

# The Corticosterone Synthesis Inhibitor Metyrapone Prevents Hypoxia/Ischemia-Induced Loss of Synaptic Function in the Rat Hippocampus

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**Background and Purpose**—Ischemia is accompanied by abundant corticosterone secretion, which could potentially exacerbate brain damage via activation of glucocorticoid receptors. We addressed whether manipulating steroid levels during ischemia affects hippocampal synaptic function along with neuronal structure. Moreover, we established whether pretreatment with the glucocorticoid receptor antagonist RU38486 is as effective in preventing deleterious effects after ischemia as is the steroid synthesis inhibitor metyrapone.

**Methods**—Rats underwent 20 minutes of unilateral hypoxia/ischemia (HI). Convulsions were monitored after HI, and 24 hours later, field potentials were recorded in vitro in the hippocampal CA1 area in response to stimulation of the Schaffer collateral/commissural fibers. Morphological alterations were determined in brain slices from the same animals. Data were correlated with steroid treatment before HI.

**Results**—Metyrapone suppressed plasma corticosteroid levels during HI, whereas corticosterone treatment significantly elevated plasma steroid levels. These treatments affected the incidence of visible seizures after HI: corticosterone treatment resulted in the highest incidence, whereas metyrapone attenuated the occurrence of seizures. Moreover, the HI-induced impairment in synaptic transmission in the CA1 area in vitro was exacerbated by concomitant corticosteroid treatment and alleviated by pretreatment with metyrapone. In parallel, degenerative changes in the hippocampus after HI were most pronounced after corticosterone treatment, whereas metyrapone reduced these alterations. RU38486 was effective only in reducing the incidence of seizures shortly after ischemia.

**Conclusions**—We tentatively conclude that synaptic function along with cellular integrity is preserved after HI by preventing the ischemia-evoked rise in corticosteroid levels rather than blocking the glucocorticoid receptor. (*Stroke*. 2000;31:1162-1172.)

**Key Words:** cerebral ischemia ■ evoked potentials ■ hippocampus ■ hormones ■ rats

Corticosteroid hormones are secreted from the adrenal glands, enter the brain, and bind to intracellular receptors: the mineralocorticoid receptor (MR) with high affinity for corticosterone, and the lower-affinity glucocorticoid receptor (GR). Both receptors are present in large amounts in the rat hippocampus, which is a principal target for corticosteroid hormones.<sup>1,2</sup> Considerable evidence indicates that glucocorticoids increase cellular vulnerability in the hippocampus when combined with additional challenges such as ischemia.<sup>3,4</sup> This glucocorticoid endangerment of neurons is thought to involve an energy-dependent elevation of extracellular glutamate concentrations and the subsequent mobilization of toxic intracellular Ca<sup>2+</sup> levels.<sup>5–10</sup> Both in vitro and in vivo studies point to the GR as a mediator of the damaging effects of corticosterone.<sup>10–12</sup>

Neurological insults, like ischemia, activate the hypothalamo-pituitary-adrenal axis, with a subsequent rise in

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steroid levels.<sup>13,14</sup> Consequently, if glucocorticoids increase neuronal vulnerability in response to ischemia, then one might predict that attenuating the rise in corticosteroid levels would improve the outcome of the insult. Indeed, it was previously shown that preventing the rise in corticosteroid levels during ischemia preserves cell survival after ischemia and prevents the occurrence of seizures.<sup>11,15,16</sup> Although these studies show that preventing the production of corticosterone during ischemia alleviates histological damage in the hippocampus later on, it remains to be determined whether this reflects a preservation of functional properties. The first aim of our present study was therefore to establish whether inhibiting the production of corticosterone, by using the 11 $\beta$ -hydroxylase inhibitor metyrapone, preserves neuronal functioning in parallel with cell survival after hypoxia/ische-

Received September 7, 1999; final revision received February 9, 2000; accepted February 9, 2000

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mia (HI). Second, because corticosteroids are thought to exert their detrimental effects via the GR, we studied the possible neuroprotective action of the GR antagonist RU38486.

## Materials and Methods

### Animal Model

The committee on Animal Bio-Ethics of the University of Amsterdam approved all experiments. Male Wistar rats (325 to 375 g; Harlan CPB, the Netherlands) were housed individually under a light/dark regimen (light on at 8 AM and off at 8 PM) and fed ad libitum. Surgical procedures were performed between 9 and 11 AM (when basal plasma corticosterone levels are low). HI was induced by using a modified Levine model in which temporary unilateral carotid artery ligation is combined with hypoxic ventilation.<sup>17,18</sup> This procedure reduces cerebral blood flow on the occluded side during HI, thereby inducing a unilateral global hemispheric ischemia. Animals were anesthetized with halothane (2%) in a mixture of 30% O<sub>2</sub> and 70% N<sub>2</sub>O. The right femoral artery was cannulated for continuous blood pressure monitoring during normoxia and the HI periods (Hewlett-Packard pressure transducer). Animals were intubated and subsequently ventilated (infant ventilator MK2, Loosco) for 10 minutes with 30% O<sub>2</sub> and 70% N<sub>2</sub>O (first normoxic period), for the next 20 minutes with 10% O<sub>2</sub> and 90% N<sub>2</sub>O (hypoxia), and finally for 5 minutes with 30% O<sub>2</sub> and 70% N<sub>2</sub>O (second normoxic period). During the hypoxic period, the right carotid artery was occluded. The rectal temperature was maintained between 36.0°C and 37.0°C during the surgical procedure. Corticosterone levels were determined in all animals from a blood sample taken at the end of the second normoxic period and after decapitation 24 hours later. In the metyrapone- and vehicle-pretreated animals, progesterone and deoxycorticosterone levels were also determined. The blood samples were transferred to centrifuge tubes containing 10 μL of heparin solution (500 U/mL) and centrifuged for 20 minutes at 3500g. The supernatant was stored at -20°C until analysis.

In an additional series of 5 metyrapone- and 4 vehicle-treated animals (see also the Treatments section), brain temperature was monitored through a small subdural probe during both normoxic periods, as well as during HI. The data presented in this article (Results) are based on the average brain temperature during the last 5 minutes of the HI period. From the same animals, a blood sample was taken from the femoral artery at the end of the hypoxic period to determine blood glucose levels.

### Treatments

One hour before HI, 16 animals received an injection with sesame oil (ischemia+vehicle group; 0.25 mL) while 14 animals (ischemia+corticosterone group) received an injection with corticosterone (40 mg/kg SC) to obtain very high plasma corticosteroid levels during HI.<sup>3,17</sup> Ten animals (ischemia+metyrapone) received an injection with metyrapone (150 mg/kg SC) 4 hours before HI at a dosage known to inhibit the stress-induced rise in plasma corticosterone levels.<sup>17,19</sup> Eighteen animals (ischemia+RU38486) received an injection with RU38486 1.5 hours before HI at a dosage (25 mg/kg SC) known to block the GR.<sup>20</sup> Moreover, 8 animals, which served as controls for surgery, received an injection with vehicle 1 hour before surgery and were exposed to normoxia only (normoxia+vehicle). In an additional series, 5 metyrapone- and 4 vehicle-pretreated animals were subjected to ischemia to determine postocclusion blood glucose levels and brain temperature during HI.

### Seizures

HI evokes convulsions after the insult.<sup>15,17</sup> To establish whether modulation of steroid levels or blockade of the GR affects the incidence of these convulsions, we recorded the number of animals per group that displayed visible seizures from the period after HI until decapitation 24 hours later by using a video camera.

### Electrophysiological Procedures

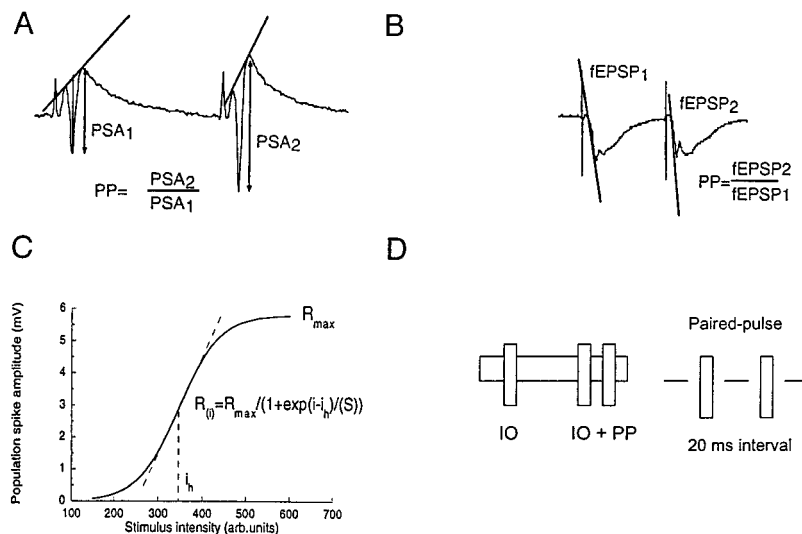
Twenty-four hours after HI, the animals were decapitated and trunk blood was collected. The brains were quickly removed and chilled (4°C) in carbogenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) artificial cerebrospinal fluid (aCSF) containing (in mmol/L): NaCl 120, KCl 3.5, MgSO<sub>4</sub> 1.3, NaH<sub>2</sub>PO<sub>4</sub> 1.25, CaCl<sub>2</sub> 2.5, D-glucose 10, and NaHCO<sub>3</sub> 25. Next, hippocampal slices (400 μm) were prepared with a Vibratome (Campden Instruments Ltd) and stored at room temperature in carbogenated aCSF. After an equilibration period of at least 1 hour, 1 slice at a time was transferred to a recording chamber, which was continuously perfused (2 to 3 mL/min) with carbogenated aCSF. The temperature in the recording chamber was maintained between 30°C and 32°C. The slice was fixed between 2 nylon meshes and kept submerged.

Schaffer collateral/commissural fibers were stimulated by a bipolar stainless steel stimulation electrode (60-μm diameter) that delivered 150-ms pulses.<sup>21</sup> The evoked field potentials were recorded with aCSF-filled glass electrodes (impedance ≈ 2 MΩ). The population spike (PS) and field excitatory postsynaptic potential (EPSP) were recorded in the stratum pyramidale and stratum radiatum, respectively. The amplitude of the PS and slope of the field EPSP were determined as shown in Figures 1A and 1B.

The stimulation protocols, data acquisition, and analysis of the evoked potentials were performed with an Atari computer with in-house-developed software. A schematic overview of the experimental protocols is illustrated in Figure 1D. Recording of field potentials was started at least 15 minutes after placing the stimulation and recording electrodes. To determine the stimulus intensity that evoked half-maximal and maximal field responses, an input/output (IO) curve was constructed that comprised 10 stimulus intensities (interval of 10 seconds) ranging from threshold to maximal stimulus intensities. The relationship between stimulus intensity and the evoked response was fit by a sigmoidal function:  $R_{(i)} = R_{\max} / \{1 + \exp[(i - i_{50}) / (-S)]\}$ , where  $R_{(i)}$  is the response at intensity  $i$ ,  $R_{\max}$  is the maximal response,  $i_{50}$  is the intensity at which half-maximal response is observed, and  $S$  represents an index proportional to the slope of the IO curve (Figure 1C). Subsequent stimuli were delivered at half-maximal intensity determined at the start of the experiment, and field potentials were recorded for 10 minutes with stimuli delivered at an interval of 30 seconds. In all experiments discussed in this study, PS amplitudes and field EPSPs varied by <15% during this period. After this period, a second IO curve was measured. The electrophysiological data shown represent the values of these IO curves. Subsequently we recorded the response to paired-pulse stimulation, delivered at half-maximal stimulus intensity with an interstimulus interval of 20 ms. The ratio of the PS amplitudes or slopes of the field EPSPs measured after paired-pulse stimulation was determined as indicated in Figures 1A and 1B.

### Histology

In addition to the brain slices used for electrophysiological recordings, 4 slices, ie, two 400-μm sections from both the ipsilateral and contralateral sides, were kept in 4% paraformaldehyde in 0.05 mol/L phosphate buffer (pH 7.4) for 24 hours to determine morphological alterations. After overnight fixation, 1 slice each from the ipsilateral and contralateral side was transferred to 0.1 mol/L phosphate-buffered saline (PBS; pH 7.4) containing 0.1% NaN<sub>3</sub>. In these sections, we studied changes in the immunocytochemical distribution of the cytoskeletal protein microtubule-associated protein-2 (MAP2). The 2 remaining sections were used for Nissl staining. The 400-μm sections were cryoprotected by overnight storage in 30% sucrose in 0.1 mol/L phosphate buffer (pH 7.4), cut into 30-μm-thick coronal sections at -15°C on a cryostat microtome, and collected in 4% paraformaldehyde in 0.05 mol/L phosphate buffer (pH 7.4) for cresyl violet staining, or in 0.1 mol/L PBS containing 0.1% NaN<sub>3</sub> for MAP2 immunocytochemistry. Immunocytochemical staining for MAP2 was performed on free-floating sections as described below, all steps being identical for all animals. The tissue sections were preincubated for 20 minutes in 0.1% H<sub>2</sub>O<sub>2</sub> in 0.05 mol/L PBS, subsequently rinsed in PBS, and immersed in 5% normal sheep serum (Sigma) in PBS for 30 minutes to reduce background staining.



**Figure 1.** Twenty-four hours after ischemia, hippocampal sections were cut. Field potentials were recorded in the hippocampal CA1 area after stimulating the Schaffer collateral/commissural fibers at least 1 hour after the rats were killed. PS amplitude and slope of the field EPSP were measured as depicted in A and B, respectively. Formulas show calculation of paired-pulse responses. C, Relationship between stimulus intensity and evoked response was fit by a sigmoidal function:  $R(i) = R_{max} / (1 + \exp((i - i_h) / (-S)))$ , where  $R(i)$  is response at intensity  $i$ ,  $R_{max}$  is maximal response,  $i_h$  is the intensity at which half-maximal response is observed, and  $S$  represents an index proportional to the slope of the IO curve. D, Schematic overview of stimulation procedures. First, an IO curve of the PS amplitude and slope of the field EPSP were measured to determine half-maximal stimulation intensity ( $i_h$ ). Next, brain slices were stimulated for 10 minutes at  $i_h$ , followed by measurement of a second IO curve. Subsequently, paired-pulse (PP) responsiveness (interstimulus interval of 20 ms) was recorded after stimulation at  $i_h$ .

Next, the sections were incubated with the first antibody (monoclonal mouse anti-MAP2; clone HM-2, Sigma) diluted 1:500 in PBS and 1% normal sheep serum overnight at 4°C under gentle movement of the incubation medium. After the primary antibody incubation, sections were rinsed in PBS and again preincubated with 5% normal sheep serum for 30 minutes before exposure to biotinylated sheep anti-mouse IgG (Amersham, diluted 1:200) in PBS for 2 hours at room temperature. Thereafter, the sections were thoroughly rinsed in PBS and incubated in streptavidin-horseradish peroxidase (Zymed, diluted 1:200) in PBS for 2 hours at room temperature. Finally, after subsequent rinsing in PBS and 0.05 mol/L Tris buffer (pH 7.4), the sections were processed by the diaminobenzidine- $H_2O_2$  reaction (30 mg of diaminobenzidine and 0.01%  $H_2O_2$  per 100 mL of 0.05 mol/L Tris buffer), guided by a visual check.

The present histological procedures did not allow accurate quantitative analysis of the observed damage. Rather, we settled for a conservative qualitative analysis in which 2 investigators examined the Nissl-stained and MAP2-immunostained sections from each animal in parallel in a blinded fashion. Histological sections were assigned to 1 of the following classes: (1) No damage: histological analysis of the ipsilateral side did not reveal any difference with respect to the contralateral side from the same animal and with control sections from normoxia-treated animals. (2) Moderate damage: Nissl staining revealed loss of integrity in the dentate gyrus; Nissl staining and MAP2 immunostaining in the dentate gyrus and CA1 area were faint in the ipsilateral compared with the contralateral side. No apparent irregularly shaped pyramidal CA1 neurons were present in the ipsilateral side when compared with the contralateral side. (3) Severe damage: Nissl staining showed pyknotic cells in the dentate gyrus and irregularly shaped pyramidal CA1 cells; MAP2 immunostaining was absent in (part of) the dentate gyrus and faint in the CA1 area.

### Statistics

All data are expressed as mean  $\pm$  SD. Blood pressure and rectal temperature were analyzed by ANOVA with repeated measures. The other parameters (plasma corticosteroid levels, maximal values of the PS amplitude and slope of the field EPSP, slope factor  $S$ , half-maximal stimulation intensity of the IO curves, and paired-pulse

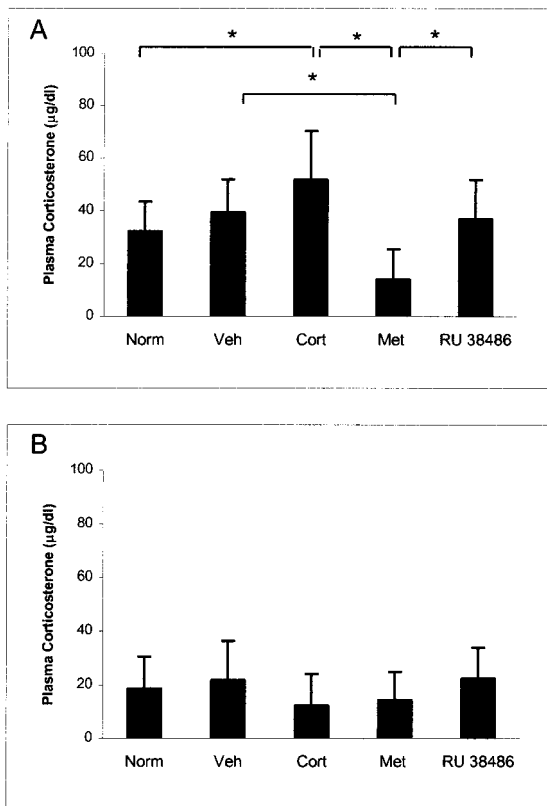
responses) were analyzed by 1-way ANOVA. A post hoc Bonferroni/Dunn test was performed to detect the level of significance between the different groups.  $P$  values  $<0.05$  were considered to be significant.

## Results

### Physiological Values and Plasma Corticosterone Levels

During HI, blood pressure dropped significantly when compared with that in the animals exposed to normoxia but returned to pre-HI levels during the second normoxic period (data not shown). As reported previously, this results in reduced blood flow in the hemisphere ipsilateral to the carotid occlusion.<sup>18</sup> No differences in blood pressure were found between the different groups exposed to HI. Rectal temperature was also comparable between the experimental groups during surgery (data not shown). Brain temperature, as monitored in a separate experiment, was significantly lower than rectal temperature during the ischemic period ( $34.0 \pm 0.7^\circ\text{C}$  and  $36.6 \pm 0.2^\circ\text{C}$  for brain and rectal temperatures, respectively, in 4 vehicle-treated ischemic animals). Metyrapone-pretreated animals displayed comparable brain and rectal temperatures ( $33.7 \pm 1.3^\circ\text{C}$  and  $36.8 \pm 0.4^\circ\text{C}$ , respectively;  $n=5$ ). Also, blood glucose levels at the end of the ischemic period were not significantly different between vehicle-treated ( $7.7 \pm 1.72$  mmol/L;  $n=4$ ) and metyrapone-pretreated ( $8.6 \pm 5.3$  mmol/L;  $n=5$ ) animals.

The experimental treatments yielded striking differences in plasma corticosterone levels between the different groups, as measured immediately after HI. Animals exposed to normoxia displayed high plasma corticosterone levels (Figure 2A), which were even slightly higher in animals exposed to HI. The highest plasma corticosterone levels were found in



**Figure 2.** Plasma corticosteroid levels measured immediately after HI (A) or after decapitation, 24 hours later (B). A, Administration of corticosterone (Cort) produced the highest plasma corticosteroid levels, whereas metyrapone (Met) attenuated the rise in steroid levels. B, 24 hours after ischemia, no differences in plasma corticosterone levels were found between the 5 experimental groups. Norm indicates normoxic; Veh, vehicle. \*Significantly different at  $P < 0.05$ .

animals injected with corticosterone. Animals injected with RU38486 displayed corticosteroid levels that were similar to those in vehicle-treated animals exposed to HI. By contrast, administration of metyrapone attenuated the ischemia-induced rise in corticosteroid levels. Twenty-four hours after HI, no group differences were found in the plasma corticosteroid levels measured immediately after decapitation (Figure 2B). It is important to note that the different treatments yielded experimental groups showing different plasma corticosterone levels during ischemia. Hence, these experimental groups allowed us to study the effect of different plasma

corticosterone levels during ischemia on seizures, neuronal damage, and hippocampal functioning after ischemia.

Furthermore, in the metyrapone-pretreated animals, plasma deoxycorticosterone levels were elevated compared with those in the ischemia+vehicle group ( $406 \pm 207$  and  $30 \pm 13$  nmol/L, respectively; data not shown). Similarly, plasma progesterone levels in the metyrapone-pretreated group were significantly elevated compared with the vehicle group ( $43 \pm 19$  and  $24 \pm 9$  nmol/L, respectively).

### Seizures

HI elicited visible seizures as reported previously.<sup>15,17</sup> These seizures were characterized by spinning of the animal around its body axis, jerking movements, and/or clonic contractions of the paws. In general, these seizures were intermittent and lasted  $< 30$  seconds. The incidence of visible seizures was correlated to a large degree with the steroid levels during HI. Thus, 7 of 16 animals that were treated with vehicle before ischemia displayed visible seizures during the 24-hour survival period after HI (Table 1). In parallel with the plasma corticosteroid levels, the highest incidence of seizures was found after corticosterone treatment, whereas metyrapone reduced this incidence. RU38486 only partly attenuated the incidence of these seizures. Interestingly however, animals treated with RU38486 displayed the lowest incidence when we analyzed the seizures during the first 4 hours after ischemia (Table 1), which might point to a temporary beneficial effect of blocking the GR.

### IO Curves of PS Amplitude and Slope of the Field EPSP

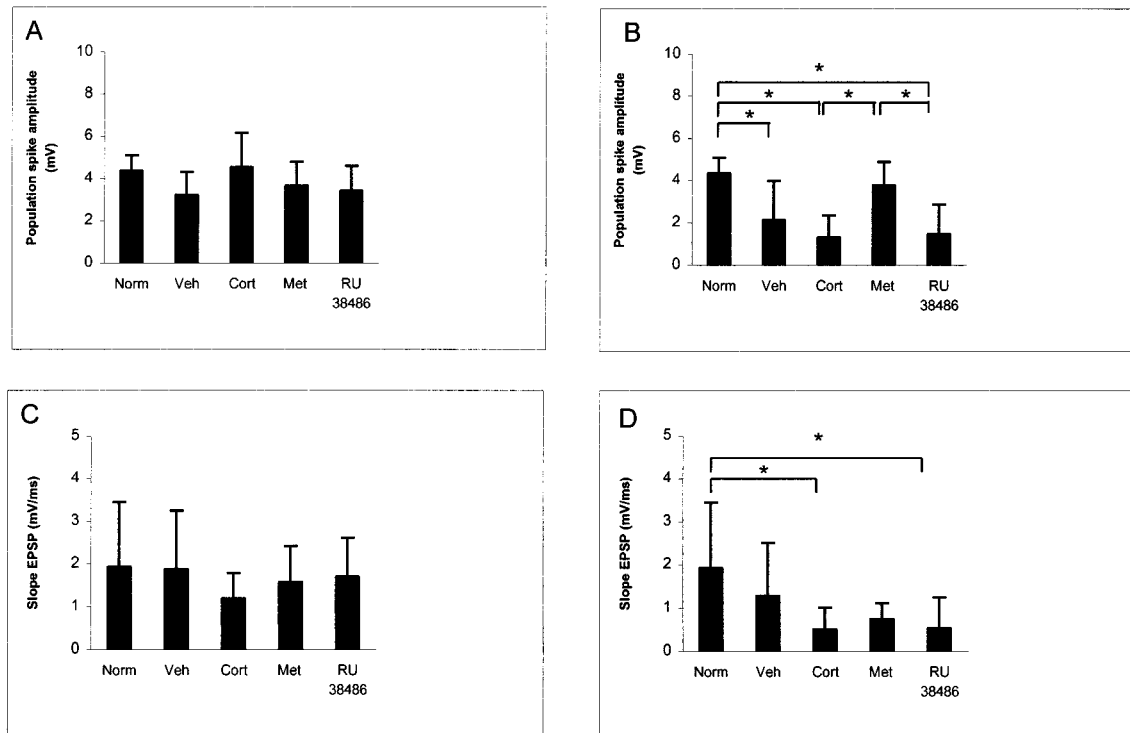
In the period 4 to 24 hours after HI, 2 animals died in both the vehicle- and the corticosterone-treated groups, while 1 animal died in the group that received RU38486. In the remaining animals, we studied neuronal functioning in the hippocampal CA1 area after HI by establishing synaptic responsiveness at both the ipsilateral and contralateral side to the occlusion. The animals reported on below exhibited a maximal PS amplitude of  $> 1.25$  mV and a maximal field EPSP slope of  $< -0.40$  mV/ms in slices from the side contralateral to the occlusion.

Synaptic responses in slices from the side ipsilateral to the occlusion were clearly affected by ischemia and corticosteroid treatment. Thus, whereas ischemia itself reduced the maximal PS amplitude in vehicle-treated animals when compared with animals exposed to normoxia (Figure 3B), the

**TABLE 1. Number of Animals Displaying Visible Seizures After HI**

Treatment	n	Incidence Over 24 Hours	Incidence Over 0–4 Hours	Incidence Before Decapitation
Normoxia+vehicle	8	0/8	0/8	0/8
Ischemia+vehicle	16	7/16 (44%)	7/16 (44%)	2/14 (14%)
Ischemia+corticosterone	14	8/14 (57%)	8/14 (57%)	0/12 (0%)
Ischemia+metyrapone	10	2/10 (20%)	2/10 (20%)	0/10 (0%)
Ischemia+RU 38486	18	6/18 (33%)	2/18 (11%)	4/17 (24%)

Numbers indicate the animals displaying seizures over the entire 24-hour period (left), during the first 4 hours after HI (middle), or immediately before decapitation, ie, 24 hours after the ischemic insult (right). After ischemia, 2 animals treated with vehicle and corticosterone died; 1 animal that received RU 38486 died.



**Figure 3.** Maximal PS amplitude (A, B) and slope of the field EPSP (C, D) on contralateral (A, C) and ipsilateral (B, D) sides. Slope values of the field EPSPs are presented as positive values. On the ipsilateral side, maximal PS amplitude after HI was severely impaired in corticosterone- (Cort) and RU38486-treated animals, whereas metyrapone (Met) prevented these alterations. Similarly, field EPSP was significantly reduced on the ipsilateral side after treatment with corticosterone and RU38486. Norm indicates normoxic; Veh, vehicle. \*Significantly different at  $P < 0.05$ .

most severe reduction in PS amplitude was found in ischemic animals treated with corticosterone. Recordings in the dendritic layer yielded a similar though somewhat less pronounced result: corticosterone treatment significantly reduced the maximal slope of the field EPSP on the ipsilateral side (Figure 3D). These results support the view that the number of principal cells that respond to Schaffer collateral/commissural stimulation after HI is reduced in animals showing high plasma corticosterone levels when compared with animals exposed to normoxia.

Metyrapone pretreatment prevented the ischemia-induced impairment in field potentials. Interestingly, the ratio between the maximal PS amplitude and the maximal field EPSP slope was significantly altered in the metyrapone-treated group compared with the vehicle controls ( $-6.5 \pm 3.3$  versus  $-2.0 \pm 1.1$ , respectively), suggesting that the neuroprotective actions of metyrapone are at least partly caused by an increased likelihood to evoke an action potential after a given synaptic input. In contrast to metyrapone, the glucocorticoid antagonist RU38486 did not prevent the reductions in field potentials after HI. In fact, the maximal PS amplitude and maximal slope of the field EPSP were reduced to the same levels as found in corticosterone-treated animals. For all of the experimental groups, no significant differences were observed with respect to the PS amplitude and field EPSP slope in slices prepared from the contralateral side (Figures 3A and 3C).

The data obtained with the IO curve protocol were fitted with a Boltzman equation  $R_{(i)} = R_{max} / \{1 + \exp[(i - i_h)/(S)]\}$ ,

yielding values for the maximal response  $R_{max}$ , the half-maximal stimulation intensity  $i_h$ , and a constant  $S$ , which is proportional to the slope of the fitted curve. Reliable fits and thus, values for  $i_h$  and  $S$  could be obtained only from those animals in which the ipsilateral PS amplitude was  $>0.50$  mV and the field EPSP slope was more negative than  $-0.15$  mV/ms. In all experimental groups except the normoxic group, some of the animals were excluded from the curve-fitting analysis on the basis of this criterion. In the animals for which IO curves were successfully fitted, ischemia and steroid treatment affected the maximal PS amplitude and field EPSP slope in a similar way as described above for all animals (data not shown). However, in these animals neither  $i_h$  nor the slope factor  $S$  revealed significant differences on the ipsilateral or contralateral side between the experimental groups (data not shown).

### Responses to Paired-Pulse Stimulation

The paired-pulse ratios were calculated only for the animals from which reliable signals could be obtained on the ipsilateral side (PS amplitude  $>0.50$  mV and slope of the field EPSP more negative than  $-0.15$  mV/ms). Analysis of these responses in the CA1 pyramidal cell layer as well as in the stratum radiatum showed facilitation of the second signal after paired-pulse stimulation (data not shown). No significant changes in facilitation in either the CA1 pyramidal cell layer or dendritic field were present between the experimental groups on the contralateral side or ipsilateral side.

**TABLE 2. Neuronal Degeneration After HI**

Treatment	Changes in Dentate Gyrus		Changes in CA1 Hippocampal Area	
	Intensity	No. of Animals	Intensity	No. of Animals
Normoxia + vehicle	0	0%	0	0%
Ischemia + vehicle	+	58%	+	33%
Ischemia + corticosterone	++	80%	++	50%
Ischemia + metyrapone	0/+	33%	0	11%
Ischemia + RU 38486	+	36%	+	21%

Intensity of histological alterations ranged from absent (0) to severe (++), as described in Materials and Methods. The number of affected animals is given as a ratio of the total number of animals per experimental group.

### Histology

Nissl staining revealed histological changes in the hippocampus 24 hours after HI when compared with animals exposed to normoxia (Table 2). At this time point, a loss of granular cells in the dentate gyrus was present on the ipsilateral (occluded) side in a number of animals (cf Figures 4A and 4C). The cell loss was randomly present in restricted parts in both the suprapyramidal and infrapyramidal blade of the granular cell layer. Histological alterations in the hippocampal CA1 area, indicated by reduced density of cresyl violet staining and an irregularly shaped appearance, were present in those animals that showed severe damage in the dentate gyrus (Figure 4D). The degenerative changes after ischemia were most prominent in the group that was treated with corticosterone (Figure 4F). When compared with the other experimental groups, this group consisted of more animals displaying evident cell loss in the dentate gyrus (Table 2). In addition, in these animals the amount of granular cell loss was most pronounced. The number of animals displaying histological alterations in the hippocampal CA1 area was also most prominent in this group. In contrast, few animals treated with metyrapone displayed granular cell loss, and the amount of damage was very limited (Figure 4G). Moreover, no obvious alterations were present in the hippocampal CA1 area (Figure 4H). Treatment with RU38486 was not as effective as metyrapone in preserving neuronal integrity.

In parallel with the findings of Nissl staining, we observed changes in the cytoskeletal protein MAP2 after HI. MAP2 is normally localized to dendrites throughout the hippocampus (Figures 5A and 5B). Exposure to HI resulted in a marked loss of MAP2 immunoreactivity that was particularly obvious in the dentate gyrus molecular layer of the ipsilateral side, thereby confirming the cell loss in this area as detected by Nissl staining (Figures 5C and 5D). In agreement with the results of Nissl staining, both the number of animals that displayed a reduction in MAP2 immunoreactivity as well as the amount of reduction was most prominent in rats treated with corticosterone before HI. By contrast, metyrapone treatment largely prevented the loss of MAP2 immunoreactivity (Figures 5E and 5F).

### Discussion

Previous studies have shown that high corticosteroid hormone levels increase neuronal vulnerability, particularly

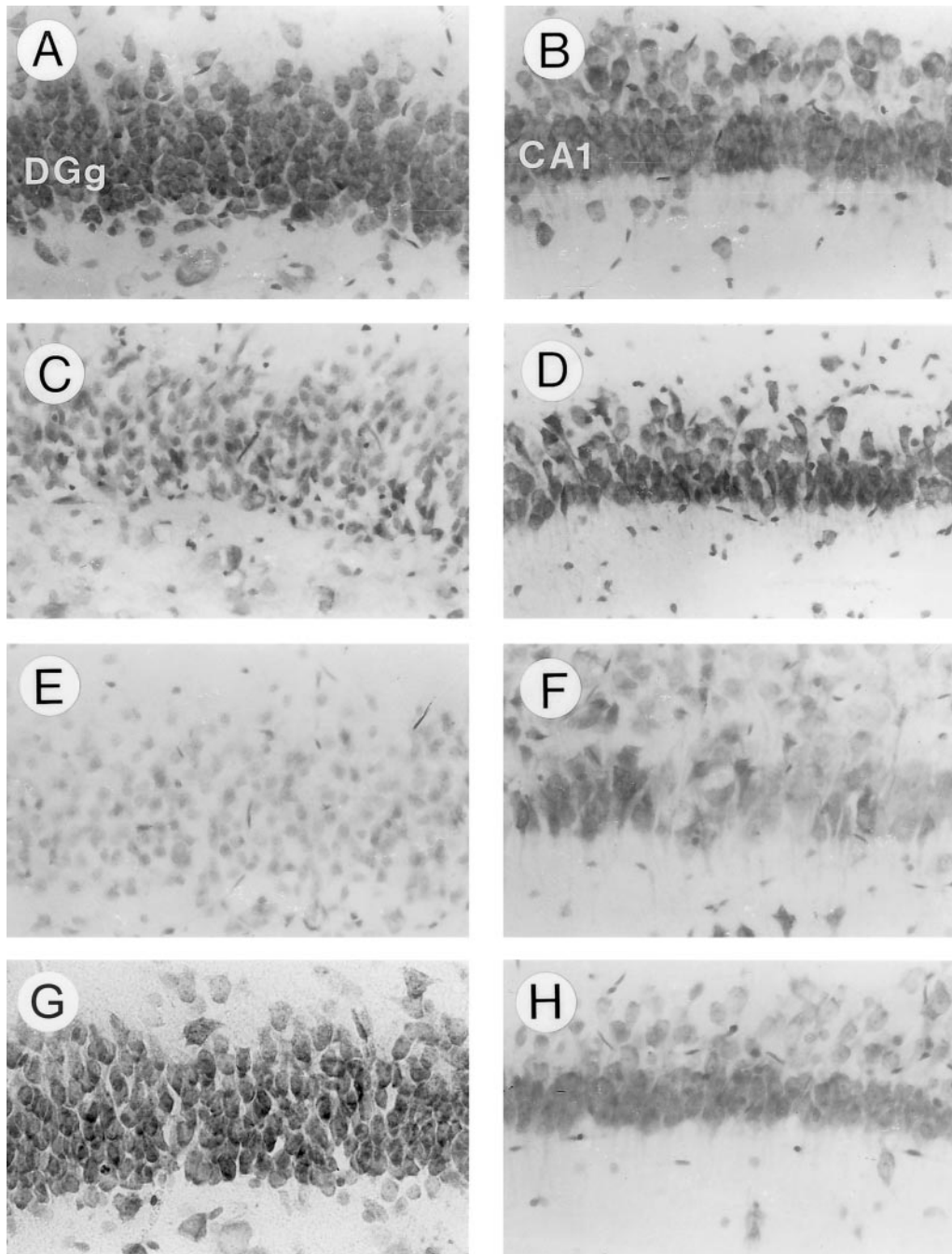
when the brain is subjected to additional challenges. A well-documented example is the corticosteroid-mediated potentiation of brain damage after ischemia.<sup>3,4,10,15–17</sup> Here we addressed 2 issues: first, whether manipulation of corticosteroid levels during HI affects synaptic transmission after ischemia, along with histological alterations, and second, because profound rises in corticosteroid levels affect GR rather than MR activation,<sup>2</sup> we implicitly hypothesized that corticosteroid effects on ischemia-induced brain damage are mediated by GRs. We therefore tested the efficacy of treatments preventing GR activation on functional parameters and damage in the hippocampus.

### Synaptic Transmission After HI

Synaptic transmission between Schaffer collateral/commissural fibers and CA1 pyramidal neurons was examined 24 hours after HI. Neurons in this area are sensitive to ischemic damage, although in our model, damage at the selected time point was still limited.<sup>15</sup> It is important to note that this allows determination of beneficial or deleterious effects of treatment. We observed that in vehicle-treated ischemic animals, only the maximal PS amplitude was significantly reduced, while the field EPSP and paired-pulse responsiveness were not significantly altered. This finding agrees with earlier studies,<sup>22</sup> pointing to an elevated spike threshold after ischemia.<sup>23</sup>

High corticosterone levels in combination with ischemia reduced both the PS amplitude and the field EPSP slope. Importantly, during the experiment, plasma corticosterone levels were comparable in all groups, so that short-term modulation of synaptic transmission by steroids<sup>24,25</sup> cannot explain group differences. The reduced synaptic function was observed only when high corticosteroid levels occurred in conjunction with HI; animals in the normoxic group, which shortly after the insult displayed plasma corticosterone levels similar to those in the ischemia+vehicle group, later on showed synaptic responses that were indistinguishable from those recorded earlier in naive controls.<sup>26</sup> Moreover, animals with high corticosterone levels did not show loss of synaptic function in the nonischemic contralateral hemisphere.

Histological observations indicate that the loss in synaptic function in ischemic animals with high corticosteroid levels may be partly due to a decrease in the number of neurons participating in the synaptic response. Although the present

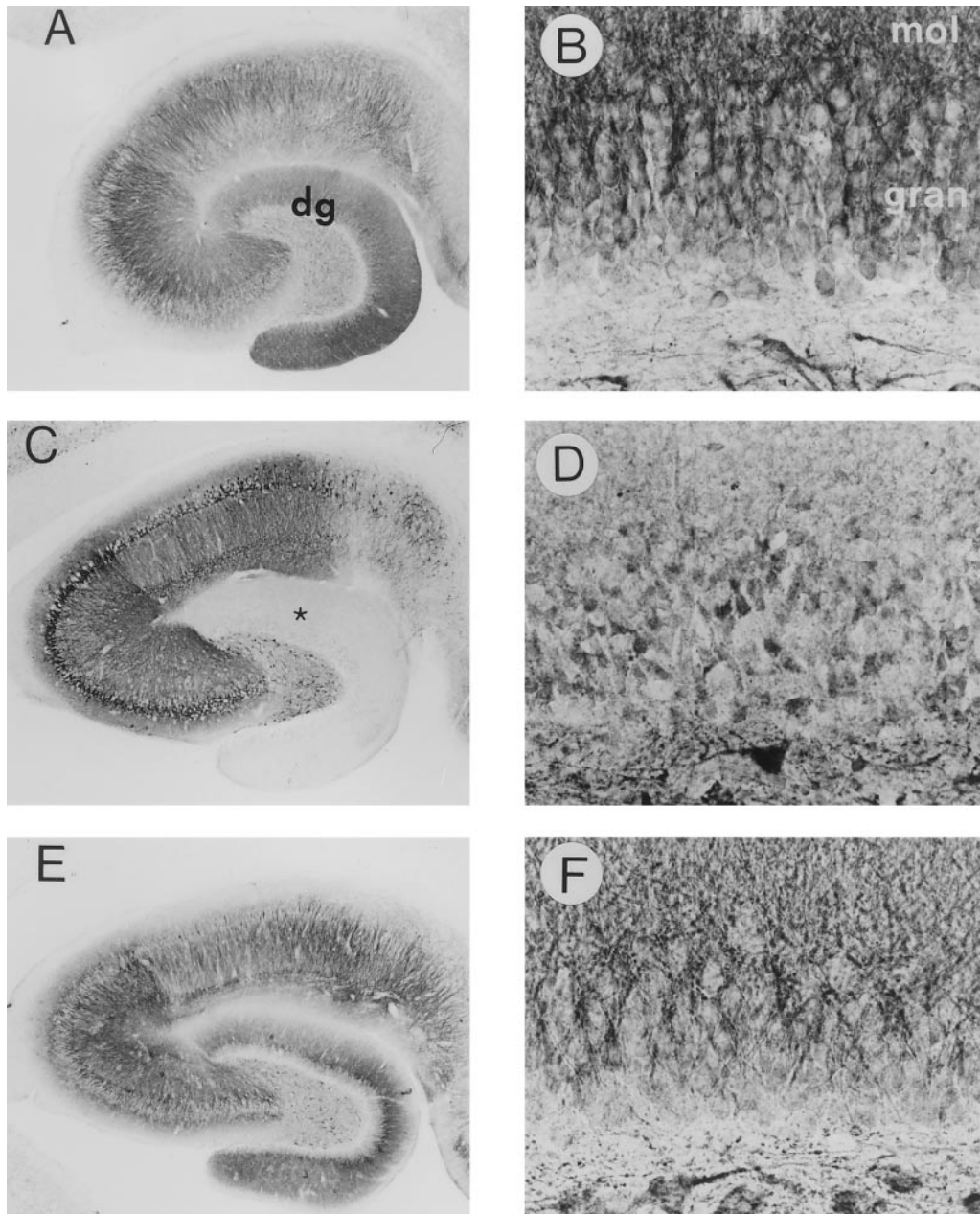


**Figure 4.** Photographs showing cell damage in dentate gyrus (left) and CA1 pyramidal cell layer (right) 24 hours after HI. In this example, HI induced loss of integrity and faint staining in dentate gyrus granular cells of a vehicle-treated animal (C); pyramidal neurons in the CA1 region show irregular shapes (D). After treatment with corticosterone, the extent of neurodegenerative changes, such as loss of integrity, was even more pronounced (E, F). However, after pretreatment with metyrapone, nearly all animals were protected against neurodegenerative changes; Nissl-stained dentate granule cells (G) and CA1 pyramidal cells (H) have a similar appearance as in normoxic controls. DGg indicates dentate gyrus granular layer; CA1: CA1 pyramidal cell layer. Magnification  $\times 350$ .

analysis was only qualitative, both the Nissl and MAP2 staining indicated that these animals revealed a high degree of structural damage, confirming earlier studies.<sup>10,15–17</sup> Because short-term rises in corticosterone, resulting in activation of GRs in addition to MRs, were found to enhance  $\text{Ca}^{2+}$  influx into CA1 pyramidal cells through voltage-gated  $\text{Ca}^{2+}$  channels while predominant MR activation seen with low corticosterone levels yielded small  $\text{Ca}^{2+}$  currents,<sup>27,28</sup> corticosteroid modulation of intracellular  $\text{Ca}^{2+}$  levels during and after

HI may contribute to the delayed neurodegeneration and functional loss observed 24 hours later.<sup>29,30</sup> Furthermore, corticosteroid-dependent depletion of energy sources, leading to excess levels of glutamate, for example, may add to the neurodegeneration.<sup>6–8,31</sup>

While the overall data support a correlation between neuronal damage and loss in synaptic function after HI, detailed data analysis indicated that histological damage is not a reliable predictor of impaired synaptic function (and



**Figure 5.** Loss of MAP2 immunoreactivity in dentate gyrus after HI was reduced after treatment with metyrapone. A, MAP2 immunoreactivity in animals exposed to normoxia (magnification  $\times 22$ ). In vehicle-treated animals exposed to HI, MAP2 immunoreactivity was reduced in the dentate gyrus granular cell layer (C, asterisk). This reduction was largely prevented by metyrapone (E). Photographs B, D, and F are higher-magnification photographs (magnification  $\times 350$ ) of dentate gyrus from animals exposed to normoxia [B], ischemia [D], and metyrapone treatment+ischemia [F]. dg indicates dentate gyrus; mol, dentate gyrus molecular layer; gran, dentate gyrus granular cell layer.

vice versa): about half of the animals showing damage in the CA1 area still displayed PS amplitudes exceeding 0.50 mV, whereas  $\approx 25\%$  of the animals showing no damage were found to have a loss of synaptic function ipsilateral to the occlusion. Conversely, in nearly half of the animals, loss of synaptic function was not associated with histological damage. The dissociation between electrophysiological and histological observations stresses that these approaches should be performed in parallel for a full appreciation of the effects. Importantly, these observations indicate that in addition to the loss of functional CA1 cells, other impairments in synaptic

function could underlie reduced responsiveness to stimulation of the afferents.

#### **Efficacy of RU38486 and Metyrapone**

Treating animals with the GR antagonist RU38486 or the steroid synthesis inhibitor metyrapone tested the hypothesis that corticosteroid effects on ischemia-induced brain damage are mediated by GRs. In contrast to metyrapone, RU38486 only partly protected against the consequences of HI. Despite a temporary alleviation of seizure activity and fewer animals with damage in the dentate gyrus, which agrees with a recent



report,<sup>32</sup> synaptic function 24 hours after the insult was severely impaired. Indirectly, this shows that loss of synaptic function 24 hours after the ischemic insult does not depend on earlier seizure activity. Thus, RU38486-pretreated rats usually did not display seizures but nevertheless developed severely impaired hippocampal synaptic function.

The poor efficacy of RU38486 can be explained in several ways. One possibility is that peripheral treatment with RU38486 blocks GRs in the pituitary much more effectively than it does brain GRs. This idea is based on a recent study showing that synthetic steroids such as dexamethasone are poorly transported across the blood-brain barrier by the *mdr-1a* protein.<sup>33</sup> If so, pretreatment with RU38486 might primarily interfere with the negative-feedback action of corticosterone at the pituitary, resulting in an excess of endogenous steroids,<sup>19</sup> rather than acting as a GR antagonist in the brain. However, several observations argue against this explanation. First, transport through the blood-brain-barrier may be seriously compromised by ischemia itself,<sup>34,35</sup> explaining earlier deleterious effects of dexamethasone.<sup>10–12</sup> Second, seizure activity shortly after HI was effectively blocked by RU38486 treatment, supporting a central effect of peripherally administered RU38486. Nevertheless, we cannot exclude that later on the ratio between RU38486 and endogenous corticosterone in the brain may have diminished, eg, due to different kinetic properties of the 2 molecules. In that case, repeated treatment with RU38486 may have a higher efficacy than the presently used single injection. An alternative explanation is based on the fact that RU38486 not only blocks GRs but also progesterone receptors.<sup>36</sup> Progesterone was recently found to protect against ischemic damage.<sup>37,38</sup> If so, blockade of progesterone receptors by RU38486 would counteract the presumed beneficial effects of GR blockade.

Unlike RU38486, metyrapone pretreatment effectively protected against ischemic damage and loss in synaptic function. These beneficial effects occurred in the absence of changes in blood glucose levels or brain temperature, 2 critical factors for the consequences of ischemic insults.<sup>16,39–42</sup> The most obvious explanation for the beneficial effects of metyrapone is the decrease in corticosteroid levels, so that extensive GR activation is prevented. In accordance, animals showing a large maximal PS amplitude ipsilateral to the occlusion (>2.5 mV) 24 hours after the insult had significantly lower corticosterone levels during ischemia than did animals with smaller PS amplitudes (26 versus 37  $\mu\text{g/dL}$ ;  $P < 0.05$ ). However, alternative explanations cannot be ruled out. In agreement with its  $11\beta$ -hydroxylase activity, metyrapone pretreatment results in accumulation of progesterone and deoxycorticosterone. Because these hormones are known to potentiate  $\gamma$ -aminobutyric acid A receptor-mediated responses and block voltage-dependent  $\text{Ca}^{2+}$  channels,<sup>43,44</sup> this might add to the neuroprotective action of metyrapone. In addition, excessive secretion of steroid derivatives through the enzyme block (eg, tetrahydrodeoxycorticosterone) could potentially reduce neuronal damage by suppressing corticotrophin-releasing hormone levels after traumatic brain injury.<sup>45,46</sup>

In summary, the present study shows that ischemia-induced impairment in CA1 synaptic function is exacerbated

by concomitant corticosteroid treatment and alleviated by pretreatment with the steroid synthesis inhibitor metyrapone. Pretreatment with a GR antagonist only has beneficial effects shortly after ischemia and antagonist treatment. We tentatively conclude that preventing the ischemia-evoked rise in corticosteroid levels rather than blocking the GR is effective in preserving synaptic function and cellular integrity after HI.

## Acknowledgments

This study was supported by a grant from Het Nationaal Epilepsie Fonds "De macht van het kleine" (grant 96-03). The authors would like to thank Lisette van Essen for her work on the corticosterone assays and Ron de Kloet for careful reading and critical comments on the manuscript.

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## Editorial Comment

Cerebral ischemia is invariably accompanied by increases in glucocorticoid secretion, and current research suggests that this neuroendocrine response may contribute to the incidence, severity, and extent of ischemic injury.<sup>1</sup> The prevailing view is that glucocorticoids aggravate neuronal vulnerability to neurotoxicants such as free radicals and excitatory amino acids.<sup>2,3</sup> The hippocampus, which is richly endowed with receptors for glucocorticoids (mineralocorticoid and glucocorticoid receptors), seems particularly prone to hypercortisolemia-induced neuronal death. This has led several laboratories to propose that hypercortisolemic states, such as those found in depression or during chronic stress, may result in reduced hippocampal volume and cell number.<sup>4</sup> Notably, however, postmortem studies on brains from depressed patients and patients who had received high therapeutic doses of corticosteroids failed to detect either gross structural changes or signs of cell loss in the hippocampus.<sup>5</sup>

Also, high doses of corticosteroid reportedly do not lead to cell loss in the nonhuman primate hippocampus.<sup>6</sup>

Hypercortisolemia appears to augment damage only during contemporaneous exposure to another severe neurotoxicant. The accompanying report strengthens this view insofar as it shows that high corticosteroid secretion exacerbates the deleterious effects of hypoxia on hippocampal neurons. The authors show that the extent of hypoxia-induced injury, including impairment of synaptic function and morphological damage to the granule cells and CA1 pyramidal subfields, can be reduced by suppressing corticosteroid biosynthesis with metyrapone. This report, which confirms and extends earlier studies, thus adds to a growing body of evidence which indicates that maintaining low adrenal steroid levels during ischemia may help reduce neuronal damage. The question that arises from these findings is whether the neuroprotective effect of metyrapone occurs as a consequence of reduced

occupancy of corticosteroid receptors. If this were the case, the immediate postischemic administration of corticosteroid receptor antagonists would, presumably, provide a straightforward strategy for reducing hypoxia-induced brain injury. Surprisingly, the authors did not observe any neuroprotection in rats treated with RU486, a glucocorticoid- (and progesterone-) receptor antagonist. This finding rejects the notion that corticosteroid receptor-mediated events amplify ischemic injury and leaves the question as to the mechanisms underlying the neuroprotective effects of metyrapone open for further investigation. In fact, metyrapone does more than simply lower corticosteroid secretion. Apart from its ability to inhibit cytochrome P450 enzymes, which endows it with antioxidative actions,<sup>7</sup> an interesting facet of metyrapone action is that its administration leads to high concentrations of the so-called neurosteroids, which themselves appear to be able to rescue neurons through their GABA<sub>A</sub>-agonistic properties as well as their ability to block voltage-dependent Ca<sup>2+</sup> channels. Inhibition of 11 $\beta$ -steroid-hydroxylase accounts for the increased levels of these steroid allosteric modulators of the GABA<sub>A</sub> receptor after metyrapone treatment.<sup>8</sup> In a related context, it is pertinent to mention that corticotropin-releasing hormone (CRH) expression was found to be elevated after cerebral ischemia or traumatic injury, leading to the suggestion that excessive CRH may also account for the size of the infarcted area,<sup>9,10</sup> and in fact, administration of a CRH-receptor antagonist was shown to reduce neuronal damage.<sup>11</sup> A link between metyrapone-elicited neurosteroids and CRH-induced brain damage can be drawn from the fact that the 2 most representative neurosteroids, tetrahydroprogesterone (often called allopregnanolone) and tetrahydrodeoxycorticosterone, have been shown to suppress CRH synthesis.<sup>12,13</sup> The important clinical implication of the current report is that metyrapone is a drug which, by interfering with glucocorticoid biosynthesis, may (at least in the short term) prove to be a useful means to improve the morphological and functional outcome of hypoxic ischemia. It is, however, important to be aware that very low corticosteroid titers over prolonged periods may also endanger neuronal survival; in experimental animals at least, minimal stimulation of hippocampal corticosteroid receptors is necessary to maintain the structural integrity of the dentate gyrus.<sup>14,15</sup>

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*Stroke*. 2000;31:1162-1172

doi: 10.1161/01.STR.31.5.1162

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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