The word rodent comes from the Latin rodere, to gnaw. The teeth of the rat are very specialised. There are a pair of incisors, and three pairs of molars in each jaw. The incisors are used for biting, fighting, holding food and for breaking up hard soil. The rat usually feeds directly off the ground but may hold the food with both hands. All food and all strange objects, as well as other rats are sniffed. The eyes of the rat are small and are placed laterally. It is capable of seeing through virtually 360°, but to see something just in front, it would need to tilt its head and use one eye only. Furthermore the rat has been shown to be color-blind (modified from R.J. Olds and J.R. Olds).

Corticosterone modifies hippocampal muscarinic receptor immunoreactivity

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Abstract

In the present study we report the effect of corticosterone in the regulation of hippocampal muscarinic acetylcholine receptor immunoreactivity (mACHR-ir) expression in rats. Adrenalectomy (ADX) or a single injection of a mineralocorticoid antagonist RU-28318 (1.0 mg/100 g b.w.) in adrenally intact rats 24 hrs prior to sacrifice revealed an increased mACHR-ir in hippocampal CA1 and CA3 areas. Corticosterone replacement (100µg/ 100g b.w.) prevented the increase in mACHR-ir of ADX animals. However, glucocorticoid receptor antagonist (RU38486) treatment in adrenally intact rats failed to affect the mACHR immunolabelling. These results point to a modulation of muscarinic receptors by corticosterone that is predominantly mediated by the mineralocorticoid receptor.

Introduction

Glucocorticoid hormones, corticosterone (in rat) or cortisol (in human), are known to influence brain processes such as mood, behaviour and memory (McEwen et al. 1986; de Kloet 1991; Bohus 1994; Korte et al. 1996). These actions of circulating corticosteroid hormones are mediated via intracellular corticosteroid receptors. At present, two different corticosterone receptor types are known in the brain: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) (de Kloet 1991). The high density of MRs and GRs in hippocampal neurons suggest that corticosteroids are important regulators of the function of this structure (McEwen et al. 1986; de Kloet 1991; van Steensel et al. 1996). Several biochemical (Gilad 1987; Takayama et al. 1987; Biegon et al. 1985) and electrophysiological studies (Hesen and Joëls 1993, 1996) have proposed a role for corticosterone in cholinergic neurotransmission in the hippocampus. The hippocampal formation and in particular the cholinergic septohippocampal system have been implicated in the neural substrate for spatial learning, predominantly through the action of acetylcholine on muscarinic acetylcholine receptors (mACHR) (Hagan et al. 1987; van der Zee et al. 1995). It has been also shown that altering levels of plasma corticosterone affect memory functions. For example, depletion of circulating corticosteroids by removal of the adrenal glands (ADX) impairs place navigation in the water maze (Oitzl and de Kloet 1992). Selective blockade of central MRs and GRs by specific antagonists disturbs different aspects of spatial learning in a conventional water maze (Oitzl and de Kloet 1992). Recently, we found that repeated blockade of MRs impaired reference memory in the hole board test, which is a free-choice food rewarded spatial learning paradigm (Douma et al. 1998b). Accordingly, both types of corticosterone receptors seem to be involved in the hormonal modulation of the process of spatial learning.

Previous results from our laboratory revealed that successful training performance in the hole board spatial discrimination task causes an increase and subcellular redistribution of mACHR immunoreactivity (mACHR-ir) in hippocampal pyramidal cells (van der Zee et al. 1995; Douma et al. 1997; Beldhuis et al. 1992). In view of the putative effects of corticosteroids on cholinergic neurotransmission and spatial memory processes, we examined cholinceptive muscarinic receptors of the hippocampal pyramidal cholinergic target cells by means of mACHR immunocytochemistry in relation to experimental manipulations of corticosteroids and their receptors. The impact
of adrenalectomy and differential MR and GR blockade in adrenally intact animals on mAChR-ir was studied in the CA1, CA3 and the dentate gyrus region of the hippocampus.

Materials and Methods

The experiments were approved by the Committee on Animal Bio-Ethics of the University of Groningen. 42 Male Wistar rats, weighing 290-340 g at the beginning of the experiments, were used. The rats were housed in groups of 6 animals per cage and kept on a 12 h lights on-light off cycle, lights on from 7.00-19.00 h. Food and water were available ad libitum. The experiments were carried out between 9.00h and 12.00h.

ADX and antagonist treatment

Six animals were sham-adrenalectomized (Sham) and twelve animals were bilaterally adrenalectomized (ADX) under ether anesthesia. Six animals of the latter group received a single subcutaneous injection (S.C.) of corticosterone in a dose of 100 µg/ 100g body weight (cortADX) producing physiological serum levels of corticosterone (de Kloet et al. 1994), whereas the other animals were injected (S.C.) with the vehicle control solution immediately after the surgical procedure.

For antagonist treatment 24 intact rats were divided into four groups of six each: Control (vehicle treated), aMR (anti-mineralocorticoid treated), aGR (anti-glucocorticoid treated), and aMR/aGR (treated with both antagonists). The MR antagonist (RU28318; 3,3-oxo-7-propyl-17hydroxy-androstan-4-en-17yl-propionic acid-lactone) (Perroteau et al. 1984) and the GR antagonist (RU38486; 17β-hydroxy-11β-(4-dimethyl amino-phenyl)17α-(1-propynyl) estra-4,9-diene-3-one) (Gaillard et al. 1984; Moguilewski et al. 1984) were kindly provided by Roussel-UCLAF, Romainville, France. Both steroids were first dissolved in ethanol and subsequently diluted in polyethylene glycol 400 (PEG; BDH chemicals Ltd., Poole England) until a final ethanol concentration of 2%. The vehicle control contained the same PEG/ethanol concentration. RU28318 was injected (S.C.) with a dose of 1.0 mg/100 g body weight, whereas RU38486 was given in a dose of 2.5 mg/100 g body weight. Injections (0.2 ml) were given at 10.30h. The effects of various treatments on mAChR’s were studied 24 hours later.

Prior to transcardial perfusion the rats were deeply anaesthetized with ether and perfused with 300 ml of a fixative consisting of 3% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB) at pH 7.4. Fixation was preceded by a prerinse with 50 ml saline solution. The brains were removed, stored overnight in 30 % buffered sucrose at 4°C for cryoprotection, and coronally sectioned on a cryostat microtome at a thickness of 20 µm. Immediately before perfusion, 0.5 ml aterial blood was taken from the left atrium to determine plasma corticosterone levels. The blood samples were immediately transferred to centrifuge tubes containing 10 µl heparin solution (500U/ml) and centrifuged for 20 min. at 3,500 G. The supernatant was stored at -20°C for the corticosterone assay.

Corticosterone was extracted from 75 µl plasma and determined by HPLC with UV detection at 254 nm according to Dawson et al. (1984) with minor changes. Briefly, plasma samples were deproteinized with methanol and centrifuged. The supernatant was further cleaned by extraction on a C8 Solid Phase Extraction Column (J.T. Baker, Deventer, The Netherlands). Corticosterone was eluted with acetone and this extract was aspirated and redissolved in 25% acetonitrile/water for subsequent injection onto the column (Nucleosil, length 10 cm, i.d. 3 mm, particle size 5 µm; Chrompack International, Middelburg, The Netherlands). The mobile phase was made by mixing 340 ml Acetonitrile to a total volume of 1 liter with water and was pumped at a rate of 0.5 ml/min. Dexamethasone was used as the internal standard. The absolute detection threshold for corticosterone in plasma was 8 ng/ml. The intra- and interassay coefficients were 3% and 8%, respectively.

For muscarinic receptor protein immunocytochemistry free floating brain sections were incubated 24 h at 4°C with phosphate buffered saline (PBS) containing mouse anti-mAChR IgM (M35; Chemunex, Paris, 1:200). Next the sections were incubated in biotinylated rabbit anti mouse IgM for 2 h at room temperature (RT) and after subsequent rinsing in PBS to streptavidin-HRP (Zymed, 1:200 2h at RT). The tissue-bound peroxidase was visualized using the diaminobenzidine (DAB) reaction (30 mg DAB in 100 ml Tris Buffer and 0.01% H2O2 ).

The distribution of M35-immunoreactivity was quantified by measuring the relative optical density (OD) by way of an image analysis system (Quanimet 600, Leica). The OD was measured in the CA1 and CA3 pyramidal cell layer and associated apical dendrites, and in the DG molecular cell layer at the anterior-posterior level I.A. 4.7 (Paxinos and Watson 1982). The OD values of the corpus callosum served as a measure for non-specific background staining. Specific staining was calculated by subtraction of the OD of the
background from the total OD values. For quantification 3 adjacent sections per animal containing the hippocampus were analysed unilaterally. The data were averaged and calculated per group. The OD of the ADX, cortADX, aMR, aGR, and aMR/aGR groups were compared with the OD of the control groups and statistically analysed using the Kruskal-Wallis ANOVA followed by the Mann-Whitney U-test. A probability level of P<0.05 was taken as statistical significance for all tests. All data are presented as means with their standard errors (SEM).

**Results**

**Plasma corticosterone**

Plasma corticosterone levels of Sham, cortADX, aMR/aGR, aMR, aGR are shown in Table 1. Plasma corticosterone levels showed the tendency to increase after aMR or aMR/aGR treatment, but data did not reach significance (P<0.06). The corticosterone levels of all ADX animals were below the detection limit of 8 ng/ml.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Plasma-Cort (µg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>6</td>
<td>7.2 ± 3.1</td>
</tr>
<tr>
<td>ADX</td>
<td>6</td>
<td>n.d.</td>
</tr>
<tr>
<td>cortADX</td>
<td>5</td>
<td>8.2 ± 1.9</td>
</tr>
<tr>
<td>aMR/aGR</td>
<td>5</td>
<td>16.3 ± 2.5</td>
</tr>
<tr>
<td>aMR</td>
<td>4</td>
<td>11.7 ± 3.5</td>
</tr>
<tr>
<td>aGR</td>
<td>5</td>
<td>6.2 ± 2.7</td>
</tr>
</tbody>
</table>

n.d. indicates not detectable

**mAChR-immunoreactivity**

The between-groups comparison indicated a significant treatment effect on mAChR-ir in CA1 and CA3 fields (P< 0.01; P< 0.04; Kruskal Wallis test), respectively. Posthoc testing revealed a significantly increased immunostaining of the ADX group in both cornu ammonis fields CA1 and CA3 (P<0.05). The ADX-induced increment, however, was reduced to control level by application of corticosterone immediately after ADX (Fig 1A). The labeling-intensity in the molecular layer of the dentate gyrus was less pronounced and non-significantly affected by ADX or cort/ADX.

![Figure 1.](image)

The effects of antagonist treatment on the changes mAChR immunostaining in CA1, CA3 and dentate gyrus molecular layer are shown in figure 1B. Kruskal Wallis analysis of the data revealed a significant effect between controls and antagonist treated animals in these hippocampal regions (P<0.01; P< 0.03; P<0.04), respectively. Administration of the MR blocker or a combination of both MR and GR antagonists profoundly enhanced the mAChR immunostaining in CA1, CA3 and although significantly less striking in dentate gyrus molecular layer. The immunolabelling was significantly (P<0.05) increased in pyramidal cell bodies and their apical dendrites in all hippocampal areas investigated. In contrast to the aMR or aMR/aGR treated animals the GR blockade alone induced no significant increase in the mAChR labeling-intensity (Fig 1B). The pattern of mAChR-ir in the CA1 area in the various
experimental groups are shown in Figure 2.

![Photomicrographs of the hippocampal CA1 area immunostained to mAChRs of Sham (A), ADX (B), cortADX (C), aMR/aGR (D), aMR (E) and aGR (F) treated rats.](image)

Figure 2. Representative photomicrographs of the hippocampal CA1 area immunostained to mAChRs of Sham (A), ADX (B), cortADX (C), aMR/aGR (D), aMR (E) and aGR (F) treated rats.

**Discussion**

In general, the present results indicate that 24 hours after adrenalectomy or selective blockade of corticosteroid receptors, mAChR immunoreactivity in the hippocampal formation of the rat is strikingly enhanced. MR blockade and adrenalectomy resulted in a similar increase in mAChR-ir, indicating that corticosterone modulates mAChRs through an MR- rather than a GR-mediated pathway. This conclusion is further supported by the finding that aGR treatment did not affect mAChR-ir. Furthermore, a combined aMR/aGR treatment resulted in the same enhancement as aMR alone. Since the occupation of MR’s is already high at low levels (i.e. base line) of corticosterone the interaction between corticosterone and hippocampal mAChRs is rather tonic than phasic (i.e. reactive) of character. The present findings are in line with the autoradiographic results of Biegon et al. (1985). In this study rats were adrenalectomized, while half of the animals were supplied with a corticosterone pellet under the skin after which the brains were processed for mAChR autoradiography. Binding of [1H]QNB in the dorsal hippocampus was reduced in adrenalectomized, corticosterone supplemented rats as compared to ADX animals.

The changes in mAChR-binding may further be interpreted in terms of changes in receptor-mediated responsiveness to mAChR-mediated agonist stimulation. Recent electrophysiological studies have shown that corticosterone dose-dependently modulates responsiveness of CA1 pyramidal neurons to the cholinergic agonist carbachol in hippocampal slices from ADX rats (Hesen and Joëls 1993; 1996). Large carbachol responses were recorded in the presence of very low doses of corticosterone, suggestive for an MR-mediated effect. Moreover moderate amounts of corticosterone significantly suppressed the depolarizing effects of carbachol. The effect of low corticosteroid levels on the excitability (Hesen and Joëls 1993; 1996) indicate that reduced corticosterone levels of ADX animals possibly result in a cholinergic supersensitivity in hippocampal neurons. The above mentioned studies and our own findings all point to an MR-mediated impact of corticosterone on the mAChR.

It remains unanswered, however, how an increase of mAChR-ir should be interpreted in terms of the functional meaning of cholinceptive mechanisms in learning and memory processes. Based on recent observations on mAChR-ir in the course of training in a spatial learning paradigm, and the impact of ADX on learning performance, we propose the following option.
Previously we reported a strongly enhanced mAChR-ir in the CA1 region after training rats and mice in a spatial orientation test (van der Zee et al. 1995; Douma et al. 1997; Beldhuis et al. 1992). Several lines of evidence now point to cholinergic activation for shifting neuronal modulation from intrinsic to external input, which may occur in the initial, attention phase of training (Dunne and Hartley 1986; Blokland 1995; Hasselmo 1995). Since ADX reportedly deteriorates spatial learning performance (Oitzl and de Kloet 1992), and at the same time enhances mAChR-ir, the increase of mAChR immunostaining could be interpreted as representing a functional state of the muscarinic receptor that renders it less sensitive to, and hence inhibits the effects of ACh in the post-attention phase of training.

In conclusion, this study shows that the mAChR-ir of hippocampal pyramidal neurons is sensitive to changes in the corticosterone plasma levels modulated by an MR- rather than GR-mediated pathway.

Acknowledgements

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