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# Intracellular $Ca^{2+}$, $Na^{+}$, and ATP Homeostasis Mediated NMDA-induced Excitoprotection in Rat Cerebellar Granule Neurons

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**Abstract** **Objective**: To study the mechanisms underlying *N*-methyl-*D*-aspartate (NMDA)-induced excitoprotection in cultured rat cerebellar granule neurons. **Methods**: Intracellular $Ca^{2+}$ ($[Ca^{2+}]i$) and intracellular $Na^{+}$ ($[Na^{+}]i$) were measured microspectrofluorimetrically with the $Ca^{2+}$ sensitive dye fura-2/Am and the $Na^{+}$-sensitive indicator SBFI/AM respectively. Cytosolic denosine triphosphate(ATP) was quantified by means of reverse-phase HPLC. **Results**: First. Glutamate rapidly induced a peak $[Ca^{2+}]i$ response in both untreated and NMDA treated neurons. In NMDA treated neurons, $[Ca^{2+}]i$ then rapidly dropped to a stable plateau concentration above that of the basal condition. Thirty minutes later, with the removal of glutamate, $[Ca^{2+}]i$ rapidly returned to near basal concentrations. In contrast, with NMDA-untreated neurons, prolonged glutamate exposure resulted in a heterogeneous $[Ca^{2+}]i$ response in many neurons, with $[Ca^{2+}]i$ either gradually or abruptly increasing. Upon glutamate removal, many untreated neurons failed to return to basal levels. Second, Glutamate rapidly induced a peak $[Na^{+}]i$ response in both untreated and NMDA-treated neurons. However, the glutamate-induced $[Na^{+}]i$ peaks during and after glutamate were all higher in untreated neurons, compared with NMDA-pretreated neurons. Third, Glutamate induced the consumption of ATP stores in the neurons. However, ATP consumption in the neurons pretreated with NMDA was significantly reduced, compared with neurons not pretrect. **Conclusions**: NMDA-induced excitoprotection in cerebellar granule neurons is associated with enhanced $[Ca^{2+}]i$ and $[Na^{+}]i$ homeostasis by reducing ATP consumption.

**Subject headings** glutamate; neurons; calcium; sodium; MK801; excitatory amino acids, *N*-methylaspartate; neuroprotective agents

# 胞内 $Ca^{2+}$、$Na^{+}$ 和 ATP 的稳定性介导 NMDA 诱导兴奋保护作用

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摘 要 目的:研究 *N*-甲基-*D*-天冬氨酸(NMDA)诱导大鼠小脑颗粒神经元兴奋毒性保护作用的机制。方法:分别用 $Ca^{2+}$ 和 $Na^{+}$ 敏感的染料 Fura-2/AM 和 SBFI/AM 测定胞内 $Ca^{2+}$ 和 $Na^{+}$ 浓度[$(Ca^{2+})i$,$(Na^{+})i$]。并用反向高效液相色谱法测定胞内三磷酸腺苷(ATP)的含量。结果:①在 NMDA 预处理组与非处理组,谷氨酸均迅速触发胞内 $Ca^{2+}$ 反应高峰,两者无显著性差异。而后在 NMDA 处理组,胞内 $Ca^{2+}$ 迅速下降,并维持在高于静息 $Ca^{2+}$ 水平的平台状,撤离谷氨酸后,$[Ca^{2+}]i$ 迅速下降并恢复至静息水平;而 NMDA 非处理组则相反。②谷氨酸均诱发上述两组神经元 $[Na^{+}]i$ 升高,在 NMDA 非处理组,$[Na^{+}]i$ 均高于 NMDA 预处理组。③谷氨酸消耗胞内 ATP,而 NMDA 预处理组则减少胞内 ATP 的耗竭。结论:NMDA 诱导兴奋毒性保护作用是通过减少 ATP 的消耗而增强胞内 $Ca^{2+}$ 和 $Na^{+}$ 的自稳态。

主题词 谷氨酸;神经元;钙;钠;MK801;兴奋性氨基酸类;N-甲基-天冬氨酸;神经保护药
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Glutamate, the major excitatory neurotransmitter, is involved in many important CNS functions. However, it can also be an excitotoxin and is thought to be involved in the pathogenesis of neuronal death due to cerebral ischemia and trauma. Application of toxic concentrations of glutamate to cerebellar granule neurons can produce excessive increases in intracellular calcium ($[Ca^{2+}]i$) and results in cell death[1]. Over stimulation of both *N*-

methyl-*D*-aspartate (NMDA) and non-NMDA receptor subtypes may be involved in the excitotoxicity of glutamate. Exposure of cultured rat cerebellar granule neurons to subtoxic NMDA concentration has previously been shown to result in a neuroprotective state, as measured by subsequent exposure to toxic concentrations demonstrated by reduced susceptibility to glutamate[2]. Also, we showed that subtoxic concentrations of NMDA could shift the concentration-response for glutamate toxicity in a concentration and time-dependent manner. This effect has been shown to be dependent on protein synthesis[3]. In this study we have further characterized intracellular excitoprotection in cerebellar granule neurons including: changes in intracellular calcium ($[Ca^{2+}]i$), intracellular sodium ($[Na^{+}]i$) homeostasis and alteration of the energy metabolic state.

# 1 Materials and Methods

## 1.1 Materials

L-Glutamate, NMDA, and MK-801 were from RBI. Fura-2/AM and SBFI/AM were from molecular probes. Ionomycin was from calbiochem. Ethylene imine polymer was from fluka chemical corp. All other chemicals were purchased from Sigma.

## 1.2 Primary neurons culture

Rat cerebellar granule neurons were prepared from 8-day-old Sprague-Dawley rat pups as described previously[3]. Briefly, neurons were dissociated from freshly dissected cerebellar by mechanical disruption in the presence of trypsin and DNase, and then plated in poly-l-lysine-coated dishes. Cells were seeded at a density of $(1.5 \sim 1.8) \times 10^6$ cells/mL in basal modified Eagle's medium (BME) containig 10% fetal bovine serum and 25 mmol/L KCl. Cytosine arabinoside (10 μmol/L) was added to the culture medium after 24h to arrest the growth of non-neuronal cells. D-Glucose (5 mmol/L) was added to the cultures on day 7 and every fourth day thereafter.

## 1.3 Measurement of Intracellular $Ca^{2+}$

Measurement of $[Ca^{2+}]i$ was carried out as previously described using microspectrofluorimetry and the $Ca^{2+}$ sensitive indicator fura-2[4]. In brief, NMDA (50 μmol/L) was added to the culture medium of pretreated neurons for 24 h. Neurons were incubation with 5 μmol/L Fura-2 for 45 min. Cells were then washed twice with BME containing 25 mmol/L KCl and 10 μmol/L glycine. The cells were continuously perfused with same buffer at 37 ℃. Following that $[Ca^{2+}]i$ was measured for 5 min as a basal $[Ca^{2+}]i$ of neurons, glutamate then was perfused for 30 min. The same buffer was then used to perfuse for another 30 min to wash out glutamate. Neurons were illuminated on an inverted microscope (Nikon Diaphot). Excitation of fura-2 was at 340 and 380 nm. Cell images were digitized and stored for subsequent analysis (Universal Imaging Co, USA). Calculation of $[Ca^{2+}]i$ was carried out as described by Grynkiewicz *et al*[5].

## 1.4 Measurement of Intracellular $Na^{+}$

Measurement of $[Na^{+}]i$ was carried out as previously described using microspectrofluorimetry and the $Na^{+}$ sensitive indicator SBFI/AM[6]. Briefly, cells was loaded with SBFI/AM (10 μmol/L) and returned to the incubator for 45 min. Cells were washed 3 times and allowed ≥15 min to complete the hydrolysis of the ester linkage before starting imaging. Cells were imagined using identical conditions to that for fura-2 experiments with excitation wavelengths, 340 nm and 380 nm and emitted light monitored at 510 nm. Background intensity levels were subtracted and in situ calibrations were carried out as descried by Harootunian *et al*[7].

## 1.5 Measurement of ATP

Untreated and NMDA-treated neurons were exposed to glutamate (100 μmol/L) for 30 min. Cytosolic contents were then extracted with HCl and adenosine phosphonucleotide ([ATP]) levels were quantified with RP-HPLC before exposure to glutamate, following glutamate and removal glutamate[8].

## 1.6 Statistical Analysis

Data are presented as $\bar{x} \pm s$. Unless otherwise stated, statistical comparisons were made using student *t*-test.

# 2 Results

## 2.1 NMDA-induced excitoprotection enhances $[Ca^{2+}]i$ homeostasis

Since glutamate-induced cell death is related to increase in $[Ca^{2+}]i$[1], we measured changes in $[Ca^{2+}]i$ associated with a 30 min exposure to glutamate in NMDA-pretreated and untreated cerebellar granule neurons in cultures. In NMDA-pretreatment of granule neurons resulted in a lessees reduction in basal $[Ca^{2+}]i$ but glutamate rapidly induced a peak $[Ca^{2+}]i$ response in both untreated and NMDA treated neurons. In NMDA treated neurons, $[Ca^{2+}]i$ then rapidly lowered to a stable plateau concentration above that of the basal condition. Thirty minutes later with the removal of glutamate, $[Ca^{2+}]i$ rapidly returned to near basal concentrations. In contrast, in case of untreated neurons prolonged glutamate exposure resulted in a heterogeneous $[Ca^{2+}]i$ response with many neurons gradually or abruptly increased $[Ca^{2+}]i$. Upon removal of glutamate many untreated neurons failed to return to basal levels (Fig 1).

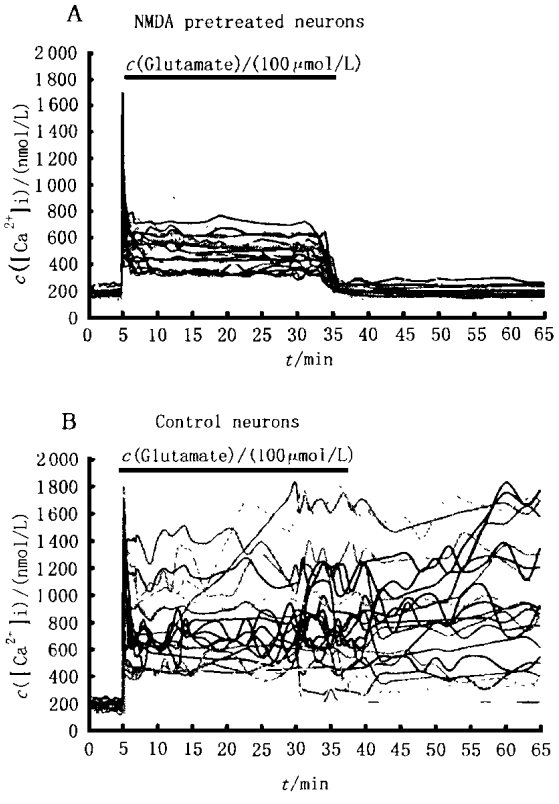


**Fig. 1 NMDA-induced excitoprotection enhances $[Ca^{2+}]i$ homeostasis**

$[Ca^{2+}]i$ was measured with the fluorescent $Ca^{2+}$ indicator Fura-2. A, following recording basal $[Ca^{2+}]i$, glutamate 100 μmol/L was applied for 30 min to cerebellar granule neurons pretreated for 24 h with NMDA (50 μmol/L). The experiment was terminated 30 min following application of glutamate. Each line (—) represent the calculted $[Ca^{2+}]i$ concentration of one cerebellar granule neuron. B as in (A) but cerebellar granule neurons were untreated with NMDA ($n=30$ in each condition)

To assess the role of the glutamate receptor subtypes in the NMDA-induced changes in $Ca^{2+}$ homeostasis, we used the noncompetitive NMDA antagonist MK-801 to block the NMDA component of the glutamate-induced rise in $[Ca^{2+}]i$. Experiments were performed as those with glutamate exposure alone except MK-801 (1 μmol/L) was perfused over the neurons for 2 min before addition of glutamate. MK-801 markedly reduced the glutamate-induced rise in $[Ca^{2+}]i$ and eliminated the differences responce between untreated and NMDA pretreated neurons in the 30 min during and after glutamate exposure.

### 2.2 NMDA-induced excitoprotection enhances $[Na^+]i$ homeostasis

To examine the effects of NMDA pretreatment on intracellular $Na^+$ ($[Na^+]i$) homeostasis, we measured $[Na^+]i$ using the $Na^+$ sensitive indicator SBFI/AM. $[Na^+]i$ of cerebellar granule neurons was monitored before, during and following exposure to 100 μmol/L glutamate. Glutamate-induced a rapid elevation of $[Na^+]i$ from basal levels (Fig 2).

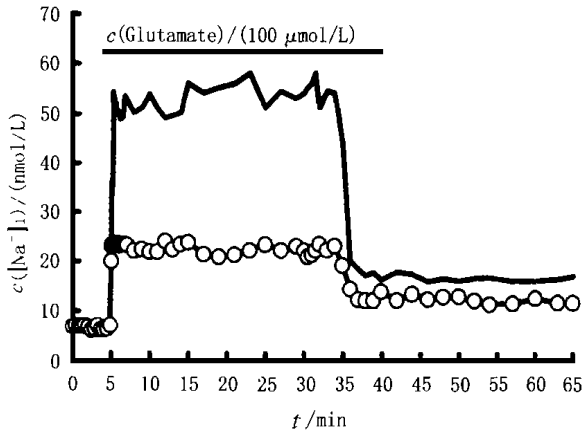


**Fig. 2 NMDA-pretreatment of cerebellar granule neurons attenuates glutamateinduced elevation of intracellular $Na^+$**

$c([Na^+]i)$ was measured with the fluorescent $Na^+$ indicator SBFI/AM. The drug application as described in Fig1. Straight-line (—) represent the mean of calculated $[Na^+]i$ concentration of NMDA-untreated cerebellar granule neurons. Circle line (-o-) represent the mean of calculated $[Na^+]i$ concentration of preexposed to NMDA (50 μmol/L) for 24 h ($n=161$ in each contion)

In both untreated and NMDA pretreated neurons, glutamate-induced a stable plateau concentration of $[Na^+]i$ above that of the basal condition. However, the

magnitude of the response differed greatly between these groups. The glutamate-induced $[Na^{+}]i$ peak, during glutamate and the post glutamate (30 min) were both greater in the untreated than in the NMDA pretreated neurons.

### 2.3 NMDA-pretreatment effects on glutamate-induced changes of ATP content

ATP content was determined on cerebellar granule neurons before glutamate, following a 30 min exposure to glutamate (100 μmol/L) and 30 min following the washout of glutamate. NMDA treatment did not alter the mean ATP (% of control) content. Exposure to glutamate induced a consumption of ATP store. However, pretreatment with NMDA reduced the consumption of ATP (18% difference) than observed in untreated cells. Even a larger degradation (28% difference) follows a 30 min recovery period in untreated cells (Fig 3).

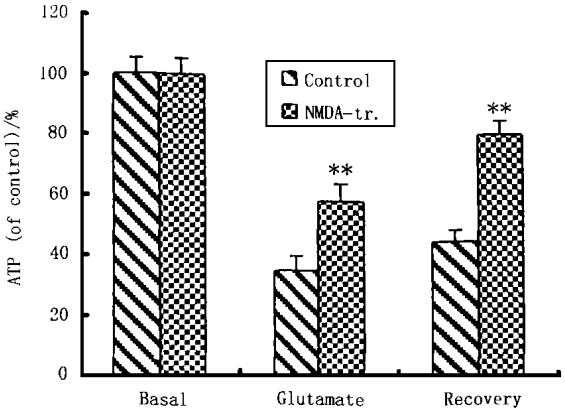


**Fig. 3 NMDA-pretreatment of granule neurons reduced glutamate-induced depletion of ATP content**

Data are from a representative experiment repeated three times with qualitatively similar results. Each point represents a mean of four replicates. * Statistically significant difference ($P < 0.05$) between. NMDA-pretreated and untreated

## 3 Discussion

In the present study we have extended our previous ly findings that preincubation of cultured cerebellar granule neurons with subtoxic concentrations of NMDA for 24 h results in the induction of exitoprotective state as measured by subsequent exposure to glutamate. We found that NMDA pretreatment-induced alterations of $Ca^{2+}$ and $Na^{+}$ homeostasis in cerebellar granule neurons. Since MK-801 can completely block glutamate toxicity at the concentrations used in this study[3], could the NMDA-induced alterations of $Ca^{2+}$ homeostasis in cerebellar granule neurons be simply due to "down-regulation" of NMDA receptors? We previous reported that exposure of NMDA results in no change of specific $[^{3}H]$ MK-801 binding to NMDA receptors and the immediate peak response in $[Ca^{2+}]i$ induced by glutamate was unaltered in cerebellar granule neurons preexposed to NMDA. Additionally, NMDA receptor-mediated neuroprotection extends to $MPP^{+}$ toxicity in cerebellar granule neurons[9]. It is suggested that the NMDA-induced excitoprotection and maintain of $Ca^{2+}$ homeostasis in cerebellar granule neurons is not due to "down-regulation" of NMDA receptors. Under both basal and pathological conditions, intracellular free $Ca^{2+}$ concentration is stringently controlled because excessive $Ca^{2+}$ influx is lethal to neurons. The transmembrane $Na^{+}$ gradient is also tightly regulated; excessive $Na^{+}$ influx which occurs under excitotoxic or ischemic conditions, may lead to inordinate excitatory amino acid efflux, as well as osmotic imbalance, cell swelling, and lysis[10]. Our data show that glutamate-induced influx of $Na^{+}$ into the cerebellar granule neurons both in NMDA treated and untreated neurons, similar pattern was observed with $Ca^{2+}$ influx. Levels of $Na^{+}$ were attenuated in NMDA treated neurons in contrast to untreated neurons. Recently, Keidrowski et al. Pointed out that glutamato-evoked increases in $[Na^{+}]i$ may play a role in $Ca^{2+}$ homeostasis destabilization. This is consistent with our observations which higher glutamate-evoked levels of $[Na^{+}]i$ in untreated neurons was associated with impaired ability to maintain $[Ca^{2+}]i$ homeostasis. Perhaps this is due to impairment of the ability of these neurons to extrude $Ca^{2+}$ via the $Na^{+}/Ca^{2+}$ exchanger. In contrast, NMDA treated neurons had lower glutamate-induced elevation of $[Na^{+}]i$ and enhanced ability to maintain $[Ca^{2+}]i$ level. At present it is not clear whether the glutamate-evoked $[Na^{+}]i$ level is higher in untreated neurons may due to enhanced $[Na^{+}]i$ influx or reduced efflux from the cell. Maintaining ATP levels is essential to maintain an effective transmembrance $Na^{+}$ gradient. Our data show

that cells treated with NMDA have the same basal ATP content as untreated neurons, but are better than untreated neurons to maintain ATP when exposed to glutamate. Lower ATP can impair ATP dependent (i. e. $Na^+/K^+$ ATPase) extraction of $Na^+$ from the cells. In addition, lower ATP may reduce activity of endoplasmic $Ca^{2+}$ ATPase thereby decreasing $Ca^{2+}$ uptake into intracellular stores and further destabilize $Ca^{2+}$ homeostasis. This may explain why $[Na^+]i$ levels are higher in untreated neurons. It is not understood what mechanism for responsibility to reducing the consumption of ATP content while exposure to glutamate in NMDA-treated neurons. There was an evidence in our previously reported that NMDA receptor-mediated neuroprotection in cerebellar granule cells requires new RNA and protein synthesis[9], suggesting that some transcription steps are involved in the excitoprotection mechanism.

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◦新成果◦

# 现代辅助生育系列技术的建立

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体外受精与胚胎移植(IVF-ET)是现代辅助生育中最有代表性的技术。正是这项技术,促进临床与基础学科紧密结合,并促进妇产科与男性科、生物学、遗传学、分子生物学等多个学科的合作;其最终的目的是研究人类生殖医学,解决难治性不育症以及人类的优生问题。本系列研究于1990年起建立:①以常规IVF-ET即第一代试管婴儿技术为起点,结合我国情况,不断改进技术,阶段性总结,从超排卵方案选择与超排卵引起的过度刺激征与多胎妊娠处理以及实验配子处理技术,积累自己的经验建立起适合我国国情,可以推广发展应用的现代辅助生育系列技术。使近年来每年700~800治疗周期的临床妊娠率保持在30%左右,出生试管婴儿达500个,成为国内最大的生殖医学研究中心。②在较充实的临床与实验基础上1995年发展单精子卵胞浆内显微注射(ICSI)受精技术,即第二代试管婴儿技术;ICSI的应用成功是治疗男性不育的一块里程碑,造福了千万个家庭。于1996年10月诞生了我国首例ICSI婴儿。③接着将显微受精与分子生物学技术相结合演进到目前已初步取得成功的人类胚胎种植前基因诊断(PGD)的人类优生第三代试管婴儿技术。第三代试管婴儿技术可以避免有遗传病孩出生,实现优生优育,减少社会和家庭负担。本项目使我国生殖医学技术迅速推上先进的国际行列。1999年荣获广东省科技进步一等奖。

(陈丽芳)