were derived from *in vitro* cytosol binding.

Finally, 24 h post-ADX the senescent rat has a pronounced reduction in [^{3}H]CORT binding in hippocampal cytosol [62, 63], which appears to be a reduction of MR and GR [64]. In senescent intact rats GR mRNA is also reduced ([65], Reul *et al.*, unpublished data), but no changes were observed in MR mRNA (Reul *et al.*, unpublished). Treatment of the senescent rat with neurotrophic substances selectively increased MR binding as shown by the *in vivo* uptake in the hippocampus as compared to the young controls [10, 64].

The abovementioned binding data were all obtained after sacrifice of the animals 24 h post-ADX. In other studies [66, 67] it was observed that the ADX-induced up- and stress-induced down-regulation was attenuated in the senescent rats. In additon GR affinity may have been increased in these old animals [68]. Taken together, the most consistent outcome of all studies is that senescent rats have reduced GR gene expression, GR receptor sites and GR plasticity in the hippocampus. MR receptors are also reduced, but have maintained plasticity in the face of neurotropic agents.

CONCLUDING REMARKS

MR and GR control adaptive responses intrinsic to neurons in the hippocampus. Electrophysiological studies showed that such a control is mediated via the genome in a time-dependent manner and antagonistic mode of action. In the hippocampal CA1 neurons, it was found that MR maintains cellular excitability while GR suppresses it with a longer time delay [69, 70]. The GR effect is of larger magnitude, and as a function of CORT concentration, it slowly overrides MR-controlled ionic

regulation. In view of the similarity in DNA binding domain, MR and GR have the potential to control simlar gene network, and are proposed to act as a binary hormone response system [12]. These recent electrophysiological and molecular studies are consistent with our concept of "tonic and feedback" action of CORT formulated in 1987 which states that CORT action via MR "contributes to hippocampal function in the interpretation of sensory information, leading to appropriate neuroendocrine and behavioural responses which are themselves subsequently subject to feedback action via GR" [9]. Recent studies on the organismic level support this concept and have shown that MR and GR exert opposite and differential control of higher brain functions in response to changing circulating CORT levels [71–74]. Finally, receptor studies have shown that MR and GR capacity may also be subject to a coordinate control by the CORT levels.

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