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Chronic corticosterone administration down-regulates metabotropic glutamate receptor 5 protein expression in the rat hippocampus

Abiye H. Iyo, Anteneh M. Feyissa, Agata Chandran, Mark C. Austin, Soundar Regunathan, and Beata Karolewicz*

Department of Psychiatry and Human Behavior, University of Mississippi Medical Center, Jackson, MS, USA

Abstract

Several lines of evidence suggest a dysfunctional glutamate system in major depressive disorder (MDD). Recently, we reported reduced levels of metabotropic glutamate receptor subtype 5 (mGluR5) in postmortem brains in MDD, however the neurobiological mechanisms that induce these abnormalities are unclear. In the present study, we examined the effect of chronic corticosterone (CORT) administration on the expression of mGluR5 protein and mRNA in the rat frontal cortex and hippocampus. Rats were injected with CORT (40 mg/kg, s.c.) or vehicle once daily for 21 days. The expression of mGluR5 protein and mRNA was assessed by Western blotting and quantitative real-time PCR. In addition, mGluR1 protein was measured in the same animals. The results revealed that while there was a significant reduction (-27% , $p=0.0006$) in mGluR5 protein expression in the hippocampus from CORT treated rats, mRNA levels were unchanged. Also unchanged were mGluR5 mRNA and protein levels in the frontal cortex and mGluR1 protein levels in both brain regions. Our findings provide the first evidence that chronic CORT exposure regulates the expression of mGluR5 and are in line with previous postmortem and imaging studies showing reduced mGluR5 in MDD. Our findings suggest that elevated levels of glucocorticoids may contribute to impairments in glutamate neurotransmission in MDD.

Keywords

hippocampus; frontal cortex; metabotropic glutamate receptor 5; metabotropic glutamate receptor 1; corticosterone; rat

Clinical and preclinical research findings strongly implicate dysregulation of central glutamatergic signaling in the neuropathology of major depressive disorder (MDD). Recently, the metabotropic glutamate receptors (mGluRs) have been proposed as attractive targets for discovery of novel therapeutic approaches against depression. We had previously shown increased expression of mGluR subtype 2/3 (mGluR2/3) (Feyissa et al., 2010) and reduced level of subtype 5 (mGluR5) in a postmortem study of the prefrontal cortex (PFC, Brodmann's area 10) from subjects diagnosed with MDD (Deschwenden et al., 2010). Our findings have been supported by neuroimaging studies showing markedly reduced binding

*Correspondence to: Beata Karolewicz, Ph.D., Department of Psychiatry, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216-4505, phone: (601) 984-5896, fax: (601) 984-5899, bkarolewicz@psychiatry.umc.edu.

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to mGluR5 in the PFC, cingulate cortex, thalamus, and hippocampus in living depressed patients (Deschwenden et al., 2010). The implications of these findings are that glutamate receptors are well-suited as potential biomarkers for depression, and are attractive targets for the discovery of novel antidepressant medications (see Palucha and Pilc, 2007; Witkin et al., 2007; Pilc et al., 2008; Sanacora et al., 2008; Hashimoto, 2009; Sanacora, 2009; Valentine and Sanacora, 2009 for reviews). Although recent postmortem and imaging data indicate an abnormal signaling at the mGluRs in MDD, the neurobiological mechanisms that lead to these abnormalities have not been characterized.

To date, eight mGluR subtypes (mGluR1–mGluR8) have been described in the mammalian brain and are classified into three groups (Conn and Pin, 1997). Group I mGluRs (mGluR1 and mGluR5) are positively linked to phospholipase C and in general function to enhance glutamate excitations. The expression of mGluR5 is high in brain regions (e.g. anterior cingulate, prefrontal cortex, thalamus, amygdala, hippocampus, substantia nigra, and striatum) that have been implicated in the pathophysiology of depression (Drevets, 2000; Ametamey et al., 2007) and other psychiatric or neurodegenerative disorders including Parkinson's disease (Johnson et al., 2009). Thus, the localization of mGluR5 receptors in key cortical thalamic/amygdaloid circuits, their expression by neurons and glia (Aronica et al., 2003), and reciprocal positive interactions with N-methyl-D-aspartate (NMDA) receptors (Alagarsamy et al., 1999), make these receptors intriguing targets for further investigation.

Behavioral stress is recognized as a major risk factor for MDD. Most animal models of depression employing chronic stress procedures are known to increase plasma corticosterone (CORT) levels (Garcia et al., 2009; Iyo et al., 2009; Kieran et al., 2010). Comparatively, many patients with MDD have elevated cortisol levels (human equivalent of rodent stress hormone: corticosterone) (Sachar et al., 1973; Gold et al., 1988a, 1988b; Reus and Miner 1985; Dinan 1994). Interestingly, patients with Cushing's disease characterized by elevated glucocorticoid levels, exhibit a high rate of MDD (Kelly et al., 1983; Brown and Suppes, 1998; Brown et al., 2004), which is often resolved upon normalization of cortisol levels. Additionally, depressive symptoms are one of the most frequent side effects of corticosteroid therapy (Patten et al., 1996). Suggesting that increased endogenous production of glucocorticoids may be one mechanism by which stressful life events precipitate depressive symptomatology. Therefore, a repeated CORT-injection paradigm may provide a useful way to study the effect of prolonged exposure to stress on various neurochemical markers implicated in the pathology of MDD. In line with this notion, repeated CORT injections have consistently shown significant increases in depressive-like behavior such as increased immobility times in the forced-swim test in rodents (Kalynchuk et al., 2004; Gregus et al., 2005; Johnson et al., 2006; Marks et al., 2009). This paradigm has also been shown to reduce weight gain, decrease sexual behavior in male rats, and reduce sucrose preference (anhedonia), all identifiable symptoms of depression in humans (Gorzalka and Hanson, 1998; Hanson and Gorzalka, 1999; Gorzalka et al., 2003; Gregus et al., 2005). Thus, based on available data it has been established that exogenous CORT administration provides a valid and reliable means to study the influence of stress and stress hormones on the etiology and treatment of depression (for comprehensive review see Sterner and Kalynchuk, in press).

The possibility that glutamate function could be altered following repeated CORT injections is consistent with previous findings indicating that chronic exogenous CORT influences extracellular glutamate levels and regulates expression of glutamate signaling markers (Venero and Borrell 1999; Autry et al., 2006; Gourley et al., 2009; Hunter et al., 2009). It has been demonstrated that CORT can regulate glutamate transmission via a dual mechanism involving rapid nongenomic and prolonged transcriptional pathways. The

transcriptional pathway is mediated by two nuclear receptors: the high affinity mineralocorticoid receptors (MR) and low affinity glucocorticoid receptors (GR) (Joëls et al., 2009). The nongenomic regulation involves binding to MRs or GRs inserted into the presynaptic membrane (Karst et al., 2005; Joëls et al., 2008; Joëls et al., 2009; Wang and Wang 2009). To date the effect of CORT on mGluRs has not been investigated. Given the importance of mGluRs in the pathophysiology of MDD, the primary aim of this study was to examine mGluR5 mRNA and protein expression in the frontal cortex and hippocampus of rats chronically treated with CORT.

EXPERIMENTAL PROCEDURES

Animals and corticosterone injections

Young, adult male Wistar rats initially weighing 200–250 g were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN). Animals were housed in groups of 2 per cage in a room maintained under standard conditions of light (12:12 h light-dark cycle), temperature (22 ± 3 °C), and humidity. Animals had ad libitum access to food and water. All procedures were carried out in accordance with the guidelines established by the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and the Animal Care and Use Committee of the University of Mississippi Medical Center.

Corticosterone (Sigma) was administered in a dose of 40 mg per kilogram of body weight, emulsified in propylene glycol (Fisher Scientific). Control animals were injected with vehicle (propylene glycol). All injections were delivered subcutaneously once per day between 09:00 and 11:30 h for 21 consecutive days. Dosage of CORT was selected based on previous studies showing that rats given 40 mg/kg (for 21 days) exhibit significant increases in depression-like behavior such as increased immobility times in forced-swim test whereas rats given lower dose do not (Kalynchuk et al., 2004; Gregus et al., 2005; Johnson et al., 2006; Marks et al., 2009). Animals were sacrificed 24 hours after the last CORT injection (between 10:00 and 12:00 h) by decapitation. The frontal cortex and hippocampus were immediately dissected and stored at -80°C until further use. In addition, the adrenal glands from each rat were harvested and weighed. We chose to investigate the frontal cortex and hippocampus, because several studies have shown that chronic exposure to exogenous CORT induces morphological alterations similar to those observed in MDD in these key brain regions implicated in the pathology of depressive disorder (Woolley et al., 1990; Watanabe et al., 1992; Wellman, 2001).

Immunoblotting and data analysis

Western blot experiments were performed as described in our previous studies (Feyissa et al., 2009; Feyissa et al., 2010). mGluR5 was labeled using anti-mGluR5 rabbit polyclonal antibody (1:2000; Millipore, Temecula, CA, USA; no. AB5675), mGluR1 was detected using anti-mGluR1 rabbit polyclonal antibody (1:500; Millipore; no. 07617) and secondary anti-rabbit antibody (1:3000; Amersham Biosciences, no. NA934). mGluR5 antibody specificity test was performed using mGluR5 blocking peptide (Millipore; no. AG374). For molecular weight visualization MagicMark XP Western Protein Standard (Invitrogen; no. LC5602) was used. Actin was used as a control for transfer and loading, and was detected on each blot using an anti-actin antibody (Millipore; no. MAB1501). Immunoreactive bands were analyzed using MCID Elite 7.0 (Imaging Research, St. Catherines, ON, Canada). The data were analyzed statistically using a two-tailed unpaired t-test (GraphPad Prism 5). The final data are expressed in relative optical density (ROD) units and presented as a ratio of mGluR5 to actin or mGluR1 to actin. A p value <0.05 was considered significant.

RNA isolation and cDNA synthesis

Total RNA was extracted from tissue samples using Trizol reagent (GIBCO BRL, Gaithersburg, MD) as described previously (Iyo et al., 2009). Briefly, tissues were homogenized in Trizol reagent using a Teflon homogenizer 3 times for approximately 30 s each. Quality and quantity of total RNA were detected spectrophotometrically using a Nanodrop spectrophotometer at 230/260/280 nm. First-strand cDNA synthesis was carried out using the Promega ImProm-II Reverse Transcription System (Promega Corporation, Madison, WI). For initiation of cDNA synthesis, oligo dT primers were used. For each reaction, cDNA was transcribed from 1 µg total RNA following an initial annealing at 25 °C for 5 min and further incubation at 42 °C for 1 h. Reactions were stopped by heating at 70 °C for 15 min to inactivate the reverse transcriptase. The cDNA synthesis was evaluated by PCR and gel electrophoresis.

Quantitative real-time PCR

Quantitative Real-time PCR (qPCR) was performed using the MyIQ single color real-time PCR detection system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Reactions were performed in a final reaction volume of 25 µl, each reaction contained 0.3 mM of each primer (Forward primer, GCAGATCAGCAGCGTAGTGA; reverse primer: TCTTTGGGGATCAGGTAGGA; NM_017012.1) and 4 mM MgCl₂, nucleotides, Taq polymerase, and buffer were included in the DNA master SYBR Green mix (Biorad, Hercules Ca). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer set was obtained from a previous publication (Iyo et al. 2009). The basic local alignment search tool (BLAST) from the National Center for Biotechnology Information (NCBI) was used in analyzing primers to preclude any homology to other sequences in the database). All qPCR experiments were performed in duplicate and melting curves were included to ascertain the formation of a single product for each gene.

Evaluation of housekeeping gene and data analysis

The MyIQ iCycler v3.0 Software for windows was used to analyze qPCR data. Copy number for each template or gene was calculated from their starting concentrations (20 ng/µl) and 1:10 serial dilutions were performed. Using these serial dilutions, a standard curve for each gene (mGluR5 and GAPDH) was generated against which cDNA samples for vehicle and CORT injected groups were compared. All samples were analyzed in duplicate and each experiment included a negative control that contained all ingredients except cDNA. The copy number data generated for each factor was normalized against GAPDH and the data imported into GraphPad Prism for further analysis. The effects of repeated CORT injections on mRNA levels of each gene were analyzed using an unpaired 2-tailed Student's t-test. Results are presented as means ± SEM and considered significant at a probability level less than .05.

RESULTS

Adrenal glands /body weights

Adrenal and body weight data indicated that the CORT dose given was effective in producing classic peripheral effects of elevated glucocorticoids (Fig 1A and B). Although the body weight of the two groups started out the same, the control group gained more weight (325.9 ± 4.8 g) than the CORT group (302.6 ± 4.8 g) representing a 7.1% increase over the former (unpaired t-test $t=3.4$, $df=22$, $p=0.0024$; Fig. 1B). When adrenal weight was compared to body weight reduction by analyzing adrenal to body weight ratios, adrenal to body weight ratio was significantly lower in the CORT group (0.05 ± 0.003 ; -51%) relative to control or vehicle group (0.108 ± 0.008 , unpaired t-test $t=6.59$, $df=22$, $p<0.0001$;

see Fig. 1C) suggesting that the reduction in adrenal gland weight (unpaired t-test $t=7.3$, $df=22$, $p<0.0001$, Fig.1A) could not be accounted for entirely by the reduction observed in overall body weight.

mGluR5 antibody specificity assay

Western blot analyses consistently revealed immunoreactive bands corresponding to the molecular mass of ~150 kDa representing mGluR5 monomer. In addition, a band with high molecular weight (outside the range of the molecular weight marker) presumably corresponding to mGluR5 dimer was observed (Fig.2). Antibody specificity experiment revealed that preincubation of the primary anti-mGluR5 antibody with a specific mGluR5 peptide completely abolished immunoreactivities for both bands, indicating specificity of the antibody (Fig. 2). Therefore, we are confident that changes in the immunoreactivity observed using anti-mGluR5 antibody truly represent changes in mGluR5 protein level given that specificity of antibody from the same source was also confirmed by others using hippocampus of mGluR5 knock-out mice (Piccinin et al., 2010). Because the primary aim of this study was to investigate mGluR5 monomer, the immunoreactivity of mGluR5 dimer was not analyzed.

Effects of chronic CORT on mGluRs protein expression

Amounts of mGluR5 protein were analyzed in the hippocampus and frontal cortex from 12 rats treated with CORT and 12 vehicle exposed controls. In the hippocampus the average mGluR5/actin ratio from CORT treated rats (1.33 ± 0.07) was significantly lower (-27%) than that of controls (1.84 ± 0.1 ; unpaired t-test $t=4.0$, $df=22$, $p=0.0006$; Fig. 3A). In contrast, the results obtained from the frontal cortex revealed that the average mGluR5/actin ratio from CORT treated rats (1.22 ± 0.09) was unchanged compared to controls (1.28 ± 0.09 ; $t=0.497$, $df=22$, $p=0.62$; Fig. 3B).

The average mGluR1/actin ratio in the hippocampus from CORT treated rats (0.35 ± 0.02) was unchanged compared to controls (0.44 ± 0.04 ; $t=1.934$; $df=22$; $p=0.07$; Fig. 4A). Similarly the mGluR1/actin ratio in the frontal cortex from CORT treated rats (0.50 ± 0.03) was unchanged compared to controls (0.55 ± 0.05 ; $t=0.75$; $df=22$; $p=0.45$, Fig. 4B).

Effects of chronic CORT on mGluR5 mRNA expression

The mean copy numbers for mGluR5 mRNA expression in both the hippocampus and cortex from CORT treated animals and vehicle treated controls are shown in Fig. 5. No significant differences were found between the two groups in both brain regions (Fig 5A and 5B; $p=0.98$ and 0.85 , respectively).

DISCUSSION

This study is the first to examine the expression of group I metabotropic glutamate receptors in rats exposed to chronic CORT injections. The results demonstrate that CORT induces a robust reduction in the levels of mGluR5 protein in the hippocampus with no change in mRNA. Interestingly, mGluR1 protein was unchanged in the same CORT treated rats. These observations coincide with the recent imaging and postmortem studies showing reduced binding and protein expression of mGluR5 in the brains of depressed individuals (Deschwenden et al., 2010). Overall, the CORT-induced reductions in adrenal and body weights seen in our paradigm are consistent with previous studies demonstrating the effects of glucocorticoids, and support the effectiveness of the CORT dose used in our experiments (Marks et al., 2009; Magarinos and McEwen, 1995).

Although no previous studies on the expression of mGluRs in a repeated CORT-injection paradigm exist, an earlier study investigating the effect of chronic mild stress (CMS) found reduced mGluR5 protein expression in the CA3 region of the hippocampus (Wierońska et al., 2001). The CMS paradigm has also been shown to reduce glutamate AMPA receptor subunit GluR1 levels in the dorsal dentate gyrus (Toth et al., 2008). Interestingly, the reduced GluR1 levels in the hippocampus coincided with decreased BDNF levels suggesting a depression-like neuropathology (Toth et al., 2008). Given that most animal models of depression employing chronic stress procedures are known to increase plasma CORT levels (Garcia et al., 2009; Iyo et al., 2009; Kieran et al., 2010) it is likely that elevated CORT levels could contribute to glutamatergic abnormalities induced by chronic stress paradigms. In support of this, a recent study by Hunter et al (2009) showed that chronic CORT regimen up-regulates genes for the glutamate kainate receptor subunit 1 (KA1) in the hippocampus, where this receptor is known to control both excitatory neurotransmission and the presynaptic modulation of neurotransmitter release (Chittajallu et al., 1996). Interestingly, it has been shown that corticosteroids (Autry et al. 2006) and chronic restraint stress (CRS) (Reagan et al., 2004) up-regulate glutamate transporter (GLT-1) in the rat hippocampus. Along these lines, it has been shown that exposure to CRS increased neuronal presynaptic glutamate uptake as well as glutamate release (Fontella et al., 2004). It is conceivable that the reduced mGluR5 protein expression observed herein could be an adaptation to increased glutamate transmission in the hippocampus of CORT treated rats. These data suggest that elevated cortisol levels could contribute to glutamate system abnormalities in MDD and is consistent with the fact that 50–80% of severely depressed patients show a significant hyperactivity of the HPA axis (Holsboer, 2000; Pariante and Miller, 2001).

While we have observed a significant decrease in the levels of mGluR5 protein, mRNA levels remained unchanged. The lack of correlation between protein and mRNA expression is not unusual since complex regulatory mechanisms including different time course of transcription and translation as well as post-transcriptional and post-translational processes may differentially regulate mRNA and protein levels (Gry et al., 2009). As mRNA synthesis always precedes protein synthesis, it is possible that our single time point at 24 hours after 21 days of CORT treatment may have missed the changes in mRNA levels. Recently, Ouattara et al. (2010) have demonstrated that changes in mGluR5 mRNA precede protein abnormalities. Thus, measurement of mRNA levels at different days of exposure to CORT as well as various withdrawal periods would reveal whether the changes seen in mRNA levels come before or parallel protein changes. In an attempt to clarify the discrepancy between mGluR5 protein and mRNA regulation we have performed simple *in vitro* studies using cultured primary rat astrocytes. We chose astrocytes, given that astrocytes express mGluR5 and thus provide a useful system to study factors which regulate mGluR5 expression. The dose of CORT (1 μ M) was selected to mimic the range of plasma CORT levels attained after stress (Reul and de Kloet, 1985). Our preliminary results show that 24 hours of exposure to CORT markedly down-regulated mGluR5 protein expression (–38%) while mRNA levels remained unchanged. On the other hand, following 5 hours of CORT exposure a robust reduction (–45%) in mRNA expression was observed (data not shown). These preliminary results demonstrate that down-regulation of mGluR5 mRNA by CORT is fast and precedes protein down-regulation. Thus, based on these preliminary *in vitro* studies we will design future *in vivo* experiments to investigate mGluR5 protein and mRNA at different time points of exposure to CORT and/or withdrawal periods to better understand the basic mechanisms behind mGluR5 receptor regulation.

The potential limitation of this study is that we measured mGluR5 immunoreactivity and RNA expression in total cortical and hippocampal tissue homogenates containing a mixture of many different cell types: such as neurons (pyramidal and nonpyramidal) and glia which express mGluRs and respond to their activation (Aronica et al., 2003). Therefore, we are

unable to define which specific anatomical subregions and/or populations of cells are affected. Thus, a next logical step would be to explore mGluR5 regulation in both *in vitro* and *vivo* studies focusing on individual cell types residing in specific anatomical subregions using autoradiography and in situ hybridization methods. Also, our future studies will be designed to elucidate whether CORT regulates mGluR5 via transcriptional mechanisms involving GR or MR receptors or via indirect pathways e.g. involving its scaffolding or regulatory proteins (Mao et al., 2005; Paquet et al., 2006). Another limitation of the present study is that mGluR5 was measured as a total pool of proteins that included intracellular pools of unassembled proteins and receptors expressed at the cell membrane, complicating the prediction of functional significance. In fact, it has been demonstrated that 40–70% of the total immunoreactivity for mGluR1 or mGluR5 subtypes in primate brain are intracellular, whereas 30–60% are located within the plasma membrane (Kuwayama et al., 2004; Paquet and Smith, 2003). Thus, reduced total protein of mGluR5 in rat brain may reflect reduced density of functional receptors due to reduced total protein concentration.

CONCLUSION

Our major findings revealed markedly reduced mGluR5 protein expression in the hippocampus in CORT treated rats, while hippocampal mGluR1 protein was unchanged. These data indicate that hippocampal mGluRs are selectively regulated by exogenous CORT, an observation that may represent an underlying biological mechanism associated with depressive symptomatology and neuropathology. Thus, it is likely that elevated corticosteroids may contribute to glutamate system abnormalities in MDD and further reinforce the notion that targeting both “stress response” systems may provide novel therapeutic strategies against depression.

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Figure 1.

Effects of repeated corticosterone injection (40 mg/kg for 21 days) on adrenal (A) and body weights (B). Adrenal to body weight ratio (C) was significantly lower in the CORT group (−51%) relative to control group after 21 days. These well-known effects of glucocorticoids confirm the effectiveness of the CORT dose administered. Values (circles) and mean values (horizontal lines) are presented. CORT, rats treated with corticosterone.

Figure 2. mGluR5 antibody specificity test using mGluR5 blocking peptide. Lanes 1–8 were loaded with 10 μ g of total hippocampal protein. Lanes 1–4 show mGluR5 immunoreactivity (monomer at ~150 kDa and presumably dimer at ~300 kDa) detected using anti-mGluR5 antibody. In lanes 5–8 mGluR5 immunoreactivity was completely abolished after incubation with anti-mGluR5 antibody in the presence of mGluR5 blocking peptide. Lower panel shows immunoreactive actin (lanes 1–8) probed with anti-actin antibody on the same blots as a control for protein loading and transfer. For molecular weight visualization 10 μ l of MagicMark XP Western Protein Standard (Invitrogen; cat. no. LC5602) was loaded into lane 0.

Figure 3.

Effects of repeated corticosterone injection on mGluR5 protein in the hippocampus (A) and frontal cortex (B). Representative immunoblots of mGluR5 and actin from six experimental animals (upper panels) and scatter plot of mGluR5 protein levels normalized to actin (lower panels) are shown. In the hippocampus the average mGluR5/actin ratio from rats treated with CORT (n=12) was significantly lower (−27%) than that of controls (n=12). In the frontal cortex the average mGluR5/actin ratio from rats treated with CORT (n=12) was unchanged as compared to control group (n=12). Normalized values (circles) and mean values (horizontal lines) are presented. CORT, rats treated with corticosterone.

Figure 4.

Effects of repeated corticosterone injection on mGluR1 protein in the hippocampus (A) and frontal cortex (B). Representative immunoblots of mGluR1 and actin from six experimental animals (upper panels) and scatter plot of mGluR1 protein levels normalized to actin (lower panels) are shown. In both brain regions the average mGluR1/actin ratios from subjects treated with CORT (n=12) were unchanged as compared to controls (n=12). Normalized values (circles) and mean values (horizontal lines) are presented. CORT, rats treated with corticosterone.

Figure 5. Effects of repeated corticosterone on mGluR5 mRNA expression in the hippocampus (A) and frontal cortex (B). mGluR5 mRNA levels in both the hippocampus and frontal cortex were unchanged in CORT treated rats (n=12) as compared to controls (n=12). Normalized values for the individual rats (circles) and mean values (horizontal lines) are presented. CORT, rats treated with corticosterone.