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Effects of L-trans-pyrrolidine-2,4-dicarboxylate, a Glutamate Uptake Inhibitor, on NMDA Receptor-mediated Calcium Influx and Extracellular Glutamate Accumulation in Cultured Cerebellar Granule Neurons

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Glutamate uptake inhibitor, L-trans-pyrrolidine-2,4-dicarboxylate (PDC, 20 μM) elevated basal and N-methyl-D-aspartate (NMDA, 100 μM)-induced extracellular glutamate accumulation, while it did not augment kainate (100 μM)-induced glutamate accumulation in cultured cerebellar granule neurons. However, pretreatment with PDC for 1 h significantly reduced NMDA-induced glutamate accumulation, but did not affect kainate-induced response. Pretreatment with glutamate (5 μM) for 1 h also reduced NMDA-induced glutamate accumulation, but did not kainate-induced response. Upon a brief application (3-10 min), PDC did neither induce elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) nor modulate NMDA-induced $[Ca^{2+}]_i$ elevation. Pretreatment with PDC for 1 h reduced NMDA-induced $[Ca^{2+}]_i$ elevation, but it did not reduce kainate-induced $[Ca^{2+}]_i$ elevation. These results suggest that glutamate concentration in synaptic clefts of neuronal cells is increased by prolonged exposure (1 h) of the cells to PDC, and the accumulated glutamate subsequently induces selective desensitization of NMDA receptor.

Key words : Glutamate, L-trans-pyrrolidine-2,4-dicarboxylate, NMDA (N-Methyl-D-aspartate), Calcium influx, cGMP, Cerebellar granule neurons

INTRODUCTION

Glutamate is a predominant excitatory neurotransmitter in the mammalian CNS and induces a variety of physiological and pathological neuronal responses. Glutamate is in high concentration (approximately 100 mM) in glutamatergic synaptic vesicles and present at concentrations of 10 mM in the cytoplasm of neurons (Talor *et al.*, 1992). Hippocampal extracellular glutamate concentration greatly increases almost immediately after onset of ischemia up to 50 μM (Globus *et al.*, 1991; Matsumoto *et al.*, 1991; Shimada *et al.*, 1993).

Removal of glutamate from the synaptic cleft is an essential component of the transmission process at glutamatergic synapses. In contrast to most non-amino acid transmitters, the action of glutamate is terminated by leaving the extracellular synaptic space via uptake into the surrounding cells, both neurons and glia. This mechanism of glutamate uptake is sodium dependent and high affinity transport systems (Watkins and Evans, 1981; Flott and Seifert, 1991).

Dihydrokainate, a glutamate uptake inhibitor, increases the N-methyl-D-aspartate (NMDA) receptor-mediated excitatory postsynaptic currents (EPSCs) in CA1 neurons of hippocampal slices, but it fails to affect non-NMDA component evoked EPSCs (Hestrin *et al.*, 1990). However, dihydrokainate is a weak inhibitor of glutamate uptake and directly activates postsynaptic excitatory amino acid (EAA) receptors (Bridges *et al.*, 1991). Preincubation with another glutamate uptake inhibitor, DL-threo-3-hydroxy-aspartate produces a time-dependent and selective inhibition of glutamate-mediated cGMP synthesis, but not significant inhibition of kainate-mediated cGMP formation (Marini and Novelli, 1991). The specific glutamate uptake inhibitor, L-trans-pyrrolidine-2,4-dicarboxylate (PDC) has been developed as a compound having no activation or inhibition of ionotropic EAA receptors (Bridges *et al.*, 1991). Sarantis *et*

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al., (1993) reported that PDC decreased the NMDA component of EPSCs, whereas it either unaffected or slightly reduced the non-NMDA-induced EPSCs. Recently, PDC was reported to depress excitatory synaptic transmission via a presynaptic mechanism in cultured hippocampal neurons (Maki *et al.*, 1994). The present study was aimed to see the effects of PDC on NMDA receptor-mediated Ca^{2+} influx and extracellular glutamate accumulation in primary cultures of cerebellar granule neurons. Primary cultures of rat cerebellar neurons were used in this study, because they are mostly glutamatergic granule cells and used widely to study the physiological, biochemical and toxicological effects of EAAs.

MATERIALS AND METHODS

Materials

NMDA, kainate and PDC were purchased from RBI (Natick, MA, U.S.A.). Fura-2/acetoxymethyl ester (fura-2 AM) was purchased from Molecular Probes (Eugene, OR, U.S.A.). Bovine calf serum and fetal bovine serum were purchased from Hyclone (Logan, UT, USA) and JCR Bioscience (Lenexa, KS, USA), respectively. All other chemicals used were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cerebellar granule cell culture

Cerebellar granule neurons were cultured as described (McCaslin and Morgan, 1987; McCaslin and Ho, 1994) with slight modifications. Briefly, 8-day-old rat pups (Sprague-Dawley) were decapitated, and the heads were partially sterilized by dipping them in 95% ethanol. The cerebellum was dissected from the tissue and placed in culture medium which lacks serum and bicarbonate. Dissociated cells by mechanical trituration were grown in Dulbecco's modified Eagle's medium (DMEM) containing sodium pyruvate (0.9 mM), glutamine (3.64 mM), sodium bicarbonate (40 mM), glucose (22.73 mM), 6% bovine calf serum and 6% fetal bovine serum on glass slides coated with poly-L-lysine. After 2 days incubation (37°C; 10% CO_2), growth medium was aspirated from the cultures and new growth medium (5 ml/dish, 300 mOsm) containing 25 mM KCl was added with 5 μM cytosine arabinoside to prevent proliferation of nonneuronal cells (McCaslin and Ho, 1994). The cells were then incubated for 14 days at 37°C under 10% CO_2-90% filtered room air. At this stage, less than 10% of the cells show glial fibrillary acidic protein immunoreactivity, as described previously (McCaslin and Ho; 1994)

Glutamate measurement

Experiments were performed using cultures grown for 10-14 days after plating on glass slides. Cells were washed to remove medium and placed in a physiological saline HEPES (PSH) buffer solution containing the following mM concentrations: 135 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 40 bicarbonate, 10 glucose and 5 HEPES (pH 7.4, 300 mOsm). After a 30 min equilibration period in PSH buffer, cells were reincubated in the presence of various concentrations of compounds at 37°C for 1 h. The quantities of glutamate secreted into the buffer (3 ml) was separated and quantified by high performance liqid chromatography (HPLC) with an electrochemical detector (Ellison *et al.*, 1987). Briefly, after a small aliquot was collected from the culture dish, amino acids were separated on a Rainin (particle size, 5 μm; 4.6×150 mm) C18 column (reverse-phase) after prederivatization with o-phthaldialdehyde/2-mercaptoethanol. Derivatives were detected by electrochemistry at 20 nA/V, and the reference electrode was set at 0.70 V. The column was eluted with mobile phase (pH 5.25) containing 0.1 M sodium phosphate buffer with 37% (v/v) HPLC-grade methanol at a flow rate of 1.0 ml/min.

Measurement of intracellular Ca^{2+} levels ($[Ca^{2+}]_i$)

Intracellular Ca^{2+} levels were determined by ratio fluorometry as described (Grynkiewicz, 1985; Cai and McCaslin, 1992). Cells grown for 10-14 days on glass slides were loaded with 10 μM frua-2 AM (dissolved in dimethylsulfoxide (DMSO)) for 1 h in PSH buffer at 37°C, and then washed with PSH buffer. The DMSO concentration never exceeded 0.1% and this concentration of DMSO had no effect on the dye signals. Cell culture slides were cut and mounted into spectrophotometer cuvettes containing 2.5 ml PSH buffer (without bicarbonate). Fluorescence was measured with a FLUOROLOG-2 spectrophotometer (SPEX Ind. Inc., Edison, NJ) equipped with a Xenon lamp, and wavelength was alternated between 340 and 380 nm for excitation using a chopper system and 505 nm for emission using a filter system. Baseline of $[Ca^{2+}]_i$ was measured for 60 sec before the addition of various experimental compounds. Ionomycin and EGTA (final concentration 10 μM and 20 mM, respectively) were added at the end of experiments to determine the emission of dye saturated with Ca^{2+} and free of Ca^{2+}, respectively. Calcium concentrations were calculated according to the method of Grynkiewicz *et al.* (1985) using a KD of 224 nM by using TM 3000 software (SPEX).

Statistical analysis

Statistical differences were analyzed by analysis of variance (ANOVA) followed by the Duncan's test. $p < 0.05$ was considered as a statistical significance.

RESULTS

Cells were incubated for 1 h with PDC (20 μM) and one of EAAs, NMDA or kainate. PDC alone caused elevation of extracellular glutamate levels 3 times over control levels (control; 0.60±0.03 μM, PDC; 1.98±0.31 μM). And NMDA or kainate at 100 μM also produced significant elevation of glutamate concentration (Fig. 1). To study the effect of PDC on glutamate accumulation induced by EAAs, PDC and NMDA or kainate were simultaneously added to cells and incubated for 1 h. PDC showed an synergic effect on NMDA-induced glutamate accumulation. But it did not augment kainate-induced glutamate accumulation (Fig. 1).

Pretreatment with PDC for 1 h prior to the addition of EAAs induced reduced NMDA-induced glutamate accumulation (from 2.00±0.54 to 1.04±0.47, μM), but did not affect kainate-induced glutamate accumulation (Fig. 2). This result suggests that the reduction of NMDA-induced glutamate accumulation may be due to prolonged increase in glutamate induced by PDC. To further determine the involvement of glutamate accumulated in extracellular fluid by prolonged treatment of PDC on NMDA-induced glutamate accumulation, the effect of exogenous glutamate (5 μM) was examined. Similar to the effects of PDC, pretreatment of glutamate for 1 h also reduced the NMDA-induced glutamate accumulation (from 2.39±0.60 to 0.61±0.06, μM). But, this treatment did not affect kainate-induced glutamate accumulation

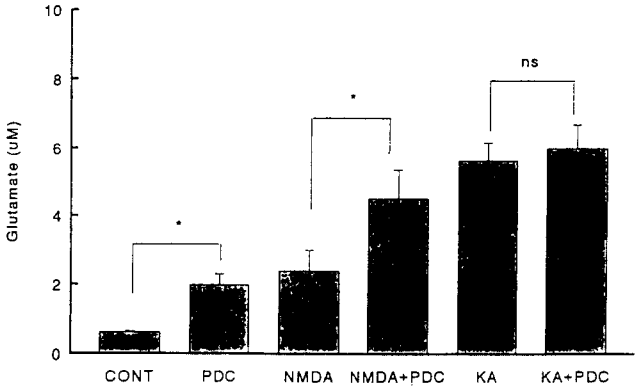

Fig. 1. Effect of PDC on NMDA- or kainate-induced glutamate accumulation. Cultured cerebellar granule cells were incubated with NMDA or kainate (KA), 100 μM each, with or without PDC (20 μM) for 1 h. Values represent the mean SEM of μM in 3 ml of extracellular buffer. Data were analyzed by an ANOVA followed by the Duncan's test. * $p < 0.05$, ns (no significant), (n=6-8)

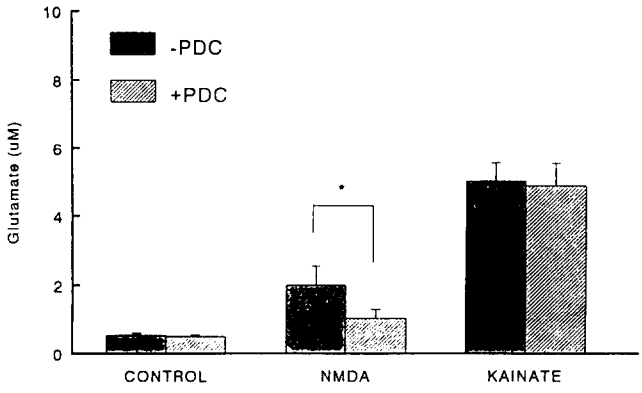

Fig. 2. Effect of pretreatment with PDC on NMDA- or kainate-induced glutamate accumulation. Cultured cerebellar granule cells were incubated with PDC (20 μM) for 1 h and washed, and reincubated with NMDA or kainate, 100 μM each, for 1 h. Values represent the mean±SEM of μM in 3 ml of extracellular buffer. Data were analyzed by an ANOVA followed by the Duncan's test. * $p<0.05$, (n=6-8)

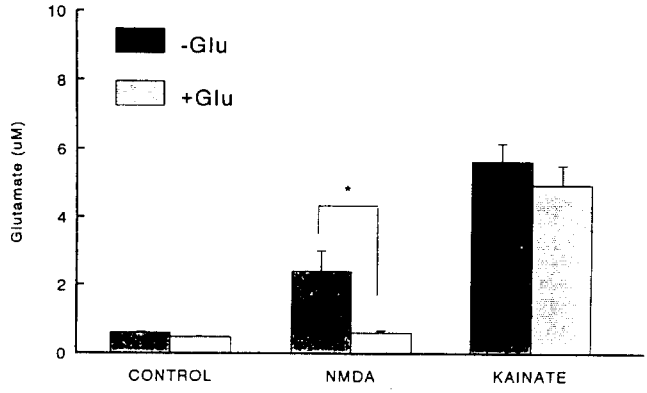

Fig. 3. Effect of pretreatment with glutamate on NMDA- or kainate-induced glutamate accumulation. Cultured cerebellar granule cells were incubated with exogenously added glutamate (5 μM) for 1 h and washed, and reincubated with NMDA or kainate, 100 μM each, for 1 h. Values represent the mean±SEM of μM in 3 ml of extracellular buffer. Data were analyzed by an ANOVA followed by the Duncan's test. * $p<0.05$, (n=6-8)

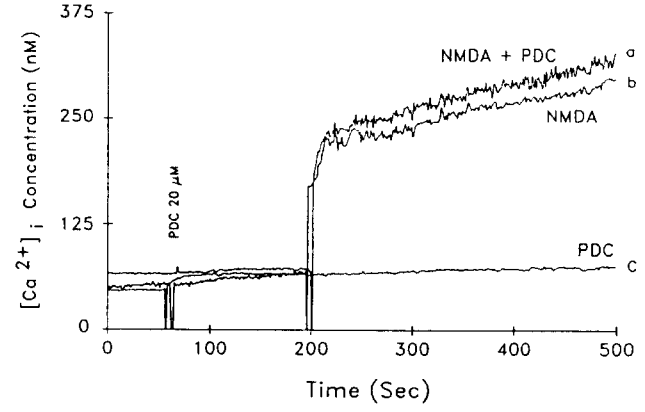

Fig. 4. Effect of PDC on the basal or NMDA-induced intracellular Ca^{2+} levels ($[Ca^{2+}]_i$). Cultured cerebellar granule cells were loaded with fura-2 AM for 60 min, washed with PSH buffer, and stimulated with PDC (20 μM) at 60 sec for curve a and c, and NMDA (50 μM) was added at 200 sec for curve a and b. Each curve represents the average of three experiments

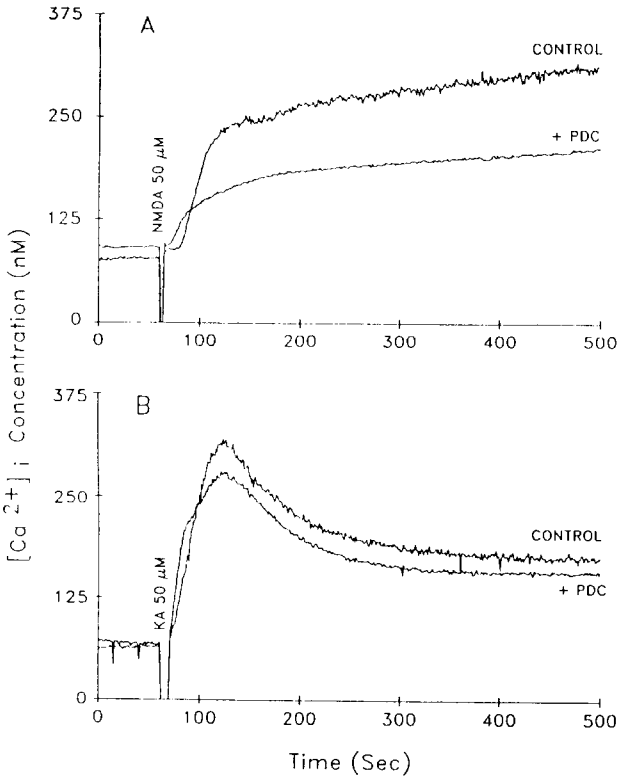

Fig. 5. Effect of PDC pretreatment on NMDA- and kainate-induced intracellular Ca^{2+} levels ($[Ca^{2+}]_i$). After cultured cerebellar granule cells were loaded with fura-2 AM for 1 h, cells were incubated with or without 20 μM PDC for 1 h, and washed with PSH buffer. And NMDA (A) or kainate (KA; B), 50 μM each, was added at the break in the curve. Each curve represents the average of four experiments.

(Fig. 3).

NMDA and kainate receptors are ligand-gated/voltage-gated ion channels and have high permeabilities to Ca^{2+}. Because it is possible that accumulation of glutamate by uptake blocker may induce NMDA receptor desensitization, changes in $[Ca^{2+}]_i$ induced by EAAs were measured with or without PDC pretreatment. In short-term (3-10 min) pretreatment, PDC affected neither basal $[Ca^{2+}]_i$ (Fig 4, c) nor NMDA-induced elevation of $[Ca^{2+}]_i$ (Fig. 4, a) compared to NMDA alone (Fig. 4, b). However, pretreatment with PDC for 1 h prior to application of EAAs reduced NMDA-induced elevation of $[Ca^{2+}]_i$ (control; 304±10. 20 vs pre-PDC; 211±15.44, nM), but did not kainate-induced elevation of $[Ca^{2+}]_i$ (Fig. 5).

DISCUSSION

To maintain CNS healthy, an appropriate glutamate's action as an excitatory neurotransmitter is required. But, glutamate, as a potent excitotoxin, in as much as prolonged exposure of most cultured neurons to micromolar concentrations, leads to either rapid or delayed neurotoxicity (Choi, 1988). But it has been reported that relatively high concentrations of NMDA or glutamate fail to induce acute neurotoxicity of cultured cerebellar granule cells (Lysko *et al.*, 1989; Kato *et al.*, 1991). For this reason, understanding the mechanism of the change of glutamate receptor function is likely to provide an insight into normal and diseased CNS processes. Receptor desensitization has been considered as a mechanism for modulating glutamate responses.

In this experiment, long-term pretreatment of neuronal cells with PDC decreased NMDA receptor-mediated elevation of calcium influx into the cell and glutamate accumulation in extracellular fluid, but did not affect kainate receptor-mediated responses. Exogenously added glutamate also reduced the NMDA-induced glutamate accumulation. If PDC directly activate NMDA receptors, it may augment the NMDA-induced elevation of $[Ca^{2+}]_i$ in short-term exposure. However, PDC treatment of short term affected neither basal $[Ca^{2+}]_i$ nor NMDA-induced elevation of $[Ca^{2+}]_i$ (Fig. 4). And, co-application of PDC in a brief period with NMDA to cells augmented NMDA-induced glutamate accumulation, since reuptake of glutamate released by NMDA receptor activation was blocked by PDC (Fig. 1). Thus, it was presumed that increased extracellular glutamate by PDC, a glutamate reuptake blocker, selectively desensitized the NMDA receptors. In support of this idea, Issacson and Nicoll (1993) suggested that the PDC-induced modulation of current is due to an elevation of ambient concentrations of glutamate rather than direct activation of NMDA receptor.

Each glutamate receptor subtype shows different features in their desensitization. Chizhmakov *et al.*, (1992) suggested that the NMDA receptors display different features in a desensitized state when tested after pre-incubation of the neuron in aspartate alone or in aspartate with glycine. Aspartate alone induces desensitization of the NMDA receptor manifested as a reduced maximal response with no change in apparent Kd, whereas a combination of both co-agonists induces a desensitization of the NMDA receptor which is accompanied by an increase in affinity of receptor for both co-agonists. Inhibition of glutamate uptake, and the subsequent accumulation of extracellular glutamate, could have both presynaptic and postsynaptic consequences. Rapid desensitization of the AMPA receptors (Trussel and Fischbach, 1990) by ambient concentrations of glutamate could result in a decreased amplitude of the EPSC. An increase in the NMDA component of the EPSC may also be expected, given the high affinity of the NMDA receptor for glutamate (Patneau and Mayer, 1990). Trussell *et al.*, (1993) indicated that AMPA receptors were desensitized by neurotransmitter and that this desensitization depended on the number of quanta in the

synaptic cleft. Though there is a couple of observation that kainate responses do desensitize (Huettner, 1990; Arvanov and Usherwood, 1991), it is not consistent in all vertebrate CNS neurons. Also in the present experiment, kainate receptor failed to show desensitization (Fig. 2, 5).

One form of NMDA receptor desensitization is voltage- and Ca^{2+}-dependent with both the rate and degree of desensitization increasing with hyperpolarization and higher extracellular Ca^{2+} concentrations (Clark *et al.*, 1990). Whether Ca^{2+} promotes desensitization by acting intracellularly or extracellularly is unclear. Although a role of desensitization is to protect cells from the deleterious effects of prolonged or repeated stimuli, whether NMDA receptor desensitization, under physiological conditions, plays a protective role remains to be determined.

In summary, glutamate reuptake inhibitor, PDC, augmented NMDA-induced glutamate accumulation in short-term application. However, pretreatment of cells with PDC reduced NMDA-induced glutamate accumulation and elevation of $[Ca^{2+}]_i$, but did not affect kainate-induced responses. Similar to the pretreatment effect of PDC, exogenous glutamate pretreatment reduced NMDA-induced glutamate accumulation.

REFERENCES CITED

Arvanov, V. L. and Usherwood, P. N. R., Concentration jump studies of intracellularly dialysed Xenopus oocytes show desensitization of kainate receptors. *Neurosci. Lett.*, 129, 201-204 (1991).

Bridges, R. J., Stanley, M. S., Anderson, M. W., Cotman, C. W. and Chamberlin, A. R., Conformationally defined neurotransmitter analogues. Selective inhibition of glutamate uptake by one pyrrolidine-2, 4-dicarboxylate diastereomer. *J. Med. Chem.*, 34, 717-725 (1991).

Cai, Z. and McCaslin, P. P., Amitriptyline, desipramine, cyproheptadine and carbamazepine, in concentrations used therapeutically, reduced kainate- and N-methyl-D-aspartate-induced intracellular Ca 2+ levels in neuronal culture. *Eur. J. Pharmacol.*, 219, 53-57 (1992).

Chizhmakov, I. V., Kiskin, N. I. and Krishtal, O. A., Two types of steady-state desensitization of N-methyl-D-aspartate receptor in isolated hippocampal neurons of rat. *J. Physiol.*, 448, 453-472 (1992).

Choi, D. W., Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci.*, 11, 465-469 (1988).

Clark, D. D., Clifford, D. B. and Zorumski, C. F., The effect of agonist concentration, membrane voltage and calcium on N-methyl-D-aspartate receptor desensitization. *Neuroscience*, 39, 787-797 (1990).

Ellison, D. W., Beal, M. F. and Martin, J. B., Amino acid neurotransmitters in postmortem human brain analyzed by high performance liquid chromatography with electrochemical detection. *J. Neurosci. Metho.*, 19, 305-315 (1987).

Flott, B. and Seifert, W., Characterization of glutamate uptake systems in astrocyte primary cultures from rat brain. Glia, 4, 293-304 (1991).

Geoffroy, M., Lambolez, B., Carvalho, L. P., Rossier, J. and Stinnakre, J., Concanavalin A potentiates NMDA-evoked responses in the Xenopus oocyte expression system. *Eur. J. Pharmacol.*, 166, 355-356 (1989).

Globus, M. Y., Busto, R., Martinez, E., Valdes, I., Dietrich, W. D. and Ginsberg, M., Comparative effect of transient global ischemia on extracellular levels of glutamate, glycine, and gamma-aminobutyric acid in vulnerable and nonvulnerable brain regions in the rat. *J. Neurochem.*, 57, 470-478 (1991).

Grynkiewicz, G., Poenie, M. and Tsien, R. Y., A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, 260, 3440-3450 (1985).

Hestrin, S., Sah, P. and Nicoll, R. A., Mechanisms generating the time course of dual component excitatory synaptic currents recorded in hippocampal slices. *Neuron*, 5, 247-253 (1990).

Huettner, J. E., Glutamate receptor channels in rat DRG neurons: activation by kainate and quisqualate and blockade of desensitization by Con A. *Neuron*, 5, 255-266 (1990).

Issacson, J. S. and Nicoll, R. A., The uptake inhibitor L-trans-PDC enhances responses to glutamate but fails to alter the kinetics of excitatory synaptic currents in the hippocampus. *J. Neurophsiol.*, 70, 2187-2191 (1993).

Kato, K., Puttfarcken, p. S., Lyons, W. E. and Coyle, J. T., Developmental time course and ionic dependentce of kainate-mediated toxicity in rat cerebellar granule cell cultures. *J. Pharmacol. Exp. Ther.*, 256, 402-411 (1991).

Lerma, J., Herranz, A. S., Herreras, O., Abraira, V. and Martin Del Rio, R., In vivo determination of extracellular concentration of amino acids in the rat hippocampus; a method based on brain dialysis and computerized analysis. *Brain Res.*, 384, 145-155 (1986).

Lin, S. S. and Levitan, I. B., Concanavalin A: a tool to investigate neuronal plasticity. *Trends Neurosci.*, 14, 273-277 (1991).

Lysko, P. G., Cox, J. A., Vigano, M. A. and Henneberry, R. C., Excitatory amino acid neurotoxicity at the N-methyl-D-aspartate receptor in cultured neurons: pharmacological characterization. *Brain*

Res., 499, 258-266 (1989).

Maki, R., Robinson, M. B. and Dichter, M. A., The glutamate uptake inhibitor L-trans-pyrrolidine-2,4-dicarboxylate depresses excitatory synaptic transmission via a presynaptic mechanism in cultured hippocampal neurons. *J. Neurosci.*, 14, 6754-6762 (1994).

Marini, A. and Novelli, A., DL-threo-3-hydroxyaspartate reduces NMDA receptor activation by glutamate in cultured neurons. *Eur. J. Pharmacol.*, 194, 131-132 (1991).

Matsumoto, K., Ueda, S., Hashimoto, T. and Kuriyama, K., Ischemic neuronal injury in the rat hippocampus following transient forebrain ischemia: evaluation using *in vivo* microdialysis. *Brain Res.*, 534, 236-242 (1991).

Mayer, M. L. and Vyklicky, Jr, L. V., Concanavalin A selectively reduces desensitization of mammalian neuronal quasqualate receptors. *Proc. Natl. Acad. Sci. USA*, 86, 1411-1415 (1989).

McCaslin, P. P. and Ho, I. K., Cell culture in neurotoxicology, In Hayes, A. W. (eds.). *Principles and methods of toxicology*. Raven Press, New York, pp. 1315-1334, 1994.

McCaslin, P. P. and Morgan, W. W., Cultured cerebellar cells as an in vitro model of excitatory amino acid receptor function. *Brain Res.*, 417, 380-384 (1987).

Patneau, D. K. and Mayer, M. L., Structure-activity relationship for amino acid transmitter candidates acting at N-methyl-D-aspartate and quisqualate receptors. *J. Neurosci.*, 10, 2385-2399 (1990).

Raulli, R., Danysz, W., and Wroblewski, J. T., Pretreatment of cerebellar granule cells with concanavalin A potentiates quisqualate-stimulated phosphoinositide hydrolysis. *J. Neurochem.*, 56, 2116-2124 (1991).

Sarantis, M., Ballerini, L., Miller, B., Silver, R. A., Edwards, M., Attwell, D., Glutamate uptake from the synaptic cleft does not shape the decay of the non-NMDA component of the synaptic current. *Neuron*, 11, 541-549 (1993).

Shimada N., Graf, R., Rosner, G. and Heiss, W., Ischemia-induced accumulation of extracellular amino acids in cerebral cortex, white matter, and cerebrospinal fluid. *J. Neurochem.*, 60, 66-71 (1993).

Talor, C. P., Geer, J. J. and Burke, S. P., Endogenous extracellular glutamate accumulation in rat neocortical cultures by reversal of the transmembrane sodium gradient. *Neurosci. Lett.*, 145, 197-200 (1992).

Trussell, L. O. and Fischbach, G. D., Glutamate receptor desensitization and its role in synaptic transmission. *Neuron*, 3, 209-218 (1989).

Trussell, L. O., Zhang, S. and Raman, I. M., Desensitization of AMPA receptors upon multiquantal neurotransmitter release. *Neuron*, 10, 1185-1196 (1993).

Watkins, J. and Evans, R. H., Excitatory amino acid transmitters. *Ann. Rev. Pharmacol. Toxicol.*, 21, 165-204 (1981).