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Nerve growth factors prevent glutamate toxicity in cortical neuronal cultures[1]

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KEY WORDS calcium; cerebral cortex; cultured cells; glutamates; intracellular fluid; nerve growth factors; neurons

AIM: To determine if nerve growth factors (NGF) can protect against glutamate-induced cortical neuron damage. **METHODS**: Neuron viability and lactate dehydrogenase (LDH) efflux in the bathing medium in primary cultures from 17-d-old mouse fetal cortex were measured to assay NGF effect. Imaging of the calcium indicator dye Fura-2 was used to measure the $[Ca^{2+}]_i$. **RESULTS**: The LD_{50} for NGF-free glutamate was 0.2 mmol·L^{-1} (95 % confidence limits 0 - 1.6 mmol·L^{-1}). In the presence of NGF 60 μg·L^{-1}, 59 % of the neurons survived in glutamate 1.6 mmol·L^{-1}. The protective effect afforded by NGF was maximal at 60 μg·L^{-1}, at which it prevented the elevation in $[Ca^{2+}]_i$. **CONCLUSION**: NGF protect cortical neurons against glutamate-induced toxicity via "stabilizing" $[Ca^{2+}]_i$ level or suppression of the rise in $[Ca^{2+}]_i$.

Persistent stimulation of glutamate receptors causes neurotoxicity and neuronal death. At least 5 subtypes of glutamate receptors have been identified. Some of these are ligand-gated ion channels and others are coupled to the breakdown of inositol phospholipids. Activation of ion channel-glutamate receptors results in an influx of Ca^{2+} into the neuron. The main routes of Ca^{2+} entry into the neuronal cytoplasm include voltage-sensitive calcium channels (VSCC) and excitatory amino acid (EAA) receptor channels. Toxic doses of glutamate result in an increase of intracellular free Ca^{2+} ($[Ca^{2+}]_i$), which persists after the removal of the amino acid from the medium[1]. It seems that the disruption of intracellular Ca^{2+} homeostasis leads to neuronal death. However, the molecular mechanisms involved are not well understood.

Recent data indicated that calcium channels and $[Ca^{2+}]_i$ can be modulated by growth factors. In dorsal root ganglion neurons nerve growth factors (NGF) was found to regulate the Ca^{2+} component of the action potential[2]. In PC12 cells NGF increased both Ca^{2+} current and Ca^{2+} channel number and caused Ca^{2+} channel redistribution[3]. Furthermore, a study in PC12 cells indicated that NGF causes the phosphorylation of a novel Ca^{2+} channel[4]. The other findings have shown that growth factors can protect CNS neurons against axotomy-induced death[5]. Similarly, Hyman *et al*[6] showed that brain-derived neurotrophic factor (BDNF), but not NGF or basic fibroblast growth factor (bFGF), protects rat ventral mesencephalic dopaminergic neurons against MPP^+ (1-methyl-4-phenylpyridinium) toxicity, and *in vivo* intra-cerebral implantation of genetically engineered NGF-secreting fibroblasts has been shown to afford similar protection against EAA neurotoxicity[7].

The present study was designed to investigate whether the neuroprotective potential of NGF, a neurotrophic factor, was against glutamate insults and to explore modulation of intracellular calcium levels and cell survial by the NGF in primary neuronal cultures.

MATERIALS AND METHODS

Neuronal cultures Cortical neurons were prepared from 17-d-old mouse fetuses by incubation of the dissected hemi-spheres in trypsin 0.5 g·L^{-1} and edetic acid 0.53 mmol·L^{-1} at 37 ℃ for 10 min and subsequent mechanical disruption of the tissue in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution[8]. The cell pellet was resuspended in Eagle's Minimum Essential Medium (Gibco) supplemented with 10 % heat-inactivated fetal bovine serum, *L*-glutamine 2,

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sodium bicarbonate 10, KCl 20, pyruvate 1, insulin 5, and glucose 20 $mmol \cdot L^{-1}$. Cells were seeded onto L-polylysine-coated plates at 37 ℃, the medium containing unattached cells was removed and fresh medium was added 4 h later. The cell density was 6.4×10^8 $cells \cdot m^2$ of culture surface. To prevent proliferation of non-neuronal cells, cytosine arabinoside (10 $\mu mol \cdot L^{-1}$) was added 24 h after seeding. Cells were incubated at 37 ℃ in 5 % CO_2 atmosphere. The culture medium was renewed every 3 – 4 d.

Experiments with glutamate and NGF Experiments were carried out 12 – 14 d after seeding. Determination of glutamate neurotoxicity was carried out[8]. Before application of monosodium glutamate (Sigma) the culture medium was removed and the cells were washed once with Locke's solution without Mg^{2+} (NaCl 154, KCl 5.6, $NaHCO_3$ 3.6, $CaCl_2$ 2.3, HEPES 5 $mmol \cdot L^{-1}$, pH 7.4) containing glucose 5.6 $mmol \cdot L^{-1}$. Cells were incubated with glutamate in this modified Locke's solution at 37 ℃ for 4 h. The incubation was terminated by washing 3 times the monolayer with the modified Locke's 3-(4, 5-dimethyl-thiazoyl-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) staining and LDH efflux assay were determined immediately. For preincubation of neurons with NGF (Mouse 7s-NGF, Sigma), the culture medium was removed and, after washing, the NGF was added in the modified Locke's solution and incubated at 37 ℃ for 30 min, then glutamate was added.

MTT stains and LDH efflux assay For staining with MTT (Sigma) 10 μL of PBS (0.1 $mol \cdot L^{-1}$, pH 7.2) containing the dye (5 $g \cdot L^{-1}$) was added to 96-well plates (100 μL cell supernatent per well) and incubated at 37 ℃ for 4 h. SDS 10 % 100 μL was added to each well overnight to extract the blue formazan. Enzyme-linked immunosorbent assay was read at a wavelenghth of 570 nm[9]. Neurotoxicity was estimated by LDH activity released into media from damaged neurons[8].

Measurement of intraneuronal calcium Procedures for fluorescence of the calcium indicator dye Fura-2[10] were modified. The suspension cells were loaded with Fura 2-AM (Sigma) 2 $\mu mol \cdot L^{-1}$ at 37 ℃ for 45 min and collected by centrifugation at $1000 \times g$ for 5 min. The loaded cells were washed twice and incubated for 60 min prior to determinations of intraneuronal calcium with Locke's solution. The $[Ca^{2+}]_i$ was determined from the ratio of the fluorescence emission using 2 excitation wavelengths (350 and 380 nm) with Spex AR-CM-MIC cation measurement system. The system was calibrated according to:

$$[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)](F_D/F_S).$$

All data were presented as $\bar{x} \pm s$.

RESULTS

Effects of NGF on glutamate neurotoxicity

Glutamate-induced neurotoxicity was readily apparent by a reduction of neuron number and loss of neurites. It can be seen that a marked increase of cell number and clear neuron bodies inconnected by a dense fibre network when cultures were incubated with NGF (60 $\mu g \cdot L^{-1}$) prior to glutamate (1 $mmol \cdot L^{-1}$). In the absence of NGF, the LD_{50} for glutamate was 0.2 $mmol \cdot L^{-1}$. At 1.6 $mmol \cdot L^{-1}$ only 19 % of the cells survived. In the presence of NGF 59 % of neurons survived at 1.6 $mmol \cdot L^{-1}$ glutamate (Fig 1).

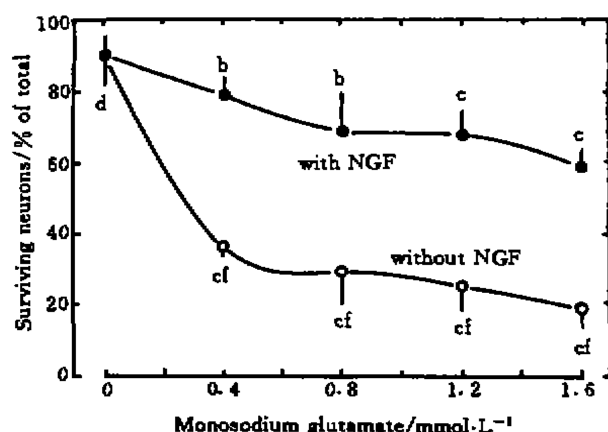

Fig 1. Neuronal death in primary cultures treated for 4 h with monosodium glutamate (○) and pretreated with NGF 60 $\mu g \cdot L^{-1}$ (●). n = 4 different plates per point. $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ vs control; $^dP>0.05$, $^fP<0.01$ vs NGF group.

The maximal protective effect was afforded by NGF 60 $\mu g \cdot L^{-1}$. Glutamate caused an efflux of LDH 325 ± 30 % of control after 3 h of exposure. Efflux of LDH was markedly diminished by pretreatment with 60 $\mu g \cdot L^{-1}$ NGF (Fig 2).

Effect of NGF on $[Ca^{2+}]_i$ Stimulation of Fura-2-loaded neurons with glutamate 1 $mmol \cdot L^{-1}$ produced a sustained increase of $[Ca^{2+}]_i$ which reached a maximum within 10 s. No measurable change in the $[Ca^{2+}]_i$ was observed after glutamate stimulation when NGF 60 $\mu g \cdot L^{-1}$ was added prior to glutamate (Fig 3).

DISCUSSION

The present data suggest that neuronal systems for calcium homeostasis are a common focus for the

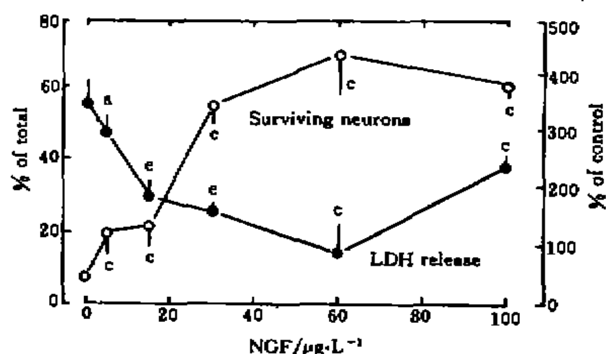

Fig 2. Effect of NGF against glutamate-induced neuronal death. $n = 4$ experiments. $^a P > 0.05$, $^c P < 0.01$ vs NGF-free group.

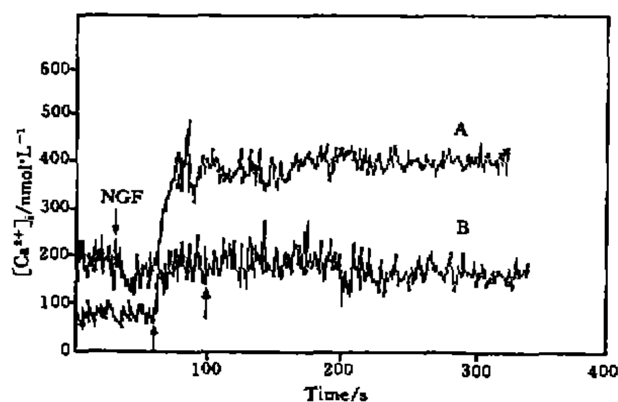

Fig 3. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) response to glutamate 1 mmol·L^{-1} (A), and pretreated with NGF 60 μg·L^{-1} prior to 1 mmol·L^{-1} glutamate (B). "↑" indicated addition of glutamate.

neurotoxic action of EAA and the neuroprotective effects of growth factors. Glutamate is the main EAA in the brain but under certain conditions it can become neurotoxic, which induces a receptor-mediated rise in the cytosolic free Ca^{2+} concentration[11]. A large body of evidence has associated delayed glutamate-induced neuronal death with sustained increase in $[Ca^{2+}]_i$[12], and this is a key step in the process of neuronal death which we proved. The findings indicate that NGF significantly prevent the toxicity of glutamate in cultured cortical neurons by suppressing intracellular calcium accumulation that caused cell damage. In other words NGF can "stabilize" $[Ca^{2+}]_i$ and thereby protect neurons against glutamate insults. However, in peripheral neurons NGF increases $[Ca^{2+}]_i$ and brings the calcium concentration to a level conductive to cell survival[10]. Taken together, these data suggest that NGF can prevent aberrant elevations or reductions in $[Ca^{2+}]_i$. These actions of NGF are very importance in their neuroprotective actions both in normal development of the nervous system and in pathological conditions. Focusing research on it may prove valuable for the development of preventative and therapeutic strategies for an array of neurological disorders.

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神经生长因子在培养的皮质神经细胞中抑制谷氨酸毒性

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关键词 钙；大脑皮质；培养的细胞；谷氨酸；细胞内液；神经生长因子；神经元

A 目的：确定 NGF 是否防止原代培养的神经细胞中谷氨酸引起的损伤. 方法：采用皮质神经细胞体外培养及形态学观察，测定神经细胞的生存力和 LDH 的释放来分析 NGF 的作用，并利用钙指示剂 Fura-2 来检测 $[Ca^{2+}]_i$ 的变化. 结果：NGF 阻止 $[Ca^{2+}]_i$ 的增加，并且对谷氨酸引起的皮质细胞的损伤具有拮抗作用，最大拮抗剂量为 60 μg·L^{-1} 结论：NGF 通过稳定 $[Ca^{2+}]_i$ 水平，或阻止 $[Ca^{2+}]_i$ 的升高来保护大脑皮质细胞抗谷氨酸毒性.

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Effect of phencyclidine on dog coronary artery

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KEY WORDS phencyclidine; dextromethorphan; coronary vessels.

AIM: To study the effect of phencyclidine (Phe) on dog coronary artery. **METHODS**: Contraction of spiral strips of dog coronary artery in bioassay and coronary artery blood flow (CBF) using electromagnetic flowmeter on anesthetized dogs were observed. **RESULTS**: Phe 0.1 – 100 μmol·L^{-1} induced contraction of strips in a concentration-dependent manner. Dextromethorphan (Dex) 10 μmol·L^{-1}, an antagonist of Phe receptor, antagonized the action of Phe. *In vivo*, Phe 10 mg·kg^{-1} increased flow of left circumflex coronary artery of anesthetized dogs from 334 ± 35 mL·kg^{-1}·min^{-1} to 510 ± 58 mL·kg^{-1}·min^{-1}, and both left ventrical pressure (LVP) and blood pressure (BP) rose slowly after medication. Dextromethorphan (Dex) 5 mg·kg^{-1} also antagonized the effect of Phe. **CONCLUSION**: The regulation of Phe on coronary artery *in vivo* differs from that *in vitro*, which may result in the contradictory effects.

Phencyclidine (Phe) receptors existed in porcine coronary artery[1]. Phe receptor agonists induced contraction of spiral strips of porcine coronary artery; Phe receptor antagonists, Dextromethorphan (Dex) and haloperidol (Hal), antagonized the effect of Phe[2]. In isolated perfused hearts of guinea pigs, Phe reduced coronary flow which was antagonized by Dex and Hal[3]. The effect of Phe on rat electrocardiogram (ECG) has been observed[4]. Phe induced a rapid elevation of T wave in ECG when injected iv, but no change of ECG when injected intracerebroventricularly. To obtain the direct evidence of Phe on coronary artery blood flow (CBF) *in vivo*, the experiments were proceeded in anesthetized dogs.

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