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Metyrapone alleviates ischemic neuronal damage in the gerbil hippocampus

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Abstract

Transient forebrain ischemia was induced in gerbils, and the effect of a pre-ischemic treatment with metyrapone (100 mg/kg) on delayed neuronal death in hippocampal CA1 neurons was evaluated. The effect of metyrapone on the ischemic release of amino acids in the CA1 region was also examined by microdialysis. Hippocampal slices were used for the evaluation of the hypoxia-induced intracellular Ca^{2+} increase by microfluorometry. The metyrapone treatment morphologically improved the damage provoked by 3 min of ischemia, although it did not alleviate the damage by 5 min. Ischemia for 3 min produced a 306% increase in the glutamate concentration in perfusates, and metyrapone suppressed the peak value to 42% of that in the control group. The extent of the increase in fluorescence intensity by intracellular Ca^{2+} was lower by 16% in slices from metyrapone-treated animals than in controls 600 s after induction of hypoxia. The removal of Ca^{2+} from the perfusion medium suppressed the hypoxic Ca^{2+} increase, and the increase was further reduced in slices pretreated with metyrapone. The increase in the level of endogenous glucocorticoids, which occurs in cerebral ischemia, may aggravate ischemic neuronal damage.

Keywords: Ca^{2+}; Cerebral ischemia; (Gerbil); Glucocorticoid; Glutamate; Hippocampus; Metyrapone

1. Introduction

The secretion of endogenous glucocorticoids is facilitated in various stress conditions such as cardiac arrest and cerebral ischemia (Munck et al., 1984; Fassbender et al., 1994). However, the increase in the plasma concentration of endogenous glucocorticoids may aggravate neuronal injury caused by cerebral ischemia, because exogenous glucocorticoids have been shown to exert a deleterious effect on neuronal injury provoked by seizure, hypoglycemia, and ischemia (Koide et al., 1986; Sapolsky, 1990; Adachi et al., 1998). Metyrapone is a potent and rapid inhibitor of the synthesis of glucocorticoids. It blocks the 1β-hydroxylation step, and reduces the plasma concentration of endogenous glucocorticoids (Temple and Liddle, 1970). This agent has been shown to reduce brain injury induced by focal and global ischemia and seizure (Smith-Swintosky et al., 1996). Further, metyrapone has been reported to reduce neuronal damage produced by kainate (Stein and Sapolsky, 1988), which harms neurons by its structural similarity to glutamate. Thus, the beneficial effect of metyrapone seems to be related to the decrease in the plasma concentration of endogenous glucocorticoids. In the present study, we investigated the effect of metyrapone on the release of neurotransmitter amino acids and the increase in intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in ischemia, both of which are crucial factors in the development of ischemic neuronal damage (Jørgensen and Diemer, 1982; Benveniste et al., 1984; Mitani et al., 1994). Then, we correlated these findings to the histologic outcome.

2. Materials and methods

2.1. Animals

This study was approved by the Committee on Animal Experimentation at Ehime University School of Medicine, Ehime, Japan. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by Ehime University School of Medicine. Male Mongolian gerbils weighing 60 to 80 g (Seiwa Experimental Animals, Fukuoka, Japan) were housed in groups in a room controlled at 23 ± 1°C and maintained in an alternating 12-h light/12-h dark cycle (lights on at 0600 h).

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Animals were deprived of food at least 6 h before the start of experiments because of the influence of the plasma concentration of glucose (Myers and Yamaguchi, 1977; Siemkowicz and Hansen, 1978).

2.2. Experimental protocols

In experiment 1, the effect of pre-ischemic treatment with metyrapone on the histologic outcome was examined by assessing the delayed neuronal death. In experiment 2, the effect of metyrapone on changes in the release of aspartate, glutamate, and glycine was examined by microdialysis. In experiment 3, microfluorometry was applied to investigate the effect of a pretreatment with metyrapone on the hypoxia-induced accumulation of $[Ca^{2+}]_i$ in hippocampal slices.

2.3. Experiment 1: histologic outcome

In this experiment, 36 gerbils were divided into six groups: nonischemic (sham-operated) groups injected with saline or metyrapone, 3-min ischemic groups injected with saline or metyrapone, and 5-min ischemic groups injected with saline or metyrapone. Metyrapone (100 mg/kg) or saline was administered intraperitoneally 60 min before ischemia. The animals were anesthetized and maintained with 2% halothane in balanced 50% O_2 and 50% N_2O. The experiment was performed under spontaneous ventilation. Both common carotid arteries were exposed, and silk threads (4.0) were looped around these arteries. After the animal was placed in a stereotaxic apparatus in the prone position, a thermocouple needle probe was inserted into the left hemisphere through a small burrhole on the skull. An identical probe was inserted into the rectum. Brain and rectal temperatures were carefully maintained at 37–38°C with a heating lamp. Sixty minutes after the administration of metyrapone or saline, transient forebrain ischemia for 3 or 5 min was achieved by pulling the threads around the bilateral common carotid arteries with 8 g weights, while maintaining the brain and rectal temperatures at 37.5 ± 0.2°C (Mitani et al., 1991). After the threads were cut to restore the blood flow, the brain and rectal temperatures were further maintained for 30 min under anesthesia. Then, the skin incision was sutured, and the animal was recovered from anesthesia. Seven days after ischemia, the brains were perfused with heparinized saline and fixed with buffered formalin. Brain slices, 5 μm thick, were stained with hematoxylin and eosin, and the numbers of preserved neurons in the hippocampal CA1 field per 1 mm length of stratum pyramidale were counted in the same level of coronal section (1.5 mm posterior to the bregma) in a single blinded manner. The average of values on both sides was then obtained for each animal.

2.4. Experiment 2: measurements of amino acids by microdialysis

In this experiment, 18 gerbils were prepared and then assigned to the control and metyrapone groups (nine animals each). We used a microdialysis technique as described previously (Chen et al., 1998). In brief, after the preparation of forebrain ischemia by the same procedure as that described in experiment 1, an additional burr hole was drilled in the right hemisphere (2.0 mm posterior and 2.0 mm lateral to the bregma). The microdialysis probe (1 mm long, 0.22 mm outside diameter, Eicom, Kyoto, Japan) was implanted in the right hemisphere (2 mm posterior and 2 mm lateral to the bregma, and 2.5 mm below the brain surface). Ringer's solution was perfused at a rate of 2 μl/min, and brain perfusates were collected every 3 min. Transient forebrain ischemia for 3 min was induced 60 min after the administration of metyrapone (100 mg/kg) or saline. At the end of the experiment, the animals were decapitated and the location of the probe was verified.

Amino acid concentrations were determined using a cation exchange high-performance liquid chromatography (HPLC) system coupled with postcolumn fluorescent derivatization as described previously (Chen et al., 1998). An in vitro recovery test was performed by inserting the dialysis probe into Ringer's solution containing 10 μM of each amino acid at 37°C. The recovery rates of aspartate, glutamate, and glycine in the perfusate were 7.7 ± 0.9%, 7.6 ± 0.9%, and 9.3 ± 1.3% (mean ± S.D., $n = 4$), respectively, when the probe was perfused at 2 μl/min.

2.5. Physiologic variables

Another group of 10 gerbils was prepared to determine the physiologic variables that may influence the extent of the neuronal damage in ischemia. Five animals were given metyrapone (100 mg/kg) intraperitoneally, and the remaining five animals were given saline. Sixty minutes after the administration, the animal was anesthetized and the blood sample was collected from the abdominal aorta to analyze serum glucose, electrolytes, and arterial blood gas levels according to routine laboratory procedures.

2.6. Experiment 3 $[Ca^{2+}]_i$ by microfluorometry

In this experiment, six gerbils were prepared and assigned to the control and metyrapone groups (three animals each). Animals in the metyrapone group were injected metyrapone (100 mg/kg) intraperitoneally, and control animals were injected saline. The increase in $[Ca^{2+}]_i$ provoked by in vitro hypoxia was observed by microfluorometry as described previously (Liu et al., 1997). In brief, gerbils were anesthetized and decapitated 60 min after the administration of metyrapone or saline. Hippocampal slices, 300 μm thick, were incubated in an artificial cerebrospinal fluid (mM: NaCl 124; KCl 5; $CaCl_2$ 2; $MgCl_2$ 2; NaH_2PO_4 1.25; $NaHCO_3$ 26; glucose 10) equilibrated with a 95% O_2/5% CO_2 gas mixture for 60 min at 26°C, then preloaded with a fluorescent indicator, rhod-2 acetoxymethyl ester (20 μM). The slice was transferred to a flow-through chamber mounted on the fluorescence microscope and perfused with normoxic medium equilibrated

with a 95% O_2/5% CO_2 gas mixture at a rate of 3 ml/min at 36.5°C. The slice was excited with 550 nm light, and the fluorescence signals (> 580 nm) were captured and processed (Argus-100; Hamamatsu Photonics, Hamamatsu, Japan).

After 15 min, in vitro hypoxia was induced by switching the normoxic medium to a glucose-free hypoxic medium equilibrated with a 95% N_2/5% CO_2 gas mixture. The fluorescence intensity of each pixel was divided by the fluorescence intensity of the corresponding pixel that had been taken before the induction of hypoxia. Thus, the ratio of fluorescence intensity of $[Ca^{2+}]_i$ was obtained every 10 s.

The effect of in vitro hypoxia in a condition free from extracellular Ca^{2+} was examined. After perfusion with Ca^{2+}-containing normoxic medium for 15 min, the medium was changed to Ca^{2+}-free normoxic medium that had been prepared by replacing $CaCl_2$ with $MgCl_2$ and adding 0.5 mM ethylenediaminetetraacetic acid. After 10 min, the medium was switched to Ca^{2+}-free hypoxic medium.

2.7. Drugs and chemicals

Metyrapone was purchased from Sigma (St. Louis, MO, USA). Halothane was obtained from Takeda Chemical Industries (Osaka, Japan). Rhod-2 acetoxymethyl ester was obtained from Dojin (Kumamoto, Japan). Other chemicals were all of reagent grade.

2.8. Statistical analysis

The data from the histology were evaluated with the Kruskal–Wallis test followed by the Mann–Whitney test. The data from microdialysis and microfluorometry were analyzed using repeated measures two-way analysis of variance to detect differences among groups. When differences were found, Fisher's protected least-squares difference test was used post hoc to compare each value with that in the control group. The data from blood analysis and the onset latency of the increase in $[Ca^{2+}]_i$ were evaluated by analysis of variance with Bonferroni's adjustment.

3. Results

3.1. Histology

As shown in Fig. 1, the number of hippocampal CA1 pyramidal neurons in the nonischemic (sham-operated) group was 235 ± 3/mm (mean ± S.D., $n = 5$). The treatment with metyrapone did not affect the number of preserved neurons in the nonischemic group (231 ± 9/mm, $n = 5$). Transient forebrain ischemia for 3 or 5 min provoked a severe damage in hippocampal CA1 pyramidal neurons after 7 days in saline-injected animals. Almost all neurons were degenerated, and the numbers of preserved neurons were 8 ± 6/mm ($n = 8$) and 5 ± 2/mm ($n = 5$), respectively. The pre-ischemic administration of metyrapone significantly reduced the damage provoked by 3 min of ischemia (166 ± 63/mm, $n = 8$). However, the treatment did not improve the damage provoked by 5 min of ischemia (5 ± 3/mm, $n = 5$).

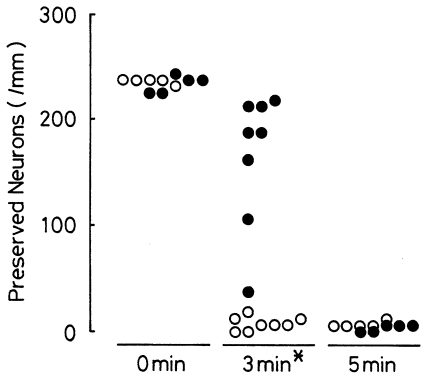

Fig. 1. Effects of metyrapone (100 mg/kg, intraperitoneally) administered 60 min before ischemia on the delayed neuronal death of CA1 pyramidal neurons. CA1 pyramidal neurons were examined 7 days after the operation, and the number of pyramidal cells (ordinate) was determined. Values obtained from individual animals are shown; saline-injected animals (○) and metyrapone-injected animals (●). *$P < 0.01$ compared with each corresponding saline-injected group.

3.2. Amino acid release

There were no significant differences in the values of amino acids in dialysates before ischemia between the control and metyrapone groups (Fig. 2). In the control group, transient ischemia for 3 min produced increases in amino acids immediately after the start of ischemia, and the levels of aspartate, glutamate, and glycine reached 231%, 406%, and 178% of their pre-ischemic values, respectively. The values decreased immediately after reperfusion. The pretreatment with metyrapone affected the time course of the glutamate concentration and suppressed the peak value to 42% of that in the control group.

3.3. Physiologic variables

There were no differences in plasma glucose, electrolytes, and arterial blood gas levels between the two groups (Table 1).

3.4. Microfluorometry

When the hippocampal slices were perfused with hypoxic medium in the in vitro experiment, almost no increase in $[Ca^{2+}]_i$ was observed in the hippocampal CA1 field within 250 s after the beginning of hypoxia (Fig. 3). Subsequently, a large increase in $[Ca^{2+}]_i$ spread throughout the CA1 field, and the ratio of fluorescence intensity reached a plateau. The onset of the beginning of the

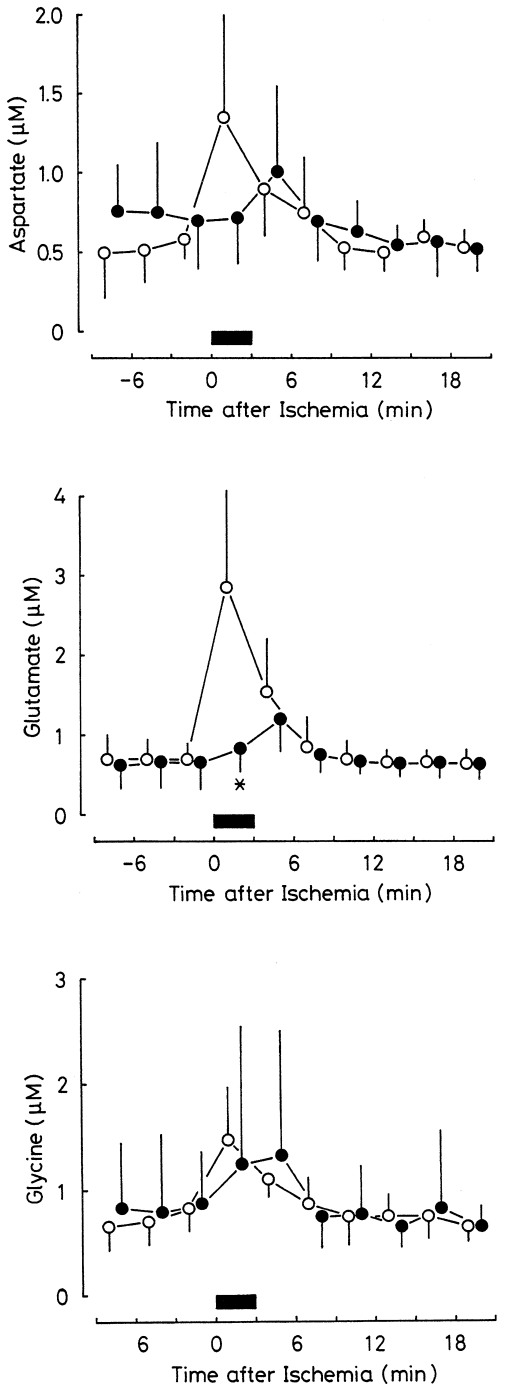

Fig. 2. Effects of metyrapone (100 mg/kg, intraperitoneally) administered 60 min before ischemia on changes in the concentration of amino acids in dialysates. Solid rectangles represent the duration of transient forebrain ischemia. The control group (○), the metyrapone (100 mg/kg)-treated group (●). Each value represents the mean ± S.D. of nine animals. Changes in the glutamate concentration by metyrapone were significant: $F = 6.2$, $P = 0.02$ for the drug, $F = 22.0$, $P < 0.0001$ for time, and $F = 13.3$, $P < 0.0001$ for the drug × time interaction. $^{*}P < 0.05$ compared with the respective values in the control group.

increase in $[Ca^{2+}]_i$ was 297 ± 19 s ($n = 10$). The ratio 600 s after the start of hypoxia reached 3.7 times the value observed immediately before hypoxia. The pretreatment with metyrapone exert an influence on the time course of

Table 1
Blood analysis

	Control	Metyrapone
Glucose (mg/dl)	124 ± 45	116 ± 28
Na^+ (mM)	145 ± 4.4	146.6 ± 1.8
K^+ (mM)	3.9 ± 0.4	3.4 ± 0.3
Ca^{2+} (mM)	0.96 ± 0.18	1.00 ± 0.12
pH	7.430 ± 0.035	7.382 ± 0.078
PCO_2 (mm Hg)	45.2 ± 7.3	44.0 ± 3.3
PO_2 (mm Hg)	229.3 ± 45.7	264.5 ± 37.0
HCO_3^- (mM)	29.2 ± 2.7	25.7 ± 4.0
Base excess (mM)	4.6 ± 1.4	0.7 ± 4.9

Metyrapone (100 mg/kg) or saline was administered intraperitoneally. Then, 1 ml blood sample was collected through the abdominal aorta under halothane anesthesia 60 min after injection. Each value represents the mean ± S.D. of five animals.

$[Ca^{2+}]_i$. The onset latency of the increase in $[Ca^{2+}]_i$ in the metyrapone group was later than that in the control group, the value being 338 ± 26 s ($P < 0.01$). The extent of the increase in fluorescence intensity of $[Ca^{2+}]_i$ in the metyrapone group after 600 s was suppressed to 84% of that in the control group.

When the slices were perfused with Ca^{2+}-free hypoxic medium, an increase in $[Ca^{2+}]$ was more gradual and occurred to a lesser extent compared with the increase in the Ca^{2+}-containing condition. The metyrapone treatment further reduced the increase in fluorescence intensity of $[Ca^{2+}]_i$ by perfusion with Ca^{2+}-free hypoxic medium. The onset of the beginning of the increase in the $[Ca^{2+}]_i$ was later in the metyrapone group than in the Ca^{2+}-free control

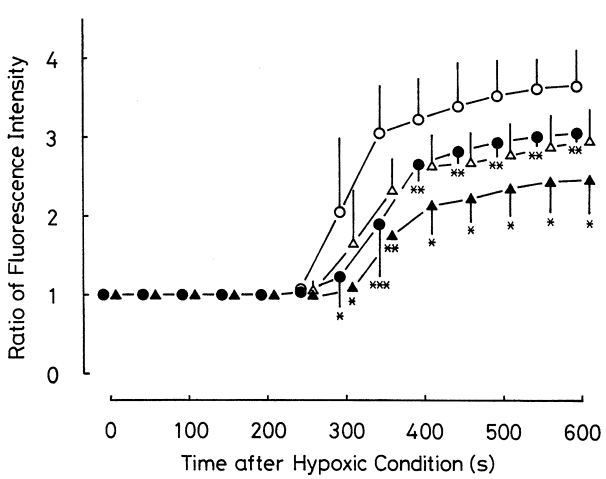

Fig. 3. Changes in the ratio of $[Ca^{2+}]_i$ in slices of the gerbil hippocampal CA1 field which underwent in vitro hypoxic conditions. The control group (○), the metyrapone (100 mg/kg)-treated group (●), the Ca^{2+}-free control group (△), and the Ca^{2+}-free metyrapone-treated group (▲). Each value represents the mean ± S.D. of 10 slices. Changes in the ratio by metyrapone were significant: $F = 17.5$, $P = 0.0006$ for the drug, $F = 249.8$, $P < 0.0001$ for time, and $F = 8.4$, $P < 0.0001$ for the drug × time interaction in the Ca^{2+}-containing condition; $F = 10.6$, $P = 0.004$ for the drug, $F = 214.2$, $P < 0.0001$ for time, and $F = 5.5$, $P < 0.0001$ for the drug × time interaction in the Ca^{2+}-free condition. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the respective values in each corresponding control group.

slices. The values in the control and metyrapone groups were 290 ± 35 and 324 ± 21 s, respectively ($P < 0.05$). The extent of the increase in fluorescence intensity in the metyrapone group after 600 s was 84% of that in the Ca^{2+}-free control group.

4. Discussion

In the present study, we observed a beneficial effect of metyrapone on hippocampal neurons, and an inhibition of the ischemic increase in the extracellular concentration of glutamate in metyrapone-treated animals. We also observed a suppression of the hypoxic increase in $[Ca^{2+}]_i$ in slices from metyrapone-treated animals.

The central nervous system contains virtually no reserve supply of oxygen and only small stores of glucose or energy-rich compounds, which are essential to maintain neuronal activity. In ischemia, an energy failure triggers excess release of excitatory neurotransmitters such as glutamate and aspartate (Jørgensen and Diemer, 1982; Benveniste et al., 1984), which causes a marked influx of Ca^{2+} into postsynaptic neurons(Mitani et al., 1994). These provoke the catastrophic enzymatic process leading to irreversible neuronal injury and cerebral edema. Therefore, the inhibition of the ischemic increase in the extracellular concentration of glutamate observed in the present microdialysis experiment may contribute to the improvement of histologic outcome by metyrapone. Further, the neurotoxic effect of glucocorticoids in ischemia has been reported to be prevented by the blockade of NMDA receptors (Armanini et al., 1990), suggesting that the deleterious effect is dependent on the excitotoxicity of glutamate. These findings, taken together with our results, imply that the beneficial effect of metyrapone is due to the suppression of the glutamate release.

Since glucocorticoids inhibit glucose uptake into hippocampal neurons and glia both in vivo and in vitro (Kadekaro et al., 1988; Horner et al., 1990; Virgin et al., 1991), endogenous glucocorticoids probably also deplete neurons of their energy. Thus, the removal of endogenous glucocorticoids by metyrapone administration may make neurons relatively energy-rich by enhancing glucose uptake. Since a glutamate release during ischemia occurs when the energy store is depleted, the beneficial effect in histology may be caused by the improvement of the intracellular energy state by metyrapone. On the other hand, released glutamate is mainly taken up into the glia, and glucocorticoids have been reported to inhibit the uptake of glutamate into astrocytes (Virgin et al., 1991; Chou et al., 1994). Because the treatment with metyrapone reduces the plasma concentration of endogenous glucocorticoids, metyrapone may also take part in the removal of the extracellular glutamate by facilitating the uptake. Thus, metyrapone may remove the extracellular glutamate by both inhibiting the release and enhancing the uptake. Furthermore, since the plasma concentration of corticosterone has been demonstrated to remain high even when the blood flow resumed (Fassbender et al., 1994), metyrapone may contribute to the improvement by alleviating the energy crisis in the postischemic period as well as in an ischemic event.

An acute and sudden increase in $[Ca^{2+}]_i$ in the CA1 field was observed in the present in vitro experiment. This finding is in good agreement with the selective vulnerability in this area, because the elevation of $[Ca^{2+}]_i$ following ischemia is a crucial factor in the development of neuronal damage (Rothman and Olney, 1995). The initiation of the increase in $[Ca^{2+}]_i$ was delayed by the pretreatment with metyrapone, and the extent of the increase was also suppressed by metyrapone. These findings are in parallel with changes in the extracellular glutamate in the microdialysis experiment. Since excess release of glutamate in ischemia induces the influx of Ca^{2+} into neurons (Stein and Sapolsky, 1988), the diminution of the extracellular concentration of glutamate by metyrapone may be a mechanism responsible for the suppression of the hypoxic $[Ca^{2+}]$ increase.

Two mechanisms are conceivable for the increase in $[Ca^{2+}]_i$. One is the influx of Ca^{2+} from the extracellular space, and the other involves the efflux of Ca^{2+} from the intracellular Ca^{2+} stores such as the endoplasmic reticulum and mitochondria. In the present experiment, the increase in $[Ca^{2+}]_i$ was still observed in the Ca^{2+}-free hypoxic condition, although the extent of the increase was smaller than that in the Ca^{2+}-containing condition. Therefore, the $[Ca^{2+}]_i$ increase observed in the Ca^{2+}-free condition seems to be caused by the element from intracellular Ca^{2+} stores. Since the metyrapone treatment further suppressed the increase in $[Ca^{2+}]_i$ in the Ca^{2+}-free hypoxic condition, metyrapone may reduce the release of Ca^{2+} from intracellular Ca^{2+} stores as well as the influx from the extracellular space. Furthermore, glucocorticoids have been shown to increase basal $[Ca^{2+}]_i$ in a nonischemic state, and this increase has been thought to be caused by impairment of the cytosolic Ca^{2+} efflux to the extracellular space (Elliott and Sapolsky, 1993). Therefore, the reduction of the action of endogenous glucocorticoids by metyrapone may suppress the $[Ca^{2+}]_i$ elevation by reducing the basal $[Ca^{2+}]_i$.

In conclusion, the blockade of the synthesis of endogenous glucocorticoids by metyrapone inhibited the release of glutamate and the increase in $[Ca^{2+}]_i$ in ischemia, then improved neuronal damage. The increase in the level of endogenous glucocorticoids in ischemia may partly take part in the aggravation of ischemic neuronal damage.

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