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Neuroprotection provided by dietary restriction in rats is further enhanced by reducing glucocorticoids

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Abstract

Glucocorticoids (GC)--corticosterone (CORT) in rodents and cortisol in primates--are stress-induced hormones secreted by adrenal glands that interact with the hypothalamic pituitary axis. High levels of cortisol in humans are observed in neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD), as well as in diabetes, post-traumatic stress syndrome, and major depression. Experimental models of diabetes in rats and mice have demonstrated that reduction of CORT reduces learning and memory deficits and attenuates loss of neuronal viability and plasticity. In contrast to the negative associations of elevated GC levels, CORT is moderately elevated in dietary restriction (DR) paradigms which are associated with many healthy anti-aging effects including neuroprotection. We demonstrate here in rats that ablating CORT by adrenalectomy (ADX) with replenishment to relatively low levels (30% below that of controls) prior to the onset of a DR regimen (ADX-DR) followed by central administration of the neurotoxin, kainic acid (KA), significantly attenuates learning deficits in a 14-unit T-maze task. The performance of the ADX-DR KA group did not differ from a control group (CON) that did not receive KA and was fed ad libitum (AL). By contrast, the sham-operated DR (SHAM-DR KA) group, SHAM-AL KA group, and ADX-AL KA group demonstrated poorer learning behavior in this task compared to the CON group. Stereological analysis revealed equivalent DR-induced neuroprotection in the SH-DR KA and ADX-DR KA groups, as measured by cell loss in the CA2/CA3 region of the hippocampus, while substantial cell loss was observed in SH-AL and ADX-AL rats. A separate set of experiments was conducted with similar dietary and surgical treatment conditions but without KA administration to examine markers of neurotrophic activity, brain-derived neurotrophic factor (BDNF), transcription factors (pCREB), and chaperone proteins (HSP-70). Under these conditions, we noted elevations in both BDNF and pCREB in ADX DR rats compared to the other groups; whereas, HSP-70, was equivalently elevated in ADX-DR and SH-DR groups and was higher than observed in both SH-AL and ADX-AL groups. These

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results support findings that DR protects hippocampal neurons against KA-induced cellular insult. However, this neuroprotective effect was further enhanced in rats with a lower-than control level of CORT resulting from ADX and maintained by exogenous CORT supplementation. Our results then suggest that DR-induced physiological elevation of GC may have negative functional consequences to DR-induced beneficial effects. These negative effects, however, can be compensated by other DR-produced cellular and molecular protective mechanisms.

Introduction

Dietary restriction (DR), also known as calorie restriction, reliably extends lifespan in a wide variety of species (Masoro, 2003), increases resistance to stress (Mattson, 2003), reduces the risk for development of cancer, diabetes, and stroke, and may reduce the risk for the development of Parkinson's and Alzheimer's diseases (Mattson, 2010). The specific cellular and molecular mechanisms underlying the neuroprotective effects of DR remain to be established. It has been postulated that DR functions as a moderate stressor to stimulate a hormetic response in the brain, resulting in increased expression of stress resistance proteins, such as heat-shock protein 70 (HSP-70), as well as neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) (Mattson 2003; Mattson et al., 2004; Arumugam et al., 2010). Both HSP-70 and BDNF are well known for promoting neuronal survival and may increase neuronal resistance to neurodegenerative diseases (Mattson, 2003).

Acute and long-term DR regimens are also known to cause moderate elevation of plasma levels of glucocorticoids (GC). These stress-induced hormones are secreted peripherally by the adrenal cortices representing corticosterone (CORT) in rodents and cortisol in primates including humans (Lupien et al., 2009). In both rodents and primates, GCs follow a diurnal rhythm that is controlled by the hypothalamic pituitary axis (HPA) in the central nervous system. In particular, both cortisol and CORT are secreted in response to the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, while ACTH secretion is controlled by hypothalamic peptide corticotrophin-releasing hormone (CRH).

GC is critical to a wide variety of functions in mammals. In response to stress, GCs are elevated slightly so that the organism may respond to perceived or actual stressful events. Once the stress subsides, GC secretion returns to normal levels. Such fluctuations during stressful events can provide neuroprotection (Abraham et al., 2006). However, when levels of GC are chronically elevated or chronically decreased in brain and other organs, the long-term effects are often detrimental. Slightly elevated levels of CORT in plasma (200–270 nM range) in the rat have been reported to attenuate N-methyl-D-aspartate (NMDA) and amyloid- β toxicity, while chronic elevation (310–650 nM range) potentiated the neurotoxic effects in the nucleus basalis magnocellularis (Abraham et al, 2006). Moreover, absence of CORT for 3 days due to adrenalectomy (ADX) can result in apoptosis of granule cells of the dentate gyrus (DG) in adult rats (Greiner et al., 2001). Reduction of CORT in ad libitum fed rats by ADX produced hypophagia and satiety (Torres et al., 2008) and decreased locomotor activity (Duclos et al., 2009). By contrast, administration of these substances as steroids results in hyperphagia in humans (Tataranni et al., 1996). Besides the direct effects of ADX on neuronal survival, the lack of CORT also enhances the vulnerability of neurons to excitotoxic insults (Abraham et al., 2006).

GCs are secreted by the adrenal cortex and readily pass the blood brain barrier where they bind to intracellular mineralcorticoid (MR) and GC receptors in both neurons and glia (Abraham et al., 2006). GCs act in a feedback loop with the HPA to stop stress-induced HPA activation. The hippocampus (HC), an important structure for learning and memory, has an abundance of GC receptors. Extended exposure of this structure to a high elevation of

GC in both baboons and rats has been reported to cause hippocampal neurodegeneration (Sapolsky et al., 1990; Reagan et al., 1999). Studies in rats reveal that a high dose of CORT exacerbates the vulnerability of neurons to excitotoxic insult and induces morphological reorganization in the HC, such as reversible atrophy of neuronal dendritic spines in hippocampal CA3 region (Magarinos & McEwen, 1995; Bisagno et al., 2000). Increased plasma cortisol levels in the elderly (Lupien et al., 1994) and individuals with diabetes, as well as experimentally elevated CORT in rodents, demonstrate the detrimental effects on learning ability and brain function. In a large study of humans ages 50–70 years, elevated salivary levels of cortisol were found to be correlated with poor cognitive function across a broad range of domains including language, processing speed, eye-hand coordination, executive function, verbal learning, and memory and visual memory (Lee et al., 2007). Many rodent studies have reported a robust, age-related increase in plasma CORT levels accompanied by declines in GC receptors in the hippocampus (Eldridge, 1989; Hibberd, 2000; Landfield and Eldridge, 1989; Masoro, 2007). Results for other rodent studies have also demonstrated that reducing CORT by ADX in a diabetic model attenuates learning deficits in a complex maze task (Stranahan et al., 2007, 2008). Blocking 11-B-hydroxysteroid dehydrogenase type 1 with carbenoxolone to reduce cortisol production improved verbal fluency in normal elderly men and verbal memory in diabetics (Sandeep et al., 2004).

It is well established that young rodents on DR regimens have higher circulating levels of CORT, yet increased levels of neuroprotection against a variety of stressors (Masoro, 2007). Reviewers of this literature have noted the paradox between these observations and reports that elevated levels of CORT can lead to neurodegeneration (Masoro, 2007; Patel and Finch, 2002). The current study was designed to address this paradox in a systematic manner. Specifically, we manipulated CORT levels surgically via ADX prior to initiating DR imposed by an every-other-day (EOD) feeding regimen versus maintenance of ad libitum (AL) feeding. CORT was replenished in ADX animals via drinking water immediately after ADX to produce a level about 30% below that of AL fed rats. Rats were maintained on their respective AL or DR regimens for 14 weeks prior to receiving either bilateral intracerebroventricular administration of the neurotoxin, kainic acid (KA), or vehicle, phosphate buffered physiological saline (PBS). Previous studies have demonstrated that ADX rats have greater hippocampal neuronal loss following similar KA treatments compared to intact animals (Stein and Sapolsky, 1988). Moreover, rats and mice on EOD regimens show greater protection against KA neurotoxicity (Anson et al., 2003; Bruce-Keller et al., 1999; Duan et al., 2001). As a functional measure of KA-induced damage and CORT manipulation, rats were tested in a complex T-maze learning task, in which performance is sensitive to KA-induced hippocampal damage (Duffy et al., 2008), as well as to manipulation of CORT (Stranahan et al., 2007, 2008). To assess anatomical damage following the treatments, stereological assessment of neuronal cell loss in hippocampal subregions CA2/3 was evaluated in each of the treatment groups. In a final set of experiments to identify possible mechanisms involved in neuroprotection, we assessed the effect of ADX and the diet treatments on the expression of neurotrophic and transcription factors including BDNF, HSP-70 and pCREB without exposure to KA.

Methods

Subjects

Male Sprague-Dawley rats at the age of around 3 months old and body weight about 300–350g were obtained from Harlan Laboratories (Frederick, MD). Rats were individually housed with food and water provided AL upon arrival. The colony was maintained under SPF conditions in a vivarium at the Gerontology Research Center (Baltimore, MD) on a 12 h light/dark cycle and with a constant ambient temperature of 21° C. Two weeks after

arrival, rats received either sham-operated surgery (Sham) or adrenalectomy (ADX). After 2 weeks recovery, rats were randomly divided into four groups: Sham-AL, Sham-DR, ADX-AL, and ADX-DR (n=15 /group), and then maintained on either an AL or DR regimen. In addition to the above 4 groups, there was a group of rats (n=10) designated as controls for the kainic acid (KA) injection. The control rats did not receive any surgical manipulation and were maintained on AL feeding regimen until all other rats were ready for intracerebroventricular injection.

Panel A in Figure 1 presents the experimental schedule and timeline for the study. Rats maintained on the DR regimen were kept on an every-other-day (EOD) fasting protocol. Food was placed into the cages at 8:00 am and removed 24 hours later for a 24-hour fast. The measurements for food and water consumption were performed daily after rats received the ADX or Sham surgical treatments. Body weight (BW) was recorded weekly prior to and during the 14 weeks of AL/RD regimens. The experimental protocol for these studies was reviewed and approved by the Institutional Animal Care and Use Committee of the Intramural Research Program of the National Institute on Aging.

Surgery

The bilateral ADX or Sham-operated surgery was performed under isoflurane anesthesia (1.5–2% vaporized) via small bilateral dorsal incisions to the abdominal cavity. The completeness of the adrenalectomy was verified by visual inspection during surgery and later by the assessment of plasma corticosterone (CORT). Immediately after adrenalectomy, ADX rats were supplied with CORT (Sigma, St. Louis, MO) in their drinking solution (0.9% saline) at a concentration of 25 µg/mL. This supplementation of CORT leads to basal GC levels (Akana & Dallman 1997), which have previously been reported to be sufficient to maintain the survival of mature hippocampal granule neurons (Cameron & Gould, 1994). For rats receiving Sham surgery, the adrenals were touched by the surgical instrument but not removed. At the time of sacrifice, the kidney and surrounding tissues were visually examined to verify a complete removal of the adrenal gland in ADX rats. Rats with any trace of adrenal tissue at the site were excluded from the study.

Assessment of Glucose and Corticosterone Levels in Blood

Fourteen weeks after rats had been maintained on either an AL or DR regimen, the blood glucose level was measured using a Freestyle glucose meter (Therasense, Alameda), and the CORT concentration was assessed in plasma obtained from blood samples collected from tail vein. For DR rats, the samples were assessed when the rats were at the end of 24 hr fasting period. For AL rats, the samples were taken after the rats received the same hours of acute food deprivation as DR rats. The level of CORT in plasma was measured using a commercially available radioimmunoassay kit (ICN Diagnostics, Costa Mesa, CA).

Intracerebroventricular Kainic Acid Injection

With rats maintained under isoflurane anesthesia (1.5–2% vaporized), KA (Sigma, St. Louis, MO) was injected bilaterally via Hamilton syringe and needle into the lateral ventricles using the following coordinates at 1.30 mm caudal to bregma, 1.8 mm left/right lateral to the mid line and 3.2 mm ventral to the surface of the brain. KA solution (0.15µg/µl in PBS) was injected at a rate of 0.2ul/min using an auto injector. After the infusion was completed, the needle was maintained in place for an additional 5 min before being slowly retracted. Control rats were subjected to the same protocol, except that PBS solution (2 µl) was injected instead.

Behavioral Assessment Using 14 Unit T- maze

The details of the layout, construction, and the behavioral protocol for the 14-Unit T-maze can be found in previous publications (Spangler et al., 1986; Pistell et al., 2009). Briefly, the maze was separated into five sections by guillotine doors that prevented animals from backtracking into previous sections of the maze (Figure 1 B). Infrared photocells were positioned throughout the maze and were wired in series to a microprocessor that recorded movement through the maze, time elapsed from start to goal, and time between photocell interruptions. Data collected from the photocells were analyzed by a microprocessor which calculated the number of errors and runtime for each section of the maze.

Prior to acquisition training in the maze, each rat received pretraining in one-way active avoidance in a footshock-motivated straight runway. Each rat was trained to a criteria of 13/15 correct avoidances to a mild footshock (Coulbourn Instruments E13–08: 0.8mA; maximum duration 120 s) for a maximum of 30 trials. Acquisition training in the 14 unit T-maze was performed one day following completion of pretraining. For DR rats, training in one-way active avoidance was conducted on a feeding day, and the performance of 14 unit T-maze was assessed on a fasting day.

In each trial, the rat was first placed into a black start box and positioned at the start location of the maze. The rat was then pushed gently into the first section of the maze, and the guillotine door was closed. A manual switch initiated a clock that controlled the shock contingency. The rat then had 10 s to pass through the first guillotine door and enter the next section. Failure to meet this criterion resulted in a scrambled foot shock (0.8mA) that was terminated when the rat passed through the door or a maximum of 300 s elapsed, resulting in the termination of the trial (testing was discontinued after two terminated trials). The shock contingency was reset after the rat passed through each guillotine door, completing each of the five maze sections and entering the goal box. A 90-s intertrial interval was used, during which time the box was placed in a holding area while the grid floor was cleaned with a 95% ethanol solution. Each rat received a total of 15 massed trials. The data were subsequently collapsed into five blocks of three trials for purposes of statistical analysis and data presentation. The errors committed as assessed by specialized software designed for the apparatus was examined as the primary performance variable.

Tissue Processing

Each rat was deeply anesthetized and perfused transcardially with chilled saline after the the(?) 24 hr fast. The whole brain was then removed and bisected by slicing through the central commissure. One hemisphere was post-fixed in 4% paraformaldehyde (PFA) for 48h followed by 30% sucrose at 4°C, and then it was frozen in a dry ice/methyl butane bath and stored at –80°C. Brains were sliced on the freezing microtome at 50µm. The HC was isolated on ice from the other hemisphere of the brain, frozen immediately on dry ice, and stored in a –80°C freezer.

Stereological Analysis

To examine the effect of DR on neuronal death induced by KA, random sampling in combination with the optical fractionator method was used to quantify the total number of pyramidal neurons in the CA2/3 region of the HC following standard methods of design-based stereology (West et al., 1991). The hippocampal regions including CA1 and dentate gyrus were not examined due to the absence of obvious cell loss. The anatomical definition of the CA2/3 region was based on Paxinos and Watson (1998) rat atlas. Around 5–6 sections including hippocampal CA2/3 region per rat were selected for counting (Mouton et al., 2002).

Western-blot and ELISA

Tissues were solubilized in SDS-PAGE sample buffer, and the protein concentration in each sample was determined using a Bio-Rad (Hercules, CA) protein assay kit with bovine serum albumin as the standard. Proteins (50 g protein per lane) were then resolved on 5–12% SDS polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat milk in TBST (Tris-HCl-based buffer with 0.2% Tween 20, pH 7.5) and then incubated overnight at 4°C in the presence of primary antibodies: HSP70 (1:1000, Cell Signaling Technology, Denver, MA), phospho-CREB (Ser133) (pCREB) or CREB (1:1000, Cell Signaling Technology, Denver, MA) at 4°C. The membranes were then washed three times in PBS buffer containing 1% Tween-20 and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (Pierce) for 1 h at 20–25°C. Bound antibodies were visualized by ECL™ Western Blotting Detection kit (GE Healthcare, Piscataway, NJ). Western blot analysis was performed in at least three separate experiments, and each experiment used five rats per group. Expression levels of each protein were quantified by densitometric analysis. The level of BDNF in the hippocampus was measured using a commercially available ELISA kit (Chemicon, Billerica, MA).

Statistical Analysis—Analysis of variance (ANOVA) followed by Student-Newman-Keuls test or Student's *t*-test for individual comparisons were computed for each variable, as appropriate, except for the body weight and maze performance scores which were analyzed using repeated-measures ANOVA followed by Student-Newman-Keuls tests. The biochemical data were analyzed using one-way ANOVA followed by Student-Newman-Keuls post-hoc tests. Statistical significance was determined as $p < 0.05$.

Results

Physiological Effects in ADX and AL/DR Regimen Fed Rats

Weekly mean body weight for DR and AL fed rats are displayed in Figure 2. The data were collected prior to and after maintaining the rats on their AL or DR regimen for a period of 14 weeks before icv administration of the neurotoxin KA and subsequent assessment of learning in the 14-unit T-maze. The DR regimen consistently reduced body weights in DR rats including Sham-DR and ADX-DR animals compared to rats maintained on the AL regimen over the 14-week period ($p < 0.05$). More importantly, neither the Sham-AL group (491.1 ± 9.71) nor the Sham-DR group (412.2 ± 10.85) significantly differed in their body weights from their ADX-AL group (469.6 ± 9.84) or ADX-DR (403.0 ± 9.76) counterparts. These results demonstrated that low level CORT replacement was effective in controlling food intake and body weight.

After 14 weeks on their respective diets, DR significantly reduced the blood levels of glucose compared to AL feeding, (Table 1). Both Sham-DR and ADX-DR rats had significantly lower levels of glucose compared to Sham-AL and ADX-AL rats ($p < 0.05$). Consistent with results from previous studies, DR elevated plasma CORT levels in Sham-DR rats compared to Sham-AL rats ($p < 0.05$). The surgical removal of the adrenal glands effectively eliminated the plasma CORT. However, with a lower dose of CORT supplement provided in drinking water, blood CORT concentration was maintained at lower levels (about 33% less) compared to those in rats with intact adrenal glands ($p < 0.01$) enabling physiological activity and survival.

Lowering CORT in DR Rats Exposed to icv KA Attenuates Maze Learning Deficits

One week after bilateral icv KA injection, rats were pretrained in the footshock-motivated straight runway task followed by a single day of testing in the 14-unit T-maze. Figure 3A

presents the mean errors for each group recorded for each of 5 blocks of 3 trials. A repeated measures ANOVA test indicated significant block and group effects ($p < 0.01$). The reduction in errors across the blocks of trials for all groups indicated that learning occurred. However, the PBS group followed by the ADX-DR KA group demonstrated significantly better learning/acquisition performance compared to the other three groups, and this observation was confirmed by post-hoc tests ($p < 0.05$). There was no significant difference between the PBS and ADX-DR KA groups ($p > 0.05$). Alternatively, the error data are presented as mean errors per trial (total of 15 trials) for each group in Figure 3B, Rats in Sham-AL, Sham-DR, and ADX-AL groups committed more errors per trial than did either the PBS and ADX-DR groups ($p < 0.05$). Thus, lowering levels of CORT in the ADX-DR group significantly attenuated learning deficits in rats that received KA. Other measures including runtime, shock duration, and number of shocks received were similarly affected among groups (data not shown).

DR Combined with ADX Produced a Larger Neuroprotection Against KA-induced Insult

All rats receiving KA injection exhibited neuronal loss as targeted in the CA2/3 regions of the HC. Figure 4A shows representative images of the CA2/3 region within the HC for each of the groups. The quantitative analysis of the intact neurons in CA2/3 region using the optical fractionator technique is presented in Figure 4B. Significant neuronal loss was produced by KA injections in Sham-AL and ADX-AL groups compared to the PBS group ($p < 0.05$). The DR regimen provided protection to neurons in the CA2/3 region. Rats in Sham-DR and ADX-DR had more spared neurons compared to Sham-AL and ADX-AL, respectively ($p < 0.05$). However, ADX produced additional protection against the KA. Compared to Sham-DR rats, ADX-DR rats had a significantly higher number of neurons spared, which resulted in a lack of a significant difference between the PBS and ADX-DR groups.

DR and ADX Increased pCREB, BDNF and HSP-70 Expression in the Hippocampus

An additional four groups of rats ($n=6$) were given either sham or ADX surgical manipulation without KA injection and maintained on either an AL or DR feeding regimen for 14 weeks. As previously reported (Mattson 2003; Mattson et al., 2004; Arumugam et al., 2010), DR increased several protective proteins including BDNF and HSP-70. A combination of DR and ADX appeared to further enhance the protective effect. The levels of phosphorylation of CREB (pCREB, Figure 5) and BDNF (Figure 6) in the HC were significantly elevated only in the group of ADX+DR ($p < 0.01$ or 0.05 , respectively). However, the level of HSP-70 was increased significantly in both Sham+DR and ADX+DR groups ($p < 0.05$, Figure 7).

Discussion

Studies of DR in rodent models have provided consistent support for the hypothesis that a substantial (20–40%) decrease in calories consumed both sustains neuronal viability and neuroprotection when faced with an insult, such as stroke or exposure to a neurotoxin (Martin et al., 2007; Mattson, 2010). The findings have been deemed paradoxical, as both acute and chronic DR cause a moderate increase in GC production in both rodents and primates including humans (Patel and Finch, 2002), although recent results appear less certain in regard to elevated GC in human and nonhuman primates on long-term DR (Downs et al., 2008; Weiss et al., 2006). Regarding this issue in rodent studies, our own previous studies have clearly demonstrated that DR increases circulating CORT levels (Wan et al., 2003; Mager et al., 2006). However, although both acute and chronic fasting moderately increase CORT in rats, only chronic fasting conditions, including daily DR as well as DR induced by EOD feeding produced consistently mild elevated CORT in rats (Mager et al.,

2006). It is important to note that mild elevations can be defined as those approximating 30–50% increases in plasma CORT levels versus marked elevations at over 100% increases associated with more severe stressors. As examples, we have previously reported around a 32% increase in plasma CORT comparing young AL to DR rats (Wan et al., 2003a), which contrasts to over a 100% increase comparing rats undergoing restraint stress to controls (Wan et al., 2003b). In the current study, there was a 43% increase in plasma CORT comparing AL to DR groups.

Numerous studies have documented that long-term, unrelieved, marked increases in GC are associated with damage to brain regions important to learning and memory including the HC which has an abundance of GC receptors (Sapolsky et al., 1990; Reagan et al., 1999). The impact of GC on hippocampal pyramidal neurons, long term potentiation, and cognition are well established (Maggio & Segal, 2007; Lee et al., 2007). However, transient increases in GC can also be beneficial to learning behavior in rats (Masoro, 2007). Chronically elevated high levels of GC, however, are detrimental to the brain, particularly the HC (Sapolsky et al., 1990).

The apparent paradox that DR provides neuroprotection at the same time that it produces sustained and moderately elevated GC has resulted in several hypotheses related to GC, DR and aging, including a glucocorticoid hypothesis of DR and the slowing of brain aging (Patel and Finch, 2002) as well as a revision of this hypothesis (Landfield et al., 2007). A hormesis hypothesis proposed by Masoro (2003) and a corollary neurohormesis hypothesis (Mattson, 2008) implicate DR as a long-term mild or low-intensity stressor that moderately increases CORT across the life span by increasing plasma free CORT. Presumably the moderately elevated CORT provides neuroprotection from a wide variety of stressors by activating a network of stress protective pathways.

The results of the present study indicate that a reduction in circulating CORT levels achieved by ADX combined with replacement of CORT to a level around 30% that of SHAM-AL rats, in DR-fed rats exposed to KA can provide additional benefit against a toxic insult beyond the neuroprotection achieved by DR alone. Although the KA injection produced neuronal loss in all rats, the ADX-DR group clearly displayed the least loss among all KA-treated groups. The functional effect of the ADX-generated protection was observed in the analysis of performance in the 14 unit T-maze. Rats from both Sham-AL and ADX-AL performed poorly in complex maze learning. Although the rats from Sham-DR group did better than those maintained on AL regimens, only the ADX-DR rats performed significantly better and did not differ from the PBS control group.

The effect of DR-induced protective efficacy apparently resulted from cumulative effects following initiation of the chronic dietary regimen. Our previous studies had monitored several physiological and behavioral parameters, such as heart rate, blood pressure, and general locomotor activity and biochemical assessments, such as CORT and blood glucose, following the initiation of DR including daily reduction in calories as well as EOD regimens in rats (Wan et al., 2003a, Mager et al., 2006). The results of previous studies indicated that the EOD regimen induced reduction in these physiological parameters, particularly heart rate and blood pressure, and those changes fluctuated initially following the daily feeding schedule. As the EOD regimen progressed, a general decline of those responses was established until an asymptotic level was reached around 4–6 weeks after the DR regimen was initiated. From that stage onward, reductions in these physiological parameters were maintained steadily regardless of the availability of food. Another study also indicated that reductions in other physiological and behavioral parameters in EOD fed rats were maintained similarly across feeding and nonfeeding days. In response to repeated body immobilization stress given over 5 consecutive days, EOD rats showed consistent adaptation

to the daily stress regardless of feeding schedule (Wan et al., 2003b). In the present study, EOD rats were behaviorally trained across feeding and nonfeeding days. Maze performance was assessed in DR rats on a fasted day. According to our previous studies, the performance of DR rats was very unlikely affected by their feeding condition after they had been maintained on the same feeding regimen for at least 14 weeks.

Our additional experiments assessing other cellular signaling events in the HC typically associated with DR provided further evidence for the beneficial effects of CORT reduction combined with DR. Examining HSP-70 levels, we noted increased levels associated with DR as previously reported (Mattson 2003; Mattson et al., 2004; Arumugam et al., 2010); however, the DR-induced increase was equivalent between SHAM and ADX groups. Therefore, it would appear that elevations in this stress protein cannot account for the improved performance and increased neuronal protection associated with reductions in CORT.

In contrast to effects of DR on HSP70 levels, our results showed clearly that both pCREB and BDNF levels were uniquely increased in the ADX-DR condition. These increases could not be directly related to CORT levels because CORT levels of ADX-AL and ADX-DR groups were equivalent (Table 1); therefore, it would appear that other DR-invoked mechanisms are in play that are normally inhibited by CORT. CREB is a transcriptional factor regulating a broad array of stress responses, including activation of BDNF (Marini et al., 2004; Mattson, 2005). It is also involved in DR-mediated activation of SIRT1 (Noriega et al., 2011). In the brain DR-mediated actions on glutamate receptors in the nucleus accumbens have been linked to CREB activation (Haberny and Carr, 2005), and CREB activation is also involved in mitochondrial neuronal protection against a wide variety of oxidative stress (Ryu et al., 2005). pCREB has also been well documented and studied for its role in memory formation (Silva et al., 1998; Bourtchuladze et al., 1994; Trofimiuk et al., 2010). Interestingly, we did not observe an increase in pCREB in the SHAM-DR group relative to SHAM-AL, and we could find no other reports indicating higher HC levels of pCREB in rodents on DR.

BDNF elevation has been observed in EOD regimens and other DR paradigms, as well as additional interventions including exercise, cognitive stimulation, and pharmacological treatment with antidepressants (Mattson et al., 2004). BDNF elevation has been well documented for its role in the regulation of synaptic plasticity, neurogenesis, neuronal survival, maturation, and neuronal protection in the brain. In fact, results linking BDNF elevation induced by DR have not always been entirely consistent, probably due to the extent and duration of DR and interactions with other experimental manipulations including rat strain. For example, Newton et al. (2005) found no age-related decline in HC BDNF levels in BNxF344 rats, but long-term DR did slightly elevate BDNF levels in CA1 regions but not in the CA3 or dentate gyrus. Khabour et al. (2010) found that both voluntary exercise and the combination of voluntary exercise and EOD feeding in Wistar rats upregulated BDNF after 6 weeks on diet, while EOD feeding alone did not elevate BDNF over the same relatively short-term exposure. Moreover, the exercise and EOD feeding plus exercise groups experienced improved spatial learning and memory performance in a water version of the radial arm maze, but EOD feeding alone group did not improve performance in this task.

In the current study we observed a 20% increase in HC levels of BDNF in the SHAM-DR group compared to SHAM-AL animals; however, this difference did not prove to be statistically significant. A similar, nonsignificant increase in BDNF expression was observed in ADX+AL rats. The magnitude of the effect in ADX+DR rats was nearly a 40% increase in BDNF expression compared to SHAM+AL animals, which was statistically significant.

Thus, it would appear that elevated levels of CORT can inhibit BDNF expression as previously reported (Schaaf et al., 2000; Zhou et al., 2000), which drives down expression in DR animals despite other mechanisms that might drive it up; whereas, marked reductions in CORT in the ADX+DR rats allow these mechanisms to drive up BDNF expression. However, because there were no significant effects on BDNF expression in the ADX-AL group, which had identical levels of circulating CORT as the ADX-DR group, it is clear that inhibitory effects of CORT on BDNF expression is operative through other mechanisms within the DR milieu. While this study would have been greatly enhanced if different doses of CORT could have been administered, the consistency of the behavioral, neuroanatomical, and biochemical results clearly demonstrate that reduction of CORT provides additional benefit to the brain beyond DR alone.

That a reduction in CORT in DR models would be additionally beneficial to hippocampal neurons was postulated by Finch (2004). Recent studies appear to provide support for this hypothesis. Adrenalectomized mice and rats with induced diabetes had both greater neuroprotection and improved learning in the Stone T-maze used in the current study (Stranahan et al., 2007, 2008). Also, the literature on rodent aging indicates that approximately 30% of aged rodents fed AL experience hypercorticism, and these animals tend to be impaired in learning and memory tasks (Issa et al., 1990; Lupien et al., 2009).

The unfavorable effects of DR-induced elevation of CORT appear to be contradictory to the widely accepted conclusion that DR provides protection to numerous organ systems and extended lifespan in many species. DR-elevated CORT is a normal condition resulting from a physiological response to this metabolic stress, i.e., chronic hunger. CORT elevation has become one of the major markers of DR that include reduction of glucose, increase in insulin sensitivity, reduced plasma leptin, and elevated plasma adiponectin. Elevated free CORT has been observed to be due to a 33% reduction from plasma CORT binding globulin (Sabatino et al., 1991) in DR animals. However, there are a number of studies showing that DR induces major beneficial effects that compensate or minimize undesirable side-effects associated with DR. In one study, rats subjected to an EOD regimen for 3 months induced 20% reduction of GC receptor mRNA in CA1 region without an effect on mineralocorticoid receptors (Lee et al., 2000). This reduction may partially compensate for the deleterious effect due to the elevated free CORT resulting from a DR regimen.

In recent years evidence has accumulated indicating that the beneficial effects produced by DR are derived by upregulating a number of protective elements/factors peripherally and centrally. Beneficial effects of DR include decrease glucose levels, increased insulin sensitivity, and improved signaling through insulin transduction pathways, which may all contribute to functional improvements at the cellular level. Our recent studies indicate that DR stimulates a number of neural growth factors, such as BDNF and bFGF, increased heat shock protein chaperones such as HSP-70, HRP-78 as well as HO-1, elevated anti-inflammatory cytokines, and decreased pro-inflammatory cytokines, all of which have been observed in the brains of mice subjected to at least 3 months alternate-day feeding paradigm (Lee et al, 2000; Arumugam et al, 2010). Thus, beyond the involvement of CORT in mediating neuroprotective effects of DR, there are clearly several other pathways involved.

In summary, studies in a wide variety of invertebrate and vertebrate species have demonstrated the health and life extension benefits of DR. Regarding brain function however, the results are more mixed. The experiments reported here provide evidence that elevated CORT induced by long-term DR may be detrimental to learning and could possibly explain the mixed results observed in longitudinal studies evaluating learning and memory in aged rodents on DR (Gallagher et al., 2011; Spangler et al., 2010). However, the present results and many other studies provide evidence showing that this unfavorable effect can be

well compensated by numerous protective mechanisms also induced by DR, which is indicated by long-term functional improvements. A recent study in humans indicated that DR could improve memory in elderly humans (Witte et al, 2009). The moderate elevation of CORT results from a normal endocrine response to DR regimens. Although an experimental reduction of CORT by ADX appeared to enhance neuroprotection, this experimental reduction by removing the entire adrenal gland is likely to affect the status of homeostasis in animals, and those potential changes have not been evaluated at all. Moreover, the current study did not include an analysis of dose response to CORT to assess effects on the parameters observed. While ADX is severe and lethal if maintenance doses of GC are not administered, pharmacological blockade may be worth considering as an additional intervention. Thus, although the current results indicate that elevated levels of CORT can attenuate many favorable responses to DR, Further studies are required to investigate these perspectives in more detail.

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A **B** **Figure 1.**

A. Diagram depicts time course and flow of experimental procedures employed in the experimental procedures including initial surgeries (ADX or Sham), initiation of diets (DR or AL feeding), second surgery (KA or Sham), and behavioral testing in complex maze paradigm. **B.** Schematic diagram showing the configuration of the 14-unit T-maze. Arrows indicate the correct pathway. Errors are defined as any deviation from the correct pathway with more than half of the rat's body within the incorrect alley. S: Start box; G: Goal box, — Guillotine door, -- False guillotine door

Figure 2. DR reduced body weight in the rats regardless of ADX surgical condition
 Body weight was assessed weekly prior to and 14 weeks after dietary treatment but before KA injection. Values were the mean $\pm$ S.E.M. * $p < 0.05$ Sham+AL and ADX+AL compared to Sham+DR and ADX+DR.

Figure 3. KA significantly impaired acquisition performance in a 14-unit T-maze. DR attenuated KA-induced impairment only in the ADX group

Panel A depicts mean errors per trial at each of 5 blocks of 3 trials for the 15 massed practice trials (2 min ITI) of acquisition training. Panel B depicts mean errors per trial for each group for the 15 trials. No significant difference emerged between the ADX+DR KA group and Control PBS group ($p > 0.05$). However, both Control PBS and ADX+DR KA exhibited superior acquisition performance, i.e., made fewer errors, compared to Sham+AL KA, Sham + DR KA, and ADX+AL KA groups. * $p < 0.05$

Figure 4. Intracerebroventricular injection of KA produced significant neuronal loss/damage in the hippocampal CA2/3 region

Panel A: Representative 10× photomicrographs of Nissl-stained neurons in hippocampal CA2/3 region. A: Control PBS; B: Sham-AL KA; C: Sham-DR KA; D: ADX-AL KA; E: ADX-DR KA. Scale bar=100μm. F: High-power magnification of Nissl-stained sections. This figure displays typical images observed for each group during stereological estimations of total neuron number in hippocampal CA2/3 region. Arrows indicate neurons which meet the criteria for being included. Panel B displays the quantitative assessment of total number of neurons in hippocampal CA2/3 region for each group. Values were mean ± SEM. * p<0.05 compared to Control PBS; # p<0.05 compared to Sham+AL KA; † p<0.05 compared to ADX+AL KA.

Figure 5. ADX plus DR increased the expression of phosphorylated CREB in hippocampus
 Panel A depicts the expression of pCREB/CREB protein in hippocampus from the four groups by western blot. Panel B shows the densitometric quantitative analysis of ratio of pCREB/CREB in the hippocampus of the four groups. ADX + DR was elevated compared to each of the other groups, * $p < 0.05$.

Figure 6. ADX plus DR increased the level of BDNF in the hippocampus
 BDNF protein level was quantified using a BDNF ELISA kit. ADX + DR was higher than either Sham + DR or ADX + AL * $p < 0.05$ and ADX + DR was also higher than the Sham + AL group ** $p < 0.01$.

Figure 7. DR regimen increased HSP-70 level in the hippocampus

Panel A depicts HSP-70 expression by western blot. Panel B shows the densitometric quantitative analysis of levels of HSP70 protein in the hippocampus. Both Sham + DR and ADX + DR had elevated levels of HSP70 compared to the Sham + AL and ADX + AL groups, * $p < 0.05$.

Table 1

Dietary restriction (DR) significantly reduced blood glucose levels compared to ad libitum (AL) fed counterparts. Because total adrenalectomy (ADX) eliminates endogenous corticosterone, exogenous corticosterone was supplemented in the drinking water. As a result, plasma corticosterone levels were maintained for physiological activity and survival with concentrations of corticosterone sustained at low levels in ADX compared to those in rats with intact adrenal glands (Sham).

	Sham+AL	Sham+DR	ADX+AL	ADX+DR
Glucose (mg/dl)	76.2±1.0	63.1±2.1 [*]	68.3±1.6	57.2±1.8 [#]
Corticosterone (ng/ml)	215.6±30.1	309.5±30.9 [*]	143.3±18.1 [†]	142.5±23.2 [†]

^{*} p<0.05 compared to Sham+AL

[#] p<0.05 compared to ADX+AL

[†] p<0.01 compared to Sham+AL and Sham+DR