Activity of Putative Cognition Enhancers in Kynurenate Test Performed with Human Neocortex Slices

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Accepted for publication March 16, 1999 This paper is available online at http://www.jpet.org

ABSTRACT

Some cognition enhancers were previously shown to potently prevent antagonism of the N-methyl-D-aspartate (NMDA)-evoked release of norepinephrine (NE) brought about in slices of rat hippocampus by kynurenic acid, an endogenous NMDA receptor blocker. We have examined the impact of putative nootropic agents in the kynurenate test performed with slices of human cerebral cortex from patients undergoing neurosurgery. In slices of human neocortex, local application of NMDA evoked release of [3H]NE; the effect of NMDA was antagonized by several NMDA receptor antagonists, including kynurenic acid. The antagonism of the NMDA-evoked [3H]NE release produced by 300 μM kynurenate was potently (EC₅₀, 10 μM) prevented by most of the nootropics tested, including aniracetam, oxiracetam, d-cycloserine, and the glutamate analog CR 2249 (but not its enantiomer CR 2361). Nicotine or tacrine (up to 10 μM) did not show any effect in the kynurenate test. Nicotine (30–100 μM) itself increased the release of [3H]NE; interestingly, the nicotine-evoked overflow was blocked not only by the nicotinic receptor antagonist mecamylamine but also by NMDA receptor antagonists, suggesting an indirect mechanism mediated by glutamate/aspartate release. To conclude, the similarities between the data obtained here with human neocortex slices and those previously obtained in the rat indicate that the kynurenate test performed with rat brain slices may represent a useful biochemical assay to study cognition-enhancing drugs.

A biochemical assay for putative cognition enhancers, termed the kynurenate test, was recently introduced (Pittaluga et al., 1997). In this test, the N-methyl-D-aspartate (NMDA)-evoked release of norepinephrine (NE) in rat hippocampal slices is antagonized by kynurenic acid, and putative cognition enhancers are tested for their ability to counteract the kynurenate antagonism. Several behaviorally active compounds have been evaluated, including putative cognition enhancers thought to act mainly through glutamatergic or nonglutamatergic mechanisms. Drugs known to affect the cholinergic system, such as nicotine or tacrine, were inactive; in contrast, other drugs displayed impressive potency in the kynurenate test, which may therefore represent a useful assay for putative cognition enhancers acting through the glutamate system via NMDA receptors (Pittaluga et al., 1997).

A better characterization of the kynurenate test is clearly needed to confirm its possible usefulness as a functional in vitro assay in the development of learning- and memory-enhancing agents. In particular, it is most important to establish whether compounds displaying activity in the rat brain respond similarly in a kynurenate test performed with human brain tissue.

NMDA receptors mediating elevation of NE release exist in the human cerebral cortex (Fink et al., 1992; Pittaluga et al., 1996). Therefore, in our investigation, human brain cortical slices from patients undergoing neurosurgery were labeled with [3H]NE, exposed to NMDA in the presence of kynurenic acid, and the following cognition-enhancing compounds were tested for their ability to prevent the kynurenate antagonism: aniracetam (Moos et al., 1988), oxiracetam (Paoli et al., 1990; Belfiore et al., 1992), D-cycloserine (Thompson et al., 1992), and the glutamate analog CR 2249 (Garofalo et al., 1996; Lanza et al., 1997). Nicotine and tacrine, two cognition-enhancing drugs believed to act through cholinergic mecha-

ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CGS 19755, 4-phosphonomethyl-2-piperidinecarboxylic acid; CR 2249, (S)-4-amino[4,4-dimethylcyclohexyl]aminopentanoic acid; d-AP5, d-2-amino-5-phosphopentanoate; GBR12909, 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride; NE, norepinephrine; NMDA, N-methyl-D-aspartate.

Received for publication December 21, 1998.

1 This work was supported by grants from National Research Council Target Project on Biotechnology and from the Italian Ministry of Health “Progetto AIDS” 1997 (contract 30A.0.58).
nisms (Davis et al., 1992b; Arneric et al., 1995), were also investigated.

**Materials and Methods**

**Human Brain Tissue.** Fresh human cortex specimens were obtained from patients undergoing neurosurgery, each on a different day, either to remove deeply located tumors (n = 18) or to treat epilepsy resistant to antiepileptic drugs (n = 14); each experiment was performed with tissue obtained from one patient. Because no significant differences between results obtained from two groups of patients were observed, data have been pooled. The tissue samples represented parts of frontal (n = 12) and temporal (n = 20) lobes obtained from 11 female and 21 male patients, ages 23 to 65 years. The experiments described were approved by the local ethical committee.

Immediately after removal, the cortical specimens were placed in aerated ice-cold physiological solution. Slices (0.4-mm thick), prepared by a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Surrey, UK) within 1 h after surgical removal, were placed in a physiological salt solution (see below) at 2 to 4°C and rinsed for 0.1 h. Slices (0.4-mm thick), pre-treated with 0.15 mM [3H]NE (20 min at 37°C) in a physiological salt solution with the following composition: 125 mM NaCl, 3 mM KCl, 1.2 mM MgSO4, 1.2 mM CaCl2, 1 mM NaH2PO4, 22 mM NaHCO3, and 10 mM glucose (aeration with 95% O2 and 5% CO2; pH 7.2 to 7.4). The incubation medium contained 0.1 μM of the selective serotonin uptake inhibitor 6-nitroquipazine and 0.1 μM of the selective dopaminergic uptake inhibitor 1-[2-(3-fluorophenyl)-2-methoxyethyl]-4-(3-phenylpropyl) piperazine dihydrochloride (GBR12909) to prevent false labeling of serotonergic and dopaminergic terminals, respectively. After washing with tracer-free medium, slices were transferred to parallel superfusion chambers (one slice per chamber) and superfused (1 ml/min) with Mg2+-free medium. Eight 5-min samples of the superfusate were collected into vials containing 100 μl of a protective solution (1.5% EDTA/1% ascorbic acid/0.001% unlabeled NE) before chromatographic separation of [3H]NE from [3H]-labeled metabolites.

The radioactivity present as [3H]NE in the release samples and superfused slices was measured by chromatography on 2.5 × 0.4-cm columns of Biorex 70 (300—400 mesh, Na+ form) equilibrated in phosphate buffer (0.2 M, pH 6.1) and eluted with 2.2 ml of 1 N HCl/1 N HCOOH (15:85 v/v) according to the procedure described by Smith et al. (1975). Radioactivity was measured by liquid scintillation spectrometry.

**Calculation.** The amounts of total tritium or [3H]NE released into each superfusate fraction were expressed as percentages of the total tissue content at the start of the respective collection period (fractional efflux). Differences between basal outflow and the drug-induced release over time were analyzed by two-way ANOVA followed by Newman-Keuls multiple-comparisons test. Drug effects, expressed as percent increase over basal outflow, were evaluated by calculating the ratio between the percent efflux in the fraction corresponding to the maximal effect and the efflux in the first fraction collected. This ratio was compared to the corresponding ratio obtained under control conditions. Multiple comparisons were analyzed with ANOVA followed by Dunnett’s test. Effects were considered significant at P < .05.

**Chemicals.** [3H]NE (specific activity, 39 Ci/mmol) was purchased from Amersham Radiochemical Center (Buckinghamshire, UK). Kynurenic acid, mecamylamine, nicotine, and d-cycloserine were obtained from Sigma Chemical Co. (St. Louis, MO). NMDA, d-2-amino-5-phosphopentanoate (d-AP5), and 7-Chl-kynurenate were obtained from Tocris Cookson (Bristol, UK). The following substances were gifts from the companies indicated: tacrine and 4-phosphonomethyl-2-piperidinecarboxylic acid (CGS 19755; NOVARTIS, Summit, NJ); dizocilpine (MK 801; Merck, Sharp & Dohme, Harlow, Essex, UK); aniracetam (Prodotti Roche, Milan, Italy); oxiracetam (Smith-Kline Beecham, Milan, Italy); 6-nitroquinazapine maleate (Duphar, Amsterdam, The Netherlands); GBR12909 (Gist Brocades, Delft, The Netherlands); (S)-4-amino-[1,4,4-dimethylclohexylaminono]pentanoic acid (CR 2249) and its enantiomer form CR 2361 (Rotta Research Laboratorium, Milan, Italy). Aniracetam (10 mM) was dissolved in EtOH/water (1:10 v/v) and diluted to the final concentration in medium. 7-Chl-kynurenate (10 mM) was dissolved in the minimum amount of NaOH (0.1 M) and then diluted in the physiological solution.

**Results**

Slices of human neocortex prelabeled with [3H]NE have been shown to release tritium when exposed in superfusion to NMDA in the absence of Mg2+ (Fink et al., 1992; Pittaluga et al., 1996). To improve the selectivity of the labeling in our experiments, human cortical slices were first incubated with [3H]NE in the presence of selective blockers of the dopamine and serotonin transporters to prevent labeling of nonnoro-drenergic terminals. Subsequently, based on the concentration-response curve reported for NMDA by Fink et al. (1992), slices were stimulated by applying a 3-min pulse of 1 mM NMDA during superfusion with Mg2+-free medium. Finally, the radioactivity released in the superfusate fractions was analyzed by chromatography. Figure 1 illustrates the time course of the drug-induced release of [3H]NE.
course of the release of tritium and of [³H]NE before, during, and after the NMDA pulse. By comparing the curves shown in the figure, it can be seen that the NMDA-evoked tritium overflow (total minus basal outflow) largely consists of unmetabolized [³H]NE. Therefore, we refer to the NMDA-induced tritium overflow as NMDA-induced [³H]NE overflow. Actually, the fact that the experiments were all performed in the absence of monoamine oxidase inhibitors suggests that glutamate activation of NMDA receptors in human neocortex probably leads to enhanced noradrenergic transmission through a potentiation of NE release.

The presence of antagonists at the recognition site (d-AP5), at the ion channel (dizocilpine), or at the glycine-recognition site (7-CI-kynurenate) of the NMDA receptor prevented the NMDA-evoked release of [³H]NE from human cortical slices (1 mM NMDA + 10 μM glycine = 85.6 ± 17.40; 1 mM NMDA + 10 μM glycine + 100 μM d-AP5 = 9.20 ± 2.30, P < .01; 1 mM NMDA + 10 μM glycine + 1 μM dizocilpine = 26.1 ± 8.7, P < .05; 1 mM NMDA + 10 μM glycine + 100 μM 7-CI-kynurenate = 14.4 ± 10.1, P < .01), confirming previous results (Fink et al., 1992). Because our aim was to perform the kynurenate test on human brain tissue, kynurenic acid was evaluated as an NMDA antagonist. Kynurenic acid could prevent the NMDA-evoked [³H]NE release also in human brain (1 mM NMDA + 10 μM glycine = 85.6 ± 17.4; 1 mM NMDA + 10 μM glycine + 100 μM kynurenic acid = 52.1 ± 10.2; 1 mM NMDA + 10 μM glycine + 300 μM kynurenic acid = 15.1 ± 7.1, P < .01); a concentration of 300 μM was used in all of the subsequent experiments because it sufficiently antagonized NMDA without affecting non-NMDA receptors whose blockade, based on data with rat brain slices (A.P. and M.R., unpublished observations), requires much higher kynurenic concentrations.

Before evaluating the putative cognition enhancers under study in the kynurenate test, it was important to ascertain whether the compounds themselves could affect the basal or NMDA-evoked release of [³H]NE. Table 1 shows that aniracetam (10 μM), oxiracetam (10 μM), d-cycloserine (50 μM), nicotine (10 μM), and tacrine (10 μM) had no effect on their own. CR 2249 (10 μM) and nicotine (100 μM) significantly enhanced the basal efflux of tritium from superfused slices. Aniracetam and oxiracetam exhibited activity in the kynurenate test when present at concentrations at least two orders of magnitude lower than kynurenate. In particular, 1 μM oxiracetam almost completely counteracted the antagonism produced by 300 μM kynurenic acid (Fig. 2). Clearly less potent than aniracetam or oxiracetam, d-cycloserine prevented the kynurenate antagonism only when added at 50 μM (Fig. 3).

The recently introduced nootropic agent CR 2249 largely counteracted the kynurenate blockade when added at 1 μM and completely abolished it at 10 μM. The action of CR 2249 was stereoselective in that its enantiomer CR 2361 was completely inactive at 10 μM (Fig. 4).

As shown in Fig. 5, nicotine itself and in a concentration-dependent manner (inset) increased the release of authentic [³H]NE from human cortical slices. The drug had no effect at 10 μM. It was therefore added at this concentration during the kynurenate test and found to be devoid of activity (Fig. 6). Similar results were obtained with 10 μM tacrine.

The nicotine (100 μM)-evoked release of [³H]NE was prevented by 100 μM of the nicotine receptor antagonist mecamylamine (Fig. 7). Figure 7 also shows that the effect of nicotine was almost abolished in the presence of the NMDA receptor antagonists dizocilpine (1 μM) and CGS 19755 (10 μM).

**Discussion**

The glutamatergic system is considered to play a major role in cognitive processes. Long-term potentiation, a model for synaptic changes underlying learning and memory, involves the activation of glutamate receptors of the NMDA type (Bliss and Collingridge, 1993). Accordingly, administration of NMDA receptor antagonists is known to produce amnesic effects (Morris et al., 1986; Miserendino et al., 1990), and activation of NMDA receptors appears to be necessary for certain kinds of learning (Davis et al., 1992a).

NE is implicated in neuronal modulation of higher cognitive functions such as attention and learning (McGaugh, 1989; Coull et al., 1997). A significant reduction in the nor-

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**Table 1**

Effects of cognition-enhancing drugs on basal and NMDA-induced release of tritium from human cortical slices prelabeled with [³H]NE

<table>
<thead>
<tr>
<th>Drug</th>
<th>Control</th>
<th>NMDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td>3.2 ± 4.2</td>
<td>49.4 ± 10.1</td>
</tr>
<tr>
<td>Aniracetam, 10 μM</td>
<td>1.4 ± 2.2</td>
<td>44.7 ± 14.3</td>
</tr>
<tr>
<td>Oxiracetam, 10 μM</td>
<td>5.2 ± 1.3</td>
<td>35.3 ± 7.7</td>
</tr>
<tr>
<td>d-Cycloserine, 50 μM</td>
<td>1.9 ± 2.7</td>
<td>59.8 ± 12.3</td>
</tr>
<tr>
<td>CGP 36742, 10 μM</td>
<td>2.6 ± 1.8</td>
<td>67.5 ± 13.6</td>
</tr>
<tr>
<td>CR 2249, 10 μM</td>
<td>12.6 ± 2.5*</td>
<td>47.8 ± 9.7</td>
</tr>
<tr>
<td>Nicotine, 10 μM</td>
<td>8.3 ± 5.2</td>
<td>45.5 ± 8.3</td>
</tr>
<tr>
<td>Nicotine, 100 μM</td>
<td>30.1 ± 9.3*</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tacrine, 10 μM</td>
<td>9.5 ± 5.6</td>
<td>48.5 ± 6.5</td>
</tr>
</tbody>
</table>

When tested alone, the compounds were present from 73 min until the end of superfusion. When tested for their effect on the NMDA-induced release, they were present from 30 min until the end of the NMDA (1 mM) stimulus. Data are expressed as percent increase of tritium release over basal and are means ± S.E. from three to six experiments run in triplicate (three superfusion chambers for each condition). n.d., not determined.

* P < .05 versus control, Dunnett’s test.
adrenergic innervation of cortex and hippocampus was observed in Alzheimer-type dementia (Chan Palay, 1991).

Interactions between glutamatergic and noradrenergic systems in mechanisms related to learning and memory have been reported (Huang and Kandel, 1996, and references therein). In this context, the established enhancement of brain NE release produced by activation of NMDA receptors may be particularly relevant. This effect has been observed in vitro with slices and synaptosomes of rat cortex or hippocampus (Jones et al., 1987; Fink et al., 1989, 1990; Pittaluga and Raiteri, 1990, 1992) and human cortex (Fink et al., 1992; Pittaluga et al., 1996) and during in vivo microdialysis of rat cortex (Lehmann et al., 1992).

The kynurenate test used in our study originates from observations on kynurenic acid made in several laboratories. Kynurenic acid is an endogenous antagonist of ionotropic glutamate receptors, particularly of the NMDA type (for review, see Stone, 1993). Kynurenate levels appear to be particularly high in human brain (Moroni et al., 1988; Turski et al., 1988) and may increase under conditions usually associated with cognitive disturbances, including aging and HIV-1 infection (Moroni et al., 1988b; Heyes et al., 1990). We postulated that cognition enhancers could act by relieving excessive endogenous antagonism of NMDA receptors when kynurenic acid levels increase. To verify this hypothesis, we set up a simple biochemical assay, the kynurenate test, in which nootropics were tested for their ability to counteract the antagonism by kynurenic acid of the NMDA-evoked release of \(^{[3H]}\)NE in rat hippocampal slices (Pittaluga et al., 1997). Several compounds that had been reported to improve learning and memory in various cognitive tasks displayed potent activity in the kynurenate test. Interestingly, the best responders were generally drugs likely to act through the glutamate system.

Our current results indicate that the kynurenate test, usually performed with rat hippocampal slices, can be reproduced well in human neocortical slices. As previously observed by Fink et al. (1992), the \(^{[3H]}\)NE-releasing effect of NMDA, although clearly receptor-mediated, is, for unknown reasons, much less pronounced in human than in rat brain tissue. Nonetheless, all of the nootropic compounds we tested in human cortical slices behaved in a manner qualitatively identical to that in rat hippocampal slices. Their potencies appear about 10-fold lower in human than in rat slices, possibly because of the higher concentration of kynurenic acid used in the kynurenate test performed with human tissue.

Despite this decrease in potency, most of the drugs tested were able to completely prevent the antagonism by 300 \(\mu\)M kynurenate when added to human cortical slices at concentrations \(\leq 10\) \(\mu\)M. Note that aniracetam is in therapeutic use in Europe to alleviate the cognitive disturbances of the elderly at the recommended oral dose of 1.5 g/day. This dose appears based on the established action of aniracetam at ionotropic glutamate receptors of the AMPA (\(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) type, where the drug has been shown to potentiate AMPA-evoked currents through a reduction in the rate of receptor desensitization at concentrations of 1 to 5 mM (Tang et al., 1991; Partin et al., 1996). If aniracetam acts at NMDA receptors in human cortical slices at \(< 10\) \(\mu\)M (Fig. 2), the above dose of 1.5 g/day should be carefully reconsidered. In fact, based on our findings, aniracetam is likely to exert a dual effect on glutamatergic transmission, namely, modulation of NMDA receptors at low micromolar concentrations and modulation of AMPA receptors at millimolar concentrations.

Besides aniracetam, other compounds displaying activity in the kynurenate test with human cortical slices have been proposed to interact directly with the glutamatergic system, particularly with NMDA receptors. These include oxiracetam (Pugliese et al., 1990), \(\beta\)-cycloserine (Hood et al., 1989; Pittaluga et al., 1997), and the novel putative cognition enhancer CR 2249 (Garofalo et al., 1996; Lanza et al., 1997; Pittaluga et al., 1997). As previously discussed, results with synaptosomes support the idea that all these compounds act at NMDA receptors, although it seems unlikely that they prevent kynurenic antagonism by binding at the strychnine-insensitive glycine site (see Pittaluga et al., 1997).

The cholinergic cognition enhancers nicotine (for review,
see Arneric et al., 1995) and tacrine (Davis et al., 1992b) were unable to decrease the kynurenate antagonism of NMDA-evoked NE release when added up to 10 μM. At higher concentrations, nicotine itself caused NE release through activation of mecamylamine-sensitive mechanisms. Previously, the same concentrations of nicotine were found to elicit release of NE from slices of rat hippocampus (Sershen et al., 1997). Although nicotine receptors mediating increases in NE release probably exist on noradrenergic nerve terminals of rat brain (Clarke and Reuben, 1996), nicotine does not seem to act directly at noradrenergic terminals in human cortex. In fact, the release of NE elicited by nicotine in human cortical slices was largely prevented by the NMDA receptor channel blocker dizocilpine, at a concentration (1 μM) at which it is unlikely to affect nicotinic receptors, and by CGS 19755, a selective antagonist at the NMDA-recognition site, suggesting an indirect mechanism whereby nicotine elicits glutamate release onto NMDA receptors that mediate release of NE. Nicotine has indeed been reported to increase glutamate release in various preparations, and this release has been implicated in the cognitive properties of nicotine (Gray et al., 1996; Wonnacott, 1997; Fedele et al., 1998).

In conclusion, several compounds proposed to improve cognition exhibited activity in the kynurenate test performed in human cortical slices. The same compounds had provided positive responses when tested in rat hippocampal slices (Pittaluga et al., 1997). The kynurenate test carried out in rat brain slices could therefore be considered a simple assay, useful in the identification and preliminary characterization of putative cognition enhancers acting via NMDA receptors.

Acknowledgments

We thank Maura Agate for editorial assistance in preparing the manuscript.
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