British Journal of Pharmacology (2000) 130, 1664–1670 © 2000 Macmillan Publishers Ltd All rights reserved 0007–1188/00 $15.00

www.nature.com/bjp

Pharmacological characterization of metabotropic glutamate receptor-mediated high-affinity GTPase activity in rat cerebral cortical membranes

[1]Nobuyuki Nishi, *,[1]Yuji Odagaki & [1]Tsukasa Koyama

[1]Department of Psychiatry, Hokkaido University School of Medicine, North 15, West 7, Sapporo 060-8638, Japan

1 Activation of heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins) functionally coupled to metabotropic glutamate receptors (mGluRs) was assessed by agonist-induced high-affinity GTPase (EC3.6.1.-) activity in rat cerebral cortical membranes.

2 L-Glutamate (1 mM) stimulated high-affinity GTPase activity to the same extent throughout the incubation period up to 20 min, in a Mg^{2+}-dependent manner. The addition of 1 mM L-glutamate augmented V_{max} of the enzyme activity (1670 to 3850 pmol mg^{-1} protein 15 min^{-1}) with slight increase in K_M value (0.26 to 0.63 μM).

3 The high-affinity GTPase activity was stimulated by the following compounds with a rank order of potency of (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV) > (2*S*,1′*S*,2′*S*)-2-(carboxycyclopyropyl)glycine (L-CCG-I) > L-glutamate ⩾ 2*R*,4*R*-4-aminopyrrolidine-2,4-dicarboxylate [(2*R*,4*R*)-APDC] > 1*S*,3*R*-1-aminocyclopentane-1,3-dicarboxylate [(1*S*,3*R*)-ACPD] > (*S*)-4-carboxy-3-hydroxyphenylglycine [(*S*)-4C3HPG] > (*S*)-3-carboxy-4-hydroxyphenylglycine [(*S*)-3C4HPG] > ibotenate, but not by L-(+)-2-amino-4-phosphonobutyrate (L-AP4), (*RS*)-3,5-dihydroxyphenylglycine [(*RS*)-3,5-DHPG], quisqualate, or L-serine-*O*-phosphate (L-SOP), indicative of involvement of group II mGluRs, in particular mGluR2.

4 (2*S*)-α-Ethylglutamate (EGLU), a presumably selective antagonist against group II mGluRs, inhibited DCG-IV-stimulated high-affinity GTPase activity in a competitive manner with an apparent K_B of 220 μM.

5 L-Glutamate-stimulated activity was eliminated by pretreatment of the membranes with sulfhydryl alkylating agent *N*-ethylmaleimide (NEM) at 30–50 μM, indicating that G-proteins of the G_i family are involved.

6 These results indicate that mGluR agonist-induced high-affinity GTPase activity in rat cerebral cortical membranes may be used to detect the functional interaction between group II mGluRs, in particular mGluR2, and NEM-sensitive G_i proteins.

British Journal of Pharmacology (2000) **130,** 1664–1670

Keywords: Glutamate; metabotropic glutamate receptors; G-proteins; high-affinity GTPase activity; (2*S*)-α-ethylglutamate (EGLU); *N*-ethylmaleimide (NEM)

Abbreviations: (1*S*,3*R*)-ACPD, 1*S*,3*R*-1-aminocyclopentane-1,3-dicarboxylate; (2*R*,4*R*)-APDC, 2*R*,4*R*-4-aminopyrrolidine-2,4-dicarboxylate; CHO, Chinese hamster ovary; DCG-IV, (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine; EGLU, (2*S*)-α-ethylglutamate; $\%E_{max}$, maximal per cent increase above basal value; G-protein, guanine nucleotide-binding regulatory protein; IAP, islet-activating protein; L-AP4, L-(+)-2-amino-4-phosphonobutyrate; L-CCG-I, (2*S*,1′*S*,2′*S*)-2-(carboxycyclopropyl)glycine; L-CCG-II, (2*S*,1′*R*,2′*R*)-2-(carboxycyclopropyl)glycine; L-SOP, L-serine-*O*-phosphate; MCCG, (2*S*,3*S*,4*S*)-2-methyl-2-(carboxycyclopropyl)glycine; mGluR, metabotropic glutamate receptor; NEM, *N*-ethylmaleimide; (*RS*)-3,5-DHPG, (*RS*)-3,5-dihydroxyphenylglycine; (*S*)- and (*R*)-3C4HPG, (*S*)- and (*R*)-3-carboxy-4-hydroxyphenylglycine; (*S*)- and (*R*)-4C3HPG, (*S*)- and (*R*)-4-carboxy-3-hydroxyphenylglycine; (*S*)- and (*R*)-4CPG, (*S*)- and (*R*)-4-carboxyphenylglycine; (*S*)- and (*R*)-3HPG, (*S*)- and (*R*)-3-hydroxyphenylglycine; (*S*)- and (*R*)-MCPG, (*S*)- and (*R*)-α-methyl-4-carboxyphenylglycine; TED buffer, 5 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4

Introduction

Glutamate, a major excitatory amino acid in the mammalian central nervous system, is an important neurotransmitter, the actions of which are not only mediated through ligand-gated cationic channels NMDA, AMPA, and kainate receptors, but also through receptors coupled to guanine nucleotide-binding regulatory proteins (G-proteins) (Pin & Duvoisin, 1995; Conn & Pin, 1997). Such G-protein coupled receptors are designated as metabotropic glutamate receptors (mGluRs) and eight genes coding for these receptors (mGluR1 through mGluR8) have been cloned thus far. Based on their amino acid sequence identity in conjunction with their transduction mechanisms and pharmacological characteristics, mGluRs have been divided into three subgroups (groups I–III). Group I comprises mGluR1 and mGluR5, and has been shown to stimulate phospholipase C as revealed by an increase in phosphoinositide turnover and Ca^{2+} release from internal stores. The latter two groups, i.e., group II (mGluR2 and mGluR3) and Group III (mGluRs4, 6, 7, and 8), have been generally considered to couple to the same intracellular signal transduction, namely, inhibition of adenylyl cyclase activity, but with different pharmacological characteristics.

*Author for correspondence.

Although it is most likely that heterotrimeric G-proteins mediate the above-mentioned signal transduction mechanisms evoked by activation of mGluRs, there have been few reports focused directly on the functional coupling between mGluRs and G-proteins, particularly in native brain membranes. We previously showed the use of agonist-induced increase in high-affinity GTPase (EC3.6.1.-) activity in native brain membranes especially when associated with adenylyl cyclase inhibition (Odagaki & Fuxe, 1997). In fact, our preliminary report regarding the L-glutamate-stimulated high-affinity GTPase activity in rat brain membranes indicated that this response in the cerebral cortex is probably mediated through group II mGluRs (Odagaki *et al*., 1996), which are known to be coupled to the inhibition of adenylyl cyclase activity. However, because of limitations of the number of compounds applied to this study, the exact pharmacological characterization of the mGluR(s)-mediated high-affinity GTPase activity was unclear. Therefore, in the present study, we have examined a series of agonists either selective or nonselective for the individual mGluR groups, on the high-affinity GTPase activity in rat cerebral cortical membranes, and extended the preliminary results in our previous report (Odagaki *et al*., 1996).

Methods

Membrane preparation

Male Sprague-Dawley rats were sacrificed by decapitation and their brains were quickly removed. All of the following manipulations were done speedily on ice and centrifugations were performed at 4°C. Cerebrocortical tissues dissected from each rat were homogenized in 5 ml of ice-cold TED buffer (5 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4) containing 10% (w v^{-1}) sucrose using a motor-driven Teflon/glass tissue grinder (20 strokes). The homogenate was centrifuged at $1000 \times g$ for 10 min, and the supernatant was kept on ice while the pellet was resuspended in 5 ml of TED/sucrose buffer and centrifuged again at $1000 \times g$ for 10 min. The combined supernatant was then centrifuged at $9000 \times g$ for 20 min. The pellet was resuspended in 10 ml of TED buffer and centrifuged at $9000 \times g$ for 20 min. The resulting pellet was resuspended in 10 ml of TED buffer and maintained on ice for 30 min. Finally the suspension was centrifuged at $35{,}000 \times g$ for 10 min and the pellet was suspended in 3.0 ml of 50 mM Tris-HCl buffer (pH 7.4). The homogenate equivalent to 1.6–3.2 mg protein ml^{-1} was divided into 150-μl aliquots, frozen quickly on powdered dry ice, and stored at −80°C until use. The protein concentration was determined by the method of Lowry *et al*. (1951), using BSA as a standard.

Pretreatment of the membranes with N-ethylmaleimide (NEM)

The thawed membranes were diluted to 1 ml with 50 mM Tris-HCl buffer (pH 7.4), which contained NEM at various concentrations. The homogenate was incubated at 4°C for 15 min, followed by the addition of dithiothreitol at 1 mM. The NEM-treated membranes were centrifuged at $5000 \times g$ for 10 min and washed with 1 ml of the buffer. The final pellet resulted from the same centrifugation was resuspended in 1.5 ml of 50 mM Tris-HCl buffer (pH 7.4), 25-μl aliquots of which were used for GTPase assay.

GTPase assay

The activation of GTP hydrolysis was determined by measuring $^{32}P_i$ liberated from [γ-^{32}P]-GTP, as described in detail elsewhere (Odagaki & Fuxe, 1997). In brief, the thawed membranes were diluted in 50 mM Tris-HCl (pH 7.4) and the aliquots equivalent to 4–8 μg protein were incubated at 30°C for the indicated period (15 min under standard assay condition) in 100 μl of the incubation medium containing the following constituents (mM): Tris-HCl (pH 7.4) 50, $MgCl_2$ 2, EDTA 0.1, EGTA 0.2, dithiothreitol 0.2, 3-isobutyl-1-methylxanthine 0.5, ATP 0.5, 5′-adenylylimidodiphosphate 0.5, phosphocreatine 5, cyclic AMP 0.5, NaCl 100, 0.3 μM [γ-^{32}P]-GTP, 5 u creatine phosphokinase, 50 μg BSA, and the compound under test at various concentrations. In the GTP dilution experiments, the membranes were incubated with 0.1 μM [γ-^{32}P]-GTP and unlabelled GTP at various concentrations. The effect of Mg^{2+} was examined in the presence of various concentrations of $MgCl_2$ in the assay buffer. The low-affinity GTPase activity was determined in the presence of 100 μM unlabelled GTP, which was subtracted from the total activity to define the high-affinity GTPase activity. The incubation was terminated by chilling all tubes in ice-cold water, followed by an addition of 500 μl of 20 mM phospholic acid containing 5% (w v^{-1}) of activated charcoal. The tubes were centrifuged at $13{,}000 \times g$ for 10 min, and an aliquot (200 μl) taken from the supernatant fraction was poured onto the solid scintillator, Ready Cap (Beckman; Fullerton, CA, U.S.A.). The radioactivity of liberated $^{32}P_i$ was measured as c.p.m. with a liquid scintillation spectrometer. Each experiment was performed in duplicate and repeated with at least three different membranes.

Data analysis

All results were presented as means ± s.e.mean. When appropriate, the increase in high-affinity GTPase activity elicited by a mGluR agonist was analysed by computer-assisted non-linear regression program originally designed for enzyme reactions in accordance with the Michaelis-Menten equation to determine the maximal per cent increase above basal value ($\%E_{max}$) and EC_{50}.

Chemicals

[γ-^{32}P]-GTP (30 Ci $mmol^{-1}$) was purchased from New England Nuclear (Boston, MA, U.S.A.). 2*R*,4*R*-4-aminopyrrolidine-2,4-dicarboxylate [(2*R*,4*R*)-APDC] was a generous gift from Lilly Research Laboratories (Indianapolis, IN, U.S.A.). L-Glutamate was from Kanto Chemical Co. (Tokyo, Japan). (2*S*)-α-Ethylglutamate (EGLU) and the following glycine derivatives and phenylglycine derivatives; (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV), (2*S*,1′*S*,2′*S*)-2-(carboxycyclopropyl)glycine (L-CCG-I), (2*S*,1′*R*,2′*R*)-2-(carboxycyclopropyl)glycine (L-CCG-II), (*S*)- and (*R*)-4-carboxy-3-hydroxyphenylglycine [(*S*)- and (*R*)-4C3HPG], (*S*)- and (*R*)-3-carboxy-4-hydroxyphenylglycine [(*S*)- and (*R*)-3C4HPG], (*S*)- and (*R*)-4-carboxyphenylglycine [(*S*)- and (*R*)-4CPG], (*S*)- and (*R*)-3-hydroxyphenylglycine [(*S*)- and (*R*)-3HPG], (*RS*)-3,5-dihydroxyphenylglcine [(*RS*)-3,5-DHPG], (2*S*,3*S*,4*S*)-2-methyl-2-(carboxycyclopropyl)glycine (MCCG), and (*S*)- and (*R*)-α-methyl-4-carboxyphenylglycine [(*S*)- and (*R*)-MCPG], were from Tocris Cookson (Bristol, U.K.). 1*S*,3*R*-1-Aminocyclopentane-1,3-dicarboxylate [(1*S*,3*R*)-ACPD], ibotenate, L-serine-*O*-phosphate (L-SOP) from Research Biochemical International (Natick, MA, U.S.A.). All

other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

Fundamental characteristics of L-glutamate-stimulated high-affinity GTPase activity

The time course of [γ-^{32}P]-GTP hydrolyzing activity is shown in Figure 1. The amounts of $^{32}P_i$ released from 0.3 μM [γ-^{32}P]-GTP were increased according to the incubation period (0–20 min) in a curvilinear manner. Nevertheless, the per cent increase in high-affinity GTPase activity elicited by 1 mM L-glutamate was approximately constant (~40%) throughout the incubation period up to 20 min. The incubation time of 15 min was chosen as a standard assay period in the following experiments.

As shown in Figure 2A, the amounts of $^{32}P_i$ released from 0.1 μM [γ-^{32}P]-GTP were gradually decreased by the addition of increasing concentrations of unlabelled GTP. The GTP hydrolyzing activity reached a plateau in the presence of 100 μM unlabelled GTP, indicative of the existence of nonspecific, low-affinity GTP hydrolyzing activity probably derived from some GTP hydrolyzing components other than receptor-coupled heterotrimeric G-proteins. The augmentative effects of 1 mM L-glutamate on the activity were cancelled in the presence of 100 μM unlabelled GTP. When the low-affinity GTPase activity, defined in the presence of 100 μM unlabelled GTP, was subtracted from the total value, the high-affinity GTPase activity could be determined in the absence and presence of 1 mM L-glutamate (Figure 2B). In the absence of an agonist, the cerebral cortical membranes possessed the high-affinity GTPase activity with a mean V_{max} of 1670$\pm$100 pmol mg^{-1} protein 15 min^{-1} (n=4) and a mean K_M for GTP of 0.26 μM ($-\log K_M = 6.59 \pm 0.06$). The addition of 1 mM L-glutamate augmented the V_{max} value to 3850$\pm$211 pmol mg^{-1} protein 15 min^{-1} (n=4), with a slight increase in the mean K_M value to 0.63 μM ($-\log K_M = 6.20 \pm 0.04$).

The effects of Mg^{2+} on GTP hydrolyzing activities were investigated by changing the concentrations of $MgCl_2$ added in the incubation medium. As shown in Figure 3A, the addition of millimolar concentrations of $MgCl_2$ nonselectively augmented total and low-affinity GTPase activities. In the absence of $MgCl_2$, the stimulatory effect of 1 mM L-glutamate was obscure in spite of the presence of high-affinity GTPase activity. The use of $MgCl_2$ at millimolar concentrations was necessary in order to maximize the L-glutamate-induced high-affinity GTPase activity (Figure 3B). Based on these data, we used a concentration of 2 mM Mg^{2+} in the standard assay for the following experiments.

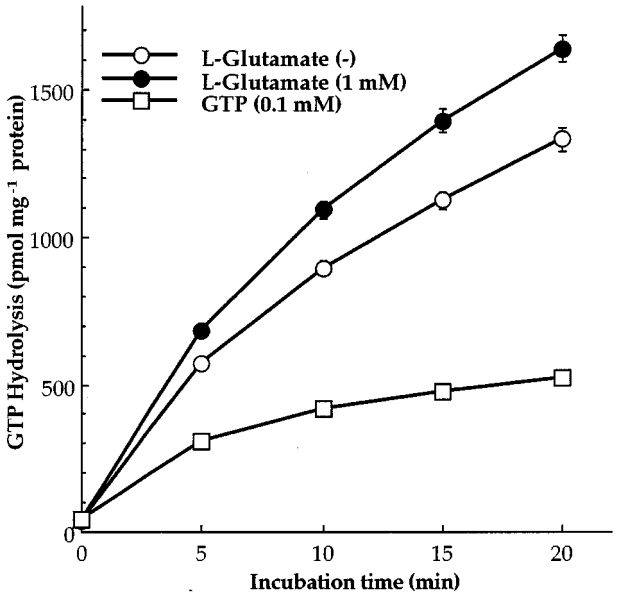

Figure 1 Time course of GTP hydrolysis in rat cerebral cortical membranes. The hydrolysis of 0.3 μM [γ^{32}P]-GTP was determined in the absence and presence of 1 mM of L-glutamate after different incubation periods at 30°C. The low-affinity GTPase activity, defined in the presence of 100 μM unlabelled GTP, was also determined. Values are means$\pm$s.e.mean of four separate experiments, each performed in duplicate.

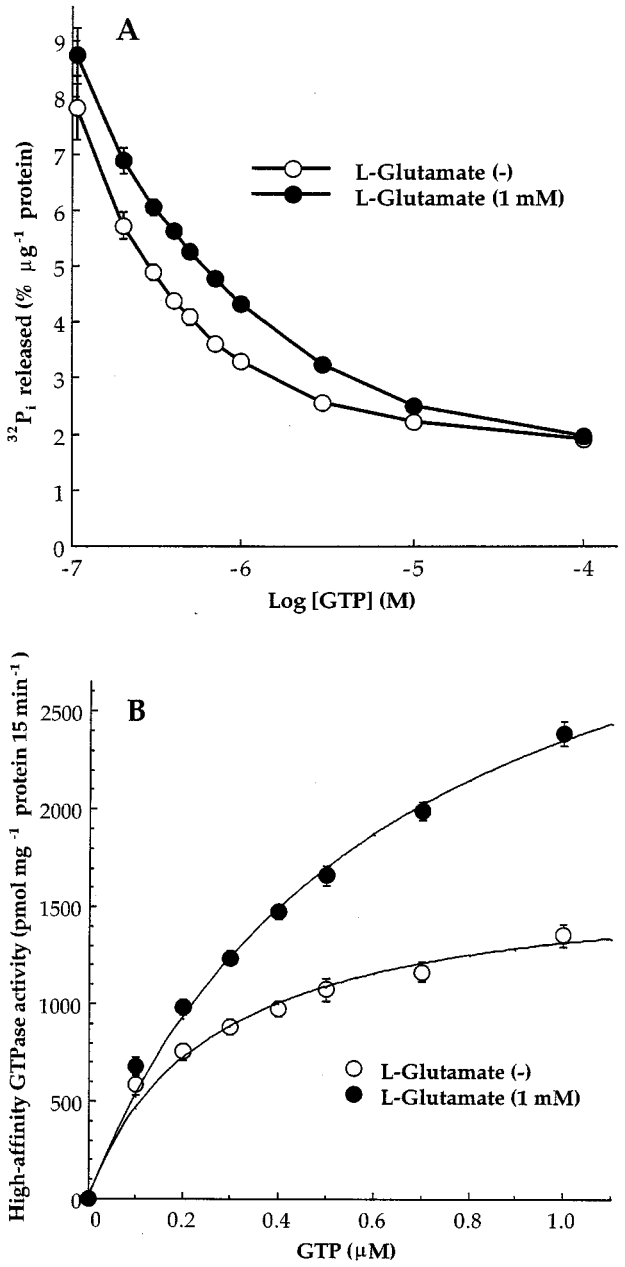

Figure 2 Isotopic dilution of GTP hydrolysis in rat cerebral cortical membranes. (A) The amounts of $^{32}P_i$ released from 0.1 μM [γ-^{32}P]-GTP in the absence and presence of 1 mM L-glutamate were determined in the presence of different concentrations of unlabelled GTP. (B) The high-affinity GTPase activity in the absence and presence of 1 mM L-glutamate was determined at various concentrations of GTP. Values are means$\pm$s.e.mean of four separate experiments, each performed in duplicate.

Pharmacological profile of the mGluR-mediated high-affinity GTPase activity

Following determination of the fundamental characteristics of GTPase activity as described above, the effects of a series of mGluR agonists were investigated. High-affinity GTPase activity was stimulated by some of the compounds examined, in a concentration-dependent and saturable manner as exemplified in Figure 4. The most potent among these compounds was DCG-IV, with a mean EC_{50} value of 0.21 μM. As summarized in Table 1, the enzyme activity was also stimulated by L-CCG-I (0.90), L-glutamate (4.8), (2*R*,4*R*)-APDC (6.8), (1*S*,3*R*)-ACPD (11), (*S*)-4C3HPG (37), (*S*)-3C4HPG (150) and ibotenate (390), with mean EC_{50} values (μM) indicated in the respective parentheses. Conversely, the addition of other compounds listed in Table 1 produced no or little stimulation of the high-affinity GTPase activity even at high concentrations [1 mM with the exception of 10 μM for L-AP4 and 300 μM for (*RS*)-3,5-DHPG]. Although some of these compounds appeared to augment the activity to some extent at the highest concentrations examined [e.g. 8.2±0.5% by 1 mM quisqualate, Figure 4; 8.2±3.8% by 2.5 mM (*S*)-4CPG;

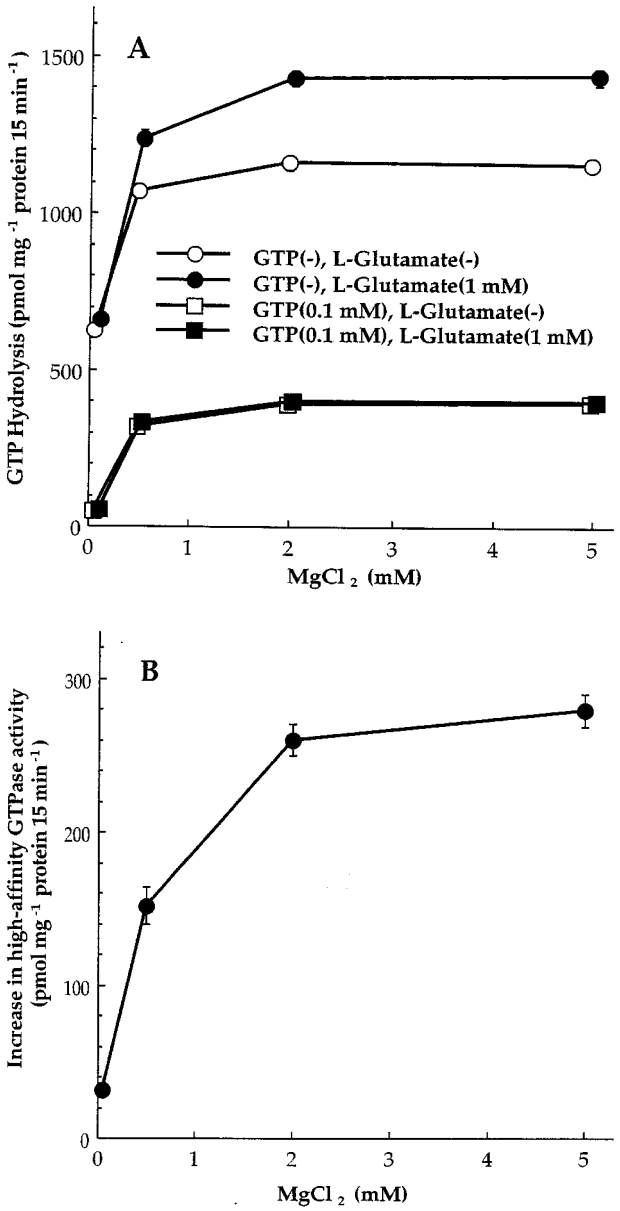

Figure 3 Effects of $MgCl_2$ on GTP hydrolysis in rat cerebral cortical membranes. (A) The hydrolysis of 0.3 μM [γ^{32}P]-GTP was determined in the absence and presence of 1 mM L-glutamate at various concentrations of $MgCl_2$. The low-affinity GTPase activity was determined in the presence of 100 μM unlabelled GTP. (B) The increase in high-affinity GTPase activity by 1 mM L-glutamate was determined at various concentrations of $MgCl_2$. Values are means ±s.e.mean of four separate experiments, each performed in duplicate.

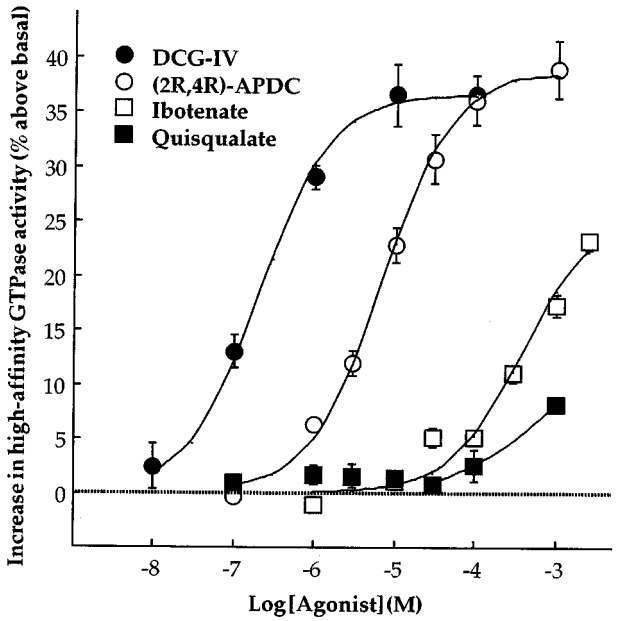

Figure 4 Representative concentration-response curves of mGluR agonist-induced high-affinity GTPase activity in rat cerebral cortical membranes. The per cent increase in high-affinity GTPase activity above the respective basal unstimulated activity was determined in the presence of increasing concentrations of DCG-IV, (2*R*,4*R*)-APDC, ibotenate, and quisqualate. Values are means±s.e.mean of 4–5 separate experiments, each performed in duplicate.

Table 1 Agonistic effects of various mGluR-related compounds on high-affinity GTPase activity in rat cerebral cortical membranes

Compound	*n*	*pEC_{50}*	*mean EC_{50}* (μM)	*$\%E_{max}$*
DCG-IV	5	6.68±0.08	0.21	36.5±1.8
L-CCG-I	5	6.05±0.10	0.90	18.7±3.2
L-Glutamate	4	5.32±0.06	4.8	32.3±4.9
(2*R*,4*R*)-APDC	5	5.17±0.01	6.8	38.8±2.6
(1*S*,3*R*)-ACPD	5	4.98±0.07	11	25.7±1.5
(*S*)-4C3HPG	5	4.43±0.02	37	18.3±1.8
(*S*)-3C4HPG	5	3.84±0.08	150	26.8±1.5
Ibotenate	4	3.41±0.03	390	25.8±1.0
L-AP4	4	–	>10	–
(*RS*)-3,5-DHPG	6	–	>300	–
Quisqualate	4	–	>1000	–
L-CCG-II	4	–	>1000	–
L-SOP	3	–	>1000	–
(*R*)-4C3HPG	5	–	>1000	–
(*R*)-3C4HPG	4	–	>1000	–
(*S*)-4CPG	5	–	>1000	–
(*R*)-4CPG	5	–	>1000	–
(*S*)-3HPG	6	–	>1000	–
(*R*)-3HPG	6	–	>1000	–
MCCG	6	–	>1000	–
(*S*)-MCPG	6	–	>1000	–
(*R*)-MCPG	6	–	>1000	–
EGLU	3	–	>1000	–

15.1±3.2% by 2.5 mM (*RS*)-3,5-DHPG], the exact EC_{50} values for these potentially weak agonists were indeterminable.

The concentration-response curve for the increase in high-affinity GTPase activity elicited by DCG-IV was shifted rightward in parallel by the addition of increasing concentrations (0.15, 0.45 and 1.5 mM) of EGLU (Figure 5A). Schild analysis of three separate experiments gave a mean apparent K_B value for EGLU of 0.22 mM ($pK_B = 3.66 \pm 0.23$), with a slope of 0.94±0.25, indicative of the competitive manner of antagonism.

Effect of NEM on L-glutamate-elicited high-affinity GTPase activity

The effect of pretreatment of the membranes with different concentrations of the sulfhydryl alkylating agent NEM for 15 min at 4°C is shown in Figure 6. The stimulatory effect of 100 μM L-glutamate on the high-affinity GTPase activity GTPase activity was reduced when the membranes were pretreated with increasing concentrations of NEM, and almost complete inhibition was achieved with concentrations of NEM above 30 μM.

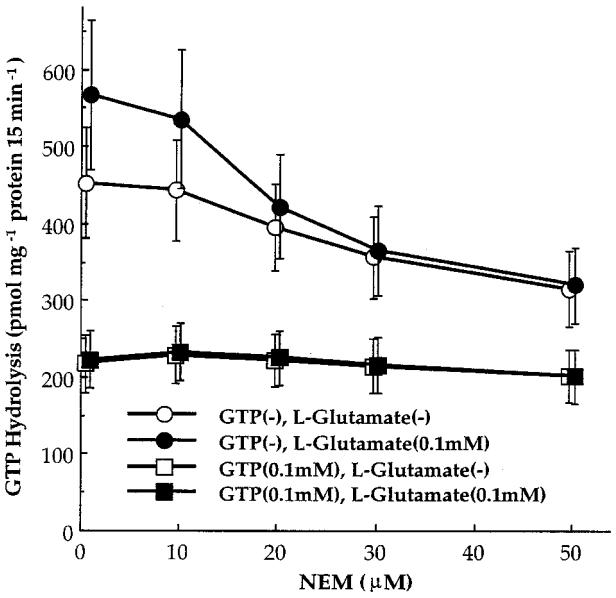

Figure 6 Effect of NEM pretreatment on GTP hydrolysis in rat cerebral cortical membranes. The hydrolysis of 0.3 μM [γ^{32}P]-GTP was determined in the absence and presence of 100 μM L-glutamate in the membranes pretreated with various concentrations of NEM at 4°C for 15 min. The low-affinity GTPase activity was determined in the presence of 100 μM unlabelled GTP. Values are means±s.e.mean of four separate experiments, each performed in duplicate.

Discussion

The fundamental characteristics of the L-glutamate-stimulated high-affinity GTPase activity in rat cerebral cortical membranes were similar to those mediated through other metabotropic receptors in rat brain membranes. The curvilinear time course of GTP hydrolyzing activity was also observed in rat hippocampal (Odagaki & Fuxe, 1995c) and striatal (Odagaki & Fuxe, 1995b) membranes. The addition of 1 mM L-glutamate increased the V_{max} of the high-affinity GTPase activity and slightly increased K_M for GTP, consistent with previous studies of agonist-induced high-affinity GTPase activities mediated through other receptors (Odagaki & Fuxe, 1995a–c). Finally, the strict requirement of millimolar concentrations of Mg^{2+} for detecting the L-glutamate-stimulated high-affinity GTPase activity was also characteristic of receptor-mediated activation of G-proteins as demonstrated previously (Odagaki & Fuxe, 1995b,c). Consequently, these features are consistent with the notion that L-glutamate-elicited high-affinity GTPase activity derives from heterotrimeric G-proteins which are coupled to and activated by the glutamate receptors associated with second messenger generating systems, namely mGluRs.

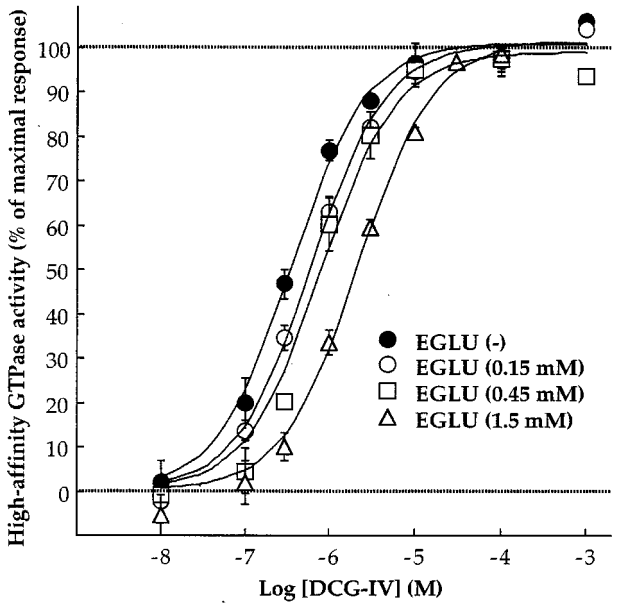

Figure 5 Antagonism of DCG-IV-stimulated high-affinity GTPase activity by EGLU in rat cerebral cortical membranes. The increase in high-affinity GTPase activity by various concentrations of DCG-IV was determined in the absence and presence of 0.15, 0.45 and 1.5 mM EGLU. The respective $\%E_{max}$ values were normalized to 100. Values are means±s.e.mean of three separate experiments, each performed in duplicate.

The involvement of group I mGluRs can be excluded by the fact that (*RS*)-3,5-DHPG and (*S*)-3HPG, both of which have been shown to be specific group I mGluR agonists (Conn & Pin, 1997; Schoepp *et al*., 1999), are devoid of stimulatory effects at least up to 300 and 1000 μM, respectively. In addition, quisqualate was an extremely weak agonist in our assay system despite its reportedly (sub)micromolar potency for group I mGluRs (Pin & Duvoisin, 1995; Conn & Pin, 1997; Schoepp *et al*., 1999). It is generally accepted that group I mGluRs are coupled to phosphoinositide turnover/Ca^{2+} release through G_q family (Pin & Duvoisin, 1995), although G-proteins of the G_i family are also likely to be involved in some cases as indicated by a partial sensitivity to pertussis toxin (islet-activating protein, IAP) (Houamed *et al*., 1991; Masu *et al*., 1991; Aramori & Nakanishi, 1992; Pin *et al*., 1992; Pickering *et al*., 1993). Because the assay for agonist-induced high-affinity GTPase activity in brain membranes seems preferable for receptors associated with adenylyl cyclase inhibition (Odagaki & Fuxe, 1997), it is possible that L-glutamate-stimulated high-affinity GTPase activity in the present study does not derive from the G_q family coupled with group I mGluRs.

The involvement of group III mGluRs is also excluded clearly by the pharmacological profile of the response. The high-affinity GTPase activity was unresponsive to L-AP4 and L-SOP up to the highest concentrations examined, i.e., 10 and

1000 μM, respectively. Both compounds have been shown to be very specific agonists for group III mGluRs with (sub)micromolar potencies (Pin & Duvoisin, 1995; Conn & Pin, 1997; Schoepp *et al*., 1999) with the exception of lower potencies (160 μM) for mGluR7 (Okamoto *et al*., 1994). The pharmacology of mGluR7 is quite unique in that L-glutamate is a very weak agonist with an EC_{50} of 1000 μM (Okamoto *et al*., 1994), and thus an involvement of this receptor subtype in the present investigation is unlikely. It is also highly unlikely that mGluR6 is involved as this receptor subtype is formed in the inner nuclear layer of the retina, but not in brain (Nakajima *et al*., 1993).

The apparent lack of involvement of group III mGluRs can not be accounted for by the preference of the assay *per se* for the interaction between inhibitory receptors and G_i associated with adenylyl cyclase inhibition (Odagaki & Fuxe, 1997), since group III as well as group II mGluRs have been presumed to functionally couple to adenylyl cyclase inhibition through IAP-sensitive G-proteins (Pin & Duvoisin, 1995; Conn & Pin, 1997). Indeed, it has been reported that activation of group III mGluRs by L-AP4 results in the inhibition of forskolin-stimulated cyclic AMP accumulation in adult rat cortical slices (Bedingfield *et al*., 1995; Kemp *et al*., 1996). On the other hand, mGluR agonists have been shown to inhibit forskolin-stimulated cyclic AMP formation to a lesser extent in mGluR4-expressing CHO cells as compared with mGluR2-expressing cells (Hayashi *et al*., 1992; 1994; Cavanni *et al*., 1994), possibly indicating that mGluR2 (group II) are functionally coupled to G_i more efficaciously than mGluR4 (group III). Saugstad *et al*. (1994) also suggested that inhibition of adenylyl cyclase might not be the predominant transduction mechanism for mGluR7. The primary intracellular signal transduction system evoked by the activation by group III mGluRs may not be adenylyl cyclase inhibition, thus involving G-proteins other than the G_i family.

Whatever the underlying mechanism(s), the agonist profile in order of potency determined in the present investigation, DCG-IV > L-CCG-I > L-glutamate $\geqslant$ (2*R*,4*R*)-APDC > (1*S*,3*R*)-ACPD > (*S*)-4C3HPG > (*S*)-3C4HPG > ibotenate, is in good accordance with that described for group II mGluRs, in particular mGluR2 (Conn & Pin, 1997; Schoepp *et al*., 1999). Of these agonists, (2*R*,4*R*)-APDC has been shown to be a highly specific group II mGluR agonist with an EC_{50} value of 12.9 μM as determined using inhibition of forskolin-stimulated cyclic AMP accumulation in the rat hippocampus (Schoepp *et al*., 1995). In the present study, (2*R*,4*R*)-APDC acted as a full agonist with a compatible EC_{50} value of 6.8 μM. The agonistic properties of some phenylglycine derivatives such as (*S*)-4C3HPG and (*S*)-3C4HPG in the present study also strengthen the above-mentioned conclusion because these two compounds have been shown to act as agonists and antagonists for mGluR2 and mGluR1, respectively, and to lack either agonistic or antagonistic effects on mGluR4 (Cavanni *et al*., 1994; Hayashi *et al*., 1994; Thomsen *et al*., 1994).

In neonatal rat motoneurones, it has been shown that (1*S*,3*S*)-ACPD-induced, but not L-AP4-induced, depression of the monosynaptic dorsal root-evoked ventral root potential is antagonized by EGLU in a competitive manner (Jane *et al*., 1996), indicating that EGLU is a selective and competitive group II mGluR antagonist. In the present study, DCG-IV-induced high-affinity GTPase activity was antagonized by EGLU in a competitive manner, further indicative of group II mGluRs involvement. The apparent K_B value of EGLU in our assay system was 220 μM, roughly comparable with the value of 66 μM reported by Jane *et al*. (1996).

The pharmacological characteristics of mGluR2 and mGluR3 resemble each other with the exception of quisqualate, which has been shown to be almost devoid of effect on mGluR2 but to act as a relatively potent agonist on mGluR3 (Pin & Duvoisin, 1995; Conn & Pin, 1997; Schoepp *et al*., 1999). The lack of effect of quisqualate in the present investigation favours the involvement of mGluR2, but not mGluR3, in L-glutamate-stimulated high-affinity GTPase activity in rat cerebral cortical membranes. However, this conclusion should remain tentative until more selective ligands are available to discriminate these two mGluRs.

Almost complete elimination of L-glutamate-stimulated high-affinity GTPase activity was observed when the membranes were pretreated with sulfhydryl alkylating agent NEM. It has been reported that treatment with NEM mimics the effect of IAP to inactivate G-proteins of the G_i family thereby resulting in uncoupling of receptor/G-protein interaction (Asano & Ogasawara, 1986; Kitamura & Nomura, 1987; Ueda *et al*., 1990; Lorenzen *et al*., 1998). Thus, the sensitivity of L-glutamate-elicited high-affinity GTPase activity to relatively low concentrations (30–50 μM) of NEM supports the notion that group II mGluRs are coupled with IAP-sensitive G-proteins of the G_i family, from which L-glutamate-stimulated high-affinity GTPase activity derives. As the G_i family comprises G_{i1-3} and G_o, it remains to be determined which subclass(es) of G_i family is(are) responsible for the response.

To summarize, the present investigation clearly indicates that activation of G-proteins by mGluRs is detectable by means of mGluR agonist-induced high-affinity GTPase activity in rat cerebral cortical membranes and that this response reflects functional interaction between group II mGluRs, in particular mGluR2, and G-proteins of the G_i family that are sensitive to NEM treatment. It should be clarified in future studies whether mGluR3 subtype is involved in this response to some extent and which subtype(s) of G_i family it derives from.

This work was supported by a Grant-in-Aid for Scientific Research (No. 09670970) from the Ministry of Education, Science and Culture of Japan.

References

ARAMORI, I. & NAKANISHI, S. (1992). Signal transduction and pharmacological characteristics of a metabotropic glutamate receptor, mGluR1, in transfected CHO cells. *Neuron,* **8,** 757–765.

ASANO, T. & OGASAWARA, N. (1986). Uncoupling of γ-aminobutyric acid B receptors from GTP-binding proteins by *N*-ethylmaleimide: effect of *N*-ethylmaleimide on purified GTP-binding proteins. *Mol. Pharmacol.,* **29,** 244–249.

BEDINGFIELD, J.S., KEMP, M.C., JANE, D.E., TSE, H.-W., ROBERTS, P.J. & WATKINS, J.C. (1995). Structure-activity relationships for a series of phenylglycine derivatives acting at metabotropic glutamate receptors (mGluR). *Br. J. Pharmacol.,* **116,** 3323–3329.

CAVANNI, P., PINNOLA, V., MUGNAINI, M., TRIST, D., VAN AMSTERDAM, F.T.M. & FERRAGUTI, F. (1994). Pharmacological analysis of carboxyphenylglycines at metabotropic glutamate receptors. *Eur. J. Pharmacol.,* **269,** 9–15.

CONN, P.J. & PIN, J.-P. (1997). Pharmacology and functions of metabotropic glutamate receptors. *Annu. Rev. Pharmacol. Toxicol.,* **37,** 205–237.

HAYASHI, Y., SEKIYAMA, N., NAKANISHI, S., JANE, D.E., SUNTER, D.C., BIRSE, E.F., UDVARHELYI, P.M. & WATKINS, J.C. (1994). Analysis of agonist and antagonist activities of phenylglycine derivatives for different cloned metabotropic glutamate receptor subtypes. *J. Neurosci.,* **14,** 3370–3377.

HAYASHI, Y., TANABE, Y., ARAMORI, I., MASU, M., SHIMAMOTO, K., OHFUNE, Y. & NAKANISHI, S. (1992). Agonist analysis of 2-(carboxycyclopropyl)glycine isomers for cloned metabotropic glutamate receptor subtypes expressed in Chinese hamster ovary cells. *Br. J. Pharmacol.,* **107,** 539–543.

HOUAMED, K.M., KUIJPER, J.L., GILBERT, T.L., HALDEMAN, B.A., O'HARA, P.J., MULVIHILL, E.R., ALMERS, W. & HAGEN, F.S. (1991). Cloning, expression, and gene structure of a G protein-coupled glutamate receptor from rat brain. *Science,* **252,** 1318–1321.

JANE, D.E., THOMAS, N.K., TSE, H.-W. & WATKINS, J.C. (1996). Potent antagonists at the L-AP4- and (1*S*,3*S*)-ACPD-sensitive presynaptic metabotropic glutamate receptors in the neonatal rat spinal cord. *Neuropharmacology,* **35,** 1029–1035.

KEMP, M.C., JANE, D.E., TSE, H.-W. & ROBERTS, P.J. (1996). Agonists of cyclic AMP-coupled metabotropic glutamate receptors in adult rat cortical slices. *Eur. J. Pharmacol.,* **309,** 79–85.

KITAMURA, Y. & NOMURA, Y. (1987). Uncoupling of rat cerebral cortical α_2-adrenoceptors from GTP-binding proteins by *N*-ethylmaleimide. *J. Neurochem.,* **49,** 1894–1901.

LORENZEN, A., LANG, H. & SCHWABE, U. (1998). Activation of various subtypes of G-protein α subunits by partial agonists of the adenosine A_1 receptor. *Biochem. Pharmacol.,* **56,** 1287–1293.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with Folin phenol reagent. *J. Biol. Chem.,* **193,** 265–275.

MASU, M., TANABE, Y., TSUCHIDA, K., SHIGEMOTO, R. & NAKANISHI, S. (1991). Sequence and expression of a metabotropic glutamate receptor. *Nature,* **349,** 760–765.

NAKAJIMA, Y., IWAKABE, H., AKAZAWA, C., NAWA, H., SHIGEMOTO, R., MIZUNO, N. & NAKANISHI, S. (1993). Molecular characterization of a novel retinal metabotropic glutamate receptor mGluR6 with a high agonist selectivity for L-2-amino-4-phosphonobutyrate. *J. Biol. Chem.,* **268,** 11868–11873.

ODAGAKI, Y. & FUXE, K. (1995a). Functional coupling between A_1 adenosine receptors and G-proteins in rat hippocampal membranes assessed by high-affinity GTPase activity. *Br. J. Pharmacol.,* **116,** 2691–2697.

ODAGAKI, Y. & FUXE, K. (1995b). Functional coupling of dopamine D_2 and muscarinic cholinergic receptors to their respective G proteins assessed by agonist-induced activation of high-affinity GTPase activity in rat striatal membranes. *Biochem. Pharmacol.,* **50,** 325–335.

ODAGAKI, Y. & FUXE, K. (1995c). Pharmacological characterization of the 5-hydroxytryptamine-1A receptor-mediated activation of high-affinity GTP hydrolysis in rat hippocampal membranes. *J. Pharmacol. Exp. Ther.,* **274,** 337–344.

ODAGAKI, Y. & FUXE, K. (1997). Agonist-induced high-affinity GTP hydrolysis as an index of receptor-mediated G protein activation in mammalian brain membranes. In *Methods in Molecular Biology vol. 83: Receptor Signal Transduction Protocols.* ed. Challis, R.A.J. pp. 133–141. Totowa: Humana Press.

ODAGAKI, Y., NISHI, N. & KOYAMA, T. (1996). Functional coupling between metabotropic glutamate receptors and G proteins in rat brain membranes. *Eur. J. Pharmacol.,* **300,** 151–154.

OKAMOTO, N., HORI, S., AKAZAWA, C., HAYASHI, Y., SHIGEMOTO, R., MIZUNO, N. & NAKANISHI, S. (1994). Molecular characterization of a new metabotropic glutamate receptor mGluR7 coupled to inhibitory cyclic AMP signal transduction. *J. Biol. Chem.,* **269,** 1231–1236.

PICKERING, D.S., THOMSEN, C., SUZDAK, P.D., FLETCHER, E.J., ROBITAILLE, R., SALTER, M.W., MACDONALD, J.F., HUANG, X.-P. & HAMPSON, D.R. (1993). A comparison of two alternative spliced forms of a metabotropic glutamate receptor coupled to phosphoinositide turnover. *J. Neurochem.,* **61,** 85–92.

PIN, J.-P. & DUVOISIN, R. (1995). The metabotropic glutamate receptors: structure and functions. *Neuropharmacology,* **34,** 1–26.

PIN, J.-P., WAEBER, C., PREZEAU, L., BOCKAERT, J. & HEIHNEMANN, S.F. (1992). Alternative splicing generates metabotropic glutamate receptors inducing different patterns of calcium release in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 10331–10335.

SAUGSTAD, J.A., KINZIE, J.M., MULVIHILL, E.R., SEGERSON, T.P. & WESTBROOK, G.L. (1994). Cloning and expression of a new member of the L-2-amino-4-phosphonobutyric acid-sensitive class of metabotropic glutamate receptors. *Mol. Pharmacol.,* **45,** 367–372.

SCHOEPP, D.D., JANE, D.E. & MONN, J.A. (1999). Pharmacological agents acting at subtypes of metabotropic glutamate receptors. *Neuropharmacology,* **38,** 1431–1476.

SCHOEPP, D.D., JOHNSON, B.G., SALHOFF, C.R., VALLI, M.J., DESAI, M.A., BURNETT, J.P., MAYNE, N.G. & MONN, J.A. (1995). Selective inhibition of forskolin-stimulated cyclic AMP formation in rat hippocampus by a novel mGluR agonist, 2*R*,4*R*-4-aminopyrrolidine-2,4-dicarboxylate. *Neuropharmacology,* **34,** 843–850.

THOMSEN, C., BOEL, E. & SUZDAK, P.D. (1994). Actions of phenylglycine analogs at subtypes of the metabotropic glutamate receptor family. *Eur. J. Pharmacol.,* **267,** 77–84.

UEDA, H., MISAWA, H., KATADA, T., UI, M., TAKAGI, H. & SATOH, M. (1990). Functional reconstitution of purified G_i and G_o with μ-opioid receptors in guinea pig striatal membranes pretreated with micromolar concentrations of *N*-ethylmaleimide. *J. Neurochem.,* **54,** 841–848.

(Received February 7, 2000
Revised May 8, 2000
Accepted May 10, 2000)