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SOURCE (OR PART OF THE FOLLOWING SOURCE):

Type	Dissertation
Title	Effects of stress and corticosterone on the hippocampus: linking gene transcription to physiology
Author	N.G. van Gemert
Faculty	Faculty of Science
Year	2008
Pages	144

FULL BIBLIOGRAPHIC DETAILS:
http://dare.uva.nl/record/270654

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Chapter 3

No effect of prolonged corticosterone over-exposure on NCAM, SGK1, and RGS4 mRNA expression in rat hippocampus

Brain Research 2006; 1093(1):161-166

No effect of prolonged corticosterone over-exposure on NCAM, SGK1, and RGS4 mRNA expression in rat hippocampus

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Abstract

Prolonged over-exposure of rats to corticosterone attenuates 5-HT_{1A} receptor-mediated responses in hippocampal CA1 cells through an unknown mechanism, not involving downregulation of 5-HT_{1A} receptor expression. We here tested if corticosterone changes 5-HT_{1A} receptor function indirectly, by altering hippocampal mRNA expression of NCAM, SGK1, or RGS4, which all modulate 5-HT_{1A} receptor function. We found that the expression of none of these candidates was affected by corticosterone treatment.

Acknowledgements

Maaike van der Mark is acknowledged for the performance of the radioimmunoassay. Siem van der Laan and Peter Steenbergen are acknowledged for their help with the in situ hybridizations.

Introduction

Activation of the hypothalamus-pituitary-adrenal (HPA) axis, e.g. by stress, triggers a cascade of events, eventually causing the adrenal glands to release corticosterone. Prolonged elevations of corticosterone levels, e.g. by hormone injections or stress during 21 days, were earlier shown to reduce hyperpolarizing responses of hippocampal CA1 cells to serotonin (5-hydroxytryptamine, 5-HT) (Karten et al. 1999; van Riel et al. 2003; van Riel et al. 2004), which develop due to 5-HT_{1A} receptor activation (Andrade and Nicoll, 1987). These attenuated responses were not accompanied by reduced 5-HT_{1A} receptor mRNA expression, indicating that processes other than transcriptional regulation of this receptor are involved. Here, the effect of repeated corticosterone administration was studied on the hippocampal expression of three candidate genes that were earlier 1) shown to be sensitive to stress or corticosterone and 2) found to affect 5-HT_{1A} receptor function.

The first candidate is the neural cell adhesion molecule (NCAM). In NCAM knockout mice, sensitization to the activation of the 5-HT_{1A} receptor was observed, caused by increased surface localization of inwardly rectifying K^+ (K_{ir}) channels, which are coupled to 5-HT_{1A} receptors through a G-protein (Delling et al. 2002; Stork et al. 1999). Different NCAM isoforms exist, i.e. $NCAM_{120}$, $NCAM_{140}$, and $NCAM_{180}$; the latter two are predominantly expressed in neurons. These isoforms are generated by alternative splicing (Barthels et al. 1988; Santoni et al. 1987), and differentially regulated by stress and corticosteroids (Sandi and Loscertales 1999; Sandi et al. 2001; Touyarot et al. 2004; Venero et al. 2002). The second candidate is the serum- and glucocorticoid-regulated kinase 1 (SGK1), which regulates the cell surface expression of several ion channels including K_{ir} channels (Gamper et al. 2002; Huang et al. 2004; Lang et al. 2003; Yoo et al. 2003). SGK1 mRNA expression was found to be regulated by glucocorticoids and stress in various cell lines and in the brain (Koya et al. 2005; Leong et al. 2003; Murata et al. 2005; Naray-Fejes-Toth et al. 2000). Finally, we examined the regulator of G-protein signaling 4 (RGS4), which - like other members of the RGS family - suppresses G-protein mediated signaling by increasing GTPase activity (Berman et al. 1996; Tesmer et al. 1997; Watson et al. 1996) and decreases 5-HT_{1A} mediated Kir currents (Beyer et al. 2004; Doupnik et al. 1997; Ghavami et al. 2004; Inanobe et al. 2001). Acute and chronic elevations of corticosterone were found to affect RGS4 mRNA expression in the locus coeruleus and in the paraventricular nucleus (PVN) of the hypothalamus (Ni et al. 1999). In the present study, hippocampal mRNA expression of these three candidates was studied in rats subjected to corticosterone injections once daily for 21 days and in vehicle-treated controls.

Materials and methods

The experiments were approved by the local Animal Experiment Committee (protocol number DED88). All efforts were made to prevent suffering of animals and to limit the number of animals used. Nine-week-old male Wistar rats (Harlan, Horst, the

Netherlands) were housed individually on a 12 h light/dark cycle, with access to food and water ad libitum. Animals received daily subcutaneous injections with oil (vehicle group; n=8) or corticosterone (Sigma, the Netherlands; 10 µg/animal) (cort group; n=8) at 9.00 a.m. for 21 days. We took care to place subcutaneous injections at different locations of the body over the course of the 21 days period, to prevent infections. Also, animals were checked daily for wounds and infections at the injection site, which were not found. In the morning of day 22, animals were rapidly decapitated and trunk blood was collected. We presently chose to decapitate rats 1 day after the last injection, to focus on the effect of prolonged elevations in corticosteroid level rather than acute rises (as caused by the last injection). This delay was earlier used too in a study where rats were subjected to 21 days of variable stress (Van Riel et al., 2003). In that study, we observed that 5-HT_{1A} receptor mediated responses were attenuated (regardless of the circulating corticosteroid levels) while 5-HT_{1A} receptor mRNA expression was unaffected, similar to what was observed when rats received corticosterone for 21 days and were killed one hour later (Karten et al., 1999). Apparently, the one day delay is not an important factor when examining 5-HT responses, although it could be important for other parameters. Plasma was stored at -20 °C until use in a radioimmunoassay (RIA; ICN Biomedicals Inc., Costa Mesa, CA, USA) to determine basal corticosterone levels. Adrenals and thymus were removed and weighed on an analytical balance (Explore, Ohaus, France). The brain was dissected out of the skull, rapidly frozen on dry ice and stored at -80 °C. Twelve µm coronal sections containing the hippocampus were cut on a cryostat and mounted with 4 sections per slide on SuperFrost Plus slides (Menzel-Glaser, Braunschweig, Germany). *In situ* hybridizations were performed as described previously (Bartsch et al. 1992; van Riel et al. 2004).

For $NCAM_{total}$, $NCAM_{180}$, and RGS4 , sense and antisense riboprobes labeled with [^{35}S]-UTP were generated with T3 or T7 polymerase from linearized cDNA clones (NCAM: (Jucker et al. 1995); RGS4: courtesy of M.R. Koelle, (Koelle and Horvitz 1996)). After fixation, 100 µl hybridization mix containing $2 \cdot 10^6$ (NCAM) or $3 \cdot 10^6$ (RGS4) cpm was applied per slide. Slides were then incubated overnight at 50 °C (NCAM) or 55 °C (RGS4). The next day, sections were washed in saline sodium citrate (SSC) to a final stringency of 2 x SSC at 50 °C for 15 min (NCAM) or 0.1 x SSC at 55 °C for 30 min (RGS4), dehydrated, and exposed to a Kodak Biomax MR film for 5 days (NCAM) or 3 weeks (RGS4). For SGK1 mRNA detection, 〈-[^{33}P]-dATP end-labeled desoxyoligonucleotide probes were used (van Riel et al., 2004). Sequences were 5′ tctggaaagagaagtgaaggcccaccaggaaagggtgcttcacat (match; reverse complement of nucleotides 456-501 of rat SGK1 coding sequence) and 5′ gctggacagagacgtgaatgcccaacaggacagggttcttcaaat (mismatch). After fixation, 100 µl hybridization mix containing $1 \cdot 10^6$ cpm was applied per slide and slides were incubated overnight in a moist chamber at 42 °C. The next day, sections were washed to a final stringency of 1 x SSC at 50 °C for 30 min, dehydrated, and film was exposed for 2 weeks.

Per animal, eight hippocampal sections were scanned, loaded into ImageJ (ImageJ 1.31v), and corrected for background. CA1, CA3 and dentate gyrus were analyzed for grey values. The value of the stratum lacunosum/moleculare of the CA3 area was used as tissue background. Grey values were averaged for all hippocampal sections of one animal. In the end, all values from one experimental group were averaged. All data are represented as mean +/- standard error of mean (SEM). Data were statistically tested by means of an unpaired Student's t-test.

Results

Body weight at the time of decapitation was not different between the two groups (see Table 1). Body weight gain tended to be smaller in the corticosterone-treated (cort) group when compared to the vehicle treated animals, but this difference was not significant. Corticosterone injections did result in significantly decreased thymus and adrenal weight. The plasma corticosterone concentration in the cort group was significantly increased compared to the vehicle group, even one day after the last injection.

Both $NCAM_{total}$ and $NCAM_{180}$ probes showed a clear distribution in the hippocampus, with moderate levels of expression in the CA1 and CA3 regions, and somewhat higher levels of expression in the DG, which was described before (Venero et al. 2002). However, $NCAM_{total}$ and $NCAM_{180}$ expression were not affected by corticosterone injections, neither in CA1, CA3, nor DG (Figure 1).

SGK1 mRNA expression was low in the CA1 region and the dentate gyrus, and relatively high in the CA3 area, as was described previously by some (Imaizumi et al. 1994; Lee et al. 2001), but not all groups (Tsai et al. 2002). Corticosterone injections for 21 days did however not affect SGK1 expression in any of the subregions tested (Figure 2).

The RGS4 probe showed a hippocampal distribution with moderate levels of expression in the CA1 and CA3 regions in comparison to thalamus and cortex, and low levels of expression in the DG, in accordance with published data (Gold et al. 1997; Ingi and Aoki 2002). Once again, no differences in RGS4 mRNA expression were found between the corticosterone and vehicle treated groups in all areas tested (Figure 3).

	Vehicle	**Corticosterone**	**P value**
Body weight (g)	353.8 ± 7.7	350.8 ± 3.8	n.s.
Body weight gain (g)	76.3 ± 6.8	67.0 ± 2.9	n.s.
Adrenal weight (mg)	15.0 ± 0.4	7.3 ± 0.9	$3.2 \cdot 10^{-6}$
Adrenal weight / 100 g body weight	4.3 ± 0.2	2.1 ± 0.3	$3.2 \cdot 10^{-6}$
Thymus weight	657.8 ± 60.4	416.5 ± 41.1	0.005
Thymus weight / 100 g body weight	184.5 ± 14.7	118.5 ± 11.2	0.003
Basal [corticosterone] (µg/dl)	1.4 ± 0.3	4.3 ± 1.5	0.05

Table 1: Body weight, thymus weight, adrenal weight, and basal plasma corticosterone concentration in animals injected with oil (vehicle) or corticosterone for 21 days. Thymus and adrenal weight were decreased, and plasma corticosterone level was increased in animals injected with corticosterone when compared to the vehicle treated animals.

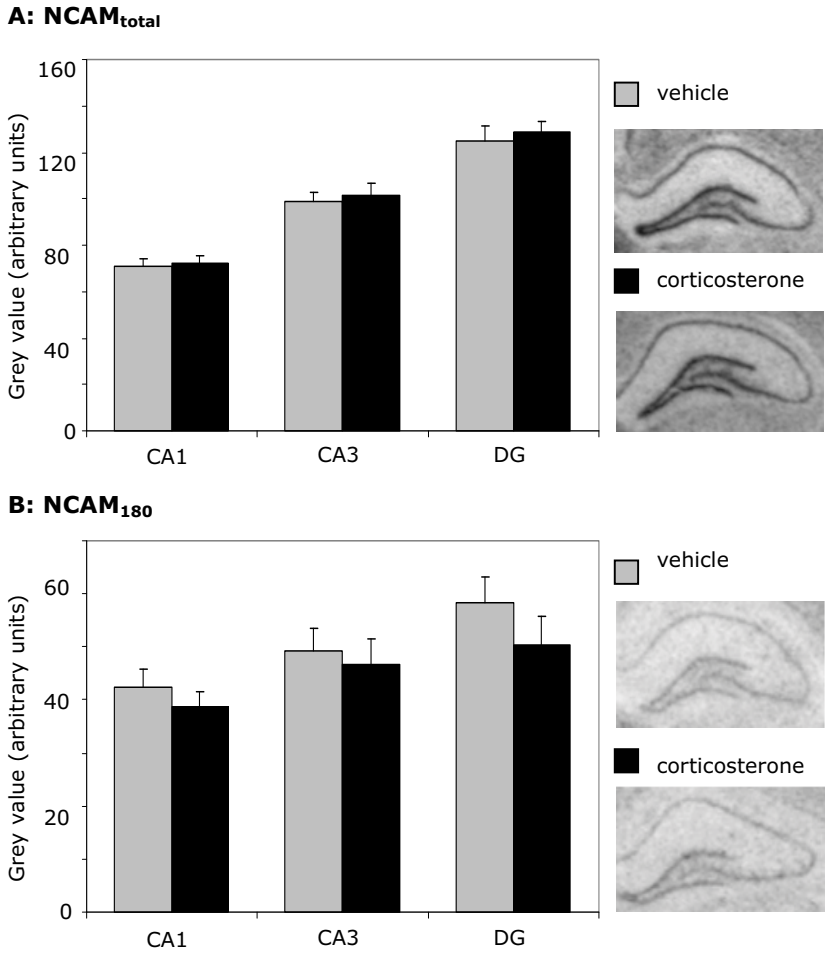

Figure 1: NCAM mRNA expression in the hippocampus of rats treated for 21 days with vehicle or corticosterone. mRNA expression of $NCAM_{total}$ (**A**) and $NCAM_{180}$ (**B**) are not affected by the 21-day injection protocol with corticosterone, in all three hippocampal subfields tested. Insets on the right show the hybridization signals for both groups. Hybridization with the sense probe did not yield a specific signal (data not shown).

Discussion

This study was initiated to examine the putative mechanism underlying attenuated 5-HT_{1A} receptor mediated responses after 21 days of elevated corticosterone levels, an attenuation that was earlier found not to be due to reduced 5-HT_{1A} receptor expression (Karten et al. 1999). We found that 21 days of corticosterone administration does not alter the transcript level of three prime candidates that can affect 5-HT_{1A} receptor function. The data can not be explained by insufficient corticosterone administration, since the paradigm we used resulted in a clearly decreased thymus and adrenal weight, and increased basal plasma corticosterone levels. These changes in HPA-axis parameters indicate that the corticosterone-injected animals were indeed subjected to high levels of corticosterone in the circulation for a prolonged period of time, whereas the parameters found in vehicle-injected animals were comparable to those found in naïve control animals of similar weight, as observed in other studies (van Riel et al. 2004).

SGK1

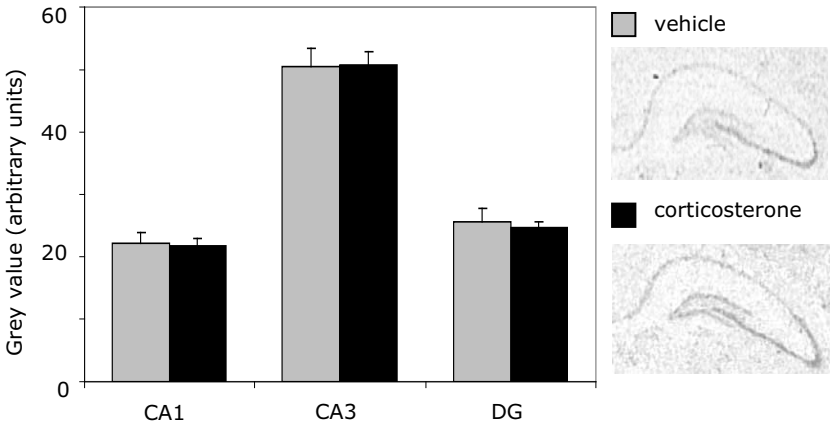

Figure 2: SGK1 mRNA expression in the hippocampal subfields tested was not affected by the high corticosterone injection protocol. Insets show the hybridization signals for both groups. Hybridization with the mismatch probe did not yield a specific signal (data not shown).

RGS4

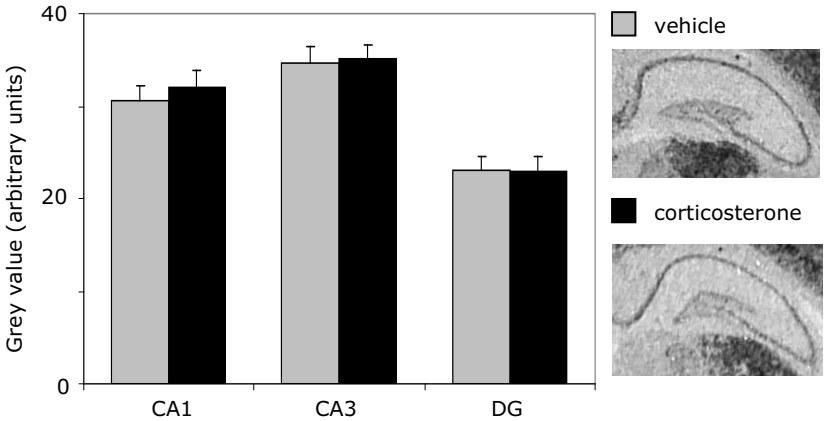

Figure 3: RGS4 mRNA was not differentially expressed in the hippocampus of vehicle versus corticosterone injected animals. Insets show the hybridization signals for both groups. Hybridization with the sense probe did not yield a specific signal (data not shown).

The first candidate we tested was NCAM. Earlier, 21-day restraint stress was found to cause a significant decrease in $NCAM_{total}$ mRNA expression in the hippocampus, whereas no changes in $NCAM_{180}$ mRNA expression were found (Sandi et al. 2001; Venero et al. 2002). Chronic psychosocial stress caused a reduction in $NCAM_{140}$ expression in the hippocampus, without affecting the expression of the $NCAM_{120}$ and $NCAM_{180}$ isoforms (Touyarot et al. 2004), suggesting that the reduction in $NCAM_{total}$ was mainly accounted for by reductions in $NCAM_{140}$ expression. This could not be addressed directly, since - in view of the overlap of $NCAM_{140}$ with other NCAM isoforms - no specific probes for $NCAM_{140}$ can be construed. For our findings, however, this is irrelevant, since we were

unable to show differences in mRNA expression of either $NCAM_{total}$ or $NCAM_{180}$ after 21 days of corticosterone administration, in line with an earlier study (Sandi and Loscertales 1999). Clearly, chronic stress and repeated injections of a high dose of corticosterone, though altering serotonin responsiveness in the same way, affect NCAM expression differently. Administration of exogenous corticosterone suppresses endogenous HPA-axis activity, whereas chronic stress causes an activation of all components of the HPA-axis. Possibly, other components of the HPA-axis are responsible for the effect of chronic stress on hippocampal NCAM mRNA expression, rather than corticosterone itself. Alternatively, it is possible that increased corticosterone levels are necessary for changed expression but not sufficient. This should be addressed by combining chronic stress paradigms with GR antagonist treatment.

SGK1 is a direct glucocorticoid target gene. Its expression was previously shown 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). Moreover, exposure to an elevated plus maze induced SGK1 mRNA expression in the ventral tegmental area, but not the prefrontal cortex in rats (Koya et al. 2005). Apparently, the regulation of SGK1 expression is region specific. Hippocampal SGK1 expression has been found to be regulated by environmental enrichment and psychophysiological stress (Lee et al. 2003; Murata et al. 2005), although it is not clear whether this was an effect of altered corticosterone levels. In our study, mRNA expression levels of SGK1 in the hippocampus were not affected by 21 days of corticosterone injections. This makes it unlikely that the effects of repeated corticosterone administration on 5-HT responses are mediated by SGK1. However, since mRNA expression levels were low in CA1 and dentate gyrus, a subtle downregulation of SGK1 expression in these areas might not be detected.

The last candidate we tested was RGS4. Chronic stress, as well as acute or 7-days corticosterone treatment, was previously found to differentially regulate RGS4 mRNA expression in the PVN and locus coeruleus (Ni et al. 1999). In other brain areas no dramatic changes in RGS4 expression were found, suggesting regional specificity in the regulation of RGS4 mRNA expression. Also in our study, using a 21-day corticosterone injection protocol, RGS4 mRNA expression levels in the hippocampus were not found to be regulated.

In summary, changes in 5-HT responsiveness in the hippocampus seen after prolonged administration of a high dose of corticosterone are not accompanied by changes in the expression of either the $5\text{-}HT_{1A}$ receptor (Karten et al. 1999; van Riel et al. 2004), $NCAM_{total}$, $NCAM_{180}$, SGK1, or RGS4 in the hippocampus. Protein levels of these candidates still may be changed by the injection paradigm and posttranslational modifications causing functional changes are conceivable, but, at least for NCAM, data so far do not support this hypothesis (Sandi and Loscertales 1999). We conclude that the attenuation of $5\text{-}HT_{1A}$ receptor mediated responses by chronically elevated corticosterone levels must involve other, less obvious candidate proteins, which are regulated by the hormone and alter $5\text{-}HT_{1A}$ receptor function.