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Research report

Glucocorticoids exacerbate insult-induced declines in metabolism in selectively vulnerable hippocampal cell fields

Anna Yusim, Olu Ajilore, Tonya Bliss, Robert Sapolsky*

Department of Biological Sciences, Stanford University, Stanford, CA 94305, USA

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Abstract

Glucocorticoids (GCs), the adrenal steroids released during stress, can compromise the ability of hippocampal neurons to survive necrotic neurological insults. This GC-induced endangerment has energetic facets, in that it can be attenuated with energy supplementation. In the present report, we studied the effects of GCs on the metabolic response of specific hippocampal cell fields to necrotic insults. We used silicon microphysiometry, which allows indirect measurement of metabolism in real time in tissue explants. Aglycemia caused a significant decline in metabolism in dentate gyrus explants, but not in CA1 or CA3 explants. When coupled with our prior report of cyanide disrupting metabolism only in CA1 explants, and the glutamatergic excitotoxin kainic acid disrupting metabolism only in CA3 explants, this demonstrates that microphysiometry can detect the selective regional vulnerability that characterizes the hippocampal response to these necrotic insults. We then examined the effects of GCs on the response to these insults, monitoring explants taken from rats that were adrenalectomized, intact, or treated with corticosterone (the GC of rats) that produced circulating levels equivalent to those of major stressors. Increased exposure to GCs worsened the decline in metabolism in dentate gyrus explants induced by hypoglycemia, and in CA1 explants induced by cyanide (after eliminating the effects of glial release of lactate for the support of neuronal metabolism). Thus, GCs worsen the metabolic consequences of necrotic insults in hippocampal explants.

Theme: Disorders of the nervous system

Topic: Neurotoxicity

Keywords: Glucocorticoids; Stress; Hippocampus; Selective Vulnerability; Metabolism

1. Introduction

Glucocorticoids (GCs), the adrenal steroids released during stress, can have adverse effects in the nervous system if secreted or administered in excess. Exposure to excessive GCs or to stress for hours to days compromises the ability of neurons to survive in vitro and in vivo models of necrotic insults (i.e. hypoxia–ischemia, excitotoxicity and energy deprivation). These ''endangering'' GC effects are most pronounced in the hippocampus, with its high concentrations of corticosteroid receptors and sensitivity to GCs (reviewed in [32]).

A central dogma exists regarding the mechanisms by which such necrotic insults are damaging. This involves the excessive accumulation of synaptic excitatory amino acids (EAA) neurotransmitters, the resulting excessive post-synaptic mobilization of free cytosolic calcium, and the activation of calcium-dependent oxygen radical formation and cytoskeletal degradation [15]. GCs worsen this degenerative cascade in an energy-dependent manner. The steroid accelerates the decline in ATP concentrations in hippocampal cells during insults [14,38]. This, in turn, compromises the costly task of containing EAA and calcium fluxes during these insults. Thus, GCs exacerbate the accumulation of extracellular EAAs, of cytosolic calcium, of oxygen radicals and of cytoskeletal degradation during these insults. Moreover, these GC effects can be blunted by supplementation with excess energy, evidence of the energetic nature of these GC actions [5–7,17,18,22–24,35–37]. As one possible cause of the

*Corresponding author. Tel.: +1-650-723-2649; fax: +1-650-725-5356.

E-mail address: sapolsky@leland.stanford.edu (R. Sapolsky)

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effects of GCs upon hippocampal energetics, the steroid can inhibit glucose uptake and utilization [4,8–10,41].

The effects of GCs upon hippocampal ATP concentrations during insults suggests that the steroid should exacerbate the effects of insults upon hippocampal metabolism as well. Silicon microphysiometry can measure extracellular acidification rates in real time. Since the products of cellular metabolism include lactic acid and carbon dioxide, the amount of metabolites extruded extracellularly can serve as an indirect measure of cellular metabolism [21]. We originally adapted microphysiometry for use in neuronal monolayer cultures [28,39] and, more recently, for use with explants from specific hippocampal cell fields from adult brain [1]. We now use microphysiometry to demonstrate the ability of GCs to worsen the disruptive metabolic effects of necrotic insults in specific hippocampal cell fields.

2. Materials and methods

2.1. Buffers/reagents

Dulbecco's modified Eagle's medium (DMEM: Gibco, Grand Island, NY, USA) was used for hippocampal dissection and microphysiometric procedures in the aglycemia and hypoglycemia experiments.

Earle's balanced salt solution without bicarbonate (EBSS; Gibco, Grand Island, NY, USA) was used for experiments with metabolic toxins. While the buffering capacity of DMEM was reduced by the substitution of 2.5 g of NaCl for every 3.7 g of $NaHCO_3$, EBSS' buffering capacity was reduced by omission of bicarbonate. While some buffering capacity remained due to the presence of 1 mM sodium phosphate monobasic, sufficient detection of pH changes due to cellular acid excretion is possible [21]. Because microphysiometry measures rates of proton extrusion, rather than absolute amount of protons in the extracellular medium, any effects of the low buffering capacity were controlled for by expressing data within explant as a function of baseline metabolic rates.

To induce hypoglycemia, the glucose concentration of the DMEM media was reduced to 1 mM from the normal 5 mM concentration. Glucose was eliminated from the DMEM in the aglycemic study. Sodium pyruvate (Gibco; 5 mM), sodium cyanide (Gibco; 4–6 mM, as indicated), and the lactate dehydrogenase inhibitor deoxycholic acid (Sigma; 2.5 g/l) were all dissolved in EBSS [39].

Corticosterone, (CORT) the predominant rat GC, was obtained from Sigma (St. Louis, MO, USA).

2.2. Animal procedure

Male Sprague–Dawley rats (Simonsen; 250–300 g) were divided into three groups: (a) adrenalectomized (and maintained on 0.9% NaCl); (b) adrenally intact; (c) intact and injected daily with 10 mg CORT (s.c., in 1 ml peanut oil); this produces sustained circulating CORT concentrations in the range seen for major stressors (20–35 μg/dl; 31). Three days later, rats were anesthesized (with 10 ml urethane/kg bw) and decapitated. Brains were rapidly removed, divided into cerebral hemispheres, and sliced into 400 micrometer thick sections. The hippocampus was isolated from a single tissue slice, and one millimeter square explants were removed from the CA1, CA3, dentate gyrus regions (Fig. 1). Explants from the desired hippocampal region from 2 to 3 sequential coronal brain slices were often combined to obtain sufficient tissue.

2.3. Microphysiometry

The microphysiometer measures the acidification rate in cells in real time which is an indicator of metabolic function; because this remains a relatively new technique, especially for use with tissue explants, it will be described in some detail. Since major products of cellular metabolism can include lactic acid and carbon dioxide, a higher rate of metabolism results in a higher rate of proton extrusion [21]. The microphysiometer also provides the advantage over conventional pH-sensitive electrodes. While capable of measuring pH, micropipette pH electrode alone would not be sufficient for the reliable measurement of the acidification rate ($d(\mathrm{pH})/dt$). The latter is more directly indicative of metabolic activity, as opposed to pH which is more related to other homeostatic functions; adapting a pH meter with a microfluidics system to accurately measure the rate of change of pH, in effect, constitutes a microphysiometer.

As originally designed, the microphysiometer indirectly measured metabolic rate in monolayer cultures. When adapted for use with explants [1], the explant of tissue, sandwiched between two porous membranes to maintain its position, is placed in a square depression at the bottom

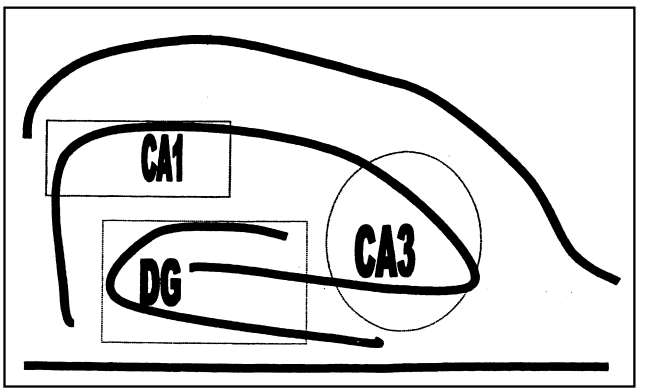

Fig. 1. Dotted lines indicate the approximate areas microdissected for use with the microphysiometer.

of a microphysiometer plunger specifically designed for tissue slices. A plastic spacer at the bottom of the plunger stabilizes the membranes to ensure that media flows directly onto the tissue slice for adequate perfusion. The assembled plunger is placed in the sensor chamber of the microphysiometer where proton efflux is measured. A debubbler–degasser membrane maintains constant temperature and oxygen content within the chamber throughout the experiment, since even small changes in these parameters may affect the microphysiometer's sensitivity. Previous studies have provided a more detailed description of the microphysiometer [1,21,28,39].

Media is pumped from a fluid supply for 60 s into the sensor chamber containing the tissue. Media flow is then halted for 60 more seconds, during which time protons accumulate in the extracellular environment. Change in pH (in microvolts) over time (in seconds) is measured by recording the rate at which protons accumulated in the sensor chamber during this static period. Since the process of ATP production is tightly coupled with cellular ATP consumption and therefore with the rate of proton extrusion from the cell, acidification rate measurements serve as indirect indicators of a tissue's metabolic rate. In the present study, explants were first allowed to equilibrate until readings had stabilized; baseline rates of metabolism were then established by averaging the acidification rates for 20 min prior to switching the fluid supply from the control media to the indicated insult. The switch from the control media to another test tube containing the same control media was defined as a sham switch, while switching from the control to a fluid supply containing the glucose-free media or media containing the indicated toxins was considered the experimental exposure. Each tissue slice was exposed, in sequence, to 2 h of control, 1 h of sham, 1 h of control and 2 h of the experimental media. Explants from adrenalectomized animals were exposed to CORT-free media throughout; explants from intact animals were exposed to media containing 10 nM CORT; explants from intact animals that had been supplemented with CORT were exposed to media containing 1 μM CORT.

The particular model of microphysiometer used in this study allows for four explants to be studied simultaneously. Explants from different treatment groups would be run simulataneously.

2.4. Statistical analysis

Data are presented as comparisons of percent metabolic decrease between sham and exposure to experimental media for each treatment (CORT-treated, untreated, adrenalectomized) and each brain region studied; this is because of the somewhat arbitrary quantitative end points generated by microphysiometry. Data are represented as mean±S.E.M; in figures in which there are no obvious error bars, variability was too small to be graphed. Statistical significance was analyzed using ANOVA and Bonferroni post-hoc tests.

3. Results

''Basal'' metabolic rates (i.e. the final twenty min during which rates had stabilized prior to the experimental manipulation) did not differ significantly by hippocampal cell field or by GC-status (data not shown).

We previously demonstrated that microphysiometry with adult hippocampal cell field explants could replicate features of the selective vulnerability that characterizes the hippocampus in vivo [1]. Specifically, we observed that exposure to cyanide significantly altered metabolic rate in CA1 but not in CA3 or dentate gyrus (thereby modeling a feature of the preferential vulnerability of CA1 to global ischemia in vivo). We also observed that exposure to the EAA kainic acid significantly altered metabolic rate only in CA3 (modeling that cell field's preferential vulnerability to excitotoxic seizures) [1]. We completed this demonstration of selective vulnerability, observing that 2 h of aglycemia caused a significant decline in metabolism in dentate gyrus explants, but not in those from CA1 or CA3 (Fig. 2).

Having established models of selective vulnerability with this system, we then examined the effects of GCs upon them. Aglycemia caused a significant decline in metabolism in dentate gyrus explants from intact animals (Fig. 3A). Glucocorticoid status (i.e. either adrenalectomy, or exposure to high CORT concentrations) had no effect on this decline. We reasoned that this represented too severe of an insult to be modulated by GCs (especially if GCs are having their adverse effects, in part, by decreasing glucose transport, an effect that would be irrelevant in an aglycemic environment). Therefore, we made dentate gyrus explants relatively hypoglycemic, by lowering glucose concentrations from 5 to 1 mM. Under these conditions, metabolic rate still showed a small but significant decline in explants from intact animals (Fig. 3B). Glucocorticoid status significantly modulated this decline in metabolism, in that adrenalectomy blunted the decline, whereas CORT treatment worsened it.

We then examined the effects of GC status on the response to metabolic poisons in CA1. In adrenally-intact rats, 4 mM, but not 6 mM cyanide caused a transient increase in metabolism, in agreement with our prior report [1]; at both concentrations, there was ultimately a significant decline in metabolism over the course of the 2 h (Fig. 4). Counter to predictions, increasing GC exposure protected explants from this decline in metabolism, rather than worsened it, whereas adrenalectomy significantly worsened the decline.

Cyanide, by inhibiting oxidative phosphorylation, is likely to cause two compensations which will influence the rate of proton efflux into the extracellular space (to be

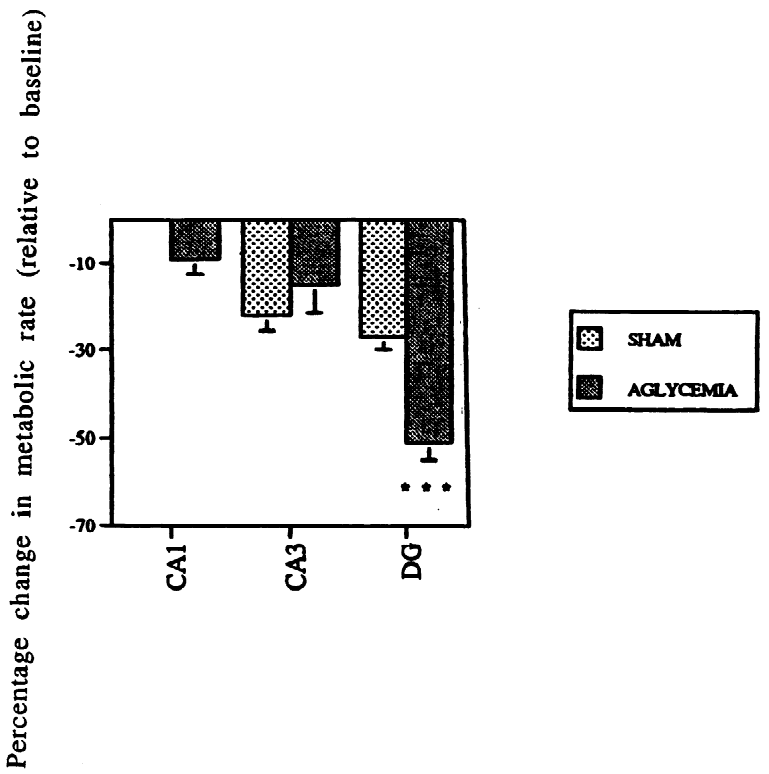

Fig. 2. Change in metabolic rate (as a percentage of baseline) in explants from hippocampal cell fields exposed to either aglycemic or normoglycemic (sham) conditions for 2 h. ***$P<0.001$, as compared to normoglycemic dentate explants (post-hoc test following one-way ANOVA). $n=11$. Change in metabolic rate in sham CA1 explants was 0%, with an error bar that was too small to be graphed. In this and subsequent figures, absolute values for controls ranged from −20 to −60 μV/s. 1 μV/s=approximately 1 millipH/min.

considered at length in the Discussion). The first is a shift to glycolysis and, with it, potentially a six-fold increase in the rate of proton efflux. The second is an extracellular accumulation of lactate released by glia [19,20]. We attempted to eliminate glycolysis by studying GC/metabolic insult interactions in CA1 explants in which we combined treatment with cyanide with substitution of glucose with pyruvate (Fig. 5). At both cyanide concentrations, this caused an even greater transient increase in metabolic rate in intact rats, followed by a decline back to baseline. Again, increasing GC exposure protected explants from this decline in metabolism, rather than worsened it.

We then attempted to eliminate both of the potential confounds in measuring proton efflux rate. Thus, we studied GC/metabolic insult interactions in CA1 explants exposed to cyanide, pyruvate (to eliminate glycolysis), and the glial LDH inhibitor sodium deoxycholate (to eliminate the glial release of lactate; [16]) (Fig. 6). Under these conditions, there was a highly significant decline in metabolism in tissue from intact rats. In this case, explants from CORT-treated animals had an accelerated decline, in that the drop in such explants was significantly greater than the other two groups during the 0–30 min interval ($P<$ 0.01 as compared to either ADX or NORM, by Newman–Keuls post-hoc test).

4. Discussion

As reviewed, GCs can compromise the ability of hippocampal neurons to survive necrotic insults. While these endangering GC actions arise in part from some non-energetic effects (reviewed in [3]), disruptive GC effects on metabolism appear contributory as well. As evidence, (a) GCs inhibit glucose transport and utilization and accelerate the decline in ATP concentrations during hippocampal insults [4,8–10,14,38,41]; (b) energy supplementation lessens the exacerbative effects of GCs upon glutamate, calcium, and calcium-dependent degenerative events during insults (reviewed in [32]); (c) overexpression of the Glut-1 glucose transporter in neurons decreases the toxicity of these insults and blunts the glutamate, calcium, and calcium-dependent degenerative events [2,12,13]. In the present report, we used silicon microphysiometry to show that GCs exacerbate the disruptive effects of necrotic insults on metabolism in explants from adult hippocampal cell fields.

Before discussing the GC effects, we first consider microphysiometric findings themselves. The rationale for studying explants of individual hippocampal cell fields, rather than from fetal hippocampal monolayer cultures, was not only the ability to manipulate GC levels physiologically in the adult source of the tissue, but to more

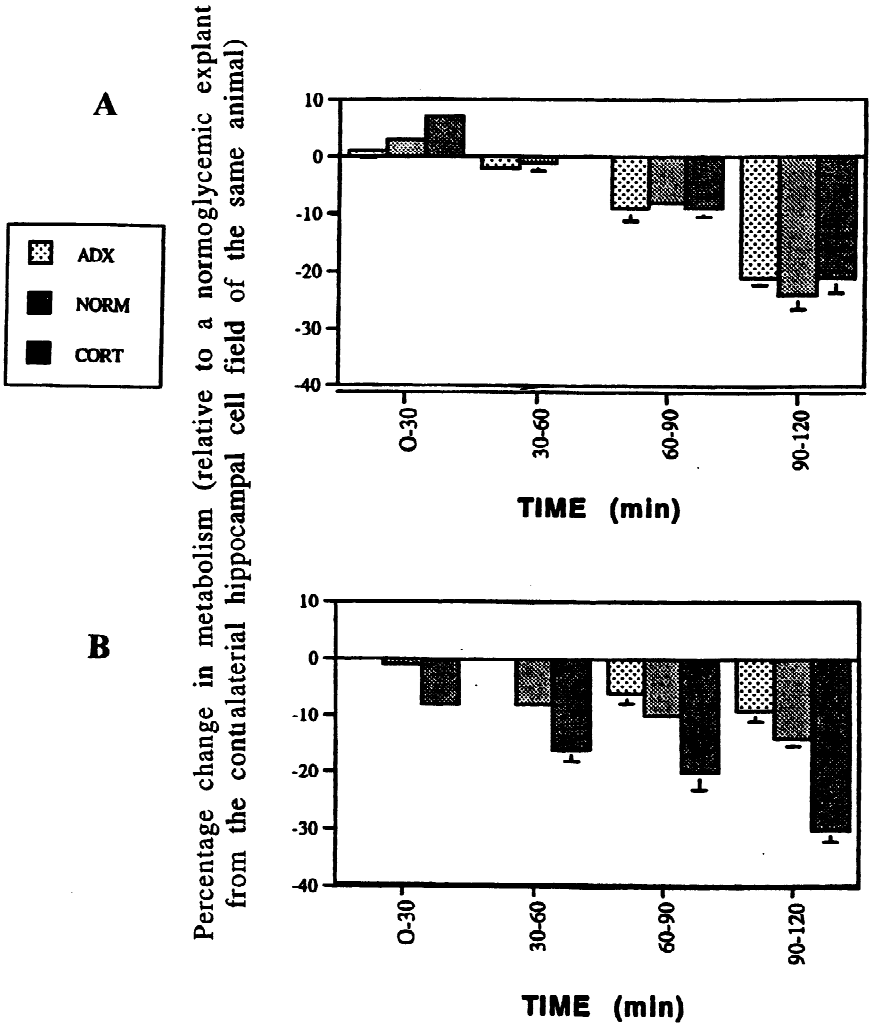

Fig. 3. (A) Effects of GCs on metabolic rate in dentate gyrus in response to aglycemia. Aglycemia itself caused a highly significant decline in metabolism over time ($P<0.01$ by two-way ANOVA) which was not effected by GC status. (B) Effects of GCs on metabolic rate in response to hypoglycemia. Metabolic rate declined significantly over time, independent of GC status ($P<0.01$, two-way ANOVA). Adrenalectomy lessened the response to hypoglycemia when compared to intact rats ($P<0.03$, two-way ANOVA), while CORT treatment worsened it, when compared to intact rats ($P<0.01$, two-way ANOVA). $n=4$–5. ADX = adrenalectomized; NORM = intact; CORT = corticosterone-treated.

closely model the necrotic insults themselves. A defining feature of necrotic injury to the hippocampus is selective vulnerability, i.e. the preferential vulnerability of CA1 to hypoxic-ischemic injury, of CA3 to excitotoxins, and of dentate gyrus to hypoglycemia. Our prior microphysiometry paper [1], plus the present data (Fig. 2) replicate elements of such selective vulnerability, in that of the cell fields examined, CA1 explants were the most metabolically vulnerable of them to cyanide, CA3 explants, to an excitotoxin, and dentate explants to aglycemia.

It is also important to discuss the complex CA1 data. Inhibition of oxidative phosphorylation with cyanide will likely effect proton extrusion in three ways: (a) a *decrease* in cellular metabolism and thus in extrusion of protons; (b) a compensatory increase in glycolysis. Glycolysis yields far less ATP per substrate molecule than does oxidative phosphorylation. Thus, either complete or partial glycolytic compensation would produce more protons per ATP molecule [21], and thus an *increase* in proton extrusion (of note, the same increase would not occur during hypo- or aglycemia). (c) Increased glial lactate release, for the purpose of bolstering neuronal energetics [19,20]. Such lactate release occurs following other necrotic insults as well, such as exposure to excitotoxins [27,34]. However, cyanide, by inhibiting oxidative phosphorylation, prevents further metabolism of lactate, causing equilibrium of lactate uptake. Lactate released by glia will accumulate extracellularly, and will be detected as an *increase* in proton extrusion.

Thus, the signal detected microphysiometrically in response to cyanide represents a combination of the insult and compensations to it; at the higher cyanide concentration this results in a transient increase in proton extru-

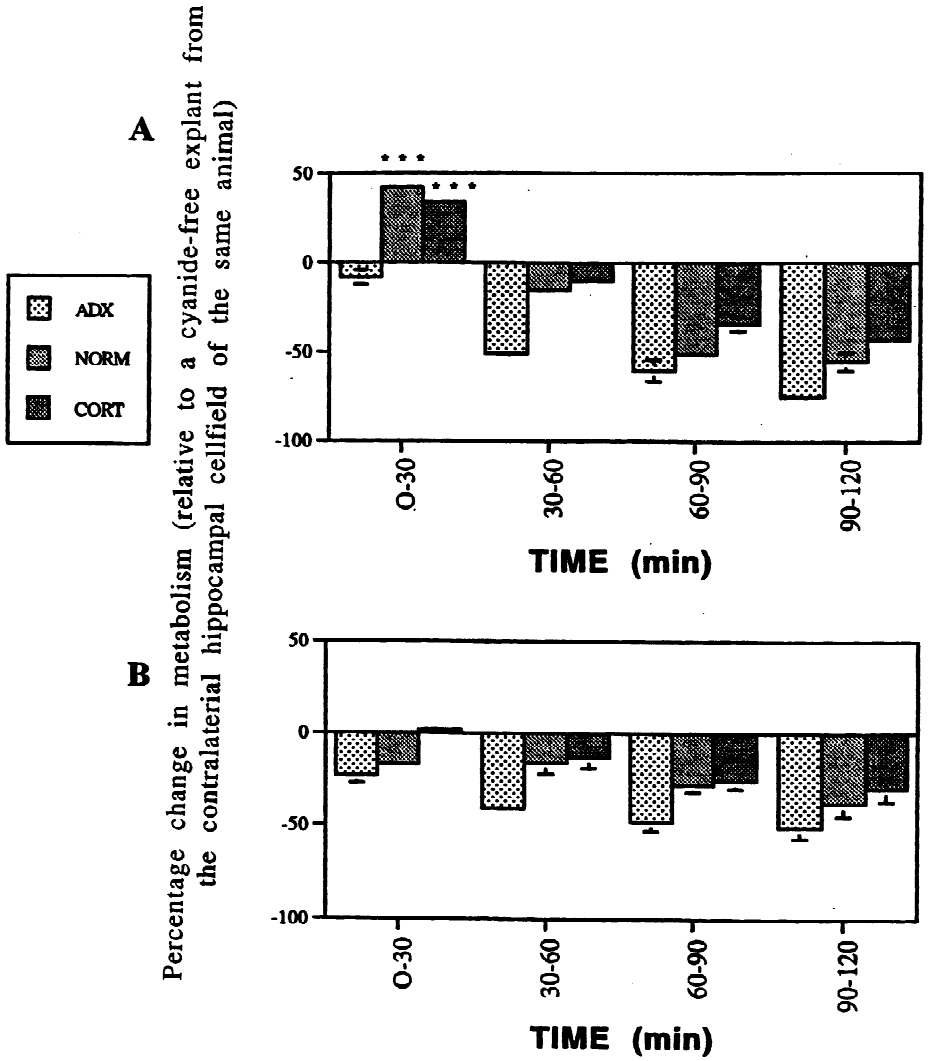

Fig. 4. Effects of GCs on metabolic rate in CA1 in response to 4 mM cyanide (A) or 6 mM cyanide (B). At both concentrations, metabolic rate, independent of steroid status, declined significantly over time ($P<0.01$ for both concentrations, by two-way ANOVA). At 4 mM cyanide, there was a transient increase in metabolism in intact and CORT-treated rats during the first 0–30 min (when compared with metabolic rates in ''control'' tissue [i.e. a cyanide-free explant from the contralateral cell field of the same animal]; ***$P<0.01$, by unpaired t-test). Adrenalectomy exacerbated the overall decline in metabolism in response to cyanide at both concentrations when compared to intact rats ($P<0.01$, 0.03 for 4 and 6 mM cyanide, respectively, by two-way ANOVA), while CORT treatment significantly lessened the decline at both concentrations when compared to intact rats ($P<0.01$ at both concentrations, by two-way ANOVA). $n=5$/group ADX = adrenalectomized; NORM = intact; CORT = corticosterone-treated.

sion (Fig. 4A; also see [1]). A weakness of microphysiometry is that it is not possible to resolve those opposing factors with the singular endpoint of proton efflux [39].

Therefore, we attempted to eliminate those confounds by treatment with cyanide, pyruvate and sodium deoxycholate. This final compound inhibits LDH-5 preferentially [16] and is thus likely to have its strongest effects in astrocytes [40]. Treatment with cyanide, pyruvate and this inhibitor eliminated the transient rise in proton extrusion, suggesting that it arose because of extracellular accumulation of lactate following cyanide exposure. The fact that the transient rise was potentiated by the addition of pyruvate (Fig. 5) suggests that pyruvate fuels glial lactate production and release.

We now discuss the GC findings. GC-status had no effect on metabolic rate in the absence of an insult in any cell field (data not shown). This agrees with the fact that the inhibition of glucose uptake by GCs in hippocampal cells is mild (on the order of 25%, in comparison to the 80% inhibition of uptake by GCs in adipocytes) [26], and that GCs do not alter basal ATP levels in cultured hippocampal cells [14,38].

However, GCs exacerbated the decline in metabolism during insults. Adrenalectomy significantly blunted the metabolic decline in hypoglycemic dentate gyrus explants, whereas CORT treatment worsened it, ultimately, to the range of explants from intact animals made aglycemic. The CORT treatment produces prolonged circulating GC concentrations in the range seen with major stressors (20–35

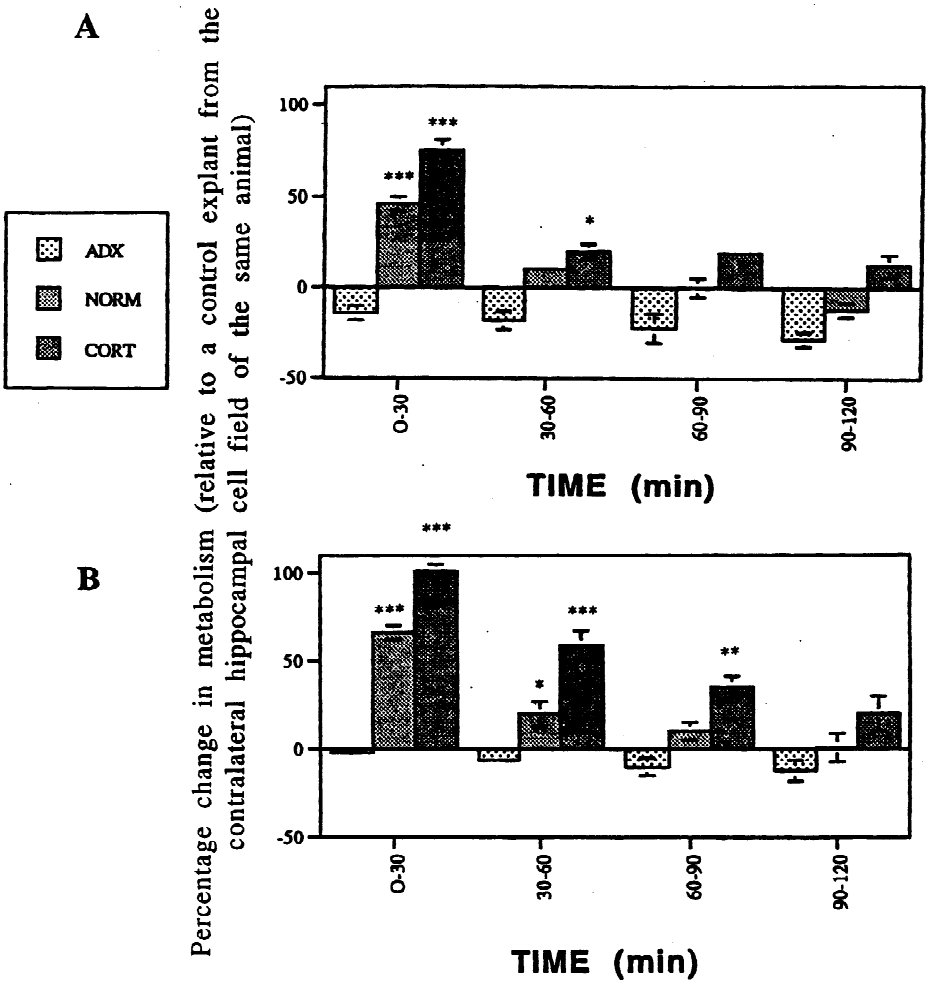

Fig. 5. Effects of GCs on metabolic rate in CA1 in response to the substitution of pyruvate for glucose, plus either 4 mM cyanide (A) or 6 mM cyanide (B). At both cyanide doses, there was a transient increase in metabolism in intact and corticosterone-treated rats (when compared with metabolic rates in ''control'' tissue [i.e., an explant from the contralateral cell field of the same animal, left in normoglycemic media without cyanide]; *, **, ***$P<0.05$, 0.01, 0.001, respectively, by unpaired *t*-test). Adrenalectomy caused a significant decline in metabolism at both cyanide concentrations when compared to intact rats ($P<0.03$, 0.01 for 4 and 6 mM cyanide, respectively, by two-way ANOVA), while CORT treatment significantly prolonged the increase in metabolism at both concentrations, when compared to intact rats ($P<0.05$, 0.03, respectively, by two-way ANOVA). $n=4–5$/group.

μg/dl; [31]), which heavily occupies the low-affinity GR receptor (of which there are ample amounts in all hippocampal cell fields [29]). Consonant with this, GC inhibition of glucose uptake is GR-mediated [9]. By worsening the metabolic decline following hypoglycemia, GCs should leave neurons less capable of containing the neurotransmitter and ionic consequences of the insult, and should, as reported [30] augment the toxicity of metabolic insults to dentate neurons.

The effects of GCs in CA1 are more complex. In explants treated with cyanide, or with cyanide and pyruvate, increasing GC exposure attenuated the decline in metabolism, counter to predictions. This is explained by the confounds discussed concerning cyanide effects on metabolic rates, on compensatory shifts to glycolysis, and on extracellular accumulation of lactate. In Figs. 4 and 5, it is impossible to tell whether GCs altered features of the insult or the compensations. With the interpretively clearer case of cyanide, pyruvate plus LDH inhibition, increased GC exposure worsened the initial decline in metabolism. Insofar as cyanide, pyruvate plus LDH inhibition provides the clearest means for monitoring the microphysiometric consequences of cyanide treatment, these data suggest that by worsening the metabolic decline in CA1, GCs compromise the ability of neurons there to survive a necrotic insult. In agreement, GCs worsen hypoxic–ischemic CA1 injury [11,33,25].

Finally, GCs augment the transiently excitatory effects on metabolism of kainic acid in CA3 explants [28]. This EAA causes release of presynaptic glutamate and mobilization of post-synaptic calcium, both of which are worsened by GCs (reviewed in [32]). Whether kainic acid ultimately caused a significant decline in metabolism (and whether that was worsened by GCs) was not tested in that study, although it would be expected.

In conclusion, the adverse, endangering effects of GCs

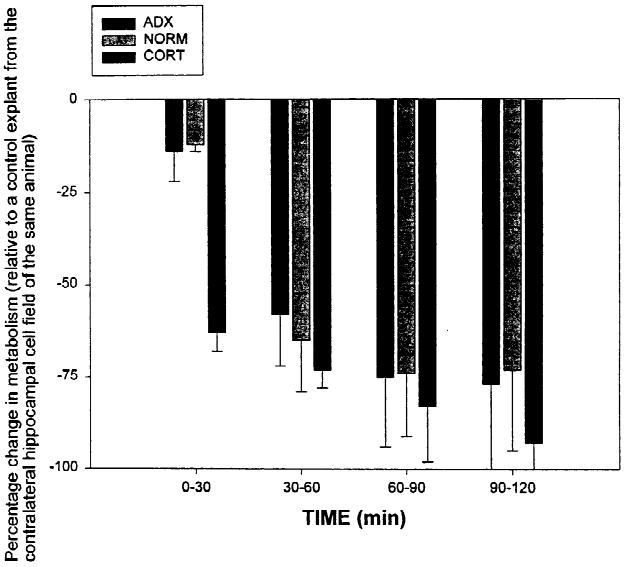

Fig. 6. Effects of GCs on metabolic rate in CA1 in response to 4 mM cyanide, substitution of pyruvate for glucose, plus the LDH inhibitor sodium deoxycholate. There was a significant decline in metabolism in intact rats over time (when compared with metabolic rates in ''control'' tissue [i.e. an explant from the contralateral cell field of the same animal, left in normoglycemic media without cyanide or sodium deoxycholate] $P<0.001$ by one-way ANOVA). Adrenalectomized rats did not differ from intact animals, whereas corticosterone treatment significantly accelerated the decline, when compared to intact rats ($P<0.05$, two-way ANOVA). $n=3$/group.

contribute significantly to the extent of hippocampal neuron loss after various necrotic insults. The present findings strengthen the notion that some of this endangerment revolves around the disruptive energetic effects of such steroids.

Acknowledgements

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