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USE OF *IN SITU* HYBRIDIZATION TO INVESTIGATE THE REGULATION OF HIPPOCAMPAL CORTICOSTEROID RECEPTORS BY MONOAMINES

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Summary—The hippocampus receives major noradrenergic and serotoninergic (5-HT) innervations which interact with corticosteroid-sensitive cells. However, the subregional localization of these actions and the corticosteroid receptor types involved have not been defined and current ligand binding techniques for estimating corticosteroid receptors are hampered by several methodological limitations. We have developed *in situ* hybridization histochemical techniques to allow specific and sensitive estimation of glucocorticoid (GR) and mineralocorticoid receptor (MR) mRNA expression in rat hippocampus. Investigation of the effects of 5,7-dihydroxytryptamine lesions of 5-HT neurons showed significantly reduced GR and MR mRNA expression in some hippocampal subregions. Both abnormal 5-HT neurotransmission and excessive corticosteroid secretion are associated with major affective disorders, particularly depression. The crucial interaction between these two systems may occur, at least in part, at the level of regulation of hippocampal corticosteroid receptor expression.

INTRODUCTION

Both corticosteroids and monoaminergic neurotransmitters are thought to act on the hippocampus affecting mood, behaviour and neuroendocrine responses [1, 2]. Elevated plasma corticosteroid concentrations due to Cushing's disease or glucocorticoid therapy are associated with a very high incidence of affective disorder [3]. Furthermore, raised circulating glucocorticoid [4] and mineralocorticoid [5] concentrations and reduced leucocyte corticosteroid receptor levels [6, 7] are state-dependent markers of depression. Because defective serotoninergic (5-HT) and catecholaminergic neurotransmission in the cortex and limbic system have been implicated in the pathogenesis of depression the effects of manipulation of corticosteroid concentrations on central monoamine biosynthesis, turnover and receptor levels have been extensively investigated [2, 8–10]. In contrast, the effects of monoamines on hippocampal mineralocorticoid (MR, type I) and glucocorticoid (GR, type II) receptors have received less attention, although there are major 5-HT and noradrenergic (NA) (and some dopaminergic) inputs to the hippocampus.

One putative modulator of hippocampal corticosteroid receptors is 5-HT, which has been suggested to provide central regulation of the hypothalamic–pituitary–adrenal axis. Thus, depletion of brain 5-HT attenuates feedback [11] and 5-HT agonists activate the axis [12]. Though the site(s) of these actions are unclear, there is a dense 5-HT pathway from the raphe to the hippocampus which interacts directly with neurons bearing corticosterone binding sites [13] and hippocampal 5-HT levels vary in parallel with the diurnal corticosterone rhythm [14]. Furthermore, potentiation of endogenous 5-HT by antidepressant drugs decreases corticosterone binding sites in hippocampal cytosol, whereas central 5,7-dihydroxytryptamine (5,7-DHT) lesions increase cytosolic receptors [15]. Thus there may be regulation of hippocampal corticosteroid receptors by 5-HT, but the hippocampal subregions and the receptor type(s) involved have not been identified.

METHODOLOGICAL CONSIDERATIONS

To date, most data on hippocampal corticosteroid receptors have been derived from radioligand binding studies. Although initial problems with ligand specificity, which precluded the clear differentiation of MR- and GR-type binding sites, have been overcome

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following the development of highly selective synthetic agonists and antagonists for both receptor types, further methodological limitations weaken this approach. Thus radioligand binding studies require prior adrenalectomy to clear steroids from otherwise occupied binding sites [2]. Adrenalectomy not only alters the state of the hypothalamic–pituitary–adrenal axis but also affects circulating levels of some catecholamines and peptides, any of which might affect neuronal corticosteroid receptor expression [2]. There are also difficulties in clearly distinguishing cytosolic (inactive) from nuclear (activated) binding sites, though these often bear an inverse relationship. Finally, quantitative studies have usually been performed on whole hippocampal extracts, whereas the available data suggest the various subregions respond differently [16] and have separate functions [17]. Although immunocytochemistry can be performed in intact animals and provides anatomically-accurate localization, there are no available MR-specific antisera suitable for use in the brain and this approach is not readily quantifiable.

IN SITU HYBRIDIZATION

Recently, cDNA clones encoding GR and MR mRNAs have been isolated. High expression of both mRNA species have been shown in the hippocampus by solution and filter hybridization [18–20]. Several groups have now developed *in situ* hybridization methods, permitting semi-quantitative analysis of subregional distributions of MR and GR mRNA [18, 21–24]. Many of the limitations of other techniques are not experienced with *in situ* hybridization; intact animals can be used, probes are fully specific and *in situ* hybridization has even been employed for MR and GR mRNA localization in human postmortem hippocampus [25] (estimation of binding sites is not reliable in postmortem brain due to their rapid degradation [26]).

Although *in situ* hybridization gives information on corticosteroid receptor gene expression rather than receptor protein content, inferences at the important level of regulation of receptor biosynthesis can be made. We have recently used this technique to examine the regulation by central 5-HT neurons of GR and MR mRNA expression in the hippocampus [24].

For *in situ* hybridization we use frozen coronal brain sections (10 μm) mounted on gelatine and poly-*L*-lysine-coated slides [24, 27]. Others have found that *in vivo* perfused, pre-fixed tissue is also satisfactory [18, 21–23]. Since synthetic oligonucleotide and random prime-labelled cDNA probes were not sensitive enough for detection of MR and GR mRNAs in tissue sections, cRNA antisense probes were employed. We have used subcloned inserts of rat MR cDNA (513 bp of steroid binding domain and 3′ untranslated region) or rat GR cDNA (674 bp of steroid binding domain) as templates for *in vitro* transcription of ^{35}S-UTP-labelled cRNA probes. We do not find that proteinase K or other pretreatments improve probe penetration or reduce background when frozen tissue sections are used. Hybridization has been optimized at 55°C for 18 h [24] after which slides are treated with RNase A, washed to high stringency (0.1 × SSC at 60°C) and exposed to film or dipped in photographic emulsion. For quantitation, silver grains are counted per high power microscopic field over the dorsal hippocampus and background (adjacent white matter) subtracted. Other approaches to quantitation have included autoradiographic film densitometry and comparison of the hybridization signal with brain paste standards containing various amounts of radioactivity [23]. Controls include hybridization with complementary "sense" cRNA of similar specific activity, RNase A pretreatment and Tm determination in the tissue of interest. If there is any doubt about probe specificity when estimating similar mRNAs, such as MR and GR, competition studies using a labelled antisense probe with excess unlabelled homologous or heterologous cRNA can also be performed.

DISTRIBUTIONS OF MR AND GR mRNAs IN HIPPOCAMPUS

We find that MR mRNA is expressed at similar high levels in all hippocampal subregions in control rats and, in general, our data confirm previous results for MR mRNA, allowing for differences in methods of quantitation [18, 21, 23]. GR mRNA expression shows a different pattern with high levels in dentate gyrus, CA1 and CA2 and significantly lower expression in CA3 and CA4. This distribution agrees with immunocytochemical findings using a monoclonal GR-specific antibody [28] and with some [21–23], but not all [29] reports of GR mRNA expression in the rat hippocampus. We find little hybridization of "sense" probes

in heterologous competition experiments, although excess cold homologous probe successfully attenuates hybridization [24].

5-HT NEUROTOXIC LESIONS

For this study we administered desipramine (25 mg/kg, i.p.) and 30 min later 5,7-DHT i.c.v. or vehicle to rats under fentanyl, fluanisone and midazolam anaesthesia, as described previously [30]. This regimen reduces hippocampal 5-HT levels by more than 60% but has little effect on NA or dopamine content [30, 31]. Using *in situ* hybridization we found [24] that 5,7-DHT lesions led to significantly reduced GR mRNA expression in dentate gyrus, CA1 and CA2, whereas MR mRNA expression fell significantly only in CA3 and CA4 (Fig. 1). The small decreases in MR mRNA expression in the remaining subregions were not statistically significant. Central 5,7-DHT lesions increase cytosolic, but reduce nuclear corticosterone binding sites in hippocampal extracts [15, 30]. Since nuclear binding probably represents activated receptors, whereas cytosolic binding may indicate inactive receptors, these data may not be entirely conflicting. Lesioned rats show reduced corticosteroid receptor mRNA expression, suggesting that biosynthetic changes are consistent with the nuclear binding studies. These data underline the importance of clearly distinguishing effects on the two receptor types and various hippocampal subregions.

The mechanisms producing the apparent subregional specificity of corticosteroid receptor regulation are unknown; indeed CA3, which shows the greatest reduction in MR mRNA expression, has the most sparse 5-HT innervation [32]. It seems likely that 5,7-DHT acts within the hippocampus, as neurophysiological studies have shown that lesions of hippocampal 5-HT inputs directly affect corticosterone-binding neurons [13]. 5,7-DHT could also affect hippocampal receptors indirectly via altered corticosteroid levels and *in situ* hybridization studies have shown that adrenocorticosteroid manipulations alter expression in some subregions [23]. However, adrenalectomy changes MR mRNA expression only in CA1-2 [23] in contrast to 5,7-DHT lesions which alter expression in CA3-4, suggesting that the mechanisms involved are distinct.

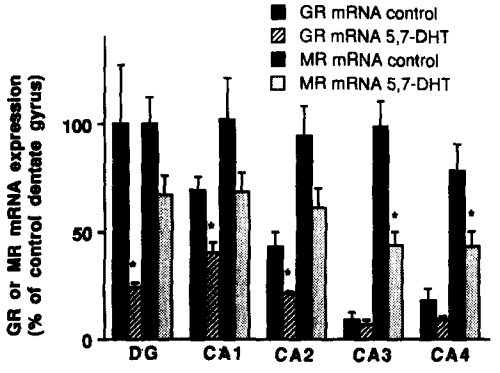

Fig. 1. GR and MR mRNA expression in hippocampal subregions in rats injected i.c.v. with either 5,7-DHT or vehicle ($n = 4$/group). DG = dentate gyrus. $^*P < 0.05$ compared with controls (ANOVA).

Whether administration of antidepressants, which potentiate monoamines by inhibiting their reuptake, affect hippocampal GR and MR mRNA expression is currently under investigation. Certainly a variety of antidepressants alter GR mRNA expression in primary fetal neuronal cultures [33]. Ligand binding data have also shown that neurotoxic lesions of central NA, and perhaps dopaminergic, pathways affect hippocampal corticosterone binding sites [34], suggesting that regulation of receptor expression may not be restricted to 5-HT.

In summary, these data show that (1) 5-HT projections to the hippocampus play an important role in maintaining the normal level of corticosteroid receptor mRNA expression and (2) this 5-HT "control" is regionally specific for GR and MR. Disordered 5-HT regulation of corticosteroid receptor mRNA expression could provide the basis for the abnormal activity of the hypothalmic–pituitary–adrenal axis which occurs in major affective disorders.

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