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Short communication

(S)-5-Fluorowillardiine-mediated neurotoxicity in cultured murine cortical neurones occurs via AMPA and kainate receptors

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Abstract

We have examined the neurotoxic effects of kainate, (*S*)-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and the novel AMPA-receptor preferring agonist (*S*)-5-fluorowillardiine in murine cultured cortical neurones. Kainate induced > 90% cell death (EC_{50} 65 μM) and (*S*)-AMPA only about 50% cell death (EC_{50} 3.1 μM), both in a monophasic dose-dependent manner. (*S*)-5-Fluorowillardiine also killed > 90% of neurones, however, in a biphasic dose-dependent manner (EC_{50} 0.70 and 170 μM). Additionally, the neurotoxic effects of (*S*)-AMPA and (*S*)-5-fluorowillardiine (high-affinity component) were attenuated by the AMPA receptor antagonist LY293558 ((3*S*,4a*R*,6*R*,8a*R*)-6-[2-(1*H*-tetrazol-5-yl)-ethyl]-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid). A component of kainate and (*S*)-5-fluorowillardiine (low-affinity component) neurotoxicity was blocked by the low-affinity kainate receptor antagonist NS-102 (5-nitro-6,7,8,9-tetrahydrobenzo[*g*]indole-2,3-dione-3-oxime). We have shown that both kainate and (*S*)-AMPA can effect substantial cell death in cortical neurones and that the novel agonist (*S*)-5-fluorowillardiine exerts its excitotoxicity through both AMPA- and kainate-preferring receptors.

Keywords: (*S*)-AMPA ((*S*)-α-amino-3-hydroxy-5-methylisoxazole-4-propionate); Cortical neuron; Excitotoxicity; (*S*)-5-Fluorowillardiine; LY293558; NS-102

1. Introduction

Glutamate-mediated excitotoxicity is thought to involve the over-activation of a family of ionotropic receptors identified by their preferred agonists: *N*-methyl-D-aspartate (NMDA), kainate and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) (Choi, 1992). Excitotoxicity has been generally considered to occur predominantly via the NMDA receptor subtype, which is slowly desensitizing and highly permeable to Ca^{2+} allowing influx of Ca^{2+} upon agonist binding (Choi, 1992). Unlike NMDA, which induces cell death rapidly after administration (Choi, 1988), the exposure of cultured neurones to micromolar concentrations of non-NMDA receptor agonists, particularly AMPA, has been reported to result in negligible cell death, in vitro, even after long term exposure (May and Robison, 1993). In some studies AMPA neurotoxicity can only be observed by using cyclothiazide to block the rapid desensitization of the receptor (May and Robison, 1993; Cebers and Liljequist, 1995). Consequently, the characterization of non-NMDA receptor-mediated excitotoxicity is well behind our understanding of NMDA receptor-mediated excitotoxicity. Recent advances in the molecular cloning of non-NMDA receptors, however, have revealed enormous subunit diversity in AMPA (Glu_1-Glu_4) and kainate (Glu_5-Glu_7, $kainate_1$ and $kainate_2$) receptor families (Hollmann and Heinemann, 1994). The subunit composition of these receptors largely determines not only agonist selectivity but also physiological function. For example, Lu et al. (1995) described subsets of non-NMDA receptors in murine cortical neurones that were highly Ca^{2+}-permeable, induced substantial Ca^{2+} influx and rapid cell death upon agonist binding. Thus non-NMDA receptors may prove to be important targets in glutamate-mediated excitotoxicity.

Recent physiological and pharmacological studies using the structurally rigid analogue of L-glutamate, (*S*)-5-fluorowillardiine, have indicated that this compound may be a preferred agonist for AMPA selective receptors. (*S*)-5-Fluorowillardiine has been shown to be a potent agonist for AMPA-preferring receptors in cultured rat embryonic hippocampal neurones (Wong et al., 1994) and (*S*)-5-[^{3}H]flu-

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orowillardiine apparently labels a high affinity AMPA receptor in rat brain synaptic membranes (Hawkins et al., 1995a). (*S*)-5-Fluorowillardiine has also been shown to selectively inhibit the binding of (*S*)-[^{3}H]AMPA, the active analogue of AMPA, to rat brain synaptic membranes (Hawkins et al., 1995b). In the present study we determined the neurotoxic profiles of (*S*)-5-fluorowillardiine and (*S*)-AMPA, the active isomer of AMPA, in cultured murine cortical neurones. In addition, we examined the neuroprotective ability of LY293558 ((3*S*,4*aR*,6*R*,8*aR*)-6-[2-(1*H*-tetrazol-5-yl)-ethyl]-1,2,3,4,4a,5,6,7,8,8*a*-decahydroisoquinoline-3-carboxylic acid), described as a systemically active, competitive AMPA receptor antagonist (Ornstein et al., 1993), and NS-102 (5-nitro-6,7,8,9-tetrahydrobenzo[*g*]indole-2,3-dione-3-oxime), described as a low-affinity kainate receptor antagonist (Johansen et al., 1993; Verdoorn et al., 1994).

2. Materials and methods

2.1. Materials

(*S*)-5-Fluorowillardiine and (*S*)-AMPA were purchased from Tocris Cookson (Bristol, UK); NS-102 was from Research Biochemicals International (Natick, MA, USA); kainate was purchased from Sigma (Sydney, Australia); LY293558 was a gift from P. Ornstein of Eli Lilly Laboratories (Indianapolis, IN, USA); Neurobasal cell culture medium was purchased from GibcoBRL Life Technologies (Melbourne, Australia). Cells were grown in Nunc (Denmark) cell culture plates, RPMI 1640 growth medium (without phenol red) and all cell culture component chemicals were purchased from Sigma and were of cell culture grade.

2.2. Cell culture

Primary cultures of murine neocortical neurons were obtained essentially as previously described (Carroll et al., 1996). Briefly, whole cerebral neocortices were removed from foetal Swiss-White mice (14–16 days gestation), taking care to remove meninges, basal forebrain and hippocampal structures. The tissue was minced, digested using trypsin (0.2 mg/ml, 5 min), mechanically dispersed using trituration (10 strokes), then plated on to poly-D-lysine (50 μg/ml) coated plates at a density of 0.2×10^6 cells/cm^2 in 24- or 96-well plates and maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 8% O_2. Cultures were grown in antioxidant rich-supplemented Neurobasal medium (Brewer et al., 1993) without the addition of L-glutamate and antibiotics. During routine culture one-half of the growth medium was changed at 3 days in vitro.

Immunocytochemical staining using antisera for neurone-specific enolase and glial fibrillary acidic protein indicated that 95% of the cells were neurones with minimal contamination by glia (data not shown). In agreement with data reported by Brewer and Cotman (1989) and Brewer et al. (1993) non-neuronal cells fail to survive more than 48 h in culture in serum-free growth medium, at low cell density, eliminating the need to use mitotic inhibitors to obtain pure neuronal cultures.

2.3. Exposure to agonists and antagonists

The effects of the experimental compounds were tested in the cultures at 6 days in vitro. All agonist and antagonist exposure was performed at 37°C in a humidified incubator (5% CO_2, 8%O_2) for 24 h at various concentrations in antioxidant-free maintenance medium. For these conditions the Neurobasal medium supplementation was modified by omitting the following components: D,L-α-tocopherol, D,L-α-tocopherol acetate, catalase, superoxide dismutase, reduced glutathione and L-glutamine. The extent of cell death in the cultures was determined using morphological examination and cell counting in conjunction with a cytotoxicity assay. After agonist exposure the cultures were examined under phase-contrast microscopy; fields ($n = 6$) were photographed for cell counting and comparison. In control cultures 1100–1200 cells were counted. Cell death was also assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, as a biochemical index of neuronal viability, essentially as described (Roehm et al., 1991) with the following modifications: MTT solutions were prepared in RPMI 1640 growth medium, 10 μl of 5 mg/ml MTT was added to 100 μl culture and after incubation at 37°C for 30 min, the medium was removed and the cells were lysed and the formazan product was dissolved by addition of 100 μl of 20% sodium dodecyl sulphate (SDS) in 40% dimethylformamide, pH 4.6 overnight. The absorbance of the wells was determined using a Ceres UV900 microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at a wavelength of 570 nm. Control cultures were included in each experiment containing either vehicle alone or vehicle with antagonist. These controls were taken as 100% neuronal viability, under the experimental conditions, and the average MTT assay absorbance (A_{570}) value was 0.55 ± 0.01 ($n = 68$). Each experiment also included control cultures with 500 μM L-glutamate, representing 100% glutamatergic neuronal cell death, with an average absorbance value of 0.09 ± 0.003 ($n = 34$). The background cell death in vehicle control cultures was 5–8%.

2.4. Data analysis

Concentration-response curves were generated using computer-assisted curve-fitting program of GraphPad Prism Version 2.0 (GraphPad Software, San Diego, CA, USA) based on a four-parameter logistic equation to describe sigmoidal concentration-response curves (Rodbard, 1974).

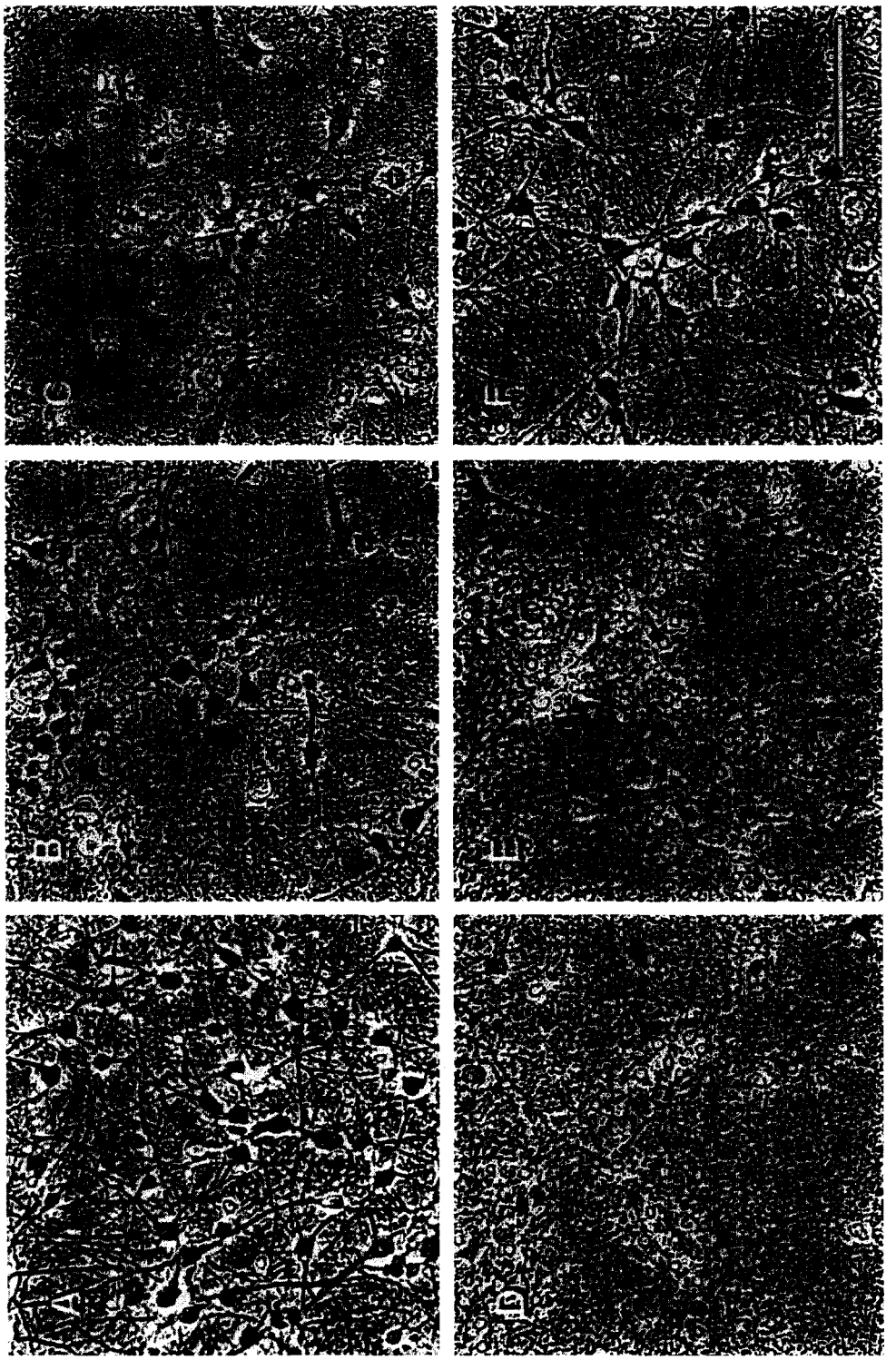

Fig. 1. Morphological evidence of neurotoxicity produced by (*S*)-AMPA, (*S*)-5-fluorowillardiine and kainate in primary cultured murine cortical neurones. Phase-contrast photomicrographs from typical representative fields of cells were taken 24 h after being transferred to antioxidant free medium and exposure to vehicle control (A), 100 μM (*S*)-AMPA (B), 10 μM (*S*)-5-fluorowillardiine (C), 100 μM (*S*)-5-fluorowillardiine (D), 100 μM kainate (E) and 10 μM (*S*)-5-fluorowillardiine + 10 μM LY293558 treatment (F). In control cultures 1100–1200 cells were counted. The calibration bar represents 10 μm.

EC_{50} values were defined as the concentrations required to produce 50% cell viability as determined from minimum and maximum neuron viability parameters of individual concentration-response curves, which yielded the components of overall cell viability sensitive to (*S*)-AMPA, (*S*)-5-fluorowillardiine and kainate. Statistical significance of the cell counting cytotoxicity data was analysed using a one-way analysis of variance (ANOVA) test with a post-hoc Dunnett's *t* test. The drug effects relative to controls were all statistically significant ($P = 0.0001$). Values are expressed as mean ± S.E.M.

3. Results

Neurotoxicity induced by (*S*)-AMPA, (*S*)-5-fluorowillardiine and kainate was assessed using both morphological and biochemical analyses. The exposure of cortical neurones to (*S*)-AMPA and (*S*)-5-fluorowillardiine for 24 h in antioxidant-free medium produced prominent neurotoxicity as shown by the observed pattern of changes in neuronal morphology (Fig. 1). As shown in Fig. 1B, neurones exposed to 100 μM (*S*)-AMPA exhibited notable characteristics of neuronal deterioration and cell death. The intricate neurite networks have extensively degenerated and there was a notable reduction in the numbers of perikarya. Cell counting indicated the loss was 56 ± 9% ($n = 6$) compared to control cultures with vehicle alone (Fig. 1A). Interestingly, cultures exposed to 10 μM and 100 μM (*S*)-5-fluorowillardiine (Fig. 1C and Fig. 1D) show a far more comprehensive destruction of neurites and cell bodies than found with 100 μM (*S*)-AMPA, with 79 ± 3% and 92 ± 5% (both $n = 6$) loss of cells, found by cell counting, respectively. Kainate was also a potent neurotoxin at 100 μM, and was shown (Fig. 1E) by similar analyses to kill 80 ± 7% ($n = 6$) of neurones. The neuroprotective effects of the AMPA receptor antagonist LY293558 (10 μM) in neurones exposed to 10 μM (*S*)-5-fluorowillardiine are shown in Fig. 1F where cell death estimated by cell counts was only 40 ± 5% ($n = 6$) compared to control cultures (vehicle alone).

The concentration-response curves for (*S*)-AMPA, (*S*)-5-fluorowillardiine and kainate were determined using the MTT assay as an index of neuronal viability and confirmed the pattern of cellular loss found in the morphological analyses described above. The EC_{50} value for (*S*)-

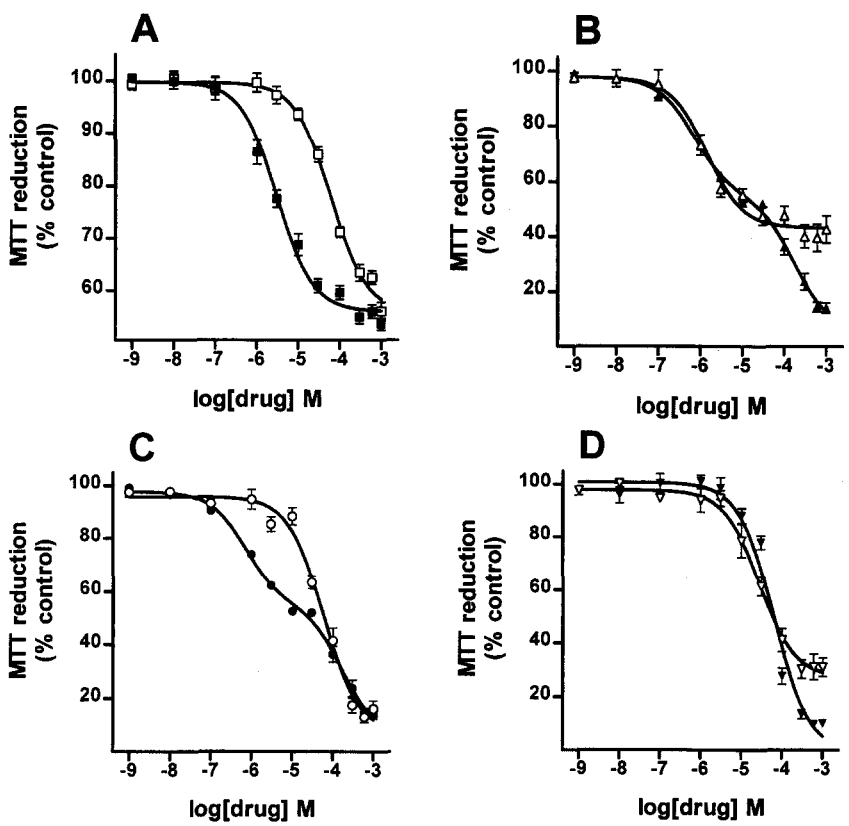

Fig. 2. Dose-dependent non-NMDA receptor-mediated excitotoxicity in murine cortical neurones. Concentration-response curves of cortical neurones exposed for 24 h to (*S*)-AMPA (A) with (□) or without (■) 10 μM LY293558, (*S*)-5-fluorowillardiine (B) with (△) or without (▲) 10 μM NS-102, (*S*)-5-fluorowillardiine (C) with (○) or without (●) 10 μM LY293558 and kainate (D) with (▽) or without (▼) 10 μM NS-102. Each point represents the means of 3–8 separate experiments (n = 12–28 cultures) ± S.E.M. Concentration-response curves were generated using computer-aided non-linear regression finding the equation that best fit the data. (*S*)-AMPA and kainate dose-response curves are monophasic and (*S*)-5-fluorowillardiine concentration-response data are biphasic. Absorbance values (n = 12–68 observations) for the experimental groups were: control (vehicle) 0.55 ± 0.01, L-glutamate (500 μM) 0.09 ± 0.003, (*S*)-AMPA (1 mM) 0.32 ± 0.006, (*S*)-5-fluorowillardiine (1 mM) 0.16 ± 0.02 and kainate (1 mM) 0.13 ± 0.01.

AMPA was 3.1 ± 0.1 μM ($n = 28$ cultures) and 62 ± 0.1 μM ($n = 16$ cultures) in the presence of LY293558 (10 μM). The EC_{50} value for kainate was 65 ± 0.1 μM ($n = 12$ cultures). LY293558 at 10 μM had no significant neuroprotective effect on the dose-dependent neurotoxicity of kainate (data not shown). As shown in Fig. 2A and Fig. 2D, the concentration-response curves for (*S*)-AMPA and kainate were monophasic indicative of activity at one binding site. Unlike AMPA and kainate, the concentration-response curve for (*S*)-5-fluorowillardiine was found to be biphasic (Fig. 2B and Fig. 2C). The EC_{50} values were 0.70 ± 0.12 μM for the high-affinity component of the curve and 170 ± 0.1 μM ($n = 28$ cultures) for the low-affinity component. The AMPA selective antagonist LY293558 was only effective against the high-affinity component of neurotoxicity induced by (*S*)-5-fluorowillardiine, indicating that this effect was likely to be via AMPA receptors (Fig. 2C). In the presence of 10 μM LY293558 the concentration-response curve for (*S*)-5-fluorowillardiine was monophasic; EC_{50} value of 57 ± 0.1 μM ($n = 12$ cultures). The recently available kainate selective antagonist, NS-102 (Johansen et al., 1993; Verdoorn et al., 1994), was used to determine whether the second phase of the (*S*)-5-fluorowillardiine concentration-response curve was due to activity at low affinity kainate receptors. Indeed, we found that NS-102 totally blocked the neurotoxicity of the low-affinity component of (*S*)-5-fluorowillardiine (upper part of the concentration-response curve unaltered) (Fig. 2B) and the low-affinity response to kainate (Fig. 2D).

4. Discussion

In this study we examined the ability of non-NMDA receptor agonists to induce excitotoxic injury in cortical neurones and found that all agonists tested could effect substantial neuronal cell death with long term exposure. In many previous reports excitotoxicity via the AMPA receptor could not be demonstrated without the use of cyclothiazide to block the rapid desensitization of the receptor (May and Robison, 1993). We have shown in this study, however, that pure cultures of murine cortical neurones are susceptible to substantial cell death (50%) with long term exposure to 100 μM (*S*)-AMPA. Previous reports indicated that (*S*)-5-fluorowillardiine was highly selective for AMPA receptors (Wong et al., 1994, Hawkins et al., 1995a,b), however our findings indicate that (*S*)-5-fluorowillardiine-mediated cell death is not exclusively at AMPA-preferring receptors in cortical neurones in vitro, but via actions at AMPA- and kainate-preferring receptors.

Three lines of evidence indicated that (*S*)-5-fluorowillardiine was acting at both high affinity AMPA and low affinity kainate receptors. First, the EC_{50} values for (*S*)-AMPA and the high affinity component of (*S*)-5-fluorowillardiine were similar being 3.1 and 0.70 μM, respectively. Furthermore, the EC_{50} values of kainate and the low affinity component of (*S*)-5-fluorowillardiine were also comparable being 65 and 170 μM respectively. LY293558 was recently reported as being AMPA receptor selective (Ornstein et al., 1993) and also neuroprotective in rat cortical and cerebellar granule neurones, acting as a competitive antagonist with preferential affinity for non-NMDA receptors (Liljequist et al., 1995). Our results also show substantial neuroprotection by LY293558 in cortical neurones; at 10 μM LY293558 was neuroprotective against (*S*)-AMPA- and (*S*)-5-fluorowillardiine (high-affinity component)-induced neurotoxicity, but ineffective against kainate (data not shown)- and (*S*)-5-fluorowillardiine (low-affinity component)-induced neurotoxicity (Fig. 2A and Fig. 2C). Finally the antagonist NS-102, selective for low-affinity kainate receptors (Johansen et al., 1993; Verdoorn et al., 1994), was found to be neuroprotective versus only the low-affinity components of kainate- and (*S*)-5-fluorowillardiine-induced neurotoxicity, indicating (*S*)-5-fluorowillardiine was acting at both AMPA and kainate receptors.

In view of the enormous diversity within the AMPA and kainate receptor families, revealed by molecular cloning, further effort is needed to fully understand the physiological role of these receptors in excitotoxic neuronal cell death. We have shown in this study that AMPA and kainate receptor activation can induce excitotoxicity in cortical neurones, effecting substantial neuronal loss and that a novel non-NMDA receptor agonist, (*S*)-5-fluorowillardiine, produces neuronal cell death via AMPA and kainate receptors. These actions are likely to be exerted via both high-affinity AMPA (Glu_1-Glu_4) receptors and low-affinity kainate (Glu_5-Glu_7) receptors.

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