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European Journal of Pharmacology 321 (1997) 171–179

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# A novel NMDA receptor antagonist protects against *N*-methyl-D-aspartate- and glutamate-induced neurotoxicity in the goldfish retina

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Received 19 September 1996; revised 20 November 1996; accepted 26 November 1996

**Abstract**

4(*R*)-(3-Phenylpropyl)-2(*S*)-glutamic acid, $C_{(3)}$, is a synthetic analogue of L-glutamate. This analogue reversibly inhibits the membrane depolarization of neurons in the CA1 region of rat hippocampal slices evoked by *N*-methyl-D-aspartate (NMDA), with an $EC_{50}$ value of 3.6 μM, whereas the depolarization of these neurons evoked by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid is not inhibited by $C_{(3)}$. Analyses of the inhibitory effect of $C_{(3)}$ on NMDA-evoked currents of dissociated rat hippocampal neurons further revealed that $C_{(3)}$ acts as a competitive antagonist of NMDA receptors and that the inhibitory action of $C_{(3)}$ is not use-dependent. Using goldfish retina as a model, we found that the neuronal damage produced by glutamate or by NMDA was effectively prevented by $C_{(3)}$. Incubation of retinas with high concentrations of $C_{(3)}$, up to 1 mM, did not induce pathomorphological changes in retinal neurons. These results suggest that $C_{(3)}$ is a useful neuroprotectant against excitotoxic damage of neurons.

*Keywords:* Excitotoxicity; NMDA receptor

## 1. Introduction

Glutamate is generally considered as the principal excitatory neurotransmitter in the central nervous system of the vertebrates. The neuronal responses elicited by glutamate are mediated by three groups of ionotropic receptors, i.e., NMDA-, AMPA-, and kainate-preferring receptors, and by a family of metabotropic receptors (Hollmann and Heinemann, 1994; Mayer and Westbrook, 1987; Monaghan et al., 1989; Nakanishi, 1994). Many studies have demonstrated that glutamate receptors are not only essential for conveying excitatory information across synapses, but are also involved in the development and in many higher functions of the brain, e.g., learning and memory (Bliss and Collingridge, 1993; Constantine-Paton et al., 1990).

Excessive stimulation of neurons by excitatory amino acids has been found to induce degeneration of central neurons (Rothman and Olney, 1987). The observation that NMDA receptor antagonists effectively prevent neuronal death induced by excitatory amino acids in many in vitro preparations suggests that NMDA receptors play an important role in the neural toxicity of excitatory amino acids (Garthwaite et al., 1986; Rothman and Olney, 1987; Choi et al., 1988; Choi, 1988b; Meldrum and Garthwaite, 1990; Regan and Choi, 1991; Randall and Thayer, 1992; Tymianski et al., 1993; Szatkowski and Attwell, 1994), although non-NMDA receptor-mediated neurotoxicity has also been reported (Meldrum and Garthwaite, 1990; Frandsen and Schousboe, 1993). In animal experiments, NMDA receptor antagonists have further been found to attenuate neuronal death associated with hypoxia/ischemia (Siesjö and Bengtsson, 1989; Simon et al., 1984; Park et al., 1988; Steinberg et al., 1988), epilepsy (Kohr and Heinemann, 1989; Dingledine et al., 1990) and trauma (Faden et al., 1989; Tecoma et al., 1989). These observations have led investigators to suggest that excitatory amino acids play an important role in the pathogenesis of the aforementioned neuronal injuries in the central nervous system (Choi,

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*PII* S0014-2999(96)00949-1

1988a, 1995; Choi and Rothman, 1990; Meldrum and Garthwaite, 1990; Lipton and Rosenberg, 1994) and that NMDA receptor antagonists may be of therapeutic use for these neuronal injuries (Meldrum, 1985; Albers et al., 1992).

We have recently reported the synthesis of a series of glutamate analogues (Hon et al., 1990) and their effects upon [$^3$H]glutamate binding to synaptic junctions isolated from porcine brain (Chang et al., 1993). In this paper, we report that one of these analogues, 4(*R*)-(3-phenylpropyl)-2(*S*)-glutamic acid ($C_{(3)}$), acts as a competitive antagonist of NMDA receptors and that $C_{(3)}$ effectively prevents glutamate- and *N*-methyl-D-aspartate (NMDA)-induced neurotoxicity in goldfish retinas.

## 2. Materials and methods

### 2.1. Materials

Goldfish were obtained from local suppliers. Sprague-Dawley rats were obtained from the Animal Center of the National Cheng-Kung University Medical School (Tainan, Taiwan). HEPES, PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]), tetraethylammonium chloride, glucose and Trizma were purchased from Sigma. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), NMDA, tetrodotoxin (TTX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and D-2-amino-5-phosphonovalerate (D-APV) were obtained from Research Biochemicals International. Other chemicals were obtained from Merck-Suchardt. $C_{(3)}$ was synthesized according to the methods described previously (Hon et al., 1990).

### 2.2. Electrophysiological studies

#### 2.2.1. Intracellular recording of CA1 neurons in rat hippocampal slices

Rat hippocampal slices, nominally 500 μm thick, were prepared from Sprague-Dawley rats as described previously (Nicoll and Alger, 1981). A submerged recording chamber (1 ml) was used and the superfusion solution was maintained at 36 ± 1°C (Tyan et al., 1996). The superfusion solution consisted of (in mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, $NaH_2PO_4$ 1.2 and glucose 11, oxygenated with 95% $O_2$/5% $CO_2$. The flow

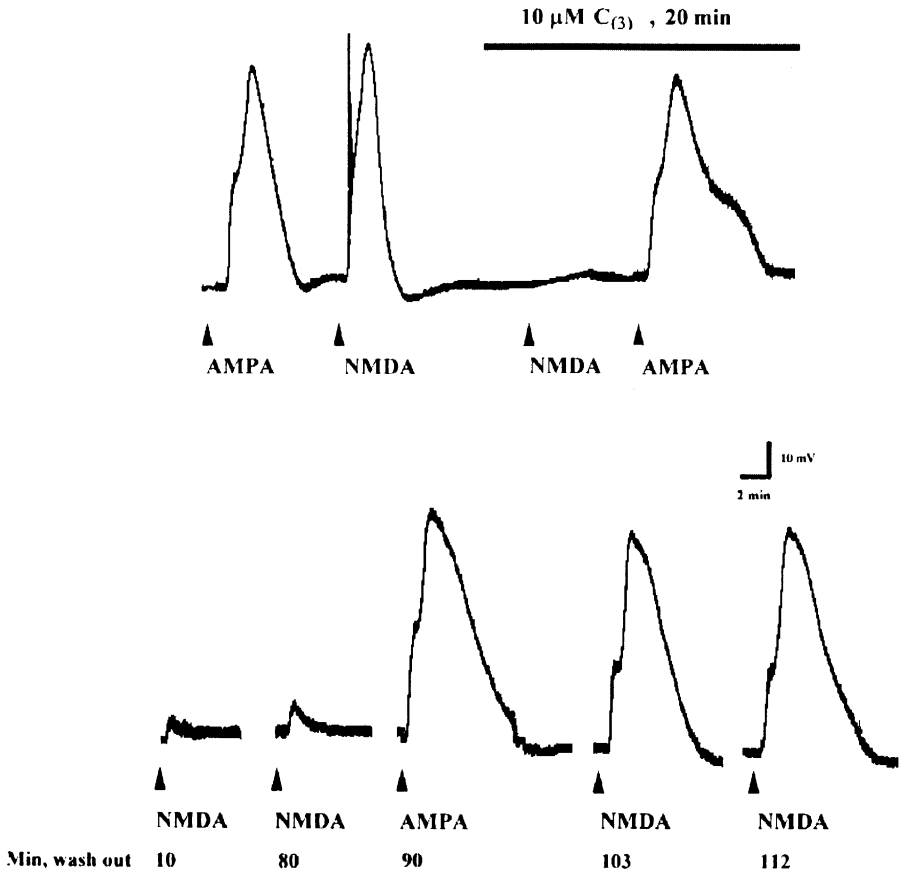



Fig. 1. NMDA-induced membrane depolarization of rat hippocampal CA1 neurons was selectively and reversibly inhibited by $C_{(3)}$. 10 μl of NMDA (2 mM) or AMPA (2 mM) was applied to the hippocampal slice by the dropping method at the time indicated by the arrowheads. $C_{(3)}$ (10 μM) was continuously present in the superfusion solution for the time indicated (solid bar). The resting membrane potential of the neuron at the beginning of this experiment was −72 mV. These results were from a single experiment representative of four.

rate of the superfusion solution was maintained at around 2 ml/min. Membrane potentials were recorded by conventional intracellular recording techniques using an Axopatch-2A amplifier. Microelectrodes were filled with 3 M KCl, and the electrode resistance was in the range between 60 and 150 MΩ. Drugs were applied either by addition to the superfusion solution or by addition directly into the recording chamber near the inlet of the superfusion solution. TTX (0.5 μM) was present in all of the experiments.

*2.2.2. Whole-cell patch-clamp recording of dissociated hippocampal neurons*

Neurons were dissociated from rat hippocampus according to the procedure of Hsu et al. (1993) with modifications. Briefly, the hippocampus of a Sprague-Dawley rat (1–2 weeks old) was chopped into small pieces (approximately 1 $mm^3$) with a razor blade. The tissue pieces were then digested in 20 ml of oxygenated PIPES solution (in mM: NaCl 120, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, glucose 25 and PIPES 20 at pH 7.2) containing 20–30 mg Pronase E (Sigma) for 30 min at room temperature. Tissue pieces were then rinsed three times with fresh PIPES solution and kept in oxygenated PIPES solution at room temperature. Cells were dissociated from these Pronase-digested pieces by repeatedly moving them, in the bath solution (in mM: NaCl 140. KCl 5, $CaCl_2$ 1, glycine 0.01, glucose 10 and Hepes 10 at pH 7.4), up and down a glass pipette with a fire-polished tip (about 2 mm in diameter). Dissociated cells were then transferred to a square recording chamber ($1 \times 1 \times 0.5$ cm) with a bottom made of a glass cover slip coated with poly-L-lysine (30 μg/ml). Before recording, cells were perfused with the bath solution containing 0.5 μM TTX for 5 min to curtail spontaneous excitation. The electrodes for patch-clamp recording were filled with a solution containing (in mM) KF 120, TEA-Cl 20, $CaCl_2$ 1, $MgCl_2$ 2, EGTA 2.25, NaATP 4 and Hepes 10 at pH 7.2, and the electrode resistance was about 3 MΩ. Transmembrane currents were recorded on a video tape with a patch-clamp amplifier (Axopatch 200, Axon) via an A/D interface (NeuroCorder DR-390, Axon). NMDA was applied to dissociated neurons by an iontophoresis unit (IP-2 pumps housed in a Neurophore BH-2, Medical Research) from a glass electrode with a resistance of about 50 MΩ and filled with 0.2 M NMDA (pH 8.0).

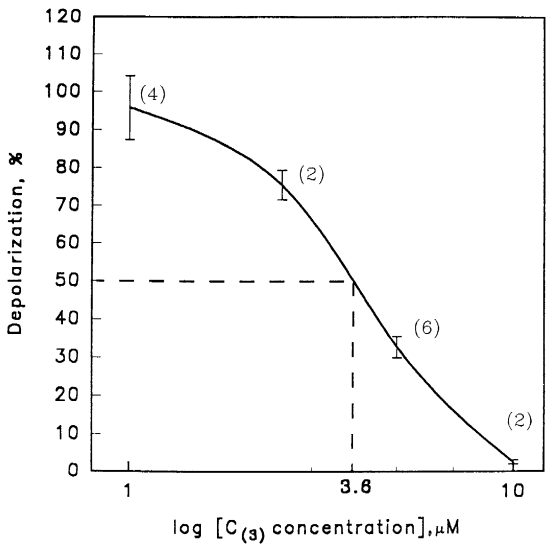



Fig. 2. Concentration-dependent inhibition of NMDA-induced membrane depolarization by $C_{(3)}$ of rat hippocampal CA1 neurons. The depolarization of individual slices was normalized, so that results are expressed as a ratio of the depolarization evoked by NMDA (10 μl, 2 mM) in the presence of various concentrations of $C_{(3)}$ to that before $C_{(3)}$ was added to the superfusion solution. Each data point represents the mean (±S.D.) of 2–6 experiments (number of experiments is shown in parentheses), and each experiment was performed with a fresh brain slice which had never been exposed to $C_{(3)}$. The curve connecting data points was generated by Sigma Plot software. The $EC_{50}$ value of $C_{(3)}$ was determined to be 3.6 μM.

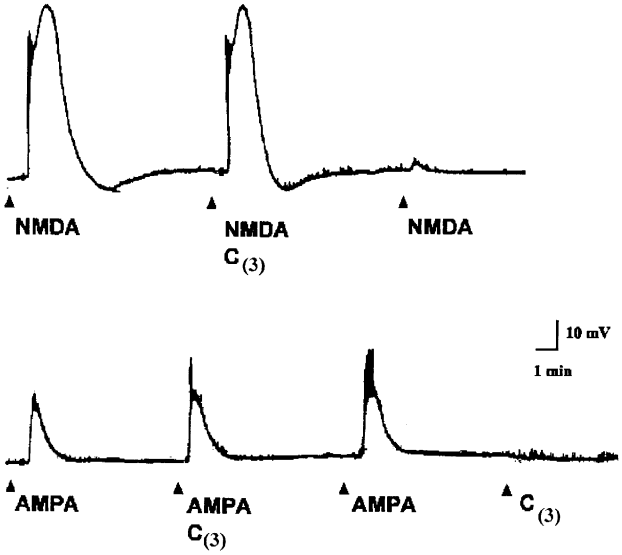
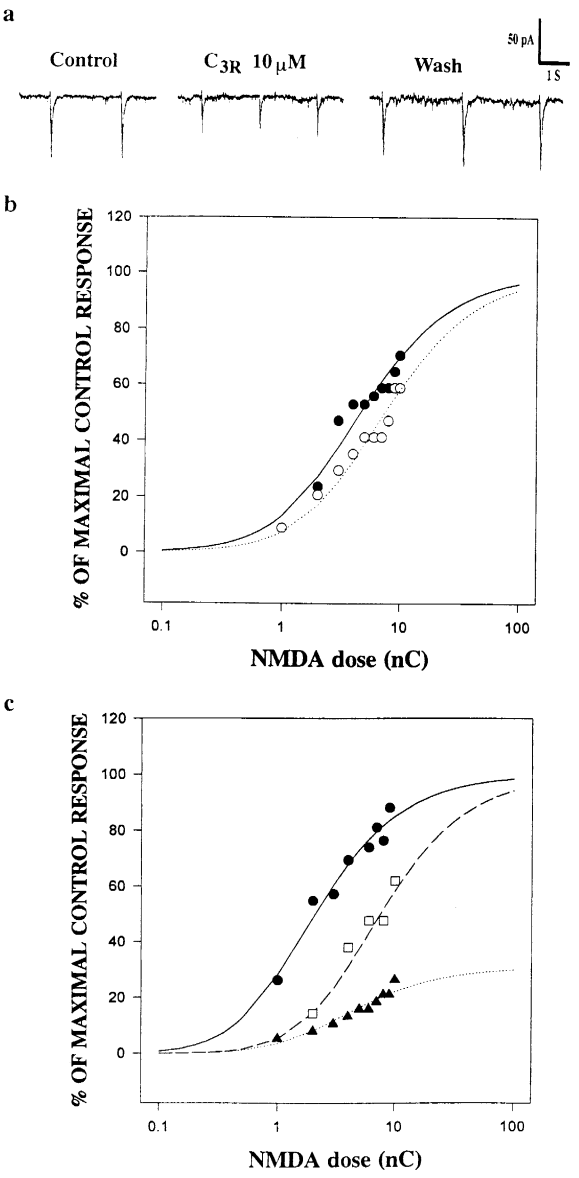



Fig. 3. $C_{(3)}$ was an antagonist of NMDA receptors with a slow onset of action. Upper panel: 10 μl of NMDA (2 mM), 10 μl of $C_{(3)}$ (2 mM) plus NMDA (2 mM), and 10 μl of NMDA (2 mM) were applied sequentially to the hippocampal slice by the dropping method at the times indicated by arrowheads. Lower panel: 10 μl of AMPA (2 mM), 10 μl of $C_{(3)}$ (2 mM) plus AMPA (2 mM), and 10 μl of AMPA (2 mM) were applied sequentially at the times indicated by arrowheads. These results were from a single experiment representative of six.

*2.3. Excitotoxicity study of goldfish retinas*

A procedure similar to that employed by Kleinschmidt et al. (1986a,b) in studying kainate neurotoxicity of goldfish retinas was used here. Goldfishes with a body length between 4 and 7 cm were dark adapted for more than 2 h before use. The eyeballs were dissected, rinsed briefly in ice-cold Ringer's solution (saturated with 95% $O_2$/5% $CO_2$), and cut horizontally into two halves. The Ringer's solution consisted of (in mM): NaCl 120, KCl 2.5, $MgSO_4$ 1.2, $CaCl_2$ 2.2, glucose 10 and Hepes 3.5 at pH 7.8 (Ishida and Fain, 1981). The upper half of the eyeball was gently shaken in ice-cold Ringer's solution to release retinal

tissues. Next, each of the resultant retinal tissues was cut into four parts, which were allowed to recover in 500 μl Ringer's solution at 25°C for 30 min under 95% $O_2$/5% $CO_2$. Following the recovery period, retinal quarters were incubated in the Ringer's solution (200 μl) at 25°C with or without different drugs for various lengths of time. After drug treatment, retinal quarters were individually placed into microcentrifugation tubes (1.5 ml), fixed with paraformaldehyde (1%) and glutaraldehyde (1.5%) in phosphate buffer (0.1 M sodium phosphate at pH 7.4) for 1 h, rinsed three times (10 min each) in the same phosphate buffer solution, and then fixed again in osmium tetroxide solution (1% $OsO_4$ in phosphate buffer) for 1 h. After being washed twice with phosphate buffer solution, fixed retinal quarters were rinsed in $H_2O$ and dehydrated sequentially in alcohol solutions containing 50, 70, 80, 90, 95 and 100% ethanol. The dehydrated tissues were finally embedded in Spurr's resin (Spurr, 1969), which was then incubated at 60°C for 12 h for solidification. Solidified sample blocks were cut into 1 μm thick sections, stained with toluidine blue (Preece, 1978) and viewed under a light microscope.




## 3. Results

### 3.1. Effects of $C_{(3)}$ on membrane depolarization induced by NMDA and AMPA of rat hippocampal CA1 neurons

Fig. 1 shows that the membrane depolarization induced by NMDA was almost completely inhibited by 10 μM of $C_{(3)}$. The $C_{(3)}$ inhibition of NMDA-induced membrane depolarization appeared to be persistent, as the neuron fully recovered from the inhibition after wash-out of $C_{(3)}$ for 100 min. In contrast, the membrane depolarization induced by AMPA of the same neuron was not inhibited by $C_{(3)}$. The dose-response curve for the inhibitory effect of $C_{(3)}$ on NMDA-induced membrane depolarization is shown in Fig. 2. The $EC_{50}$ value was estimated to be 3.6 μM (Fig. 2). When a mixture (10 μl) containing $C_{(3)}$ (2 mM) and NMDA (2 mM) was applied to the slice by the

Fig. 4. (a) The inhibitory action of $C_{(3)}$ on NMDA receptors was not use-dependent and (b and c) $C_{(3)}$ acted as a competitive NMDA receptor antagonist. (a) Membrane currents induced by iontophoretic pulses of NMDA (20 ms, 100 nA) in dissociated hippocampal neurons before (left traces) and 3 min after (middle traces) $C_{(3)}$ (10 μM) was added to the superfusion solution, and 4 min after $C_{(3)}$ was washed out with fresh superfusion solution (right traces). The results were from a single experiment representative of six. (b,c) Dose-response curves of NMDA-evoked inward currents in the absence (● in b and c) or the presence of 10 μM $C_{(3)}$ (○ in b), or 30 μM D-APV (□ in c), or 2.5 mM magnesium (▲ in c). NMDA was applied as iontophoretic pulses of 20 ms duration and intensities in the range between 5 to 500 nA. The control dose–response curves were fitted to a non-linear least-fitting program (Sigma Plot) of the equation of $I = I_{max} \times ([A]/([A] + K_d))^n$, where $A$ is the dose of NMDA in nanocoulombs. The number of sites ($n$), the maximum response ($I_{max}$) and the dissociation constant of NMDA ($K_d$) were allowed to vary to obtain the best fit (Westbrook and Mayer, 1987). The dose-response curves in the presence of $C_{(3)}$, D-APV and magnesium were fitted using the estimates for $n$, $I_{max}$ and $K_d$ from the control dose-response curve for each neuron while the dissociation constant of the antagonist ($K_i$) was allowed to vary to obtain the best fit. The dose–response curves in the presence of $C_{(3)}$ and D-APV could be fitted satisfactorily to the model for a competitive antagonist but not to the model for a non-competitive antagonist. The model for a competitive antagonist is described by the equation: $I = I_{max} \times \{[A]/([A] + (K_d \times (1 + [i]/K_i))\}^n$, and the non-competitive model is described by the equation: $I = I_{max} \times \{[A]/([A] \times (1 + ([i]/K_i)) + K_d)\}^n$. The dose–response curve in the presence of magnesium could be fitted satisfactorily to the model for a non-competitive antagonist but not to the model for a non-competitive antagonist. The control curves in b and c were from two single experiments representative of ten. The curves in the presence of $C_{(3)}$, D-APV and magnesium were from a single experiment representative of a total of five, six and two experiments, respectively.

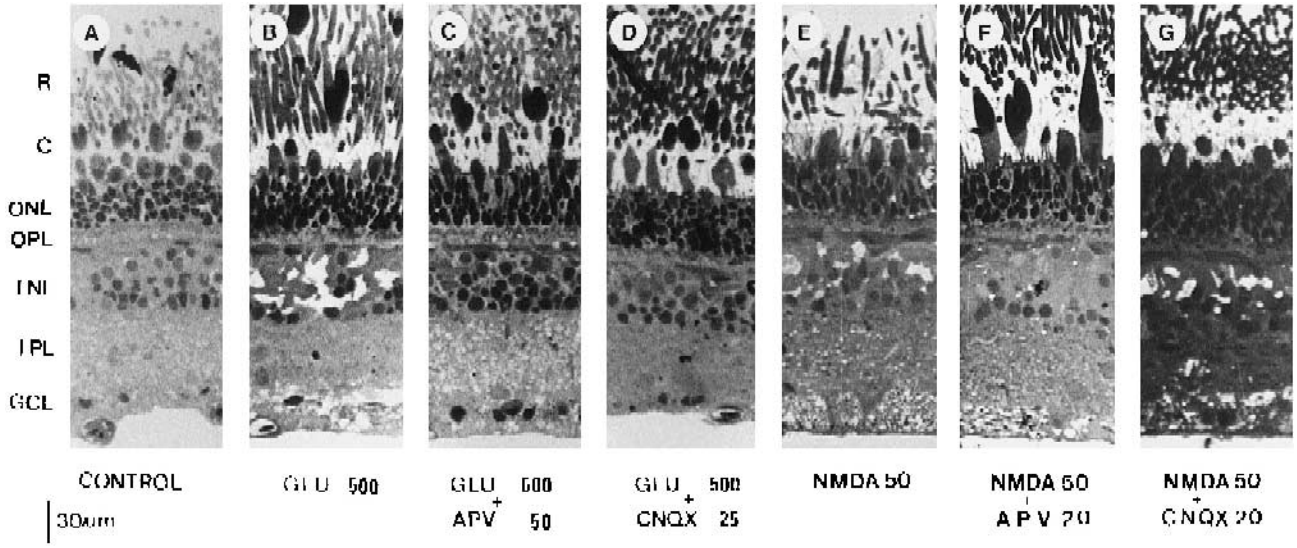



Fig. 5. Glutamate- and NMDA-induced neurotoxicity of the goldfish retina. Following surgical and recovery steps, retinal quarters were incubated with the normal Ringer's solution (A), or with the normal Ringer's solution containing (B) glutamate (500 μM), (C) glutamate (500 μM) plus D-APV (50 μM), or (D) glutamate (500 μM) plus CNQX (25 μM), or with magnesium-free Ringer's solution containing (E) NMDA (50 μM), (F) NMDA (50 μM) plus APV (20 μM) or (G) NMDA (50 μM) plus CNQX (20 μM) at 25°C under 95% $CO_2$/5% $O_2$ for 30 min. Retinal quarters were then fixed, dehydrated, embedded in resin blocks, cut into thin sections, and stained with toluidene blue. Each experiment was performed in duplicate. Abbreviations: R, rod cell; C, cone cell; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; GCL, ganglion cell layer.

dropping method, the resultant membrane depolarization was of a magnitude comparable to that induced by NMDA alone (Fig. 3). However, much less depolarization was produced by subsequent application of NMDA (2 mM). For the same neuron, application of $C_{(3)}$ (2 mM) along with AMPA (2 mM) did not inhibit the depolarizing effect of AMPA, nor was the subsequent depolarization produced by AMPA inhibited. This result would suggest that the inhibitory action of $C_{(3)}$ occurs more slowly than the depolarizing action of NMDA. Application of $C_{(3)}$ alone did not affect the resting membrane potential of hippocampal CA1 neurons (Fig. 3).

### *3.2. Mechanism for the action of $C_{(3)}$ as an antagonist of NMDA receptors*

The whole-cell voltage-clamp recording method was then used to monitor the effects of $C_{(3)}$ on the current response to iontophoretic pulses of NMDA of dissociated hippocampal neurons (Fig. 4a). When added to the bath for

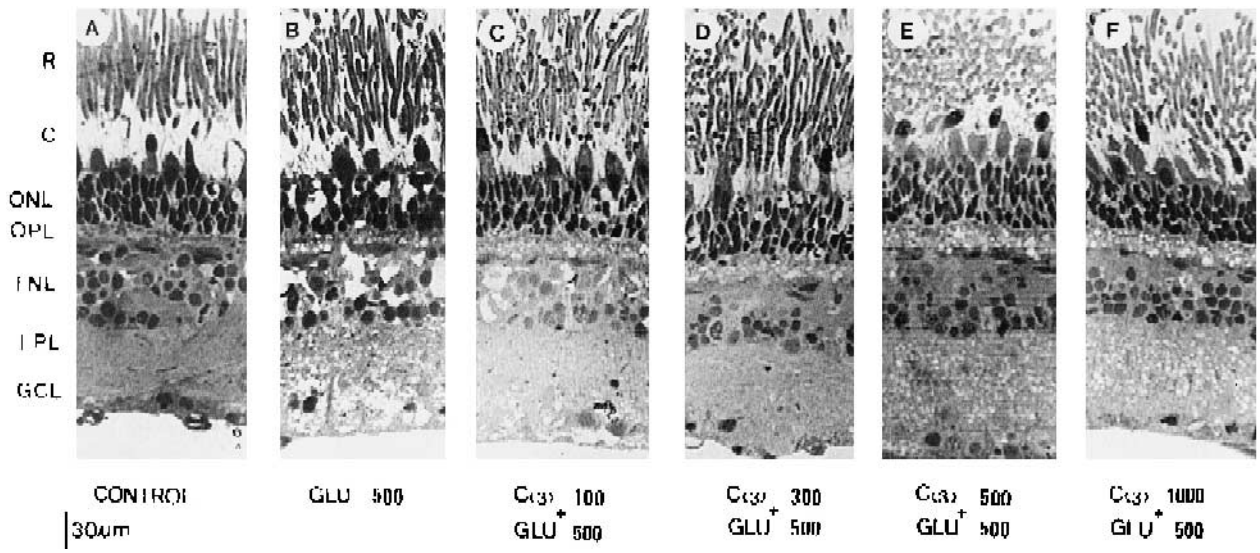



Fig. 6. $C_{(3)}$ protected goldfish retinas against glutamate-induced neurotoxicity. Retinal quarters were incubated in the normal Ringer's solution in the absence (A) or in the presence of the following drugs: (B) glutamate (500 μM), (C) glutamate (500 μM) plus 100 μM $C_{(3)}$, (D) glutamate (500 μM) plus 300 μM $C_{(3)}$, (E) glutamate (500 μM) plus 500 μM $C_{(3)}$, and (F) glutamate (500 μM) plus 1000 μM $C_{(3)}$.

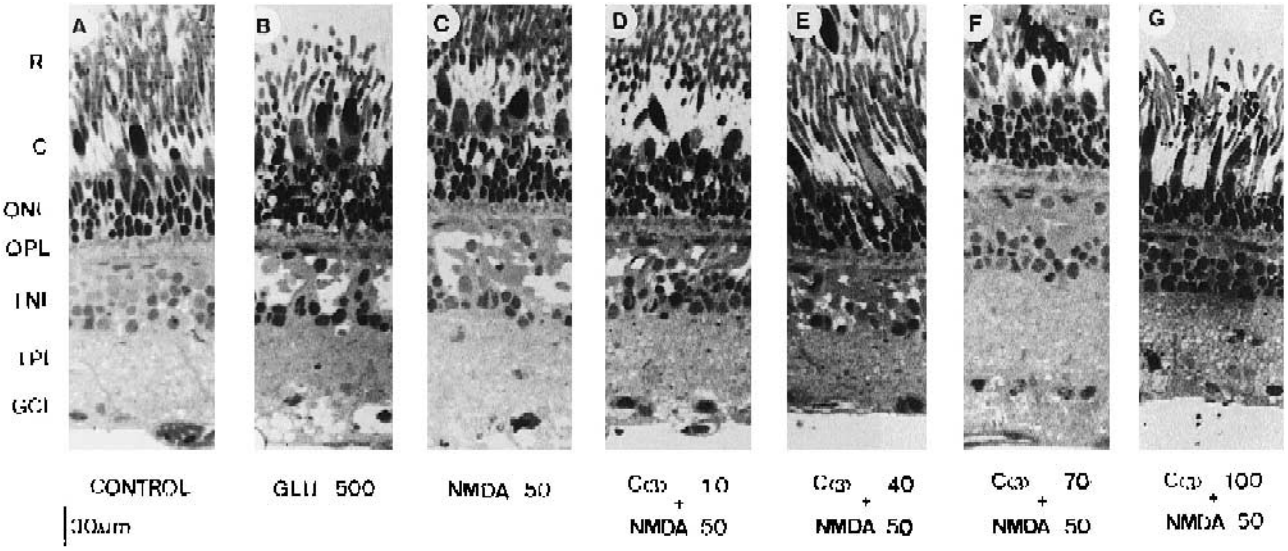



Fig. 7. $C_{(3)}$ protected goldfish retinas against NMDA-induced neurotoxicity. Retinal quarters were incubated in magnesium-free Ringer's solution in the absence (A) or in the presence of the following drugs: (B) glutamate (500 μM), (C) NMDA (50 μM), (D) NMDA (50 μM) plus 10 μM $C_{(3)}$, (E) NMDA (50 μM) plus 40 μM $C_{(3)}$, (F) NMDA (50 μM) plus 70 μM $C_{(3)}$, and (G) NMDA (50 μM) plus 100 μM $C_{(3)}$.

3 min, $C_{(3)}$ (10 μM) reduced the amplitude of the NMDA-induced current to 54% of that prior to exposure to $C_{(3)}$. The amplitude of the membrane currents induced by subsequent pulses of NMDA applied to the same neuron remained virtually unchanged (middle traces of Fig. 4a), indicating that the inhibitory action of $C_{(3)}$ on NMDA receptors was not use-dependent. The neuron fully recovered from $C_{(3)}$ inhibition after the cells were washed with fresh bath solution for 4 min. In agreement with the results obtained with the intracellular recording method in rat hippocampal slices (Figs. 1 and 2), the current response to AMPA was not affected by addition of $C_{(3)}$ to the bath solution (data not shown).

To distinguish whether $C_{(3)}$ was a competitive or a non-competitive antagonist of NMDA receptors, we fitted the dose-response curves of NMDA-induced currents in the presence of $C_{(3)}$, in the presence of a competitive NMDA antagonist, D-APV, and in the presence of a non-competitive NMDA antagonist, magnesium, to the equations for a competitive and a non-competitive model (Fig. 4b and c). The dose-response curves of NMDA in the presence of D-APV (30 μM) and $C_{(3)}$ (10 μM) could be fitted satisfactorily by the equation for a competitive model, but not by the equation for a non-competitive model (Fig. 4b). The dose-response curve in the presence of magnesium (2.5 mM) was fitted satisfactorily by the equation for

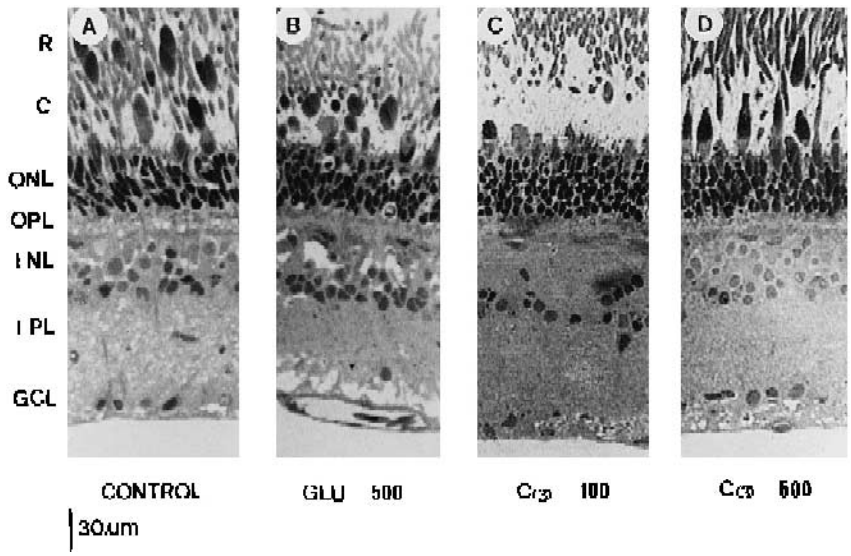



Fig. 8. Effects of $C_{(3)}$ upon goldfish retina. Retinal quarters were incubated in the normal Ringer's solution in the absence (A) or in the presence of the following drugs: (B) 500 μM glutamate, (C) 100 μM $C_{(3)}$, and (D) 500 μM $C_{(3)}$.

a non-competitive model, but not by the equation for a competitive model (Fig. 4c). These results indicated that $C_{(3)}$ acted as a competitive antagonist of NMDA receptors. The Hill coefficient, $n_H$, derived from the dose-response curves of NMDA-induced currents was $2.2 \pm 0.5$ (mean $\pm$ S.D., $n = 10$). The dissociation constant for the binding of $C_{(3)}$ to the NMDA receptor, $K_i$, was estimated to be $14.5 \pm 5.3$ μM (mean $\pm$ S.D., $n = 5$). This value was higher than the $EC_{50}$ value, 3.6 μM, of $C_{(3)}$ for inhibition of NMDA-induced membrane depolarization of CA1 neurons in brain slices (Fig. 2). This discrepancy is possibly due to different environments surrounding dissociated neurons and neurons in hippocampal slices, to different bath solutions used in intracellular recording and patch-clamp recording experiments, and to different processes for dissociating neurons from hippocampus and for producing hippocampal slices. These differences may also account for the observation that dissociated neurons recovered from $C_{(3)}$ inhibition much more rapidly than neurons in hippocampal slices did (Figs. 1 and 4).

### *3.3. Protective effects of $C_{(3)}$ on goldfish retinal neurons against excitotoxicity*

The effects of $C_{(3)}$ upon glutamate- and NMDA-induced neurotoxicity in goldfish retinas were then studied. Goldfish retinas were used here because excitatory amino acids are known to be the principal neurotransmitters in goldfish retina (Ishida and Fain, 1981; Miller and Slaughter, 1986; Tachibana and Okada, 1991), glutamate receptors of goldfish retina are recognized by anti-rat glutamate receptor antibodies (Stell and Barton, 1993), and the retina has a relatively simple structure and is easily accessible. Before studying the effect of $C_{(3)}$, the neurotoxic effects of glutamate and NMDA on goldfish retinas were characterized. Retinal neurons appeared to retain their intact cellular structure after incubation in the Ringer's solution for 30 min (Fig. 5A). In the presence of 500 μM glutamate, massive swelling of dendrites and somatas was observed in the inner nuclear layer and ganglion cell layer of the retina (Fig. 5B). This damage became noticeable as early as 15 min after the retina was exposed to glutamate, and no clear regional difference in the extent of damage was observed in the retina (data not shown). This glutamate-induced neuronal damage could be prevented by including D-APV or CNQX in the Ringer's solution (Fig. 5C and D), implying that both NMDA and non-NMDA receptors were involved in glutamate-induced neurotoxicity in goldfish retinas. In the normal Ringer's solution, which contained magnesium, NMDA-induced neurotoxicity to retinal neurons was negligible (results not shown). In the absence of magnesium, however, NMDA (50 μM) and glutamate (500 μM) induced comparable neuronal damage in the retina (only the NMDA-treated sample is shown in Fig. 5E). In the magnesium-free Ringer's solution, NMDA- and glutamate-induced neuronal damage was prevented by including D-APV, but not by including CNQX, in the incubation solution (only the NMDA-treated samples in the presence D-APV and CNQX are shown in Fig. 5F and G, respectively).

$C_{(3)}$ attenuated the neuronal damage induced by glutamate and NMDA. Fig. 6 shows that neuronal damage induced by glutamate (500 μM) was partially inhibited by adding 100 μM $C_{(3)}$ and was completely inhibited by adding 300 μM or more $C_{(3)}$ to the Ringer's solution (Fig. 6B–D). In the absence of magnesium, 70 μM or more $C_{(3)}$ was found to completely block the neurotoxic effects produced by 50 μM NMDA (Fig. 7). Similarly, the neuronal damage induced by glutamate (500 μM) in the absence of magnesium was also prevented by $C_{(3)}$ (100 μM) and by $C_{(3)}$ (100 μM) plus CNQX (20 μM) (data not shown). By itself, $C_{(3)}$ at 100 or 500 μM did not produce pathomorphological changes in the retina (Fig. 8).

## 4. Discussion

The results of our electrophysiological studies indicate that $C_{(3)}$ acts as a competitive antagonist of NMDA receptors of rat hippocampal neurons. This conclusion is in agreement with our previous findings obtained with radioligand binding techniques, namely that $C_{(3)}$ effectively displaces [$^3$H]glutamate bound to glutamate receptors on synaptic junctions isolated from porcine brain. The dissociation constant of $C_{(3)}$ for displacing [$^3$H]glutamate bound to synaptic junctions is 11 μM (Chang et al., 1993). [$^3$H]Kainate and [$^3$H]AMPA bound to porcine brain synaptic junctions are only partially displaced by $C_{(3)}$ at higher concentrations: 100 μM $C_{(3)}$ displaces $29.6 \pm 1.0\%$ ($n = 3$) and $16.4 \pm 0.4\%$ ($n = 3$) of the bound [$^3$H]kainate and [$^3$H]AMPA, respectively (Y.-C. Chang, unpublished data). These results also suggest that $C_{(3)}$ interacts primarily with NMDA receptors in porcine brain synaptic junctions.

The possibility that $C_{(3)}$ acts as a neuroprotectant against neurotoxicity induced by excitatory amino acids was tested in this study. We found that the damage of goldfish retinal INL and GCL neurons induced by glutamate and by NMDA was mainly mediated via NMDA receptors. This neuronal damage could be effectively prevented by including $C_{(3)}$ in the incubation solution. This observation not only supports the conclusion obtained with electrophysiological and radioligand binding studies, that the primary target of $C_{(3)}$ is NMDA receptors, but also raises the possibility of the potential usefulness of $C_{(3)}$ as a neuroprotectant in various neurodegenerative diseases.

Overactivation of glutamate receptors, particularly the NMDA subtype receptors, has been implicated in the pathogenesis of neuronal death in various neurodegenerative disorders including hypoxia/ischemia, seizures, hypoglycemia, brain trauma, Alzheimer's disease and Huntington's disease (Choi, 1988a, 1995; Meldrum and Garthwaite, 1990; Lipton and Rosenberg, 1994). As a result,

NMDA antagonists have been proposed as novel clinical therapy for these neurological disorders (Meldrum, 1985; Albers et al., 1992). However, application of NMDA antagonists causes psychotic reactions in humans and pathomorphological changes in rat brain (Olney et al., 1989, 1991). Furthermore, acute administration of MK801, an antagonist of NMDA receptors, to developing rats makes the brain more vulnerable to the toxic effects of excitatory amino acids (McDonald and Johnston, 1990). These side effects limit the therapeutic usefulness of various presently available NMDA antagonists. In this study, we found a new NMDA receptor antagonist, $C_{(3)}$, to be effective in protecting goldfish retinal neurons from damage induced by glutamate and NMDA. We also noticed that even at the concentration of 1 mM (Fig. 6F) or 500 μM (Fig. 8D) $C_{(3)}$ did not cause morphological damage to retinal neurons. As a result, $C_{(3)}$ is potentially useful as a neuroprotectant without pathological side effects. In addition, $C_{(3)}$ is rather hydrophobic due to the presence of three methylene groups and a phenyl ring. This physical property makes $C_{(3)}$ more likely to penetrate the blood-brain barrier in in vivo applications. Furthermore, $C_{(3)}$ has the basic structure of an α-amino acid; it may adopt the metabolic machinery of other natural α-amino acids for its own catabolism in the brain.

The results obtained in this study indicate that $C_{(3)}$ is a competitive antagonist of NMDA receptors and an effective neuroprotectant against the toxic effects of glutamate and NMDA. Further studies are needed to explore its clinical applications.

## Acknowledgements

This work was supported in part by grants (NSC 83-0208-N007-103 and NSC 85-2311-B007-006-B12) from the National Science Council of the Republic of China.

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