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RESEARCH ARTICLE

Differences in colocalization of corticosteroid-binding globulin and glucocorticoid receptor immunoreactivity in the rat brain

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SUMMARY

Endocrine regulation of central and systemic stress response as well as learning and memory are in part controlled by systemic glucocorticoid levels. So far steroids have been thought to act on the brain predominantly through nuclear receptors. However, some brain systems known to respond to glucocorticoids seem to be devoid of the respective receptor proteins (GR). It is likely that known central actions of adrenal steroids may also be mediated by non-genomic actions involving intrinsic binding globulins. In recent studies we described the intrinsic expression of corticosteroid-binding globulin (CBG) in rat, mouse and human brains. Here we report an immunohistochemical mapping study on the colocalization of CBG and of GR in the rat brain. In the nucleus accumbens, septum, hippocampus, globus pallidus, medial and basolateral amygdale nuclei, magnocellular preoptic nuclei, diagonal band of Broca high intensity of CBG immunoreactivity was accompanied by weak or moderate GR staining, and *vice versa*. In the caudate putamen, bed nucleus of stria terminalis, septohypothalamic nucleus and parvocellular subdivision of the paraventricular nucleus strong GR immunoreactivity was observed, but CBG was almost undetectable. In contrast, throughout the supraoptic nucleus and magnocellular subdivision of the paraventricular nucleus numerous strongly CBG-positive cells were observed, devoid of specific GR immunoreactivity. It is most likely that CBG in the brain may be involved in the response to changing systemic glucocorticoid levels in addition to known nuclear and membrane corticosteroid receptors, or in glucocorticoid responsive regions devoid of these receptors.

1. Introduction

Adrenal steroids are known to have great functional importance in the brain. Central and systemic stress response as well as learning and memory are likely to be controlled by glucocorticoids (GCs) and mineralocorticoids (MCs). It has been shown that GCs are involved in memory consolidation, while MCs seem to be involved in appraisal and response to novelty (Ter Horst et al., 2012). Steroids are capable of crossing the blood brain barrier thus being predestined peripheral mediators of central functions through a feedback loop. The hypothalamo-pituitary adrenal (HPA) axis is the best described circuit in this respect (Aguilera, 1994). Central actions of GCs have been thought to be mediated predominantly through nuclear glucocorticoid receptors (GR) which have been well described and characterized in various brain regions (Fuxe et al., 1985; Reul and de Kloet, 1985; Chao et al., 1989; Ahima and Harlan, 1990). However, many of the clearly GC sensitive brain systems seem to be devoid of GR under basal conditions. The effects of GCs on synthesis and release of vasopressin and oxytocin in magnocelluar perikarya of the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei (Jirikowski et al., 1993; Berghorn et al., 1995) have been demonstrated (for review, see de Kloet, 2000). However, the absence of GR suggests that GCs may act on these neurons in a nuclear GR-independent manner.

Systemic GCs are known to be transported in the blood stream bound to specific binding globulins. More than 95% of serum GC bound to corticosteroid-binding globulin (CBG), a well characterized 50–60 kDa glycoprotein (Westphal, 1986; Hammond, 1990), thought to serve as a buffer of serum steroid levels (Westphal, 1971). CBG is a member of serine proteinase inhibitor (SERPIN) family and it is mainly produced by hepatocytes (Hammond et al., 1987; Scrocchi et al., 1993). However, its extrahepatic expression (including the central nervous system) has also been demonstrated (Miska et al., 2004; Misao et al., 1994, 1999; Scrocchi et al., 1993; Del Mar Grasa et al., 2001; de Kloet and McEwen, 1976; Perrot-Applanat et al., 1984). Recent findings of various groups suggest that the functions of CBG could be much more diverse than assumed so far (for review, see Moisan, 2010; Henley and Lightman, 2011; Cizza and Rother, 2012). At the present time, CBG is believed to take part in rapid, non-genomic effects of GC in various cells (Rosner, 1991; Orchinik et al., 1997; for review, see Gagliardi et al., 2010). We have recently shown that GCs stimulate rapid secretion of CBG

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independently of GR perhaps through membrane action in neural cells *in vitro* (Pusch et al., 2009). Recently, the presence of mineralocorticoid receptors (MR) in magnocellular hypothalamic neurons has also been reported (Pietranera et al., 2012) suggesting the possibility of a co-localization of CBG with MR in these cells.

Based on these findings, the primary objective of the current study has been to perform a comprehensive mapping of CBG and GR immunoreactivity (IR) in the various regions of the rat brain. Analysis of the possible co-expression of CBG with MR was beyond the scope of the present work. In previous studies, we described the immunohistochemical distribution of CBG in human hypothalamus and in rat brain, partially co-localized with the two important peptides of stress response – vasopressin and oxytocin (Sivukhina et al., 2006; Möpert et al., 2006; Jirikowski et al., 2007). Here we paid particular attention to brain regions known to be functionally linked to GCs effects, including the limbic system and the neuroendocrine hypothalamic nuclei.

2. Materials and methods

The animal experiments performed in this study have been carried out in accordance with the *European Union Directive 2010/63/EU for animal experiments* (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm).

Intact adult male Wistar rats ($n = 3$) were killed by prolonged ether anesthesia followed by cardiac perfusion with 4% paraformaldehyde in isotonic phosphate-buffered saline (PBS). After fixation, brains were removed and post-fixed in the same fixative overnight at 4 °C. On the next day, series of frontal sections (50 μm thick) were cut in a rostral-to-caudal direction on a vibratome (Leica VT1000E, Leica Instruments, Nußloch, Germany) and collected in PBS until staining was performed.

Free-floating consecutive sections were stained with antibodies to either CBG or GR as described (Sivukhina et al., 2009). Briefly, blocking of non-specific binding sites was performed in multi-well plastic plates for 1 h at room temperature (RT) using PBS containing 0.05% Triton X-100 and 5% normal goat serum (PBS-NGS). Incubation with one of the primary rabbit polyclonal antibodies – anti-CBG (Affiland, Inc., 1:500) or anti-GR (M-20, Santa Cruz Biotechnology, Inc., 1:200) in working solution (WS; 1% NGS, 0.05% Triton-X in PBS, pH 7.4) was carried out at 4 °C for 3 days. Then sections were washed in PBS and incubated in WS with biotinylated goat anti-rabbit IgG (Vector Elite kit, Vector Laboratories, Inc., Burlingame, CA; 1:200). After washing in PBS, avidin–biotin–peroxidase complex (Vector Laboratories, CA) in PBS was applied. The reaction product was visualized with diaminobenzidine (DAB, Sigma) and H_2O_2 in PBS. After rinsing in distilled water, sections were affixed onto microscopic slides and mounted with gelatine.

To evaluate co-localization of GR with CBG double immune staining was performed. After the sections were stained for GR as described above, they were treated for 15 min in 0.1 M glycine buffer at pH 3.4 in order to remove the immunocomplexes (Sivukhina et al., 2006). Subsequently, sections were incubated for 3 days at 4 °C in rabbit polyclonal anti-CBG (1:250 in 0.5% Triton X 100-PBS containing 1% NGS) and developed with Cy3-labeled goat anti-rabbit Fab fragment (Dianova, 1:300 in 0.5% Triton X 100-PBS containing 1% NGS). After washing, sections were affixed onto microscopic slides and mounted with antifading medium (Fluoromount G, Southern Biotechnology Associates, Biozol, Eching, Germany). For immunohistochemical control, incubation with the preabsorbed anti-CBG antibodies instead of the specific antibody was performed as described above. For the preabsorption, rat CBG protein (USCN Life Sci, Inc., Cat. Nr. E91226Ra) was immobilized on cyanogen bromide activated Sepharose 4b according to the manufacturers protocol (Sigma, Munich). A 1 ml Pasteur pipette stuffed with a few fibers of glass wool served as a column. Then, 100 μl aliquots of anti-CBG, diluted 1:10 in PBS were pipetted onto the column and allowed to bind for 1 h at RT. Thereafter, the column was rinsed with 1 ml of PBS. The rinsing solution was assumed to be stripped of specific antibody.

Immunostained sections were examined under an "Olympus BX50" and "Zeiss Axio Imager.M1" microscopes. Digital cameras "Olympus DP10" and "AxioCam MRc" were used for microphotography. Digital images were adjusted for contrast and brightness in "Adobe Photoshop" (Adobe Systems, version 8.0.1). Anatomical identification of labeled structures was based on the cytoarchitectonic descriptions of the rat brain (Paxinos and Watson, 1986).

3. Results

CBG IR was found as a granulated product predominantly in cells with neuron-like morphology, confined to the perinuclear cytoplasm and to processes throughout various brain regions. The intensity of CBG-staining varied from weak to moderate. An overview of the distribution of CBG IR is given in Fig. 1. CBG-positive cells could be found in different locations of the basal forebrain such as the medial and the lateral septum, the septofimbrial nucleus, the vertical and the diagonal band of Broca as well as the nucleus accumbens (Fig. 1A and B). CBG IR was observed in numerous cells of the amygdaloid complex and in the endopiriform region (Fig. 1D and E). Large numbers of CBG-stained neuronal perikarya were also found in the CA 2 and CA 3 regions as well as in the dentate gyrus of the hippocampus (Fig. 1F). These cells appeared mostly in the pyramidal layer while CBG positive cells CA 1 region only appeared to be scattered and non-pyramidal. CBG stained cells were observed in various regions of the cerebral cortex, including the frontal, cingulate, parietal and piriform cortex (Fig. 1D). In the preoptic nucleus and in the medial preoptic area intensity of immune signal was most pronounced (Fig. 1C). In the lateral and medial hypothalamus CBG IR was detected in many cells throughout the lateral hypothalamic area, lateroanterior and ventromedial hypothalamic nucleus, lateral preoptic area, medial preoptic nucleus and anterior commissural nucleus (Fig. 1C–F). In the PVN CBG-positive cells were predominantly observed in the magnocellular portion while only few parvocellular CBG-stained neurons were detected (Fig. 2E). Numerous magnocellular CBG-immunoreactive cells were also found in the SON (Fig. 2G). In the median eminence CBG IR could be found mainly in axonal varicosities of the internal layer. Moreover, CBG-positive cells were also observed distributed throughout the thalamus, ventral pallidum, globus pallidus and in the accessory olfactory nuclei (Fig. 1B–F).

GR IR was in all cases nuclear while cytoplasm and neuronal processes remained mostly unstained. Since cellular staining patterns of CBG and GR were quite different (nuclear for GR and cytoplasmic for CBG) distinction of the fluorescent and peroxidase stained reaction products was rather easy. Distribution of both immunoreactivities is compiled in Fig. 1. In some brain regions, like the anterior and the central amygdaloid nuclei, the thalamus, cerebral cortex, ventral pallidum, endopiriform region and in the accessory olfactory nuclei both immunosignals were found to be equally distributed and mostly co-localized. Most of the colocalization of both antigens was found throughout the cerebral cortex. However, in other structures like the nucleus accumbens, the lateral and medial septum, the globus pallidus, medial and basolateral amygdaloid nuclei, magnocellular preoptic nuclei und in the diagonal band of Broca, the high intensity of CBG IR (Fig. 2A) was accompanied by weak or moderate GR-immunosignal (Fig. 2B).

Interestingly, in the caudate putamen (Fig. 2C and D), the lateral part of bed nucleus of the stria terminalis, the septohypothalamic nucleus as well as the parvocellular subdivision of the PVN (Fig. 2E and F) known to be strongly GR-positive, CBG IR was found only in few cells. In contrast, throughout the SON and magnocellular