

CHRONIC CORTICOSTERONE AFFECTS BRAIN WEIGHT, AND MITOCHONDRIAL, BUT NOT GLIAL VOLUME FRACTION IN HIPPOCAMPAL AREA CA3

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Abstract—Corticosterone (CORT), the predominant glucocorticoid in rodents, is known to damage hippocampal area CA3. Here we investigate how that damage is represented at the cellular and ultrastructural level of analyses. Rats were injected with CORT (26.8 mg/kg, s.c.) or vehicle for 56 days. Cell counts were estimated with the physical disector method. Glial and mitochondrial volume fractions were obtained from electron micrographs. The effectiveness of the CORT dose used was demonstrated in two ways. First, CORT significantly inhibited body weight gain relative to vehicles. Second, CORT significantly reduced adrenal gland, heart and gastrocnemius muscle weight. Both the adrenal and gastrocnemius muscle weight to body weight ratios were also significantly reduced. Although absolute brain weight was reduced, the brain to body weight ratio was higher in the CORT group relative to vehicles, suggesting that the brain is more resistant to the effects of CORT than many peripheral organs and muscles. Consistent with that interpretation, CORT did not alter CA3 cell density, cell layer volume, or apical dendritic neuropil volume. Likewise, CORT did not significantly alter glial volume fraction, but did reduce mitochondrial volume fraction. These findings highlight the need for ultrastructural analyses in addition to cellular level analyses before conclusions can be drawn about the damaging effects of prolonged CORT elevations. The relative reduction in mitochondria may indicate a reduction in bioenergetic capacity that, in turn, could render CA3 vulnerable to metabolic challenges. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

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Glucocorticoids (GCs) are released during stress, and serve to maintain homeostasis (Munck et al., 1984) by mobilizing energy for emergency responses, but they do so at the expense of energy storage. Although the readiness to respond is adaptive over short durations, the con-

sequences of delayed energy storage can be seen in the periphery as muscle wasting (Hickson and Marone, 1993; Sapolsky et al., 2000) and cardiac ultrastructural damage (Clark et al., 1982). Of equal concern is the possibility of damage in the CNS.

Chronically elevated GCs are found in a number of neuropsychiatric disorders (Carroll et al., 1976; Whiteford et al., 1987; Starkman et al., 1992; Seed et al., 2000; Walder et al., 2000). Whereas several of these disorders have been associated with reduced hippocampal volume (Starkman et al., 1992; Sheline et al., 1996, 1999), cortisol treatment in primates failed to produce volume loss (Leverenz et al., 1999). Even so, the prevalence of elevated GCs across neuropsychiatric disorders emphasizes the need to further understand the damage that occurs in the hippocampus after chronic elevations in GCs, because these hormones may be contributing to the complexity of the disorders.

GCs are known to influence at least two types of receptors, both cytoplasmic, and both are believed to regulate gene transcription. The low-affinity GC receptor (GR) is distributed throughout the brain, whereas the high affinity mineralocorticoid receptor (MR) has a high density in the hippocampus (De Kloet et al., 1998). Chronic GC elevations lead to unusually prolonged binding of GRs. Because the co-occurrence of binding at both receptors appears to bring about the greatest vulnerability, brain areas with high concentrations of both MRs and GRs are of particular interest. For this reason, the effects of GCs on the hippocampus have received much attention.

MR and GR receptors are expressed on astrocytes (Bohn et al., 1991; Cintra et al., 1994), although at lower levels than in neurons (Bohn et al., 1994). The co-expression of MR and GR on astrocytes may make astrocytes vulnerable to chronic elevations of GCs. GCs regulate the expression of a considerable number of genes in astrocytes including glutamine synthetase (Kumar et al., 1986), and glial fibrillary acidic protein (GFAP; Nichols, 1999). Regulation of GFAP, the major intermediate filament protein in mature astrocytes (Pixley and de Vellis, 1984), has received the most attention. Short or long-term administration of GCs decreased the concentration of GFAP in the hippocampus (O'Callaghan et al., 1989, 1991). GCs also decrease GFAP mRNA (Nichols et al., 1990; O'Callaghan et al., 1991). The association of GFAP with major alterations in the astrocytic cytoskeleton lends support to the hypothesis that GCs may be altering astrocytic structure. Because the correspondence between GFAP in mature, relatively normal animals may not be as tightly coupled

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Abbreviations: CORT, corticosterone; GCs, glucocorticoids; GFAP, glial fibrillary acidic protein; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; VEH, vehicle.

with astrocytic structure as appears to be the case during development and severe damage, alterations in GFAP are suggestive, but not necessarily indicative of a change in glial volume. To directly test whether or not glial volume is altered in response to chronic elevations in GCs, glial area fraction, an estimate of volume fraction (Weibel, 1979), was measured from electron micrographs in animals that received corticosterone (CORT), the predominant GC in rodents, or vehicle (VEH) treatment for 56 days.

In the hippocampus GR receptors are expressed in mitochondria (Moutsatsou et al., 2001) and hormone response elements are present in the mitochondrial genome (Tsiriyotis et al., 1997). The possibility that CORT affects mitochondrial function is of concern because these cellular organelles are solely responsible for cellular respiration, and are responsible for 90% of ATP production. These organelles are also responsible for calcium buffering, production of radical oxygen species, and initiation of apoptosis. In peripheral tissues, *in vitro* studies have demonstrated that CORT regulates mitochondrial gene transcription, including the transcription of genes involved in oxidative phosphorylation (Van Itallie, 1992). It has also been shown to inhibit cytochrome c oxidase activity (Simon et al., 1998). *In vivo* cortisol acetate at levels that produce atrophy of fast-twitch muscles also inhibited muscle mitochondrial function (Marone et al., 1994). Likewise, noise stress at levels sufficient to elevate CORT, produced mitochondrial structural damage in cardiac muscle (Soldani et al., 1997).

Although there is very good reason to believe that CORT would also impair mitochondrial function in brain tissue, only two studies have assessed this issue. In the first, Miller et al. (1989) found that 3-day CORT administration (20 mg/kg body weight) did not change mitochondrial surface density in hippocampal subfield CA3 in rats. In another study, Magarinos et al. (1997) used 21 days of restraint stress, which would elevate CORT. They found that CORT did not change mossy fiber terminal size, but the total area of the terminal occupied by mitochondria was greater than controls. They did not measure total number of terminals or total mitochondrial volume fraction. In the present study, we used prolonged administration of CORT to test whether it influences mitochondrial volume fraction in the apical dendritic neuropil of area CA3. Volume fraction was measured, because the size of individual mitochondrion can vary along with the number, and as a result, tissue volume fraction, which represents both, provides a better representation of mitochondrial presence within the tissue (Smith and Ord, 1983). Any changes in mitochondrial volume fraction will have implications for the mitochondrial functional capacity for oxidative metabolism, and therefore the ability of the tissue to meet metabolic demands, and withstand metabolic challenge.

Because there have been conflicting reports of whether CORT causes cell loss in CA3, the area we investigate, we have also utilized unbiased cell counting methods to estimate CA3 cell number. In addition, CA3 cell layer and apical dendritic neuropil volume was measured.

EXPERIMENTAL PROCEDURES

Subjects

Ten male Sprague–Dawley rats (380–450 g) were habituated to the colony for 6 days and handled for an additional 5 days before treatment conditions commenced. Food and water were provided *ad libitum*, but monitored for the duration of the experiment. All treatment and testing protocols were approved by the institution's animal care and use committee and conformed to National Institutes of Health guidelines. All effort was made to minimize the number of animals used. Animals were well-handled prior to the start of the experiment in order to minimize the response to handling and injections. Animal assignment to groups was designed so that body weight was equally distributed across groups. One group (CORT, $n=5$) received daily injections (s.c.) of 10–11 mg (26.8 mg per kg body weight) of CORT suspended in 300 μ l sesame oil; another group (VEH, $n=5$) received 56 daily s.c. injections of sesame oil alone. All treatments lasted 56 days, a duration previously used (Bodnoff et al., 1995).

The CORT dose used is reported to cause prolonged elevations in plasma CORT concentrations with a subsequent decline to basal levels within 24 h (Hauger et al., 1987). Injections and handling were administered at the same time daily (30 min after light onset in a 12-h light/dark cycle) to coincide with the circadian nadir of endogenous CORT production. We have previously measured this dose of CORT to produce plasma CORT concentrations of 42.63 ± 7.22 μ g/dl relative to VEH concentrations of 14.18 ± 3.19 μ g/dl (1–3 h post-injection on the 5th day of injections). These plasma concentrations of CORT following injections are similar to concentrations measured in control animals brought into a busy novel environment (Gorby, Tata and Anderson, unpublished observations).

Tissue preparation

Forty-eight hours following the final injection, the animals were anesthetized with ketamine and xylazine (80 mg/kg and 13 mg/kg i.m., respectively), and coded so that all subsequent work was done by investigators blind to the experimental conditions. Heparin (0.35 ml) was injected into the heart prior to intracardial perfusion with warm buffered saline with 4% sucrose, followed by fixative (2.5% paraformaldehyde, 1.5% glutaraldehyde in 0.1 M phosphate buffer with 4% sucrose). Hearts, adrenals, and skeletal muscles were removed, cleaned and stored in fixative. Brains were stored cold for 4 h within the head prior to dissection to avoid the production of artifacts that could be confused with CORT-induced cell damage. Upon removal from the skull, the brains were stored in fixative overnight. The left or right cerebral hemisphere was randomly chosen for anatomical analysis. Serial coronal slabs (300 μ m) were cut through the hemisphere using a vibratome. The hippocampal slabs were dissected from the coronal sections of the chosen hemisphere, osmicated (1% OsO₄ in 0.1 M phosphate buffer, pH=7.2), dehydrated in a fresh ethanol series, and then embedded in Durcupan epoxy (Fluka, Buchs, Switzerland). All hippocampal slabs were given codes that were not broken until data collection was completed. The embedded hippocampal slab thickness was verified under a dissecting microscope with a micrometer eyepiece, and the average thickness was calculated.

Structural analysis in the hippocampus

Quantitative anatomical data in the current study were collected from the dorsal CA3 subregion of the hippocampus, an area that has been the focus of many previous studies of the effects of CORT on the hippocampus (Sapolsky et al., 1985; Woolley et al., 1990; Bodnoff et al., 1995). For our purposes, the dorsal hippocampus was defined as coronal sections that contained no ventral hippocampal connections. These consisted of the first half

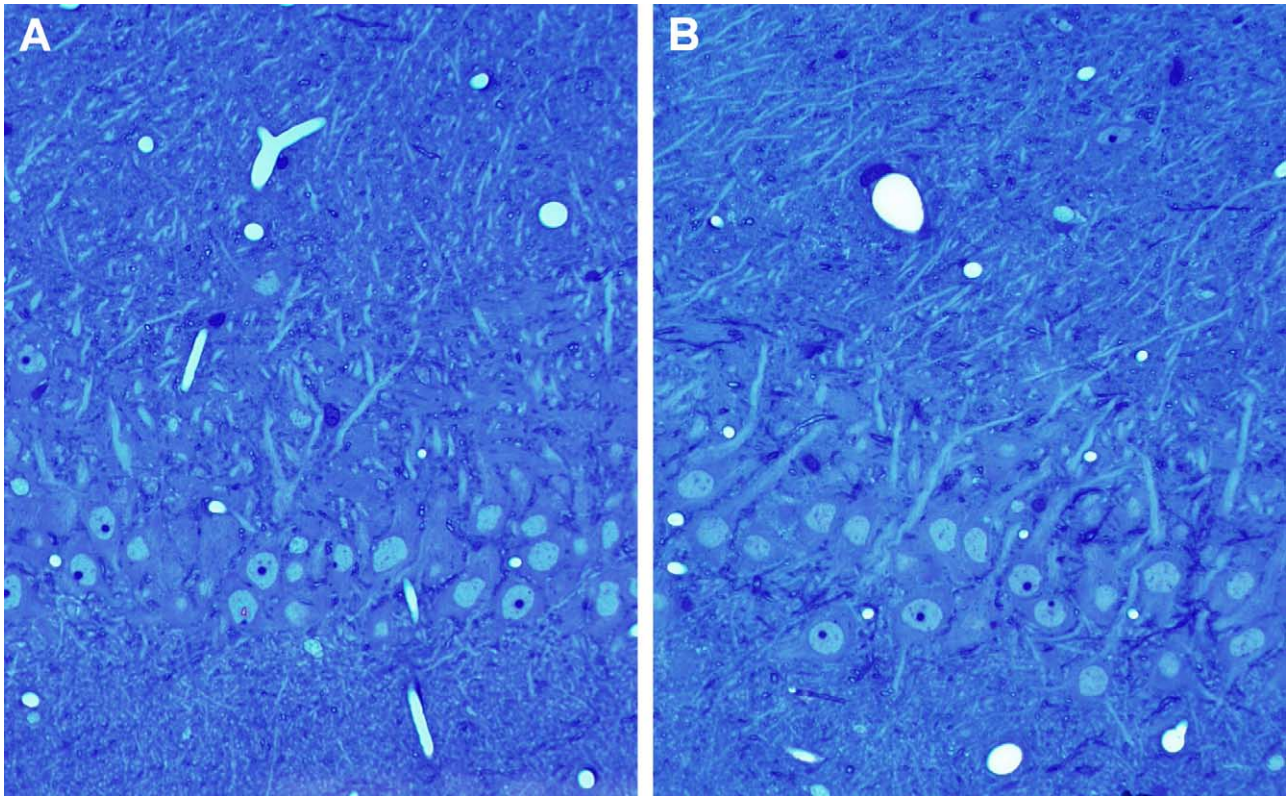


Fig. 1. Cell counts and neuropil volume were measured from serial sections of 1 μm thickness. Shown here is an example of the appearance of cell bodies and neuropil in the VEH group (a) and the CORT group (b).

of the total number of hippocampal slabs dissected from coronal sections through one hemisphere from each animal. The number of sections fitting this definition did not significantly differ across groups (CORT=8.3, VEH=8.2, $t(8)=.309$, $P=0.77$). Data were collected from the proximal and middle portions of CA3, using the nomenclature of Ishizuka et al. (1995). These regions correspond to CA3c and almost all of CA3b, the areas in which apical dendritic atrophy have been reported following 21 days of CORT treatment (Woolley et al., 1990) or restraint stress (Watanabe et al., 1992a,b, 1993; Sunanda et al., 1995).

Serial 1 μm sections were taken from the septal face of each dorsal hippocampus slab and mounted onto slides, then stained with Toluidine Blue. The area containing the CA3 pyramidal cell layer (stratum pyramidale) and neuropil layers containing pyramidal cell apical dendrites (stratum lucidum, radiatum, and lacunosum-moleculare) were calculated for each section using stereology software (Stereo Investigator; MicroBrightField, Inc., 2000). The proximal and middle CA3 cell layer and neuropil volumes were then calculated with the formula

$$\text{Volume} = \sum \text{area} \times \text{average slab thickness}$$

CA3 pyramidal cells (see Fig. 1) were counted using the physical disector method (Sterio, 1984) from detailed camera lucida drawings of the CA3 cell layer (300 \times). A counting frame with a known area: $a(\text{fr})$ was laid on the first of two drawings of adjacent semithin sections. This counting frame was used to calculate the disector volume, which is equal to the area of the disector frame multiplied by the distance between the reference and look-up sections. Only those cells that: a) did not contact the exclusionary lines of the counting frame and b) were not present in the adjacent drawing, were counted; thus providing the cell number per disector, Q^- . CA3 pyramidal cell density, was then

calculated with the formula as

$$\text{Cell density} = Q^- / \text{disector volume}$$

Sampling fields, used to calculate CA3 mitochondrial and glial cell area fraction, were chosen with the systematic random sampling method applied to the hippocampus as previously described (Geinisman et al., 1996). Although the sample sites for ultrastructural analyses are a small proportion of neuropil, the proportion of samples from each layer should correspond to the proportion that each layer contributes to the total dendritic neuropil volume. Stratum lucidum makes up a small proportion of the apical dendritic neuropil, and therefore makes up a small proportion of the sampling fields. Likewise, because the stratum radiatum accounts for a substantial proportion of neuropil, it is represented by a large proportion of the sampling fields. This method assures that the sample sites as a whole provide estimates that are representative of the whole apical dendritic neuropil. Serial ultrathin (60–90 nm) sections of the neuropil containing the sampling fields were collected from the septal face of each dorsal hippocampus slab. Data were collected from one micrograph per block, and an average of five blocks per animal. The glial profiles were identified in electron micrographs (39,000 \times) by their light cytoplasm, and an irregular shape that conformed to the surrounding profiles as described by Peters et al. (1991) and used previously by Anderson et al. (1994) (see Fig. 2). Likewise, glial profiles could not have neuro- or micro-tubules. It is expected that the large majority of these profiles represent astrocytic processes. As is seen in most electron micrographs, most glial profiles represent glial processes rather than cell bodies. Bundles of intermediate filaments were found in some astrocytic processes, but as previously described they are rarely seen in processes within

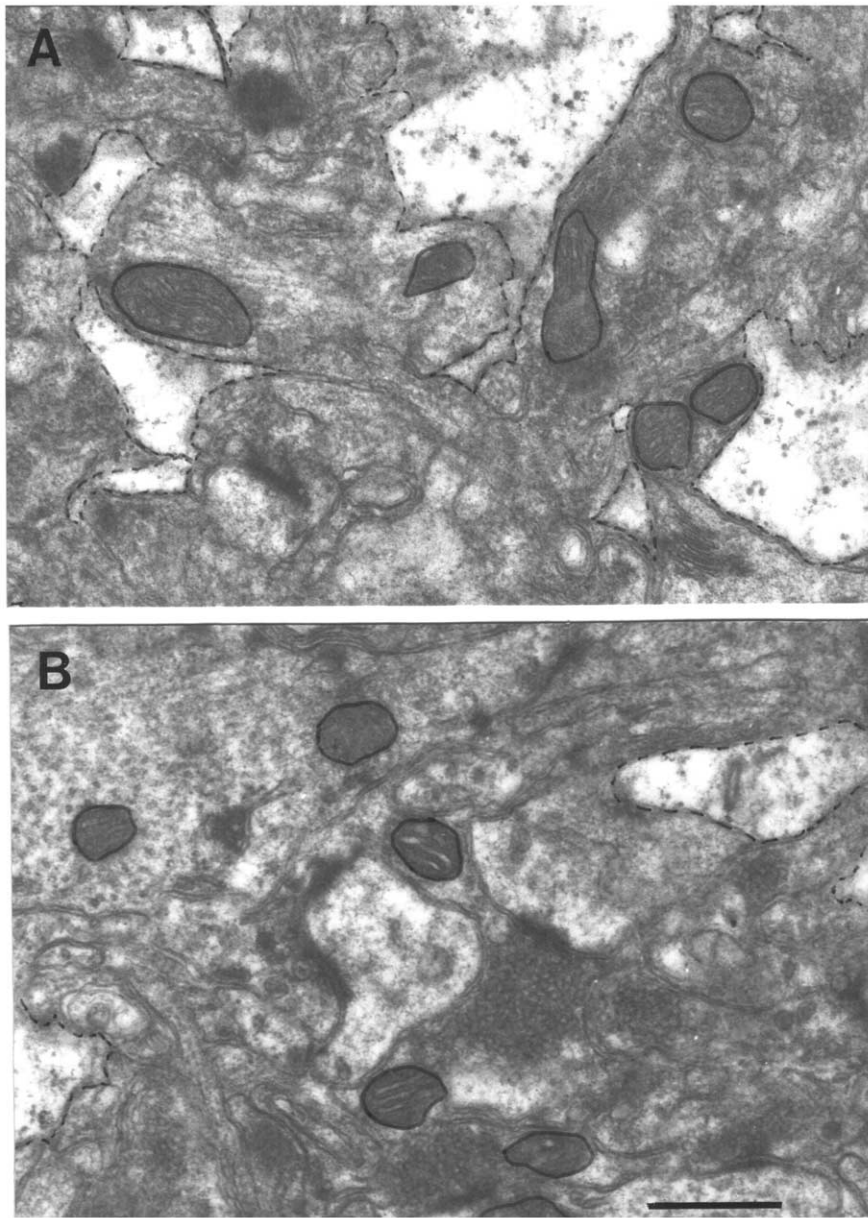


Fig. 2. Mitochondrial and glial volume fraction were estimated by measuring the fraction of area per sampling site made up by profiles of each type. Mitochondrial profiles are shown here circled by solid lines. Glial profiles are outlined by dotted lines. Adjacent sections were used to confirm the category of ambiguous profiles. These images represent the magnification from which profiles were identified and measured from A) VEH and B) CORT. The micrographs shown are approximately 17% of the size of a single sampling frame. The variation in clusters of profile types within and across sampling frames prevents visual comparisons of groups. As a result, quantification was necessary. Scale bar=500 μm .

gray matter (Peters et al., 1991). Myelin was not included. Mitochondrial profiles were identified by their cristae, dark matrix, and outer membrane. All mitochondria were included in the measurement despite the cell or process type they resided in. It should be noted, however, that most of these profiles were of neuronal origin. Very few mitochondria are seen in glial profiles, because glial cells do not consume as much energy as neurons. The identified mitochondrial and glial profiles were traced with a digitizing tablet, and the area of each, along with the total area of the micrograph, was measured using NIH Image (version 1.56). Total mitochondrial and glial area per micrograph were each divided by the total area of the micrograph to cal-

culate their respective area fractions, which provide unbiased estimates of volume fractions (Weibel, 1979).

Statistical analysis

Body weight was analyzed with a repeated measures ANOVA using weekly weight as the within subjects factor and group as the between subjects factor. Peripheral organ weights were analyzed with two-tailed Student's *t*-test ($\alpha = .05$). Treatment effects on CA3 cell layer and apical dendritic neuropil volume, cell density, mitochondrial and glial area fraction were analyzed with a one-way ANOVA.

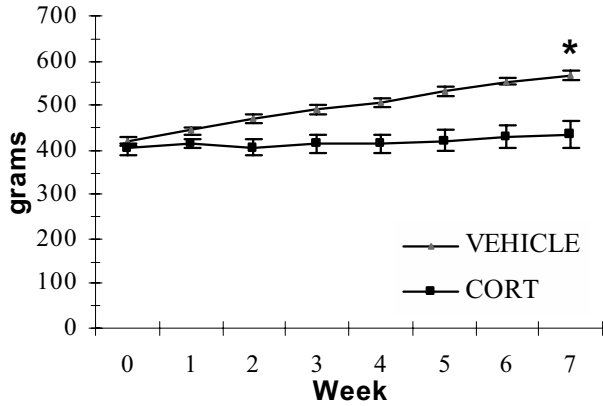


Fig. 3. Mean body weights were equal in the first week of the 56-day treatment period. However, VEH animals were 24% heavier than CORT animals by the final week (* $P < 0.01$). While CORT animals gained only 9% in body weight by the end of 8 weeks, VEH animals gained 38% more body weight. Values are means \pm S.E.M.

RESULTS

Body and organ weights

Despite the equal average body weight across groups at the beginning of treatment, the CORT group weighed less at the end of the experiment. A repeated measures ANOVA on mean body weights (with treatment as the between subjects factor and week as the within subjects factor) revealed a main effect for both treatment [$F(1,8) = 16.5, P < 0.01$; see Fig. 3] and week [$F(8,64) = 37.2, P < 0.0001$; see Fig. 3]. A significant interaction between treatment and week was also found [$F(8,64) = 15.9, P < 0.0001$; see Fig. 3]. To further investigate the interaction between treatment and week, a repeated measures ANOVA was performed separately in each group with week as the within subjects variable. This revealed a main effect of week for VEH animals [$F(8,32) = 113, P < 0.0001$] with body weight being 38% higher at the end of the 56-day treatment. There was no effect of week for the CORT group [$F(8,32) = 1.69, P = 0.16$], whose body weight gain was only 9%. At the end of treatment, VEH animals were 24% heavier than CORT.

As summarized in Table 1, the CORT group had a 25% lower heart weight [$t(8) = 4.64, P < 0.05$], and a 72% lower adrenal gland weight [$t(8) = 4.51, P < 0.005$] than the VEH group. The CORT gastrocnemius muscle, a fast oxidative muscle that is susceptible to GC damage (Hickson and Marone, 1993), weighed 35% less than VEH gastrocne-

mius [$t(8) = 6.28, P < 0.0001$; see Table 1]. CORT had no effect on soleus muscle weight [$t(8) = 1.25, P = 0.24$; see Table 1], a slow oxidative muscle reported to be resistant to GC damage (Hickson and Marone, 1993). When expressed as a percentage of body weight, the CORT group had a 62% lighter adrenal gland [$t(8) = 4.18, P < 0.005$; see Table 1] and a 14% lighter gastrocnemius muscle [$t(8) = 2.83, P < 0.05$; see Table 1]. These findings suggest that the adrenal and gastrocnemius are more susceptible to CORT treatment than the rest of the body as a whole.

Structural analysis

CORT had a 6% lower brain weight than VEH [$t(8) = 2.46, P < 0.05$; see Table 1]; a small but significant difference (see Fig. 4). This was substantially less of a difference than the group differences in body weight. As a result, the brain to body weight ratio was 24% higher in CORT than VEH animals [$t(8) = 3.11, P < 0.05$; see Table 1]. Cell layer volume was 4.64% lower in the CORT group than in VEH, a difference that was not statistically significant [$F(1,8) = .267, P = 0.619$; see Fig. 4]. Apical dendritic neuropil volume was 3.48% lower in the CORT group than VEH group, a nonsignificant difference [$F(1,8) = .101, P = 0.759$; see Fig. 4]. Cell density estimates were based on a mean of 23 disectors per animal yielding an average Q- of 335 and 313 per animal for the CORT and VEH groups, respectively. Cell density was not reduced by CORT [$F(1,8) = .002, P = 0.968$; see Fig. 4]. Although the overall brain weight was significantly lower, there were no gross structural effects in middle and proximal CA3 of the dorsal hippocampus.

At the ultrastructural level of analyses, a one-way ANOVA revealed no significant difference between groups in glial area fraction, an estimate of glial volume fraction [$F(1,8) = .173, P = 0.689$; see Fig. 5]. A one-way ANOVA revealed that the CORT animals had a significant reduction in mitochondrial area fraction, an estimate of volume fraction, when compared with the VEH rats [$F(1,8) = 6.091, P < 0.05$; see Fig. 5]. Despite this reduction, visual inspection yielded no obvious signs of damage (e.g. swollen or degenerating mitochondria).

DISCUSSION

Our findings indicate that elevating CORT for 56 days does not influence CA3 cell number or cell layer volume. These findings are consistent with previous studies utilizing unbiased counting methods (Vollmann-Honsdorf et al., 1997;

Table 1. Effects of 56 daily corticosterone injections on body and peripheral organs (means \pm SEM)

	Weight (grams)					
	Body	Brain	Adrenals	Heart	Gastrocnemius	Soleus
VEH	578 \pm 8	1.74 \pm 0.022	0.073 \pm 0.011	1.47 \pm 0.041	3.27 \pm 0.102	0.193 \pm 0.020
CORT	441 \pm 28***	1.63 \pm 0.041*	0.021 \pm 0.002**	1.10 \pm 0.068**	2.13 \pm 0.149***	0.174 \pm 0.010
Organ/body weight						
VEH		0.301 ⁻² e \pm 0.003 ⁻² e	0.126 ⁻³ e \pm 0.02 ⁻³ e	0.254 ⁻² e \pm 0.006 ⁻² e	0.565 ⁻² e \pm 0.014 ⁻² e	0.34 ⁻³ e \pm 0.004 ⁻³ e
CORT		0.374 ⁻² e \pm 0.023 ⁻² e*	0.0476 ⁻³ e \pm 0.01 ⁻³ e**	0.254 ⁻² e \pm 0.023 ⁻² e	0.486 ⁻² e \pm 0.025 ⁻² e*	0.40 ⁻³ e \pm 0.002 ⁻³ e

Sousa et al., 1998; Leverenz et al., 1999). The dose used in the present study caused no reduction in the volume of the dorsal CA3 apical dendritic neuropil. Whereas we expected to see an increase in glial volume fraction, we did not find that in this study. Either these animals did not undergo the atrophy described by Woolley et al. (1990), or some other tissue constituent increased as dendrites atrophied. Mitochondrial volume fraction was reduced by CORT. This finding suggests that the metabolic capacity of CA3 apical dendritic neuropil may be impaired after CORT elevations that are prolonged for 56 days.

The CORT treatment produced well-known peripheral effects of GCs, and reduced brain weight

The dose of CORT used in this study should produce physiologically relevant elevations in CORT. The same dose in a separate group of animals produced significant elevations in circulating plasma CORT concentrations. The elevations were equivalent to the concentrations we have seen in rats exposed to a novel environment or stress (unpublished data), and to the concentrations reported during restraint stress (Watanabe et al., 1992b). The effectiveness of the CORT dose used here can be seen by the reduction in body weight gain, and the reduction in gastrocnemius muscle and adrenal gland weight. The latter were influenced relatively more than the body weight. Although brain weight was reduced, the brain weight change was small enough relative to the body weight loss that the brain to body weight ratio was greater in the CORT group than the VEH group. These data suggest that the brain is more resistant to the effects of CORT than many other organs. Overall these findings support the effectiveness of the CORT dose and duration used in this experiment.

CORT did not reduce CA3 cell density or the volume of the cell layer or apical dendritic neuropil

Early studies utilizing traditional counting methods that are influenced by cell size reported that CORT decreases the number of pyramidal cells in the CA3 hippocampal subfield

(Sapolsky, 1985; Mizoguchi et al., 1992; Clark et al., 1995). More recent studies using counting methods that are not biased by differences in cell size have failed to show neuron loss after CORT treatment. More specifically, administration of CORT at higher doses (40 mg/kg) for 1 or 3 months failed to cause cell loss when rats were treated beyond the post-natal period (Sousa et al., 1998). Absence of neuronal loss in CA3 and CA1 hippocampal fields was also reported after 3 weeks of chronic psychosocial stress (Vollmann-Honsdorf et al., 1997), and after cortisol treatment in primates (Leverenz et al., 1999). The failure to find a reduction in cell density or neuropil volume here suggests that there is no cell loss. This conclusion is consistent with the findings of the previous reports that employed stereological counting methods. If CORT does not cause cell loss when there is a 24% difference in body weight, and an 8% difference in brain weight, it seems unlikely that any physiologically relevant parameters of CORT administration would cause cell loss in young animals. The failures to find cell loss are consistent with the failure to find evidence for greater levels of apoptosis in the subregion CA3 following postmortem analysis of depressives that had prolonged elevations in cortisol (Lucassen et al., 2001).

There was no indication of apical dendritic neuropil volume reduction. Administration of a higher dose (40 mg/kg) in adult animals has been reported to cause volume loss (Sousa et al., 1998). It seems possible that higher doses are necessary to produce volume reduction.

CORT did not alter glial volume fraction in the apical dendritic neuropil

To test whether CORT changed the composition of the apical dendritic neuropil, glial volume fraction was measured. Previous studies have focused on GC effects on GFAP, the major intermediate filament protein in mature astrocytes. *In vitro* studies of astrocyte cultures found an increase in GFAP gene expression after short (6 or 24 h) or longer (3 weeks) exposure to CORT (Rozovsky et al., 1995; Melcangi et al., 1997). However, GFAP mRNA was

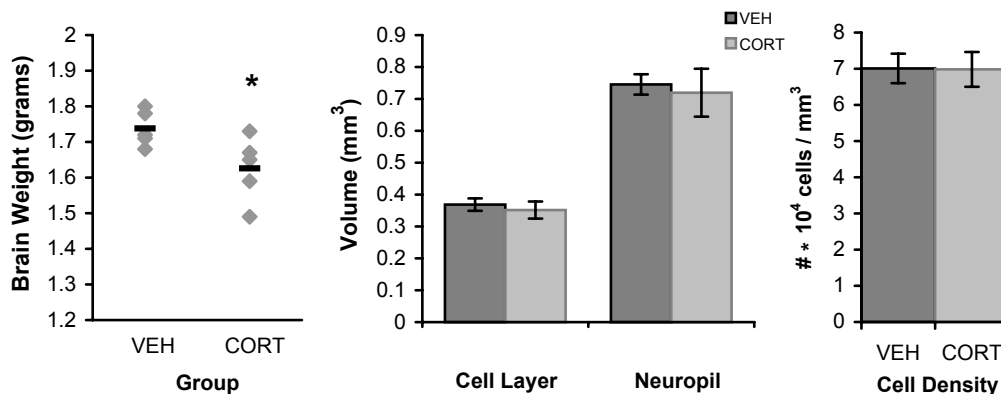


Fig. 4. Fifty-six days of CORT significantly reduced brain weight (6%; * $P < 0.05$), but did not affect cell layer volume (s. pyramidale, $P > 0.05$), or apical dendritic neuropil volume (s. lucidum, radiatum and lacunosum-moleculare, $P > 0.05$). Likewise, 56 days of CORT did not significantly reduce CA3 cell density within the cell layer ($P > 0.05$). Bars are means \pm S.E.M.

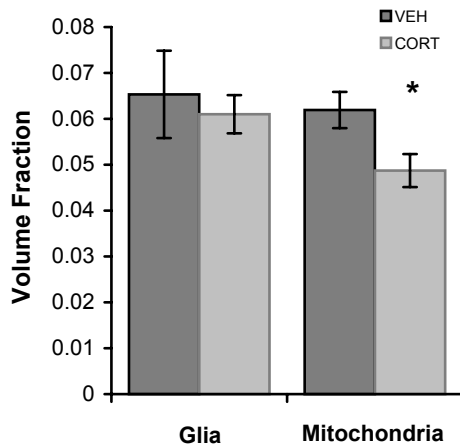


Fig. 5. Glial volume fraction was not significantly altered by the CORT treatment ($P > 0.05$). However, CORT significantly reduced mitochondrial volume fraction ($* P < 0.05$). Bars are means \pm S.E.M.

decreased when astrocytes were co-cultured with neurons (Rozovsky et al., 1995). *In vivo* studies indicate that acute or longer-term exposure to CORT decreased the concentration of GFAP. More specifically, 3 weeks of CORT treatment (50 mg/kg) in young rats (40–50 days old) decreased GFAP concentration in the hippocampus (O'Callaghan et al., 1989; Nichols et al., 1990). In human postmortem tissue from depressives and steroid-treated individuals, Muller et al. (2001) found semi-quantitative differences in the intensity of GFAP staining in glial cell bodies in CA1 and CA2, but not in glial fibers in CA3. Although GFAP measurements may provide important information regarding glial changes, they do not provide a direct estimate of astrocytic volume. Here, glial volume fraction was not changed by 56-day administration of CORT (26.8 mg/kg). The similarity of the parameters (10–11 mg/rat/day) between our study and that of Nichols et al. (1990), suggests that while GFAP may decrease, glial volume fraction does not change.

CORT reduced the mitochondrial volume fraction in the apical dendritic neuropil

In the present study, CORT caused a significant reduction in the mitochondrial area fraction. Despite the decrease, the overall neuropil volume did not change. Several earlier studies investigated CORT effects on mitochondria. Miller et al. (1989) administered 20 mg per day for 3 days and found no change in mitochondrial surface density. It is likely that the difference in duration of the two studies, the first being 3 days, and ours being 56 days accounts for the difference in findings. In another study, Magarinos et al. (1997) used 21 days of restraint stress to elevate CORT, and found that the total area of mossy fiber terminals occupied by mitochondria was greater after stress than in controls. Because mossy fibers are restricted to the stratum lucidum, which makes up a very small, albeit important part of the apical dendritic neuropil, mitochondrial area fraction within mossy fiber profiles would contribute very little to the estimated mitochondrial volume fraction for the

whole apical dendritic neuropil of dorsal CA3. The latter was measured in the present study. The reduced mitochondrial volume fraction reported here suggests that metabolism may be reduced or impaired in the neuropil overall. These results are also consistent with CORT effects in muscle, where the CORT levels elevated by chronic stress negatively correlated with muscle mitochondrial metabolism (Duclos et al., 2001), and administration of cortisol acetate impaired muscle mitochondrial functioning (Marone et al., 1994).

It is likely that the reduction in mitochondrial volume fraction is a direct effect of CORT action on mitochondria. CORT could be binding to mitochondrial GR receptors (Moutsatsou et al., 2001), which are known to regulate mitochondrial gene transcription (Van Itallie, 1992). In particular, CORT could be acting on hormone response elements suggested to be present on the cytochrome oxidase subunit I gene, a gene of mitochondrial origin (Tsiriyotis et al., 1997). The reduction in CA3 mitochondrial volume after CORT treatment, taken together with the previous research, is consistent with the hypothesis that CORT impairs mitochondrial function in CA3.

The reduction in mitochondrial volume fraction is consistent with the ability of CORT to impair hippocampal function. Hippocampal function can be assessed with tests of spatial learning. Using the animals included in this study along with additional animals, we have tested the effects of CORT on novel arm recognition in the Y-maze, a task shown to be hippocampal dependent (Conrad et al., 1996). Although the animals could recognize the novel arm after 21-day administration of CORT, they were impaired after a 56-day administration (Coburn-Litvak et al., 2003). The ability of 56-day administration of CORT to impair working memory is consistent with previous findings that restraint stress impairs spatial working memory (Luine et al., 1994; Conrad et al., 1996). The possibility that impaired mitochondrial function may play a role in impaired memory is further supported by findings that CORT and sodium azide, given at doses in which neither produced spatial learning deficits, acted synergistically to impair spatial learning and reduce brain cytochrome oxidase activity (Bennett et al., 1996). Several studies have also demonstrated an association between memory loss and mitochondrial dysfunction with aging (Liu et al., 2002; Navarro et al., 2002). Thus CORT effects on mitochondrial function may contribute to spatial learning deficits caused by chronic CORT exposure.

If the mitochondria are impaired by CORT, and the impairment includes impaired electron transport chain function, then CORT may increase radical oxygen species production. This could lead to mtDNA mutations that cascade to further impair mitochondrial function. This possibility is supported by a report of moderate levels of DNA fragmentation in some depressives that had experienced elevated GCs (Lucassen et al., 2001). However, a more direct causal relationship must be established in animal models before firm conclusions can be drawn.

The present results support the hypothesis that prolonged elevations in GCs make the hippocampus vulner-

able to other insults. There have been many studies showing that GCs elevated at the time of the insult increase the damage caused by the insult (Stein-Behrens et al., 1994). When the elevations of GCs are blocked, the CA3 damage from kainic acid can be reduced (Smith-Swintosky et al., 1996). Reduced mitochondrial volume fraction after prolonged elevations of GCs, therefore, may exacerbate the vulnerability to metabolic challenge resulting from acute elevations of GCs.

The failure to find either neuropil volume loss or glial hypertrophy was unexpected in light of reports that CORT administration at a dose of 10 mg/day for 21 days causes dendritic atrophy (Woolley et al., 1990). The dose used in the present study was equivalent to 10–11 mg/day, but in slightly larger animals. Although the dose relative to weight (mg/kg) was somewhat lower than that used by Woolley et al. (1990), our dose produced well-established peripheral effects of CORT, and has been shown to significantly elevate plasma CORT concentrations to values reported during stress. Sousa et al. (1998) used a higher dose (40 mg/kg) and found that apical dendritic neuropil volume was reduced following 30 day treatment with CORT, a finding more consistent with the possibility of dendritic atrophy. It is possible that damage from lower doses takes subtler forms, and only with higher doses may CORT produce gross morphological damage.

CONCLUSION

CORT elevations that were sufficient to reduce organ and muscle weight as well as significantly reduce brain weight, caused no gross anatomical alterations in the middle and proximal regions of dorsal CA3. Although CORT has been shown to reduce GFAP, glial volume fraction was not significantly reduced after exposure to elevated CORT for 56 days. In contrast, CORT reduced mitochondrial volume fraction, suggesting that metabolic capacity may be impaired. Because indications of damage were restricted to the ultrastructural level of analysis, conclusions about tissue health should not be based solely on gross morphological analysis. Impaired spatial working memory in the animals used in the present study (experiment 2 in Coburn-Litvak et al., 2003) suggests that memory deficits are not dependent upon gross morphological damage in CA3. CORT effects on mitochondria are consistent with previous studies showing that CORT can influence mitochondria in heart and muscle. The data suggest that chronic elevations in CORT for 56 days leave the hippocampal area CA3 vulnerable to metabolic challenge.

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