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Reduction of striatal *N*-methyl-D-aspartate toxicity by inhibition of nitric oxide synthase

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Abstract—Coronal slices of rat brain were incubated for 40 min in 300 μM kainate (KA) or 500 μM *N*-methyl-D-aspartate (NMDA). Histological examination showed neuronal degeneration accompanied by significant losses in the activity of neuron-specific enolase (NSE; EC 4.2.1.11) (−23% KA; −26% NMDA). The activity of the glial enzyme glutamine synthetase (GS; EC 6.3.1.2) was also reduced (−32% KA; −27% NMDA). Pre-incubation with 100 μM L-N^G-nitroarginine (L-N-ARG), an inhibitor of nitric oxide (NO) synthase (EC 1.14.23.—), for 20 min attenuated the toxicity of NMDA, but not KA. NSE levels after successive incubation in L-N-ARG and NMDA were 95% of controls incubated in Krebs bicarbonate medium only (GS activity 89% of controls). In contrast, pre-incubation with L-N-ARG prior to the addition of KA resulted in neuronal degeneration and significant reductions in NSE levels and GS activities. These observations suggest that the unrestricted function of NO synthase is significant in mediating NMDA neurotoxicity whereas KA toxicity is associated with alternative mechanisms not linked to NO production.

It has been demonstrated that excitatory amino acids (EAA*) and their agonists can be toxic to central nervous tissue *in vivo* and *in vitro* and might play an important role in human neurological disorders such as stroke, epilepsy and chronic neurodegenerative diseases [1–3]. *N*-Methyl-D-aspartate (NMDA) and kainate (KA) are structural analogues of glutamate and can cause excitotoxic neuronal injury both after direct injection *in vivo* [3, 4] and on incubation of brain slices *in vitro* [3, 5, 6]. It is well known that NMDA and KA each activate a different sub-class of post-synaptic glutamate receptors [2, 7, 8], and that there are other differences in their mechanisms of action. For instance, the prolonged activation of post-synaptic dendrosomal NMDA receptors leads to an accumulation of intracellular calcium and can cause severe neuronal loss by damaging mitochondria, activating proteases and stimulating several second messenger systems including the production of nitric oxide (NO) [9, 10]. Unlike NMDA, KA toxicity is dependent on an intact glutamatergic projection from the cortex [4], and KA has been shown to cause an increase in extracellular levels of glutamate [11], which may arise by leakage from the cytoplasm by means of the glutamate carrier protein [12]. Decreases in the activity of glutamine synthetase (GS; EC 6.3.1.2), an enzyme localized exclusively in glial cells [13], have been demonstrated after incubation of coronal slices *in vitro* with both KA and NMDA [6], and KA toxicity is reduced after impairment of glial cell metabolic activity [5]. It has recently been demonstrated that the activation of NMDA and KA receptors in cerebellar slices induces NO synthesis from arginine [10, 14]. NO synthase (EC 1.14.23.—) is an intraneuronal, oxygen- and calcium-dependent enzyme that is inhibited by L-N^G-nitroarginine (L-N-ARG) with IC_{50} concentrations in cerebellar slices of 0.6 μM [15, 16].

By use of histological examination of thionin-stained striatal sections and the determination of neuron-specific enolase (NSE; EC 4.2.1.11) levels and GS activities in striatal homogenates, this study investigates whether the inhibition of NO synthase by L-N-ARG influences the neurotoxic potency of NMDA and KA on rat striatal cells *in vitro*, and if so, whether NMDA and KA toxicity are affected differently.

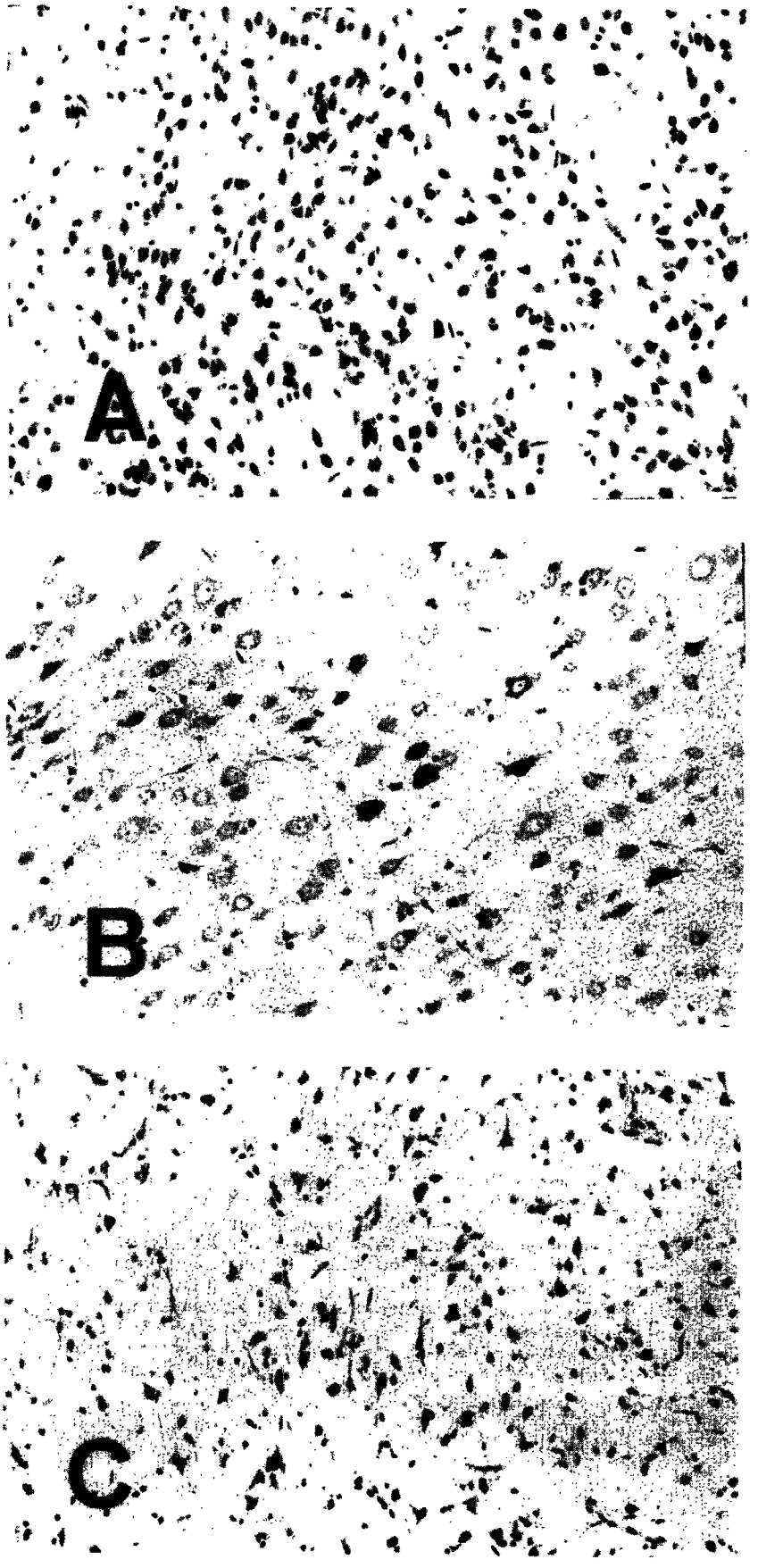
* Abbreviations: EAA, excitatory amino acids; GHA (L-glutamic acid γ-monohydroxamate); GS, glutamine synthetase; KA, kainate; NMDA, *N*-methyl-D-aspartate; L-N-ARG, L-N^G-nitroarginine; NSE, neuron-specific enolase.

Materials and Methods

Chemicals. L-N-ARG, KA and NMDA were purchased from the Sigma Chemical Co. (Poole, U.K.). The radioimmunoassay for human NSE was supplied by Pharmacia Diagnostics AB (Uppsala, Sweden) and used according to the supplier's instructions. Previous work has shown this to be adequate for detecting the enzyme in rat brain [6]. All other chemicals were supplied by BDH Ltd (Poole, U.K.).

Incubation of brain slices. The tissue-slice procedure described previously [5, 6] was used for assessing excitotoxicity *in vitro*. Briefly, coronal rat brain slices including the corpus striatum and part of the cerebral cortex were prepared. The slices were placed singly in a number of open perfusion chambers. Each chamber was perfused with oxygenated Krebs bicarbonate medium pH 7.4, at 30° at a rate of 0.5–1.0 mL/min. Control values were obtained after an incubation time of 40 min in Krebs bicarbonate medium only. Compounds under study were added in the following concentrations: KA 300 μM, NMDA 500 μM (incubation time 40 min each). Pre-incubation with 100 μM L-N-ARG for 20 min was followed by an incubation either with KA (300 μM) or with NMDA (500 μM) for 40 min each, so that five different conditions were compared (see Table 1). The slices were then removed and processed for either histological or biochemical analysis.

Assessment of striatal injury. Overall neuronal cell injury was estimated by histological examination of the tissue after fixation of each slice in 2% paraformaldehyde–1% glutaraldehyde in 0.1 M phosphate buffer, followed by dehydration and paraffin impregnation. Sections (6 μm) were stained in thionin (nuclei, nucleoli and rough endoplasmatic reticulum of neurons become visible; cytoarchitecture of glial cells is not discernible) for examination by light microscopy. Metabolic changes to glial cells were assessed by the measurement of GS activity in striatal homogenates (caudate-putamen dissected out and homogenized in 0.1 M sodium acetate buffer, pH 5.5) by the method of Wellner and Meister [17], which monitors the γ-glutamyl-transferase (EC 2.3.2.2) activity of the enzyme by the formation of L-glutamic acid γ-monohydroxamate from glutamine and hydroxylamine. To assess neuronal metabolism, NSE levels were measured [18]. The same homogenates in which GS activities were determined were first diluted 1:10 using 20 mM Tris–phosphate buffer (pH 7.4) containing 3 mM $MgSO_4$ and then centrifuged at 100,000 *g* for 1 hr. The supernatants were used for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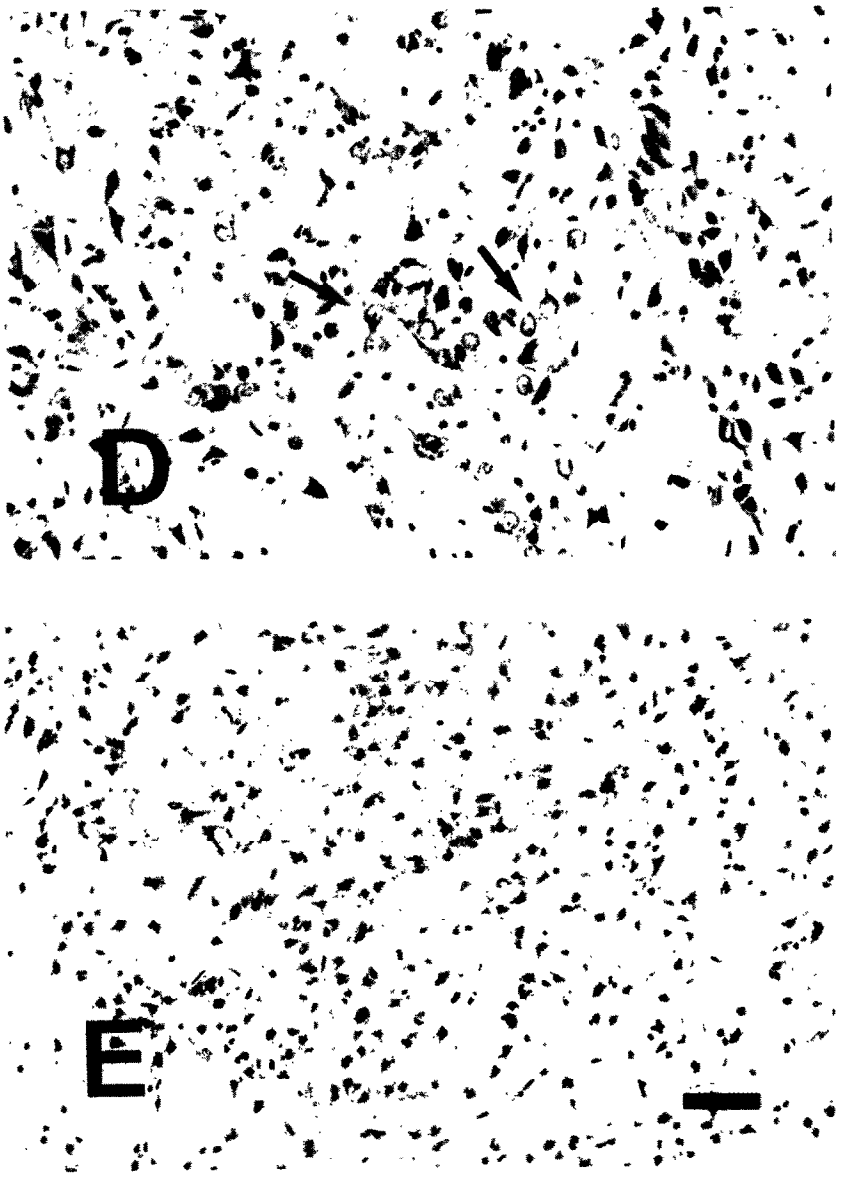

Fig. 1. Coronal rat brain slices including the corpus striatum were incubated in Krebs bicarbonate medium and with NMDA and KA for 40 min at 30°. Where appropriate, slices were pre-incubated in 100 μM L-N-ARG for 20 min. After fixation and embedding in paraffin, 6 μm sections were stained in thionin and examined by light microscopy. Scale bar = 50 μm, magnification scale is equal in photomicrographs A–E. (A) Section through unlesioned striatum after 40 min incubation in Krebs bicarbonate medium (control). (B) Corpus striatum after incubation in NMDA (500 μM), reduced number of striatal neurons, swelling of neuronal perikarya. (C) Striatum after incubation in kainate (300 μM), wide-spread karyopyknosis. (D) L-N-ARG (100 μM, 20 min) + NMDA (500 μM, 40 min), minimal lesion of striatal neurons, no perikaryal swelling, note surviving neurons (arrows) albeit lower number than in (A). (E) L-N-ARG (100 μM, 20 min) + KA (300 μM, 40 min), reduced number of neurons, many pyknotic neurons.

Table 1. Effects of inhibition of NO synthase by L-N-ARG on the toxicity of KA and NMDA in rat corpus striatum

Incubation	N	GS	%	NSE	%
Control	7	1.96 ± 0.29	100	341.33 ± 33.01	100
NMDA	7	1.43 ± 0.14*	73	253.20 ± 11.52*	74
KA	7	1.34 ± 0.10*	68	262.40 ± 21.97*	77
L-N-ARG + NMDA	5	1.74 ± 0.10†	89	323.40 ± 13.09†	95
L-N-ARG + KA	5	1.48 ± 0.06*‡	76	298.20 ± 15.71*‡	87

Coronal rat brain slices were incubated in oxygenated Krebs bicarbonate medium (control), KA (300 μM) and NMDA (500 μM) for 40 min each at 30° as described in Materials and Methods. Pre-incubation in L-N-ARG (100 μM) for 20 min was followed by a 40 min incubation period in either KA (300 μM) or NMDA (500 μM). Activities of GS (units: nmol L-glutamic acid γ-monohydroxamate formed/mg protein/hr) and levels of NSE (units: μg/L) were determined in homogenates of corpus striatum.

Values are means ± SD.

P values were calculated for multiple comparison procedures (Bonferroni *t*-tests) after analysis of variance including all groups was done.

N refers to the number of observations from different rats assayed in duplicate.

* $P < 0.05$ vs control.

† $P < 0.05$ vs NMDA only.

‡ NS vs KA only.

radioimmunoassay of γγ-enolase. The protein content of each sample was measured according to the method of Markwell *et al.* [19].

Results

Successive incubation in L-N-ARG and NMDA. Pre-incubation of coronal rat brain slices in L-N-ARG reduced the neurotoxic effect of NMDA on striatal tissue. Histological examination showed that the number of viable neurons was high and that the swelling of neuronal perikarya found after incubation in NMDA only was absent (Fig. 1B and D). The levels of NSE in striatal homogenates after successive incubation in L-N-ARG and NMDA in comparison to control values obtained after incubation in Krebs bicarbonate medium only are given in Table 1. Multiple comparison procedures revealed a significant difference in NSE levels between the conditions "NMDA only" and "L-N-ARG + NMDA", but no significant difference between the conditions "control" and "L-N-ARG + NMDA". Similar results were found with respect to the activity of the glial marker enzyme GS. Control experiments showed that pre-incubation of the slices with 100 μM L-N-ARG for 40 min had no significant effect on nerve cell viability or on the activities of GS and the immunoreactivity of NSE. Furthermore, KA, NMDA and L-N-ARG showed no direct inhibitory effects on the GS activity or NSE immunoreactivity.

Successive incubation in L-N-ARG and KA. In contrast to the significant attenuating effect of L-N-ARG on NMDA toxicity, the neurotoxic potency of KA on striatal neurons was only slightly reduced by pre-incubation with L-N-ARG. Histological analysis showed a large number of pyknotic neurons (strongly stained chromatin within striatal neurons of reduced size) and only a few nerve cells with normal morphological appearance. The lesion pattern found after successive incubation in L-N-ARG and KA was very similar to the one found after incubation in KA only (Fig. 1C and E) and was markedly different from the appearance of unlesioned corpus striatum (Fig. 1A). Both NSE levels and GS activity in striatal homogenates remained relatively low after successive incubation in L-N-ARG and KA and were significantly different from control values (Table 1).

Discussion

It has been established in various *in vitro* models of excitotoxicity that an influx of extracellular calcium, with subsequent cellular overload, can be lethal to nerve cells [3, 9]. Cytotoxic mechanisms triggered by high intracellular calcium levels include the activation of second messenger systems, the mobilization of lipases and proteases, the generation of free radicals and the depletion of energy stores [7, 9].

Our results demonstrate that the pre-incubation of coronal rat brain slices with an inhibitor of NO synthesis, L-N-ARG, prior to the addition of NMDA resulted in a marked survival of striatal neurons and an only minimal impairment of both GS and NSE activities. Similar results derived from a study on rat hippocampal slices have recently been published [20]. The finding that the inhibition of NO synthesis protects striatal and hippocampal neurons of the CA1 region from the toxic effects of NMDA suggests that NO-mediated events play a major role in nerve cell death caused by NMDA receptor activation. Whether reduced levels of NO itself or the absence of subsequent NO-triggered events are important for the protective effect cannot be elucidated from these experiments. Beckman [15] recently claimed that peroxynitrite ($ONOO^-$) formed from NO and O_2^- can decompose to the cytotoxic oxidants hydroxyl radical and nitrogen dioxide, thereby causing cerebral injury not only in neurons bearing EAA receptors but in the vicinity reached by the diffusible agents NO and $ONOO^-$ as well.

In contrast to the protective effect of L-N-ARG against NMDA toxicity, the neurotoxic potency of KA was not significantly changed by pre-incubation with L-N-ARG. Earlier studies on the effects of KA on glutamate metabolism have indicated that KA acts as a competitive inhibitor of high-affinity glutamate uptake into neurons and glial cells [21]. Since uptake inhibition is not a good indicator of neurotoxicity [2], other mechanisms of action might be more relevant to account for KA's ability to destroy neurons. KA can cause direct damage by a massive influx of sodium, chloride and water into neurons leading to osmotic lysis [9]. High concentrations of KA can activate α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors [2], alter the compartmentation of the glutamate–glutamine system in lesioned striatum [22] and reduce intraneuronal ATP and glucose levels [4]. The density of KA receptors in the corpus striatum might be higher than the density of NMDA receptors [8]. Some or all of those

mechanisms not linked to the stimulation of NO synthesis could contribute to the different vulnerability of striatal neurons to the excitotoxins NMDA and KA. We have demonstrated previously that both NMDA and KA, when present during incubation of coronal slices, influence glial cell metabolism, as evidenced by significant reductions in GS activity [6]. In terms of KA toxicity, these results have been relatively easy to explain, since astrocytes contain KA receptors [23] through which a rise in intraglial calcium levels might be mediated [24]. However, with NMDA, the possible involvement with glial cell metabolism is more obscure, mainly because there is no evidence for NMDA receptors on glial cells [23]. NMDA toxicity is presumed to take place primarily through the activation of post-synaptic NMDA receptors [2]. The experiments reported here provide a clue to an effect of NMDA on glial cell metabolism: NO is a diffusible compound that can readily travel from the site of its synthesis in the post-synaptic neuron and affect soluble guanylate cyclase (EC 4.6.1.2) activity in adjacent cells [10]. Indeed, it has been postulated by Garthwaite [10] that this is the probable sequence of events, since the NMDA-induced rise in intracellular calcium levels in the post-synaptic cell which is required to activate NO synthase also effectively inhibits the guanylate cyclase activity in this location [10]. Thus, it is possible that diffusion of NO into astrocytes provides the effective link-up between NMDA receptor activation of the post-synaptic membrane and altered glial metabolism.

In conclusion, the results of this investigation support the hypothesis that the neurotoxic potency of NMDA in rat corpus striatum *in vitro* depends on the unrestricted function of NO synthase since the inhibition of this enzyme protects striatal tissue from excitotoxic damage. The difference in the mechanisms of action between KA and NMDA as excitotoxins is once again highlighted, since KA toxicity occurs by a mechanism which is not sensitive to inhibition of NO synthase.

*Department of Biochemistry
Trinity College
Dublin 2, Ireland
‡Department of Biochemistry
University College
Dublin 4, Ireland

HARALD KOLLEGGER*†
GETHIN J. MCBEAN‡
KEITH F. TIPTON*

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† Corresponding author: H. Kollegger, Neurological Clinic, University of Vienna, Währinger Gürtel 18-20, A-1090 Wien, Austria. Tel. (43) 1-40400-3135; FAX (43) 1-40400-5735.