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Caloric Restriction Augments Brain Glutamic Acid Decarboxylase-65 and -67 Expression

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The ketogenic diet is a very low-carbohydrate, high-fat diet used to treat refractory epilepsy. We hypothesized that this diet may act by increasing expression of glutamic acid decarboxylase (GAD), the rate-limiting enzyme in γ-aminobutyric acid (GABA) synthesis. Thus, we evaluated brain GAD levels in a well-established, seizure-suppressing, rodent model of the ketogenic diet. Because the diet is most effective when administered with a modest (~10%) calorie restriction, we studied three groups of animals: rats fed ad libitum standard rat chow (Ad lib-Std); calorie-restricted standard chow (CR-Std); and an isocaloric, calorie-restricted ketogenic diet (CR-Ket). We found that GAD67 mRNA was significantly increased in the inferior and superior colliculi and cerebellar cortex in both CR diet groups compared with control (e.g., by 45% in the superior colliculus and by 71% in the cerebellar cortex; $P < .001$). GAD65 mRNA was selectively increased in the superior colliculus and temporal cortex in both CR-Std and CR-Ket diet groups compared with ad lib controls. The only apparent CR-Ket-specific effect was a 30% increase in GAD67 mRNA in the striatum ($P = .03$). Enhanced GAD immunoreactivity was detected in parallel with the mRNA changes. These data clearly show that calorie restriction increases brain GAD65 and -67 expression in several brain regions, independent of ketogenic effects. These observations may explain why caloric restriction improves the efficacy of the ketogenic diet in treating epilepsy and suggest that diet modification might be useful in treatment of a number of brain disorders characterized by impaired GAD or GABA activity.

Key words: GABA; seizures; epilepsy; ketones; ketogenic diet

The ketogenic diet is a high-fat, low-carbohydrate diet that has long been used as a treatment for intractable childhood epilepsy (Swink et al., 1997; Thiele, 2003). A substantial number of patients benefit from the diet in terms of reduction in frequency and/or severity of seizures, but the mechanism(s) involved in the diet's effects on the brain remain unclear. It has been hypothesized that, because both the ketogenic diet and starvation reduce seizures and lead to ketosis, that ketone bodies per se, particularly β-hydroxybutyrate (β-OHB), may exert direct seizure-suppressive effects. There are, however, several other hypotheses concerning the effect of diet modification on seizure activity, including diet-induced changes in energy metabolism, membrane lipid composition, brain water content, pH, neurotransmitter function, and synaptic transmission (for review see Schwartzkroin, 1999).

Interestingly, the ketogenic diet is more efficacious in both rodents (Bough et al., 1999) and humans when administered with calorie restriction (Vining et al., 1998). We recently employed a well-established animal model (Bough and Eagles, 1999; Bough et al., 1999) to study the effects of the ketogenic diet on the brain insulin-like growth factor (IGF) system and glucose transporter expression (Cheng et al., 2003). In this diet model, juvenile rats fed a normal rat chow diet ad lib are compared with groups fed calorically restricted normal rat chow and calorically restricted, seizure-suppressing, ketogenic diets. In this paradigm, we found that there were distinctly divergent effects of dietary caloric content and macronutrient composition on brain IGF systems and glucose transporter expression (Cheng et al., 2003). For example, IGF receptor and GLUT3 expression were significantly reduced by the calorie-restricted, standard chow diet but significantly increased with the calorie-restricted, ketogenic diet, suggesting that enhanced IGF1 receptor and glucose transporter expression could be instrumental in ketogenic diet-induced seizure suppression.

In the present study, we investigated the effects of diet on brain expression of glutamic acid decarboxylase

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(GAD), the rate-limiting enzyme in brain γ-aminobutyric acid (GABA) synthesis. GABA metabolism plays an important role in the origin and spread of seizure activity (Bradford, 1995; Petroff et al., 1996; Olsen and Avoli, 1997). GABA antagonists induce seizures (Concas et al., 1992), and GABA-mimetic drugs suppress seizures (Meldrum, 1996; White, 1997; Sills and Brodie, 2001). Studies on animal models of epilepsy suggest that decreases in levels of GABA and the GABA receptor are associated with increased seizure susceptibility, as well as the converse (Lasley and Yan, 1994; Homanics et al., 1997; Gernert et al., 2002). We demonstrated previously that rats fed either a ketogenic diet or a calorie-restricted, normal diet showed a significant increase in the threshold of seizures induced by pentylenetetrazole (PTZ) infusion (Bough et al., 1999). PTZ appears to act primarily by blocking GABA inhibition (Olsen, 1981). Therefore, evaluation of brain GAD expression in this same model may shed some light on the anticonvulsant effect of the diet treatment through modulation of the GABA pathway.

MATERIALS AND METHODS

Animals

The rat protocol used in this study was approved by the Georgetown University Animal Care and Use Committee. The experimental protocol and the diets have been described in detail previously (Bough et al., 1999; Cheng et al., 2003). In brief, 3-week-old, male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were randomly divided into groups to receive standard Purina 5001 rat chow ad libitum (Ad lib-Std); calorie-restricted, standard Purina 5001 chow (CR-Std); or calorie-restricted, ketogenic diets (CR-Ket). The calorie restriction was modest at 90% of calculated energy requirement, based on the relationship [(0.3 kcal/g body wt./day)(g body wt.)(1/kcal/g diet)] (Rogers, 1979). The CR-Ket diet (No. F3666; Bio-Serv, Frenchtown, NJ) was composed of fat (78%), protein (10%), carbohydrate (2%), and inert matter (10%) and had a metabolizable energy content of 7.3 kcal/g. Purina 5001 chow is composed of fat (10%), protein (25%), carbohydrate (50%), and inert matter (15%) and had an energy content of 3.9 kcal/g. Both diets are balanced for vitamins and minerals and contain at least minimal protein (10%). For the CR-Std group, food pellets that contained the correct number of daily calories were prepared. These three groups of rats were then put on their diets for 7 days. We chose an early time point in exposure to the diet to look for changes in gene expression reflecting the direct effect of the treatment, expected to occur before behavioral changes. After the diet treatments, all three groups of rats were weighed, then killed by decapitation after CO_2 anesthesia. Brains were removed, cut in the midsagittal plane, and immediately frozen in dry ice. Brain hemispheres were cut into 10-μm-thick sagittal sections at −15°C, thaw-mounted onto poly-L-lysine-coated slides, and used for in situ hybridization and immunohistochemistry analysis. The opposite hemispheres were used for RNA and protein sample preparation. Trunk blood was collected for simultaneous glucose and ketone measurement. β-OHB levels were determined spectrophotometrically by using 20 μl of plasma (GDS Technology, Elkhart, IN). Blood glucose was measured by placing a drop of trunk blood on the test strip and inserting it into the One Touch Profile glucose meter (Lifescan, Inc., Johnson and Johnson, Milipitas, CA).

In Situ Hybridization

The protocol for in situ hybridization and generation of a cRNA probe has been described in detail elsewhere (Bondy, 1991). GAD has two major isoforms with molecular weights of 65 and 67 kD that are synthesized from two independent genes (Erlander et al., 1991). The rat GAD67 and GAD65 clones used for synthesis of the cRNA probes were kindly provided by Drs. Allan J. Tobin and Niranjala Tillakaratne of UCLA. GAD67 contains a 3.2-kb cDNA fragment corresponding to the complete GAD coding region and 3′-untranslated sequence, and GAD65 contains 2.3-kb complete cDNA sequence (Erlander et al., 1991). These probes have ~64% sequence identity and recognize distinct RNA bands on Northern blot (Erlander et al., 1991). After hybridization, sections were exposed to film for 3 days and later dipped in Kodak NTB2 emulsion. Parallel sections were hybridized to sense probes and processed together with antisense hybridized sections. All sections from the different diet treatment groups were prepared, hybridized, and washed at the same time, then exposed to the same film to minimize experimental variation. The quantification of hybridization signal was carried out in a blinded fashion. Hybridization signal was captured using a monochrome video camera. Signals were analyzed with NIH Image 1.57 software for several brain structures, including superior colliculus, temporal cortex, frontal cortex, striatum, thalamus, inferior colliculus, and molecular cell layer of the cerebellum. Background signal from a sense probe was subtracted prior to further analysis. Two sections from each brain were scored, and four measurements were made in each section; thus eight measurements were obtained, and the mean was calculated for each animal. For revealing cellular localization, the sections were also dipped in Kodak NTB2 emulsion for 10 days, developed, and then counterstained with hematoxylin and eosin.

Immunohistochemistry

Immunohistochemistry was performed with the avidin-biotin-immunoperoxidase method (Cheng et al., 1998). Briefly, frozen tissue sections were first fixed in 4% formaldehyde for 10 min, then washed in 1× Tris/NaCl buffer (pH 7.5)/0.1% Triton X-100. Sections were then quenched in 3% H_2O_2 for 10 min. After being washed, sections were blocked in 10% normal goat serum in a solution containing 1% bovine serum albumin (BSA)/0.02% Na-azide/1× Tris-NaCl buffer (pH 7.5) for 45 min. Slides were then incubated overnight at 4°C with either anti-GAD67 antibodies (1:500; Chemicon, Temecula, CA) or anti-$GAD_{65/67}$ antibody (1:800; US Biological, Swampscott, MA; detecting GAD65 predominantly as examined by immunoblotting; data not shown). After being washed, the slides were incubated with biotinylated secondary antibody (1:400; New England Biolabs, Beverly, MA) at room temperature for 30 min. The signal was detected and amplified by using the ABC peroxidase method (Vector, Burlingame, CA) and visualized with 3,3′-diaminobenzidine solution (DAB Kit; Vector). Sections were counterstained with toluidine blue (Sigma, St. Louis, MO).

TABLE I. Effects of Diets on Body and Brain Weights, Serum Glucose, and Ketone Levels†

	Ad lib-Std	CR-Std	Percentage of control	CR-Ket	Percentage of control
Body weight (g)	85.3 ± 3.0	41.7 ± 2.1★	↓ 51%	42.5 ± 3.8★	↓ 51%
Brain weight (mg)	1.4 ± 0.05	1.25 ± 0.03★	↓ 10%	1.27 ± 0.02★	↓ 10%
Glucose (mg/dl)	179 ± 10.5	127 ± 15.9★	↓ 29%	57.5 ± 7.2★★	↓ 68%
Ketones (mM)	0.27 ± 0.001	0.23 ± 0.03	No change	4.4 ± 0.67★★	↑ 1530%

†The data represent means ± SEM for six animals per group.
★$P < .01$ compared with AL-Std.
★★$P < .0001$ compared with AL-Std and CR-Std.

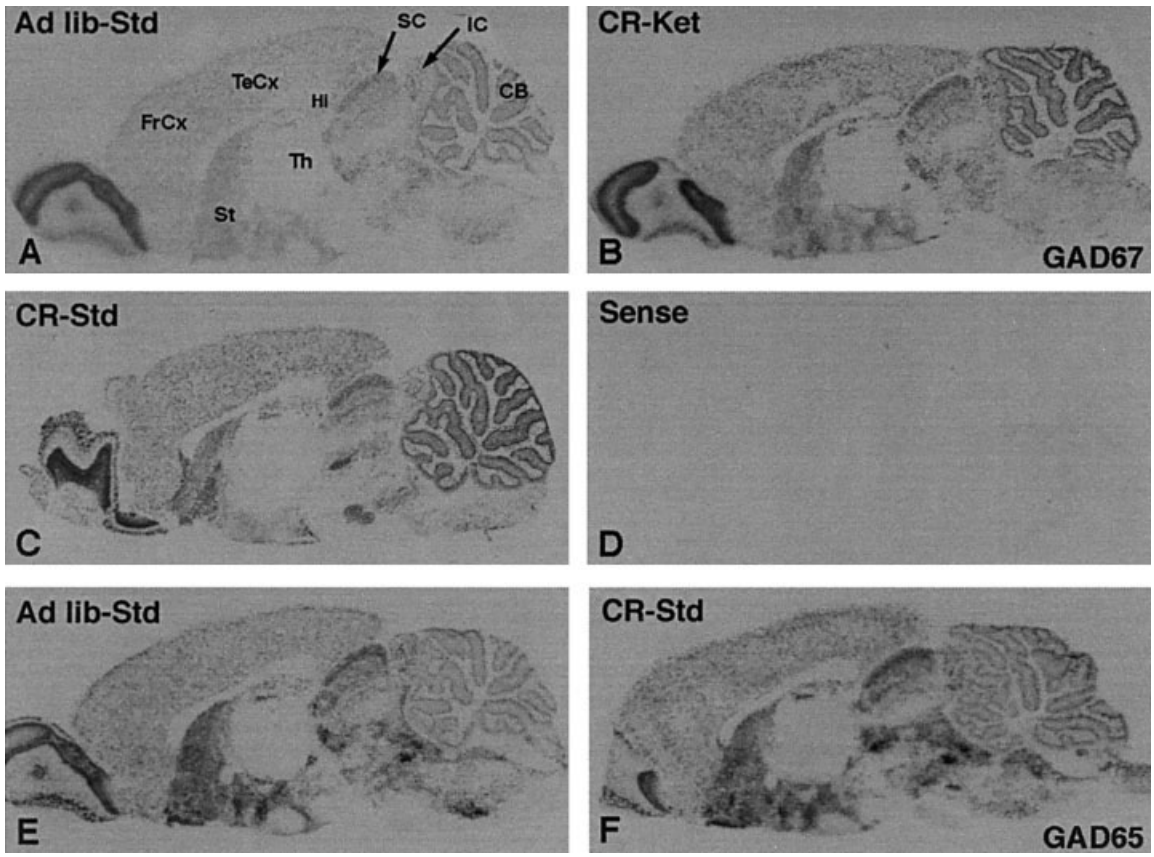

Fig. 1. Effects of dietary manipulation on GAD67 mRNA expression in the rat brain. **A–C:** Film autoradiographs of representative brain sections from animals in each of the three different diet groups hybridized to radiolabeled GAD67 cRNA probes and exposed to the same piece of film. **D:** Background signal produced by sense probe hybridization. **E,F:** Film autoradiographs showing GAD65 mRNA expression in Ad lib-Std and CR-Ket diets. CB, cerebellum; FrCx, frontal cortex; Hi, hippocampus; IC, inferior colliculus; SC, superior colliculus; St, striatum; TeCx, temporal cortex; Th, thalamus.

RESULTS

Rats fed the Ad lib-Std diet gained weight, whereas those fed either of the CR diets only maintained the weight they had at diet onset. Those fed either CR diet exhibited lower blood glucose levels than did Ad lib controls but only the CR-Ket group had elevated blood ketones (Table I). All animals maintained good coat quality, were alert and active, and engaged in vigorous "play". Both CR diet groups appeared active, well-groomed, and healthy. After 7 days on their diets, all three groups were scarified and examined for their brain GAD65 and -67 gene expression.

GAD65 and -67 mRNAs display similar expression patterns in the rat brain (Fig. 1). Hybrid signal densitometry showed that GAD67 mRNA was significantly increased in the inferior and superior colliculi and in the molecular layer of cerebellar cortex, with both CR-Ket and CR-Std diets compared with Ad lib-Std ($P = .009$, .0001, .019 respectively; Table II). Figure 2 illustrates diet-induced changes in GAD67 gene expression at the

TABLE II. Comparison of GAD67 and GAD65 mRNA Levels in Specific Brain Regions of Different Diet Groups†

Brain region	CR-Ket vs Ad lib-Std (%)	P value	CR-Std vs Ad lib-Std (%)	P value
GAD67				
Inferior colliculus	148.8 ± 17.1	.009★	141.9 ± 7.4	.021★
Superior colliculus	146.2 ± 5.0	.0001★	145.5 ± 8.8	<.0001★
Frontal cortex	119.3 ± 10.1	.331	125.6 ± 17.8	.203
Temporal cortex	127.9 ± 15.0	.177	129.0 ± 13.8	.160
Striatum	130.3 ± 8.1	.032★	112.2 ± 12.5	.358
Cerebellum	139.7 ± 12.1	.019★	171.0 ± 12.3	.0003★
GAD65				
Inferior colliculus	95.1 ± 7.0	.663	101.2 ± 13.1	.917
Superior colliculus	119.9 ± 5.9	.008★	119.2 ± 4.0	.014★
Frontal cortex	100.1 ± 11.3	.992	112.9 ± 4.3	.332
Temporal cortex	146.3 ± 19.0	.038★	151.7 ± 15.8	.029★
Striatum	92.2 ± 5.7	.357	89.0 ± 7.6	.220
Cerebellum	82.9 ± 7.2	.209	100.8 ± 11.7	.951

†Data are expressed as percentage of Ad lib-Std control (N = 6 per group). The quantification of hybridization signal was detailed in *Materials and Methods*.
★Statistically significant difference was obtained by comparing different diet to Ad Lib-Std control by ANOVA, followed by Fisher's least significant difference tests.

cellular level. The magnitude of the effect of each CR diet was similar in superior and inferior colliculi, but the increase in cerebellar GAD67 mRNA was substantially greater in response to the CR-Std diet (171%; $P = .0003$) compared with the CR-Ket diet (140%, $P = .019$) with Ad lib-Std control used as a reference point (Table II). Cerebral cortical GAD67 mRNA levels were increased in both CR diet groups, but the effects did not attain statistical significance (Table II). Finally, GAD67 mRNA was significantly increased in the striatum in response to the CR-Ket diet but was not altered in this region by the CR-Std diet.

GAD65 mRNA displayed a somewhat different response to diet modifications compared with GAD67 (Fig. 1). GAD65 mRNA was not altered in the inferior colliculus but was significantly increased in the superior colliculus and temporal cortex by both CR diets (Table II). There was no significant diet-induced change in GAD65 mRNA levels in the frontal cortex, striatum, or cerebellum.

GAD67 immunoreactivity was increased in parallel with the in situ hybridization results (Fig. 2), with selectively increased GAD67 expression noted in cerebellar cortex (Fig. 2A,B,E,F) and superior (Fig. 2C,D) and inferior (Fig. 2G,H) colliculi in the CR-Std (shown in Fig. 2) and CR-Ket (not shown) groups. Similarly, GAD65 immunoreactivity was increased in the superior colliculus and temporal cortex (data not shown).

DISCUSSION

This study shows that a brief, 7-day exposure to a modest calorie restriction enhances GAD mRNA and protein levels in several brain regions, including superior and inferior colliculi, cerebellar cortex, and temporal cortex. It is worth mentioning that these changes in brain GAD67 mRNA levels were also observed on hybridization of samples from the different diet groups to Affymetrix Rat Neurobiology U34 DNA arrays (our unpublished data). Neither ketosis nor dietary fat nor carbohydrate per se seems to play a crucial role in the regulation of brain GAD expression in these regions, in that the effects were remarkably similar in both the CR-Std and the CR-Ket diet groups. The fact that all diet groups maintained a healthy appearance and were alert and spontaneously active suggests that the changes in brain gene expression were not due to malnutrition or stress. Both CR groups had lower blood glucose levels than the ad lib controls, so relative hypoglycemia might contribute to enhanced GAD expression. An alternative way of looking at this paradigm is that animals on the ad lib diet are actually overfed and hyperglycemic, resulting in suppression of brain GAD expression. CR had differential effects on the two GAD isoforms, with GAD67 affected more prominently, being increased by ~50% or more in the inferior and superior colliculi and in the cerebellar cortex, whereas GAD65 was increased by lesser amounts and only in the superior colliculus and temporal cortex. The only effect that appeared specific for the ketogenic diet was a selective increase in GAD67 in the striatum.

The significance of these differential changes is unknown. GAD65 and GAD67 are the products of two independently regulated genes (Erlander et al., 1991; Szabo et al., 1996; Pinal et al., 1997; Yanagawa et al., 1997). The enzymes differ in sequence, molecular weight, and interaction with the cofactor pyridoxal 5′-phosphate. Both are present in most GABA-containing neurons, but GAD65 is more concentrated in nerve endings, whereas GAD67 is more widely distributed in somata and dendrites and appears to be responsible for most brain GABA synthesis (Soghomonian and Martin, 1998). In GAD67 null mice, brain GAD65 levels remain normal, but GABA levels are markedly reduced (Asada et al., 1997). On the other hand, GAD65 null mice apparently have normal brain GABA levels and exhibit only slight increases in seizure susceptibility (Asada et al., 1996; Kash et al., 1997).

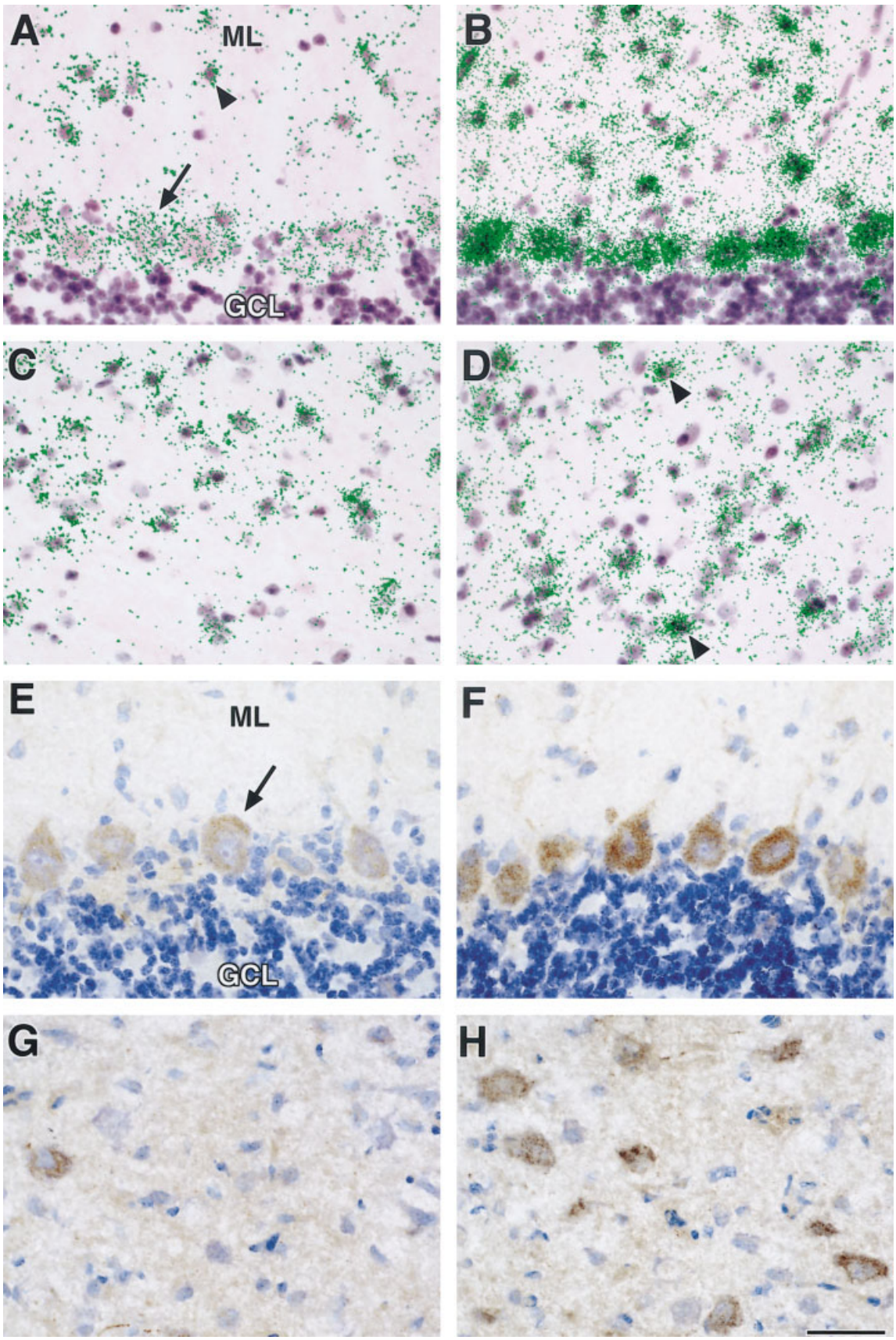

Figure 2.

We have demonstrated previously that both the CR-Std and the CR-Ket diets delay seizure onset (Bough et al., 1999). Recently it has been shown that the CR-Std diet by itself is anticonvulsant (Bough et al., 2003), and mild to moderate CR alone could significantly reduce seizure susceptibility in the EL mouse, a genetic model of idiopathic epilepsy (Greene et al., 2001). Greene et al. (2001) have shown that CR alone reduces blood glucose levels, as seen here in rats fed the CR-Ket diet, and they suggest an important role for reduced blood glucose in seizure incidence. Our data suggest that CR may exert anticonvulsant effects at least in part by increasing GAD and thus GABA synthesis, a finding consistent with reports by Yudkoff et al. (2001a,b) showing enhanced conversion of glutamate to GABA. Insofar as the actions of GABA are generally inhibitory, this may be a mechanism by which CR and/or high-fat diets elevate seizure threshold. This regulatory effect seems to be independent of macronutrient composition in the diet; the carbohydrate-rich and fat-rich diets show similar efficacy in modulating GAD gene expression. However, in considering the fact that the ketogenic diet confers a greater seizure resistance (Bough et al., 1999, 2000, 2003), it appears that other mechanisms in addition to GABA stimulation are involved in the ketogenic diet's salutary effects. We have previously shown, in support of that view, distinctly divergent effects of the CR-Std and CR-Ket diets on brain IGF system and glucose transporter expression. For example, IGF receptor and GLUT3 expression were significantly *reduced* by the CR-Std diet but significantly *increased* with the CR-Ket (Cheng et al., 2003), suggesting that enhanced IGF1 receptor and glucose transporter expression could be instrumental in differential ketogenic diet-induced seizure suppression.

Recent clinical studies suggest that neurological disorders aside from epilepsy may be associated with decreased GAD levels and GABA synthesis. For example, GAD67 is reportedly decreased in the brains of patients with schizophrenia (Hashimoto et al., 2003), autism (Fatemi et al., 2002), and Parkinson's disease (Nisbet et al., 1996). Moreover, caloric restriction appears to be beneficial for brain function and disease prevention in both animal models and humans (for review see Mattson, 2003). These observations together with the present findings suggest that diet modification may be a useful approach for prevention and/or treatment of brain disorders in addition to epilepsy.

Fig. 2. Effects of calorie restriction on neuronal GAD67 mRNA (**A–D**) and protein (**E–H**) expression levels. Panels at left (A,C,E,G) are representative micrographs from AL-Std subjects; panels at right (B,D,F,H) are from CR-Std diet subject. A,B: Representative darkfield micrographs revealing GAD67 mRNA (green dots represent oxidized silver grains) localized in Purkinje cells (arrows) and molecular layer (ML) interneurons (arrowheads) of the cerebellar cortex. C,D: GAD67 mRNA concentrated in interneurons of the superior colliculus. E,F: GAD67 immunoreactivity detected in the cerebellar cortex. Prominent cytoplasmic staining of Purkinje somata is noted. G,H: GAD67 immunoreactivity was detected in the inferior colliculus. Scale bar = 40 μm.

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