Effects of stress and corticosterone on the hippocampus: linking gene transcription to physiology
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Chapter 2

Effect of brief corticosterone administration on SGK1 and RGS4 mRNA expression in rat hippocampus

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Abstract

Acute stress and corticosterone have been found to enhance 5-HT\textsubscript{1A} receptor mediated responses in rat hippocampal CA1 cells. This altered 5-HT\textsubscript{1A} responsiveness could not be explained by changes in 5-HT\textsubscript{1A} receptor mRNA expression. We tested the hypothesis that the corticosterone targets RGS4 or SGK1 gene expression, and thus affects 5-HT\textsubscript{1A} receptor function. To that end, the effect of a single corticosterone injection on hippocampal mRNA expression of RGS4 and SGK1 was studied. Hippocampal expression of neither RGS4 nor SGK1 was affected by the corticosterone injection. Strikingly, SGK1 mRNA was strongly up-regulated in the corpus callosum. We reject however the hypothesis that the effect of corticosterone on 5-HT\textsubscript{1A} responsiveness is mediated via altered RGS4 or SGK1 mRNA expression.

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Introduction
In response to stress, the hypothalamus-pituitary-adrenal (HPA) axis is activated, eventually leading to the release of corticosterone from the adrenal glands. Corticosteroids act as transcriptional regulators, thereby affecting many physiological parameters of the cell. Corticosterone has previously been found to affect serotonin (5-hydroxytryptamine; 5-HT) 1A receptor mediated responses in CA1 cells of the rat hippocampus. Specifically, after acute stress, a single corticosterone injection, or incubation of hippocampal slices with high levels of corticosterone, increased 5-HT1A receptor mediated responses were found (Hesen and Joels 1996; Joels and de Kloet 1992; Joels et al. 1997; Karst et al. 2000). This effect could be reversed by treatment with the glucocorticoid receptor (GR) antagonist mifepristone (Hesen and Joels 1996; Joels and de Kloet 1992; Joels et al. 1997) and was found to be dependent on homodimerization and DNA binding of the glucocorticoid receptor (Karst et al. 2000), indicating that transcriptional changes are necessary for alterations in 5-HT1A receptor mediated responses to occur.

Data on the effect of corticosterone on 5-HT1A receptor mRNA expression in the hippocampus is less conclusive. Some studies report a decrease in mRNA in the hippocampal CA1 area after acute administration of a high corticosterone dose (Czyrak et al. 2002), others report no effect (Meijer and de Kloet 1994; Meijer and de Kloet 1995). In any case, no increased 5-HT1A mRNA expression in the CA1 area has been found that could account for the observed increase in responses. Therefore, we here tested if corticosterone targets the transcription of other genes that might be involved in altered 5-HT1A mediated responses. Two candidate genes were selected that were earlier 1) shown to be sensitive to stress or corticosterone and 2) found to affect 5-HT1A receptor function.

The first candidate is regulator of G-protein signaling 4 (RGS4). Like other members of the RGS family, this molecule decreases G-protein mediated signaling by increasing GTPase activity (Berman et al. 1996; Tesmer et al. 1997; Watson et al. 1996), and suppresses 5-HT1A receptor mediated inwardly rectifying K+ (Kir) currents (Beyer et al. 2004; Doupnik et al. 1997; Ghavami et al. 2004; Inanobe et al. 2001). RGS4 mRNA expression was found to be affected by acute and chronic corticosterone elevations in locus coeruleus and in the paraventricular nucleus (PVN) of the hypothalamus (Ni et al. 1999). The second candidate is serum- and glucocorticoid-regulated kinase 1 (SGK1), which regulates cell surface expression of various ion channels, including the Kc channel which is activated via the 5-HT1A receptor (Gamper et al. 2002; Huang et al. 2004; Lang et al. 2003; Yoo et al. 2003). SGK1 mRNA expression was found to be increased by stress stimuli and glucocorticoid treatment in various cell lines and in the brain (Chen et al. 1999; Koya et al. 2005; Leong et al. 2003; Murata et al. 2005; Naray-Fejes-Toth et al. 2000). In this study, mRNA expression of these two candidate genes was tested one hour after injection with a high dose of corticosterone, a treatment that increases 5-HT1A receptor mediated responses (Hesen and Joels 1996). The timepoint chosen seems highly
relevant since functional changes develop after 1-2 hours (Joels and de Kloet 1992). Any transcriptional changes resulting in functional alterations should therefore take place earlier in time.

**Materials and methods**

The experiments were approved by the local Animal Experiment Committee (protocol number DED108). All efforts were made to prevent suffering of animals and limit the number of animals used as much as possible. Male Wistar rats (n = 16; Harlan, Horst, the Netherlands) weighing 197 ± 9 g were housed individually on a 12 h light/dark cycle (lights on at 8.00 a.m.) with food and drinking water ad libitum. On the day of the experiment, at 9.30 a.m., animals received an injection with oil (vehicle group; n = 8) or corticosterone (Sigma, the Netherlands; 10 mg/animal) (cort group; n = 8). The injections were given subcutaneously in a total volume of 800 μl. At 10.30 a.m., one hour after the injection, animals were quickly decapitated. Trunk blood was collected and centrifuged at 5000 rpm for 20 min at room temperature. Plasma was stored at -20 °C until use in a radioimmunoassay (RIA; ICN Biomedicals Inc., Costa Mesa, CA, USA) to determine plasma corticosterone levels. The brain was rapidly dissected out of the skull, frozen on dry ice, and stored at -80 °C.

For *in situ* hybridization, 12 μm thick coronal sections containing the hippocampus were cut on a cryostat and mounted on SuperFrost Plus slides (Menzel-Glaser, Braunschweig, Germany). Sections were fixed in 4 % paraformaldehyde for 60 minutes and subsequently washed twice in 1x phosphate buffered saline (PBS) at room temperature. Sections were then acetylated for 10 minutes in 0.1 M triethanolamine (pH = 8.0) with 0.25 % acetic anhydride, washed in 2x saline sodium citrate (SSC) for 10 minutes, dehydrated in an ethanol series (50 %, 80 %, 100 %, 100 %; 1 minute each) and air-dried.

To study RGS4 mRNA expression, ~240 bp sense and antisense riboprobes labeled with [35S]-UTP were generated from a linearized cDNA clone (courtesy of M.R. Koelle; (Koelle and Horvitz 1996)) using T7 and T3 polymerase, respectively. Hybridization mix was prepared containing 50 % formamide, 10 % dextran sulphate, 100 mM DTT, 350 mM NaCl, 25 mM Tris-HCl, 1.2 mM EDTA, 1x Denhardt’s solution, 0.1% Na-thiosulphate, 0.1% SDS, 100 μg/ml tRNA, and 100 μg/ml hsDNA. Probes were added to the hybridization mix in a concentration of $3 \times 10^6$ cpm per 100 μl. To each slide, 100 μl of this mix was applied. Slides were then coverslipped and incubated overnight at 55 °C. The next day, coverslips were carefully removed and slides were rinsed in 2x SSC at room temperature and subsequently incubated for 30 minutes at 37 °C with RNase A in 0.5 M NaCl and 10 mM Tris-HCl. Sections were then washed at 55 °C in 2x SSC for 5 minutes, in 0.5x SSC for 10 minutes, and in 0.1x SSC for 30 minutes. Sections were dehydrated in an alcohol series, air-dried, and exposed to a Kodak Biomax MR film for 3 weeks.
Brief corticosterone administration

For SGK1 mRNA expression, α-[33P]-dATP end-labeled deoxyoligonucleotide probes were used. Sequences were 5’ tctggaagagaagggccacagaaaggtgttcatc (match; reverse complement of nucleotides 456-501 of rat SGK1 coding sequence) and 5’ gctggacagacgatgccaacaggagcgttcacat (mismatch). 0.33 pmol of the probe was end-labeled with 3.3 pmol 33P-ATP (NEN, Boston, MA, USA) using Terminal deoxynucleotidyl Transferase (TdT; Roche, Woerden, the Netherlands), purified with a chloroform extraction and ethanol-precipitated. Per slide, 100 μl hybridization mix containing 50 % formamide, 10 % dextran sulphate, 20 mM DTT, 25 mM NaSO4, 1 mM Na-pyrophosphate, 4x SSC, 5x Denhardt’s solution, 100 μg/ml poly A, 100 μg/ml hsDNA, and 1·10^6 cpm SGK1 probe was added. Sections were then coverslipped and incubated overnight at 42 °C. The next day, coverslips were removed and sections were rinsed in 2x SSC at room temperature and subsequently incubated for 30 minutes at 37 °C with RNase A in 0.5 M NaCl and 10 mM Tris-HCl. After this, sections were washed in 1x SSC twice for 30 minutes at 50 °C, and once for 5 minutes at room temperature. Slides were then dehydrated in an alcohol series, air-dried and exposed to a Kodak Biomax MR film for 2 weeks.

Eight hippocampal sections per animal were scanned, loaded into Image J (Image J 1.31v), and corrected for background. The cell layers of the CA1, CA3 and dentate gyrus regions were analyzed for grey value. Expression was further tested by analyzing grey values of the medial part of the corpus callosum directly above the hippocampus, and the average of all layers of the cerebral cortex overlaying the hippocampus. The value of the stratum lacunosum/moleculare of the CA3 area, which shows no detectable signal in either experimental condition, was used as tissue background. Per animal, the grey values for each region were averaged. After this, the values of all animals of the same group were averaged. Data are presented as mean ± standard error of mean (SEM). Since the effects of corticosterone on mRNA expression in different brain areas are independent of each other, and our question particularly addressed expression changes in the CA1 area, statistical testing was performed by means of multiple unpaired Student’s t-tests. A P-value < 0.05 was considered statistically significant.

Results

As expected, plasma corticosterone levels were significantly increased by the injection with a high dose of corticosterone (vehicle 1.3 ± 0.1 μg/dl; cort 60.0 ± 11.3 μg/dl; P < 0.001). These values are comparable to the elevated plasma corticosterone concentration that was earlier shown to increase 5-HT1A receptor mediated responses (Hesen and Joels 1996).

RGS4 mRNA expression showed a clear distribution in the hippocampus, with moderate expression in the CA1 and CA3 regions when compared to thalamus and cortex, and lower expression in the dentate gyrus (Figure 1). This is in line with previous findings (Gold et al. 1997; Ingi and Aoki 2002). A single injection with a high dose of corticosterone did not affect RGS4 mRNA expression measured one hour later in any of
the hippocampal subfields tested. In the corpus callosum and cortex, also no effect of corticosterone on RGS4 mRNA expression was found (data not shown). The hippocampal expression of SGK1 also showed a clear distribution, with low levels in CA1 and dentate gyrus, and relatively high levels in the CA3 area (Figure 2). This was previously described by most (Imaizumi et al. 1994; Inanobe et al. 2001; Lee et al. 2001) but not all groups (Tsai et al. 2002). A single corticosterone injection did not affect SGK1 mRNA expression in any of the hippocampal subfields tested or in the cortex. However, corticosterone did induce a > 5 fold increase in SGK1 mRNA expression in the corpus callosum (P < 0.001; Figure 2).

**Figure 1:** RGS4 mRNA expression in the hippocampal subfields (CA1, CA3, and DG) tested was not significantly affected by an injection with high corticosterone (CA1: P = 0.17; CA3: P = 0.39; DG: P = 0.18). Insets show the hybridization signals for both groups. Hybridization with the sense probe did not yield any specific signal (data not shown).

**Figure 2:** SGK1 mRNA expression in several brain areas after a vehicle or corticosterone injection. Expression in the hippocampal subfields (CA1, CA3, and DG), or in the cortex (CX), was not affected by corticosterone (CA1: P = 0.75; CA3: P = 0.84; DG: P = 0.78; CX: P = 0.99). In the corpus callosum (CC), SGK1 mRNA expression was significantly (P < 0.001) increased 1 hour following a single injection with a high level of corticosterone. Insets show the hybridization signals for both groups. Hybridization with the mismatch probe did not yield any specific signal (data not shown).
Discussion

This study was initiated to examine putative mechanisms underlying the increase of 5-HT$_{1A}$ receptor mediated responses one hour after corticosterone administration in hippocampal CA1 cells (Hesen and Joels 1996; Joels and de Kloet 1992; Joels et al. 1997; Karst et al. 2000), an increase that was not accompanied by increased 5-HT$_{1A}$ mRNA expression in this area (Czyrak et al. 2002; Meijer and de Kloet 1994; Meijer and de Kloet 1995). We hypothesized that transcriptional regulation of RGS4 or SGK1 was involved. Our findings however indicate that RGS4 or SGK1 expression most likely does not contribute to the observed corticosterone-induced changes in 5-HT$_{1A}$ receptor mediated responses in the CA1 area.

The first candidate we tested was RGS4. A single injection with corticosterone was previously found to differentially affect RGS4 mRNA expression in locus coeruleus and PVN 6 hours later (Ni et al. 1999). In other brain regions no dramatic changes in RGS4 mRNA expression were found, indicating that the effect of corticosterone on RGS4 expression in the brain is region specific. Also in our study, 1 hour after a single corticosterone injection no differences in RGS4 mRNA expression were found in any of the hippocampal subfields tested.

SGK1 is a direct glucocorticoid target gene, which was previously found to be upregulated by various glucocorticoid treatments and stress stimuli in cell cultures (Itani et al. 2002; Leong et al. 2003; Webster et al. 1993), in an immediate early gene-like time frame (Chen et al. 1999). Moreover, SGK1 expression in the brain was found to be upregulated after various treatments (Koya et al. 2005; Lee et al. 2003; Murata et al. 2005). After exposure to an elevated plus maze, SGK1 mRNA expression was found to be increased in the ventral tegmental area (VTA) but not the nucleus accumbens or prefrontal cortex, indicating regional specificity in the transcriptional regulation of SGK1 (Koya et al. 2005). Still, increased SGK1 expression in the hippocampus has been found after psychophysiological stress (Murata et al. 2005) and environmental enrichment (Lee et al. 2003), although it is possible that stress-related factors other than corticosterone, like corticotrophin releasing hormone or noradrenalin, induced this effect. In our study, a single injection with corticosterone did not result in elevated SGK1 mRNA levels in any of the hippocampal subfields tested.

In the corpus callosum, however, a specific and marked increase of SGK1 mRNA expression was found 1 hour after injection with corticosterone. The upregulation of gene expression was not a generic effect, since RGS4 mRNA expression in the corpus callosum was not affected (data not shown). Previously, increased SGK1 mRNA expression has been found in the corpus callosum 2 hours after transient global cerebral ischemia (Nishida et al. 2004). Also, 3 to 14 days after brain injury increased SGK1 expression was found in this area (Imaizumi et al. 1994). In the latter case, oligodendrocytes were likely to be responsible for the SGK1 mRNA upregulation; this might also be the case in our present findings. Peripheral and central glial cell types can show a strong transcriptional response to glucocorticoids (Grenier et al. 2005; Zhu et al. 1994). The cell
specific mechanisms of GR signaling found in different types of glial cells (Fonte et al. 2005; Grenier et al. 2006) may form part of the explanation for the regional specificity of the presently observed effect.

An indication that corticosterone might indirectly affect SGK1 expression in glia can be found in the observation that corticosteroids cause a strong shrinkage of cultured microglial cells (Tanaka et al. 1997). SGK1 mRNA expression has previously been found to be highly responsive to cell volume, where dehydration and shrinkage of cells led to increased SGK1 transcript levels (Wärntges et al. 2002). Whether this indeed can explain the increased SGK1 mRNA expression in corpus callosum found after corticosterone injection remains to be studied. This also goes for the functional consequences of the SGK1 mRNA upregulation. The finding does raise the possibility that the axonal information flow may be subject to regulation by corticosterone.

The objective of this study was to explain corticosterone-induced changes of 5-HT\textsubscript{1A} receptor mediated responses in CA1 cells of the hippocampus, which already occur after 1-2 hours (Joels and de Kloet 1992). Any changes in transcriptional regulation that might account for these functional changes should therefore take place earlier in time, i.e. within 1 hour after corticosterone injection. This time frame is indeed sufficient for changes in mRNA levels to occur, as demonstrated by a recent study where corticosterone was found to affect transcriptional regulation of 81 genes in the hippocampus already after 1 hour (Morsink et al. 2006). With respect to RGS4 we are not aware of such rapid transcriptional regulation, as the effect of corticosterone on mRNA levels was assessed previously after longer periods of time (Ni et al. 1999). The SGK1 gene, however, has the capacity to react to corticosteroids within 30 minutes, even at the protein level (Chen et al. 1999) – as is also evident from the effect observed in corpus callosum in the present study.

The marked effect of corticosterone on SGK1 mRNA in the corpus callosum indicates that corticosterone treatment and quality of the tissue were adequate in this study. Furthermore, in tissue of the same animals altered hippocampal (other transcript) mRNA expression has been found (Morsink et al. 2006).

In summary, the increased 5-HT\textsubscript{1A} responsiveness found in hippocampal CA1 cells after exposure to a high level of corticosterone is not accompanied by changes in the mRNA expression of 5-HT\textsubscript{1A}, RGS4 or SGK1. Therefore, we conclude that other, less obvious, candidates which are regulated by corticosterone can affect 5-HT\textsubscript{1A} receptor function.