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# Role of metabotropic glutamate receptor subclasses in modulation of adenylyl cyclase activity by a nootropic NS-105

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**Abstract**

The involvement of metabotropic glutamate (mGlu) receptors in the modulatory actions of a novel cognition enhancer, (+)-5-oxo-D-prolinepiperidinamide monohydrate (NS-105), on adenylyl cyclase activity in rat cerebrocortical membranes and primary neuronal cultures was investigated using selective antagonists and antisense oligodeoxynucleotides for mGlu receptor subclasses. In rat cerebrocortical membranes, the inhibitory action of NS-105 (0.1 μM) on forskolin-stimulated cAMP formation was blocked by a group II mGlu receptor antagonist, (±)-α-ethylglutamic acid, and by a group III antagonist, (+)-2-amino-2-methyl-4-phosphonobutanoic acid (MAP-4), but not by a group I antagonist, (±)-1-aminoindan-1,5-dicarboxylic acid (AIDA), whereas the facilitation of cAMP formation by NS-105 (1 μM) in pertussis toxin-pretreated membranes was abolished by AIDA but not by (±)-α-ethylglutamic acid or MAP-4. In primary cultured neurons of mouse cerebral cortex, the inhibitory action of NS-105 on adenylyl cyclase activity disappeared after treatment with antisense oligodeoxynucleotides for group II ($mGlu_2$ and $mGlu_3$ receptors) and group III ($mGlu_4$ and $mGlu_7$ receptors) but not group I ($mGlu_5$ receptor) mGlu receptor subclasses. These findings suggest that the inhibitory action of NS-105 on adenylyl cyclase activity is mediated through group II and group III mGlu receptor subclasses while the facilitatory action is dependent on the group I mGlu receptor subclass. 

*Keywords:* NS-105; Glutamate receptor, metabotropic; Adenylyl cyclase; Antisense oligodeoxynucleotide, knockdown; Primary neuronal culture

## 1. Introduction

(+)-5-Oxo-D-prolinepiperidinamide monohydrate (NS-105) is a novel cognition enhancer with potent anti-depressant activity (Shimidzu et al., 1997). This compound shows anti-amnesic actions in a variety of animal models of dementia, including those induced by the dysfunction of central cholinergic neurons (Nakagawa et al., 1988; Ogasawara et al., 1999), although the cellular mechanisms underlying the cognition-enhancing action are not fully understood.

Several lines of evidence have shown that the intracellular cAMP-signal transduction pathway is involved in the processes of learning and memory (Frey et al., 1993; Weisskopf et al., 1994). Stimulation of $G_s$-coupled receptors such as β-adrenoceptors in the rat brain is reported to enhance memory retention (Liang et al., 1986). It has also been shown that spatial learning in the Morris water maze task is impaired in type I adenylyl cyclase mutant mice (Wu et al., 1995). Moreover, long-term potentiation, which is considered to be an electrophysiological model that reflects certain types of learning and memory (Bliss and Collingridge, 1993), is perturbed in the hippocampal CA1 subfield of these mutant mice (Wu et al., 1995).

We have recently found that NS-105 modulates adenylyl cyclase activity in the rat brain as well as in primary cultured neurons of the mouse cerebral cortex (Oka et al., 1997a,b). Namely, it inhibited forskolin-stimulated cAMP formation in a pertussis toxin-sensitive G protein-dependent manner, while it enhanced cAMP formation after pretreatment of membranes with pertussis toxin in a cholera toxin-sensitive G protein-dependent fashion. In addition, both of these actions were blocked by a non-selective metabotropic glutamate (mGlu) receptor antagonist L-2-amino-3-phosphopropanoate (AP-3), suggesting an involvement of mGlu receptors in the modulatory actions of NS-105 on adenylyl cyclase activity.

It has been demonstrated that the activation of mGlu receptor plays a role in the induction and maintenance of

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long-term potentiation, long-term depression and learning and memory (Zheng and Gallagher, 1992; Bortolotto et al., 1994; Toms et al., 1996). The mGlu receptors are coupled both positively and negatively to adenylyl cyclase. Briefly, various mGlu receptor agonists including (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD) inhibited forskolin-stimulated cAMP formation in cultured neurons (Prézeau et al., 1994) and cells expressing recombinant mGlu receptors (Tanabe et al., 1992) in a manner dependent on pertussis toxin-sensitive G proteins. Moreover, the stimulation of mGlu receptors increased cAMP formation in slices (Winder and Conn, 1992) and synaptoneurosomes of the rat hippocampus (Musgrave et al., 1994) and cells expressing certain types of mGlu receptors (Aramori and Nakanishi, 1992). Both pharmacological and expression cloning studies have demonstrated the existence of different subclasses of mGlu receptors. To date, eight genes encoding mGlu receptors have been cloned (Nakanishi, 1992; Tanabe et al., 1992; Simoncini et al., 1993; Pin and Duvoisin, 1995), and multiplicity in this receptor family is augmented by the existence of splice variants (Pin et al., 1992; Minakami et al., 1993; Pickering et al., 1993). Based on their similarity of amino acid sequences and pharmacological profiles, mGlu receptors have been divided into three groups (Nakanishi, 1992; Schoepp and Conn, 1993; Pin and Duvoisin, 1995). Group I mGlu receptors consist of $mGlu_1$ and $mGlu_5$ receptors, which are positively coupled to both phosphoinositides hydrolysis/$Ca^{2+}$ signal transduction and cAMP formation (Aramori and Nakanishi, 1992). In contrast, the activation of group II ($mGlu_2$ and $mGlu_3$ receptors) or group III ($mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$ receptors) mGlu receptors results in the inhibition of adenylyl cyclase activity via pertussis toxin-sensitive G proteins (Tanabe et al., 1992, 1993; Wu et al., 1998).

Therefore, the present study was designed to clarify which subclasses or subgroups of mGlu receptors participate in the modulatory action of NS-105 on adenylyl cyclase activity. For this purpose, the effects of various subclass-selective mGlu receptor antagonists on NS-105-induced modulation of adenylyl cyclase activity were examined in rat brain. Furthermore, the effect of NS-105 on adenylate cyclase activity was investigated in the antisense knockdown of mGlu receptor subclasses in primary cultured neurons from the mouse cerebral cortex.

## 2. Materials and methods

### 2.1. Animals

Seven-week-old male Wistar rats (Charles River Japan, Kanagawa) and pregnant ddY strain mice were used. Rats were housed in groups of five to six in a room controlled at 21–25°C and 45–65% humidity and maintained under a 12-h light/dark cycle (lights automatically on at 0800 h). Food and water were freely given. Experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals written by the Japanese Pharmacological Society.

### 2.2. Membrane preparation from rat cerebral cortex

The rat cerebral cortex was homogenized in 10 volumes of 10 mM HEPES–NaOH (pH 7.4) buffer containing 1 mM ethylene glycol bis (β-aminoethyl-ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 1 mM $MgCl_2$, 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride (buffer A). After centrifugation at $1000 \times g$ for 10 min, the supernatant was further centrifuged at $30{,}000 \times g$ for 20 min. The pellet was washed twice with buffer A and used for cAMP assay. In a set of experiments, membranes were treated with pertussis toxin, as described previously (Oka et al., 1997a). Briefly, the pellet of cerebrocortical membranes was suspended in ADP-ribosylating buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM disodium dihydrogen ethylenediamine tetraacetate (EDTA), 1 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM adenosine triphosphate (ATP), 10 mM thymidine and 10 mM nicotine adenine dinucleotide (NAD). Seventy-five micrograms of membrane protein was incubated at 37°C for 1 h with 20 μg/ml pertussis toxin pre-activated by incubation at 30°C for 10 min with 50 mM dithiothreitol. Incubation was terminated by centrifugation at $30{,}000 \times g$ for 20 min.

### 2.3. Assay of cAMP formation in membrane fraction

Adenylyl cyclase activity in membrane fractions was measured as follow. The reaction mixture, which contained 10 μM forskolin, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.3 mM EDTA, 10 μM guanosine triphosphate (GTP), 10 μM ATP, 1 mM 3-isobutyl-1-methylxanthine (IBMX), 50 mM phosphocreatine and 50 U/ml creatine phosphokinase in 500 μl of 50 mM HEPES–NaOH (pH 7.4), was incubated for 15 min at 37°C with membrane fraction (100–200 μg of protein) in the presence or absence of NS-105 and various mGlu receptor antagonists. The reaction was terminated by addition of an equal volume of ice-cold 0.1 N HCl. After centrifugation at $30{,}000 \times g$ for 20 min, the cAMP content in the supernatant was assayed using the cAMP enzyme immunoassay system (Amersham; Buckinghamshire, UK). Simultaneously, the resulting pellet was suspended and its protein content was measured by the method of Bradford (1976) using bovine serum albumin as a standard.

### 2.4. Primary neuronal cultures

Primary neuronal cultures were prepared from the cerebral cortex of fetal mice, as described previously (Ohkuma et al., 1986; Hirouchi et al., 1992). Briefly, the neopallidum was dissected from the 15-day-old fetal mouse

brain, and the meninges were carefully removed. Tissues were minced and trypsinized in $Ca^{2+}$-free Puck's solution at 37°C for 5 min and then triturated with a Pasteur pipette. The cell suspension was centrifuged at $900 \times g$ for 2 min. The resultant pellet was resuspended in Dulbecco's modified Eagle medium (DMEM), followed by filtration through a nylon sieve (mesh size: 60 μm). The dissociated cells were seeded on 35-mm (inside diameter) tissue culture dishes precoated with poly-L-lysine, and maintained for 3 days in DMEM supplemented with 15% fetal bovine serum. On the fourth day of culture, the cortical cells were exposed to DMEM containing 20 μM cytosine arabinoside and 15% fetal bovine serum for 24 h to prevent the growth of non-neuronal cells. Thereafter, the cells were continuously cultured in DMEM containing 15% fetal bovine serum.

### 2.5. Assay of cAMP accumulation in primary neurons

The cAMP accumulation in cultured neurons was measured as described previously (Weiss et al., 1985; Oka et al., 1997b). On the 10th day of culture, neuronal cells were washed and preincubated for 10 min at 37°C (5% $CO_2$/95% air mixture) with HEPES-buffered saline (140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 11 mM glucose and 15 mM HEPES: pH 7.4). The neuronal cells were incubated for 10 min with 1 μM NS-105 in the presence of 1 μM forskolin and 1 mM IBMX. The reaction was terminated by aspiration of the incubation medium followed by addition of 1 ml of ice-cold 0.1 N HCl. Cells were scraped off with a rubber policeman. After centrifugation at $10{,}000 \times g$ for 10 min, the cAMP content in the supernatant was determined as described above.

### 2.6. Reverse transcription–polymerase chain reaction (RT–PCR) of mGlu receptor subtypes in primary neurons

The preparation of Poly(A)$^+$RNA in the primary neurons was performed using QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech). The expression of mGlu receptor subtypes such as $mGlu_2$, $mGlu_3$, $mGlu_4$, $mGlu_5$ and $mGlu_7$ receptors was predicted in the cells of cerebral cortex (Okamoto et al., 1994; Prézeau et al., 1994). In this study, the primers for each mGlu receptor subtype and β-actin were constructed on the basis of previous reports (Nudel et al., 1983; Okamoto et al., 1994; Prézeau et al., 1994). The sequences of synthesized DNA were designed as follows. [$mGlu_1$ receptor FW: 5′-AAT CTA CAG CAA TGC TGG CG-3′ (1156–1175), RV: 5′-CTT CGA TGA CTT CAT CTC TG-3′ (antisense: 1360–1341); $mGlu_2$ receptor FW: 5′-CCC ACT CTC TGC GGG CCG TG-3′ (1282–1301), RV: 5′-CTG CCT GCC CGC AGA TAG GT-3′ (antisense: 1579–1560); $mGlu_3$ receptor FW: 5′-CTC CAA CAT CCG CAA GTC CT-3′ (888–907), RV: 5′-TGT CAA TGG CCA GGT GCT TG-3′ (antisense: 1282–1263); $mGlu_4$ receptor FW: 5′-TGA GCT ACG TGC TGC TGG CG-3′ (1943–1962), RV: 5′-TGT CGG CTG ACT GTG AGG TG-3′ (antisense: 2509–2490); $mGlu_5$ receptor FW: 5′-GTC TCC TGA TGT CAA GTG GT-3′ (1254–1273), RV: 5′-GGA CCA CAC TTC GTC ATC AT-3′ (antisense: 1767–1748); $mGlu_7$ receptor FW: 5′-CGC TAT GAC TTC TTC TCT CG-3′ (968–987), RV: 5′-TTT CCA ATT CGC TCC TGT CC-3′ (antisense: 1590–1571); β-actin FW: 5′-TGG TGG GTA TGG GTC AGA AGG ACT C-3′ (131–155), RV: 5′-CAT GGC TGG GGT GTT GAA GGT CTC A-3′ (antisense: 396–372)].

The reaction of RT–PCR was performed using Takara EX Taq polymerase and a Takara RNA PCR kit (AMV ver. 2.1, Kyoto, Japan). The PCR conditions were 25 cycles of 30 s denaturation at 94°C, 30 s annealing at 54°C, and 60 s extension at 72°C, after preheating at 94°C for 2 min. PCR products were electrophoresed on 1.5% agarose gel and detected by ethidium bromide staining. The bands of PCR product were analyzed by NIH Image (ver. 1.6). In this study, the reaction was performed under the experimental condition that both the content of template mRNA and the number of amplification cycles were sufficient for a linear increase in amplified product.

### 2.7. Treatment with antisense oligodeoxynucleotides for mGlu receptor subclasses of primary neurons

In order to clarify the involvement of each mGlu receptor subclass, several antisense oligodeoxynucleotides for mGlu receptor were used in the primary neuronal cells. Namely, the knockdown of group I mGlu receptor ($mGlu_5$ receptor), group II mGlu receptor ($mGlu_2$ and $mGlu_3$ receptors) and group III mGlu receptor ($mGlu_4$ and $mGlu_7$ receptors) were examined using antisense oligodeoxynucleotides synthesized by phosphorothioate residues. The antisense sequences were indicated as follows: 5′-CCC AAG CAG TGA TTC CAT-3′ ($mGlu_2$ receptor: 209–192); 5′-CTT CAG TTC CAG TGC CTT-3′ ($mGlu_3$ receptor: 307–290); 5′-TAA AGG CTG AGG AGT AGG-3′ ($mGlu_4$ receptor: 137–120); 5′-GAT CAA CAG AAG GAC CAT-3′ ($mGlu_5$ receptor: 285–268); 5′-GAT GTT GGC CAC CAT GAT-3′ ($mGlu_7$ receptor: 901–884). These were selected as specific sequences for each targeting receptor except $mGlu_7$ receptor, sequence of which was common to $mGlu_4$ receptor (573–556), one of the group III mGlu receptor subclasses.

The antisense oligodeoxynucleotide for each mGlu receptor subtype was added at 2 μg/35-mm dish. The antisense oligodeoxynucleotides were premixed with lipofectin reagent (Life Technologies, Rockville, MD, USA) in DMEM, which is devoid of antibiotics and serum, according to the instruction manual. On the 7th day of cultured, neuronal cells were washed with phosphate-buffered saline and treated with this mixture in antibiotic-free DMEM containing 15% fetal bovine serum (Chiang et al., 1991; Chen et al., 1996). After incubation for 3 h at 37°C, the

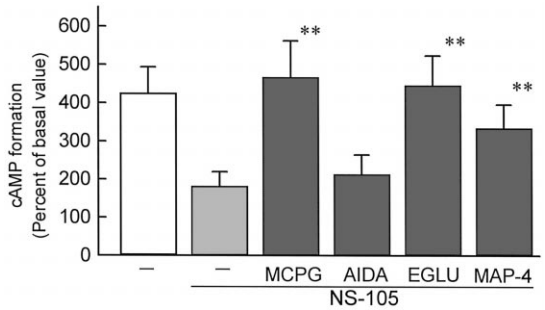


Fig. 1. Effects of various antagonists of mGlu receptor subclasses on NS-105-induced inhibition of adenylyl cyclase activity in membrane fractions of the rat cerebral cortex. NS-105 (0.1 μM) was added to the medium in the presence or absence of various mGlu receptor antagonists such as 100 μM MCPG, 10 μM AIDA, 100 μM (±)-α-ethylglutamic acid (EGLU) and 100 μM MAP-4. The cAMP content is expressed as a percentage of the basal value. Each column represents the mean ± S.E. of 6 experiments. The basal cAMP formation without forskolin stimulation was 2.4 ± 0.2 pmol/mg protein/min (mean ± S.E., $N = 6$). $^{**}P < 0.01$ as compared with NS-105 alone (Dunnett's test).

neuronal cells were washed with phosphate-buffered saline and further cultured in normal growth medium. On the 3rd day after application of the antisense oligodeoxynucleotides, neurons were used for the cAMP assay and RT–PCR analysis.

### 2.8. Statistical analysis

Data on cAMP formation were analyzed by using the SAS program (SAS/STAT, ver. 6, 4th edition, 1990, SAS Institute, Cary, NC). The statistical significance was determined by either Student's *t*-test for comparison between two groups or one-way analysis of variance (ANOVA), followed by Dunnett's test in the case of multiple comparisons. The expression of mRNA for mGlu receptor subtypes and β-actin was analyzed by NIH Image (ver. 1.6) and their statistics were determined by two-way ANOVA, followed by Dunnett's test. $P$ values of $< 0.05$ were considered as statistically significant.

### 2.9. Chemicals

NS-105 was synthesized in our laboratories. (+)-α-Methyl-4-carboxyphenylglycine (MCPG), (±)-1-aminoindan-1,5-dicarboxylic acid (AIDA), (±)-α-ethylglutamic acid, (+)-2-amino-2-methyl-4-phosphonobutanoic acid (MAP-4) and (2*S*,1′*R*,2′*R*,3′*R*)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV) were purchased from Tocris Cookson (Bristol, UK). Forskolin, 7-deacetyl-7-*O*-hemisuccinyl and IBMX were obtained from Carbiochem–Novabiochem (San Diego, CA, USA) and Sigma (St. Louis, MO, USA), respectively. Pertussis toxin was donated from Seikagaku Kogyo (Tokyo, Japan). Other chemicals used in the present experiment were all of reagent grade.

## 3. Results

### 3.1. Effects of various antagonists of mGlu receptor subclasses on NS-105-induced modulation of adenylyl cyclase activity in membrane fractions from rat cerebral cortex

The effects of various antagonists of mGlu receptor subclasses on NS-105-induced inhibitory and facilitatory actions of adenylyl cyclase activity were examined in membrane fractions from the rat cerebral cortex. As shown in Fig. 1, NS-105 (0.1 μM) inhibited forskolin-stimulated cAMP formation in membrane fractions of the rat cerebral cortex. This inhibition was counteracted by 100 μM MCPG, an antagonist of group I and group II mGlu receptor (Eaton et al., 1993), and by 100 μM (±)-α-ethylglutamic acid, a selective antagonist of group II mGlu receptor (Jane et al., 1996). In addition, 100 μM MAP-4, a selective antagonist of group III mGlu receptor (Jane et al., 1994), also reversed the NS-105-induced inhibition. However, 10 μM AIDA, a selective antagonist of group I mGlu receptor (Pellicciari et al., 1995), failed to block NS-105-induced inhibition of cAMP formation. The concentrations of NS-105 for producing a consistent inhibition of adenylyl cyclase activity (0.01–0.1 μM) were 10 times lower than those for inducing stimulation (0.1–1 μM) according to our previous report (Oka et al., 1997a). Therefore, in our present study, the concentrations of various mGlu receptor antagonists used for antagonizing the inhibitory action of NS-105 (0.1 μM) were also 10 times lower than those for blocking the facilitatory action of NS-105 (1 μM).

NS-105 (1 μM) facilitated forskolin-stimulated cAMP formation in pertussis toxin-pretreated membrane fractions of the rat cerebral cortex (Fig. 2). Both 100 μM AIDA and

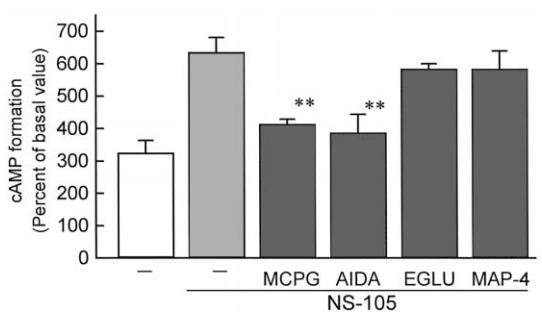


Fig. 2. Effects of various antagonists for mGlu receptor subclasses on NS-105-induced enhancement of adenylyl cyclase activity in pertussis toxin-pretreated membrane fractions of the rat cerebral cortex. NS-105 (1 μM) was added to the medium in the presence or absence of various mGlu receptor antagonists including 1 mM MCPG, 100 μM AIDA, 1 mM (±)-α-ethylglutamic acid (EGLU) and 1 mM MAP-4. The cAMP content is expressed as a percentage of the basal value in pertussis toxin-pretreated membranes. Each column represents the mean ± S.E. of 5 experiments. The basal cAMP formation without forskolin stimulation was 4.8 ± 0.5 pmol/mg protein/min (mean ± S.E., $N = 5$). $^{**}P < 0.01$ as compared with NS-105 alone (Dunnett's test).

1 mM MCPG antagonized this facilitatory action of NS-105. In contrast, the cAMP formation enhanced by NS-105 was not significantly affected by 1 mM (±)-α-ethylglutamic acid or 1 mM MAP-4.

### 3.2. Expression and knockdown of mGlu receptor subclasses in primary neurons from mouse cerebral cortex

To further clarify the involvement of mGlu receptor in NS-105-induced modulation of adenylyl cyclase activity, the effect of this compound on cAMP accumulation was examined in neurons using antisense oligodeoxynucleotides for mGlu receptor subtypes. As observed in rat cerebrocortical neurons, NS-105 has both inhibitory and facilitatory actions on adenylyl cyclase activity in primary neurons from the mouse cerebral cortex (Oka et al., 1997b). Firstly, the expression of mRNA for mGlu receptor subtypes in the primary neuronal cells of the mouse cerebral cortex was examined by RT–PCR. The expression of mRNA for $mGlu_2$, $mGlu_3$, $mGlu_4$, $mGlu_5$ and $mGlu_7$ receptors but not for $mGlu_1$ receptor was confirmed in the neuronal cells.

Subsequently, the antisense oligodeoxynucleotides for these mGlu receptor subtypes were designed as described in Materials and methods. The antisense oligodeoxynucleotide for $mGlu_5$ receptor was used to deplete group I mGlu receptor, those for $mGlu_2$ and $mGlu_3$ receptors were applied to deplete group II mGlu receptor, and those for $mGlu_4$ and $mGlu_7$ receptors were prepared to decrease group III mGlu receptor. The effects of antisense oligodeoxynucleotides for each mGlu receptor subclass were examined by semiquantitative RT–PCR. As shown in Fig. 3 and Table 1, application of antisense oligodeoxynucleotide (2 μg/35-mm dish) for group I mGlu receptor decreased $mGlu_5$ receptor mRNA without affecting the expression of other subclasses of mGlu receptor mRNAs. Antisense oligodeoxynucleotides (2 μg/35-mm dish, respectively) for group II mGlu receptor caused the selective depletion of $mGlu_2$ and $mGlu_3$ receptor mRNAs. Application of antisense oligodeoxynucleotides (2 μg/35-mm dish, respectively) for group III mGlu receptor reduced the expression of mRNA for $mGlu_4$ and $mGlu_7$ receptors without affecting the expression of group I and group II mGlu receptors. In contrast, there was no difference in the amount of β-actin mRNA among control and antisense oligodeoxynucleotide-treated groups.

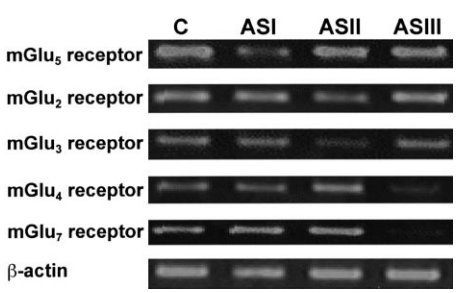


Fig. 3. RT–PCR of mGlu receptor subtypes and β-actin in primary neuronal cells pretreated with antisense oligodeoxynucleotides for mGlu receptor subclasses. The antisense oligodeoxynucleotides for each mGlu receptor subtypes were added at 2 μg/35-mm dish. C: control (no treatment); ASI: pretreatment with antisense oligodeoxynucleotide for $mGlu_5$ receptor; ASII: pretreatment with antisense oligodeoxynucleotides for $mGlu_2$ and $mGlu_3$ receptors; ASIII: pretreatment with antisense oligodeoxynucleotides for $mGlu_4$ and $mGlu_7$ receptors.

Table 1
Changes in the mRNA expression for mGlu receptor subtypes and β-actin in primary cultured neurons pretreated with antisense oligodeoxynucleotides for mGlu receptor subclasses
The density of band shown in Fig. 3 was determined using NIH Image. Each value expresses the percentage of the respective control and represents the mean ± S.E. of five experiments. ASI: pretreatment with antisense oligodeoxynucleotide for $mGlu_5$ receptor; ASII: pretreatment with antisense oligodeoxynucleotides for $mGlu_2$ and $mGlu_3$ receptors; ASIII: pretreatment with antisense oligodeoxynucleotides for $mGlu_4$ and $mGlu_7$ receptors.

| | Percent of control | | | |
|---|---|---|---|---|
| | Control | ASI | ASII | ASIII |
| $mGlu_5$ receptor | 100.0 ± 23.3 | 62.6 ± 23.3[a] | 96.5 ± 21.5 | 111.2 ± 19.8 |
| $mGlu_2$ receptor | 100.0 ± 26.9 | 104.9 ± 22.8 | 50.5 ± 15.6[b] | 129.5 ± 14.7 |
| $mGlu_3$ receptor | 100.0 ± 16.5 | 84.5 ± 18.2 | 56.1 ± 6.0[b] | 122.7 ± 20.3 |
| $mGlu_4$ receptor | 100.0 ± 13.5 | 95.8 ± 9.9 | 95.2 ± 23.5 | 45.3 ± 7.7[a] |
| $mGlu_7$ receptor | 100.0 ± 20.4 | 109.9 ± 21.7 | 105.7 ± 24.3 | 46.7 ± 15.9[a] |
| β-actin | 100.0 ± 13.1 | 98.7 ± 7.6 | 99.0 ± 14.1 | 100.9 ± 15.0 |

[a] $P < 0.01$; compared with each control (Dunnett's test).
[b] $P < 0.05$; compared with each control (Dunnett's test).

### 3.3. Effects of antisense and sense oligodeoxynucleotides for mGlu receptor subclasses on forskolin-stimulated cAMP accumulation in primary neurons

The effect of mGlu receptor subclasses on forskolin-stimulated cAMP accumulation was examined in cultured neurons treated with antisense oligodeoxynucleotides for mGlu receptor subclasses. DCG IV (3 μM), a selective agonist of group II mGlu receptor (Ishida et al., 1993), significantly reduced forskolin-stimulated cAMP accumulation (Fig. 4A). This reduction was not observed in cells pretreated with antisense oligodeoxynucleotides (2 μg/35-mm dish, respectively) for group II mGlu receptor, but was in those for group I and group III mGlu receptors (Fig. 4B–D). In contrast, the treatment with sense oligodeoxynucleotides (2 μg/35-mm dish, respectively) for group II mGlu receptor did not affect the inhibitory action of DCG IV on adenylyl cyclase activity (Fig. 5).

### 3.4. Effect of NS-105 on knockdown of mGlu receptor subclasses in primary neurons

NS-105 (1 μM) inhibited forskolin-stimulated cAMP accumulation in non-treated neurons (Fig. 6A). The in-

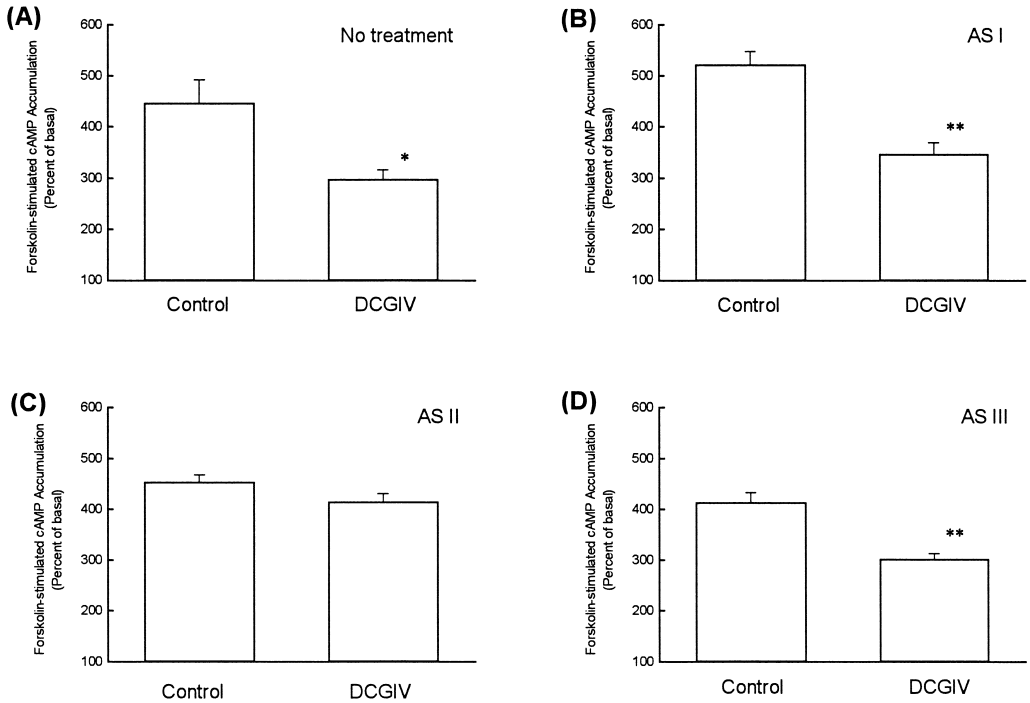


Fig. 4. Effect of pretreatment with antisense oligodeoxynucleotides for each mGlu receptor subclass on DCG IV (3 μM)-induced reduction of cAMP accumulation in primary neuronal cultures. The antisense oligodeoxynucleotides for each mGlu receptor subtypes were added at 2 μg/35-mm dish. The cAMP content is expressed as a percentage of the basal value. Each column represents the mean ± S.E. of 5 experiments. (A) No treatment. The basal value of cAMP accumulation was 2.0 ± 0.2 pmol/mg protein/min. (B) ASI: pretreatment with antisense oligodeoxynucleotide for $mGlu_5$ receptor. The basal value of cAMP accumulation was 2.7 ± 0.2 pmol/mg protein/min. (C) ASII: pretreatment with antisense oligodeoxynucleotides for $mGlu_2$ and $mGlu_3$ receptors. The basal value of cAMP accumulation was 2.3 ± 0.2 pmol/mg protein/min. (D) ASIII: pretreatment with antisense oligodeoxynucleotides for $mGlu_4$ and $mGlu_7$ receptors. The basal value of cAMP accumulation was 2.8 ± 0.1 pmol/mg protein/min. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with each control (Student's *t*-test).

hibitory action of NS-105 on forskolin-stimulated cAMP accumulation disappeared in the knockdown of group II and III mGlu receptors, but not in the knockdown of group I mGlu receptor (Fig. 6B–D).

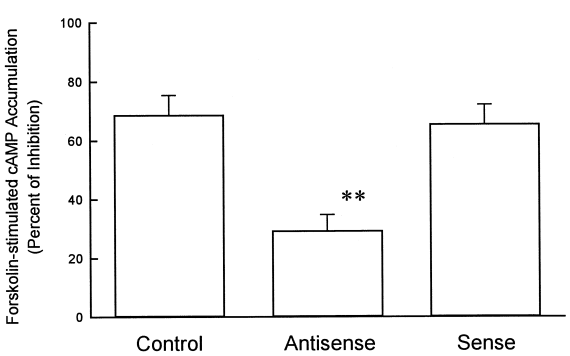


Fig. 5. Effect of pretreatment with antisense and sense oligodeoxynucleotides for group II mGlu receptor on DCG IV-induced inhibition of cAMP accumulation in primary neuronal cultures. The forskolin-stimulated value is expressed as a percentage of inhibition of cAMP accumulation by 3 μM DCG IV. The antisense and sense oligodeoxynucleotides for $mGlu_2$ and $mGlu_3$ receptors were added at 2 μg/35-mm dish. Each column represents the mean ± S.E. of 5 experiments. Control: no treatment; Antisense: pretreatment with antisense oligodeoxynucleotides for $mGlu_2$ and $mGlu_3$ receptors; sense: treatment of sense oligodeoxynucleotides for $mGlu_2$ and $mGlu_3$ receptors $^{**}P < 0.01$ as compared with control (Dunnett's *t*-test).

## 4. Discussion

We have previously reported, for membranes of the rat cerebral cortex, that NS-105 inhibited forskolin-stimulated cAMP formation, but it markedly increased cAMP formation in membranes pretreated with pertussis toxin (Oka et al., 1997a). Similar bidirectional actions of NS-105 on adenylyl cyclase activity were observed in primary cultured neurons of the mouse cerebral cortex (Oka et al., 1997b). It is probable that both the inhibitory and facilitatory actions of NS-105 are mediated by mGlu receptors, since both actions of NS-105 were blocked by AP-3 and mimicked by 1*S*,3*R*-ACPD, a mGlu receptor agonist (Oka et al., 1997a). However, a lack of selectivity of AP-3 for mGlu receptors has been claimed by several investigators (Mistry et al., 1996).

Multiple mGlu receptor subtypes have been cloned and a variety of agonists and antagonists selective for each mGlu receptor subclass or subgroup are currently available. Thus, to confirm our previous findings and to further

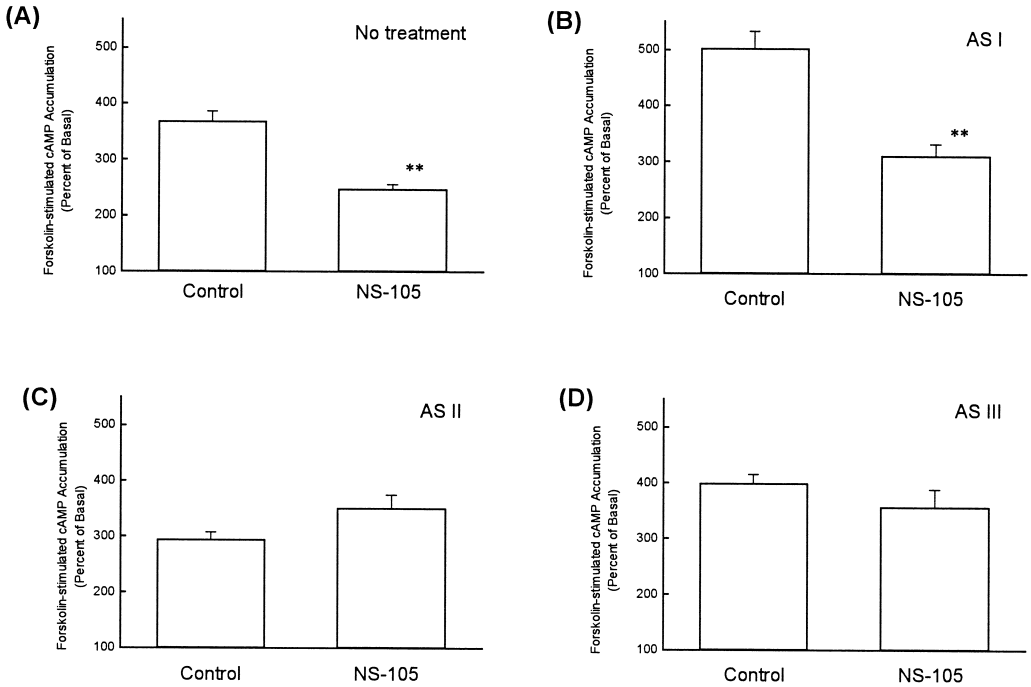


Fig. 6. Effect of pretreatment with antisense oligodeoxynucleotides for each mGlu receptor subclass on NS-105 (1 μM)-induced inhibition of cAMP accumulation in primary neuronal cultures. The cAMP content is expressed as a percentage of the basal value. The antisense oligodeoxynucleotides for each mGlu receptor subtypes were added at 2 μg/35-mm dish. Each column represents the mean ± S.E. of four to five experiments. (A) No treatment. The basal value of cAMP accumulation was 5.7 ± 0.3 pmol/mg protein/min. (B) ASI: pretreatment with antisense oligodeoxynucleotide for $mGlu_5$ receptor. The basal value of cAMP accumulation was 4.8 ± 0.2 pmol/mg protein/min. (C) ASII: pretreatment with antisense oligodeoxynucleotides for $mGlu_2$ and $mGlu_3$ receptors. The basal value of cAMP accumulation was 5.3 ± 0.3 pmol/mg protein/min. (D) ASIII: pretreatment with antisense oligodeoxynucleotides for $mGlu_4$ and $mGlu_7$ receptors. The basal value of cAMP accumulation was 4.1 ± 0.4 pmol/mg protein/min. $^{**}P < 0.01$ as compared with each control (Student's *t*-test).

clarify which subgroups or subclasses of mGlu receptors are implicated in the modulatory actions of NS-105 on adenylyl cyclase activity, the effects of several specific mGlu receptor antagonists on the actions of NS-105 were examined in membranes of rat cerebral cortex. The inhibitory action of NS-105 on forskolin-stimulated cAMP formation was blocked by the group II selective antagonist, (±)-α-ethylglutamic acid, and by MAP-4, a group III mGlu receptor antagonist, but not by AIDA, an antagonist of group I mGlu receptor. In our previous study, this inhibition by NS-105 in brain membranes and cultured neurons was dependent on $G_i/G_o$ proteins, since pretreatment with pertussis toxin completely blocked the inhibitory action (Oka et al., 1997a,b). It has been demonstrated that both group II and group III mGlu receptors are coupled to pertussis toxin-sensitive G proteins and negatively regulate adenylyl cyclase activity (Tanabe et al., 1992, 1993). Taken together, NS-105 may inhibit adenylyl cyclase activity by acting on group II and group III mGlu receptors/$G_i$ protein system.

In contrast, the stimulatory effect of NS-105 on cAMP formation in pertussis toxin-pretreated membranes was abolished by AIDA but not by (±)-α-ethylglutamic acid or MAP-4. Therefore, it is suggested that NS-105 may stimulate adenylyl cyclase activity through the activation of group I mGlu receptor. We have already shown that the enhancement by NS-105 of cAMP formation was mediated through cholera toxin-sensitive $G_s$ proteins and was not affected by calphostin-C, a protein kinase C inhibitor, and an intracellular $Ca^{2+}$ chelator, 1,2-bis (2-amino-5-fluorophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetrakis (acetoxymethyl) ester (BAPTA/AM) (Oka et al., 1997b). However, it is difficult to explain how NS-105 stimulates adenylyl cyclase activity through activation of group I mGlu receptor, since there is no direct evidence for the coupling of mGlu receptor with $G_s$ protein. Our results are generally consistent with the report that (*R*,*S*)-3,5-dihydroxyphenylglycine, a group I mGlu receptor agonist, enhances forskolin-stimulated adenylyl cyclase activity in rat cerebral cortical astrocytes where $mGlu_5$ receptor is the only source of group I mGlu receptor and that the action is not dependent on $[Ca^{2+}]_i$ or the protein kinase C activity (Balazs et al., 1998).

The involvement of mGlu receptor in the modulatory action of NS-105 on adenylyl cyclase activity was further determined. In the primary neuronal cells of the mouse cerebral cortex, the expression of mRNAs for $mGlu_2$, $mGlu_3$, $mGlu_4$, $mGlu_5$ and $mGlu_7$ receptors but not for $mGlu_1$ receptor was found by RT–PCR. This finding was generally consistent with the report by Prézeau et al. (1994), who have shown the expression of mGlu receptor subtypes in the primary cultured neurons of mouse cerebral cortex. Subsequently, antisense knockdown for mGlu

receptor subclasses was attempted using primary neurons from the mouse cerebral cortex.

Knockdown by using specific antisense oligodeoxynucleotides for certain neurotransmitter receptors has been widely used for analyzing the function of receptors (Ghelardini et al., 1999). A study of the knockdown of specific mGlu receptor subtypes has also been performed in vivo (Dorri et al., 1997). In this study, treatment with antisense oligodeoxynucleotides specific for each mGlu receptor subclass selectively decreased the expression of the mRNA for the corresponding mGlu receptor subclass. However, we did not determine the changes in protein level for mGlu receptor subtypes, since specific antibodies or radiolabeled ligands for mGlu receptor subclasses are not currently available. In the case of the $\alpha_{1B}$-adrenoceptor, it has been shown that treatment with antisense oligodeoxynucleotide weakly reduces the expression of $\alpha_{1B}$-adrenoceptor mRNA, while it showed the clear knockdown of this protein level (Gonzalez-Cabrera et al., 1998).

In the present study, the lack of functional mGlu receptors in the antisense oligodeoxynucleotide-transfected neurons was confirmed as follows. DCG IV, a selective agonist for group II mGlu receptor, no longer inhibited forskolin-stimulated cAMP accumulation in neurons transfected with antisense oligodeoxynucleotides for group II mGlu receptor, while this mGlu receptor agonist significantly inhibited the cAMP accumulation in normal cells and those transfected with antisense oligodeoxynucleotides for group I or group III mGlu receptors. In addition, the knockdown of group II mGlu receptor was specific for the antisense sequence, since the treatment of sense oligodeoxynucleotide had no influence on the DCG IV-induced inhibition of forskolin-stimulated cAMP accumulation. In these neurons, the inhibitory action of NS-105 disappeared in neurons deficient in mRNAs for group II or group III mGlu receptors but not in neurons lacking group I mGlu receptor mRNA. Therefore, it is suggested that group II and/or group III mGlu receptors are major targets of NS-105 for inhibiting adenylyl cyclase activity. Unfortunately, in the present study, we could not determine the effects of antisense oligodeoxynucleotides on NS-105-induced facilitation of cAMP accumulation in pertussis toxin-pretreated neurons because of the neuronal damage due to the pertussis toxin treatment after application of antisense oligodeoxynucleotides.

In both studies using specific antagonists and antisense oligodeoxynucleotides for mGlu receptor subclasses, the inhibitory action of NS-105 was almost completely blocked by the group II mGlu receptor antagonist, (±)-α-ethylglutamic acid (Fig. 1) or prior treatment with antisense oligodeoxynucleotides for group II mGlu receptor (Fig. 6), although the antagonist (MAP-4) or the antisense oligodeoxynucleotides for group III mGlu receptor partially attenuated the action of NS-105. Therefore, it is likely that the inhibitory action of NS-105 on adenylyl cyclase activity is predominantly mediated by group II mGlu receptor. However, we do not know at present why NS-105 did not cause any suppressive action on adenylyl cyclase activity by acting on the remaining group such as group III mGlu receptor in tissues or neurons whose group II mGlu receptor was blocked or disappeared. It seems likely that group III mGlu receptor may also participate in the inhibitory action of NS-105, provided that group II mGlu receptor is activated by this compound.

In conclusion, NS-105 inhibited forskolin-stimulated cAMP formation in rat cerebrocortical membranes as well as primary neuronal cultures of the mouse cerebral cortex, and concurrently enhanced cAMP formation in pertussis toxin-pretreated preparations. A pharmacological study using selective antagonists of mGlu receptor subgroups demonstrated that the inhibitory action of NS-105 was mediated by stimulation of group II and group III mGlu receptors. Consistent with these findings, NS-105 no longer decreased forskolin-stimulated cAMP accumulation in primary cultured neurons pretreated with antisense oligodeoxynucleotides for group II or group III mGlu receptor mRNAs. In contrast, the enhancement of forskolin-stimulated cAMP formation by NS-105 observed in pertussis toxin-pretreated membranes was due to the stimulation of group I mGlu receptor but not group II or group III mGlu receptor.

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