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BMY-14802 Antagonizes Harmaline- and D-Serine-Induced Increases in Mouse Cerebellar Cyclic GMP: Neurochemical Evidence for a σ Receptor-Mediated Functional Modulation of Responses Mediated by the *N*-Methyl-D-aspartate Receptor Complex *In Vivo*

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SUMMARY

BMY-14802 [α-(4-fluorophenyl)-4-(5-fluoro-pyramidinyl)-1-piperazine butanol], a potent σ ligand with poor affinity for dopamine and phencyclidine receptors *in vitro*, attenuated parenteral harmaline- and direct intracerebellar D-serine-induced increases in mouse cerebellar cGMP. Intracerebroventricularly injected BMY-14802 also antagonized the effects of intracerebellar D-serine, indicating a central mechanism. However, direct co-injection of BMY-14802 into the cerebellum failed to antagonize the D-serine-induced increases in cGMP, indicating a locus of action outside the cerebellum. In contrast, quisqualate-induced cGMP increases were not attenuated by BMY-14802. These results indicate a functional modulation of the *N*-methyl-D-aspartate/glycine/phencyclidine/ion channel complex-mediated events by BMY-14802, possibly through a transsynaptic mechanism, thus representing the first *in vivo* demonstration of a σ ligand modulation of a response mediated through the *N*-methyl-D-aspartate receptor complex.

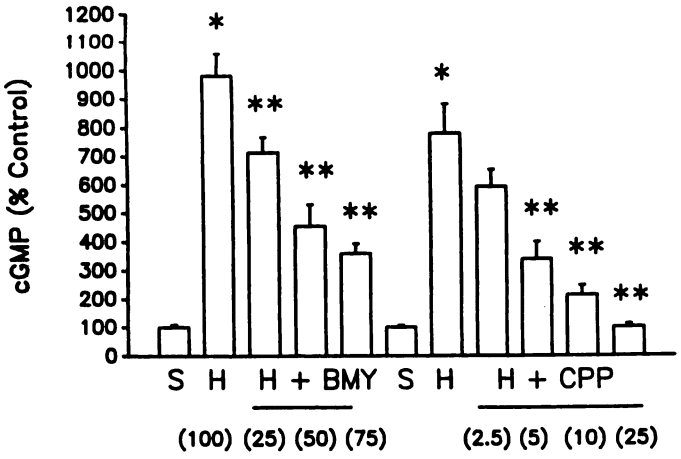
Extensive pharmacological studies have revealed the existence of multiple recognition sites on the NMDA receptor complex, a subclass of glutamate receptors. These recognition sites include a site for acidic amino acids such as glutamate, a site for the neutral amino acid glycine, a site for dissociative anesthetics such as PCP, a site for divalent ions such as magnesium and zinc (1), and a site for polyamines such as spermidine and spermine (2). An important role for the NMDA receptor complex in the pathophysiology of ischemic brain damage is also well established (1). Competitive NMDA antagonists such as CPP and CGS-19755 (3, 4), PCP agonists such as MK-801 (dizocilipine maleate) and PCP (4, 5), glycine antagonists such as HA-966 (6), and σ ligands such as dextromethorphan (7) were found to attenuate ischemic injury in several models of ischemia, suggesting a modulation of the NMDA receptor complex by these classes of compounds. Of greater significance to this investigation is the modulation of NMDA receptor-mediated events by σ ligands, besides the putative modulation by competitive NMDA antagonists and by PCP agonists. However, multiple receptor affinities of several σ ligands, including several benzomorphans that were examined as σ ligands, exemplified by dextromethorphan, make it difficult to unequivocally define potential interactions of σ and NMDA receptors *in vivo*. Furthermore, the known *in vivo* biotransformation of dextromethorphan to dextrorphan, a potent PCP ligand, may contribute to its antiischemic potential. Recently, BMY-14802 a novel antipsychotic compound, has been characterized as a selective σ ligand with poor affinity for dopamine and PCP receptors *in vitro* (8). Recent evidence indicates its potential as a novel antiischemic agent as well (9). Due to the putative role of the NMDA receptor complex in the pathophysiology of ischemia, the present investigation was aimed at elucidating the modulation of the NMDA receptor complex by σ ligands *in vivo* by examining the ability of BMY-14802 to modulate cGMP levels in the mouse cerebellum, a well characterized second messenger response mediated by the NMDA receptor complex (10–14).

Materials and Methods

Male Swiss-Webster mice (17–22 g) were used in all the studies described herein. Groups of mice were implanted, under sodium pentobarbital anesthesia, with polyethylene ICB and/or ICV cannulae and

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; CPP, 3-(2-carboxypiperizin-4-yl)propyl-1-phosphonic acid; PCP, phencyclidine; BMY-14802, [α-(4-fluorophenyl)-4-(5-fluoropyramidinyl)-1-piperazine butanol]; ICV, intracerebroventricular(ly); ICB, intracerebellar(ly); CGS-19755, (*cis*)-4-phosphonomethyl-2-piperidine carboxylic acid; HA-966, 1-hydroxy-3-aminopyrrolidone-2.

were used 3 days later. BMY-14802-1 (HCl salt) was dissolved in distilled water and the pH was adjusted to 6.5–7 before administration via the cannulae. D-Serine, an agonist at the NMDA-associated, strychnine-insensitive, glycine recognition site, increases cerebellar cGMP (10). To determine the abilities of BMY-14802 to modulate responses mediated via the glycine recognition sites, mice were injected ICB with D-serine 10 min after the ICV administration of BMY-14802, and the animals were sacrificed by focused microwave irradiation 10 min later. For direct injections into the cerebellum, BMY-14802 and D-serine were co-administered and the animals were sacrificed 10 min later. Groups of mice were also injected with D-serine (200 μg, ICB) 20 min after intraperitoneal injection of BMY-14802 (25 and 75 mg/kg) and were sacrificed 10 min later. For harmaline-reversal experiments, mice were administered BMY-14802 (intraperitoneally) 30 min before sacrifice and harmaline (100 mg/kg, subcutaneously) 10 min before sacrifice. Drug solutions were adjusted to pH 6.5–7.0 before local injections. All local injections (ICV and ICB) were made in a volume of 5 μl/mouse. Cerebella were extracted in 1 N-HCl and cGMP levels were quantified by a radioimmunoassay (NEN) (10, 11, 14).



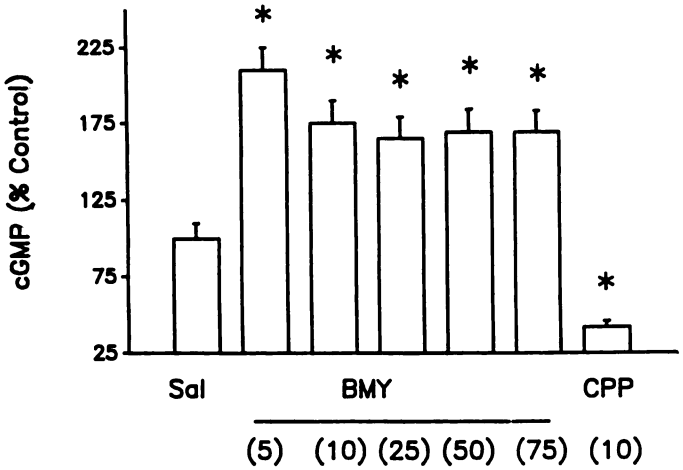


Fig. 1. Effects of BMY-14802 and CPP on basal and harmaline-induced cGMP levels. *Upper*, dose-dependent reversal of harmaline (*H*)-induced increases in cGMP by BMY-14802 (*BMY*) and CPP. cGMP levels in saline (*S*)-treated groups in these experiments [pmol/mg of protein, mean ± SE (number of mice)] were 2.5 ± 0.2 (7) and 3.1 ± 0.27 (8), respectively. *Lower*, effects of BMY-14802 (*BMY*) and CPP on basal cerebellar cGMP. cGMP levels in the saline (*Sal*)-treated group [pmol/mg of protein, mean ± SE (number of mice)] were 2.25 ± 0.17 (8). The values in parentheses represent doses (mg/kg, intraperitoneally) $^*p < 0.05$ versus control group (Dunnett's test); $^{**}p < 0.05$ versus harmaline group alone (Neuman-Keuls' test). For basal cGMP measurements, mice were injected with BMY-14802 or CPP (both intraperitoneally) 30 min before sacrifice. For the harmaline-reversal experiments, mice were injected with harmaline (100 mg/kg, subcutaneously) 20 min after the administration of BMY-14802 or CPP (both intraperitoneally) and were sacrificed 10 min later.

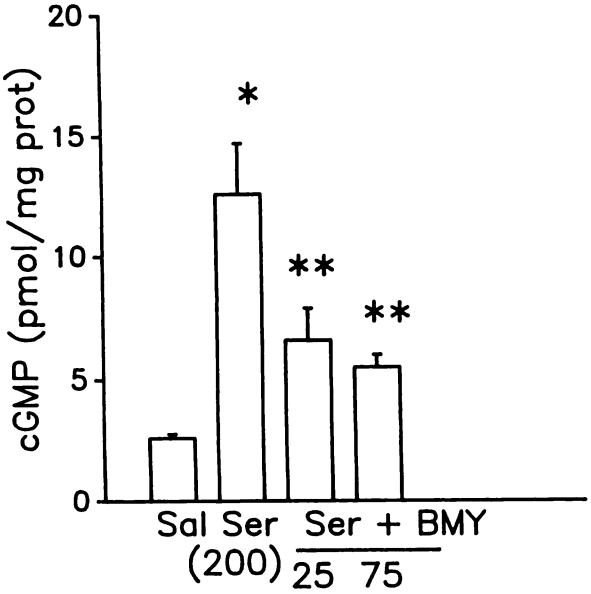


Fig. 2. Parenteral administration of BMY-14802 (*BMY*) reverses D-serine (*Ser*)-induced increases in mouse cerebellar cGMP. D-Serine (200 μg) was injected ICB 20 min after BMY-14802 (intraperitoneally, 25 and 75 mg/kg) and mice were sacrificed 10 min later. $^*p < 0.05$ versus saline (*Sal*) control group (Dunnett's test), $^{**}p < 0.05$ versus D-serine group (Neuman-Keuls' test).

BMY-14802 was a generous gift from Dr. Duncan P. Taylor (Bristol-Myers, Wallingford, CT). D-Serine was obtained from Aldrich; CPP and quisqualic acid (synthetic grade) were obtained from Research Biochemicals, Inc., and Cambridge Research Biochemicals, Inc., respectively.

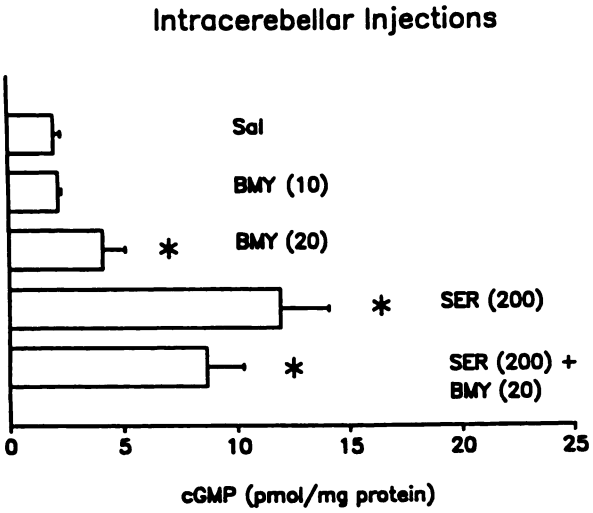
Data were analyzed by a one-way analysis of variance followed by either a Dunnett's or a Neuman-Keuls' test. Hill coefficients were calculated from the log-logit transformations of dose-response data (10, 15).

Results

BMY-14802 significantly increased basal cerebellar cGMP and this response was dose independent (Fig. 1, *lower*). However, BMY-14802 reversed the harmaline-induced increases in a dose-dependent manner, with an ED_{50} of 46.4 mg/kg, intraperitoneally, and a Hill coefficient of 1.42 (correlation coefficient, $r = 0.98$) (Fig. 1, *upper*). In contrast, CPP, a competitive NMDA antagonist, decreased basal levels and reversed harmaline-induced increases with an ED_{50} of 4.95 mg/kg and a Hill coefficient of 1.15 ($r = 0.98$) (Fig. 1, *upper*). However, statistical analysis (test for parallelism) revealed no significant difference between these slopes.

Due to the putative modulation of the NMDA receptor complex by glycine (1), the ability of BMY-14802 to attenuate ICB D-serine-induced cGMP levels was also measured. BMY-14802 (25 and 75 mg/kg, intraperitoneally) reversed the D-serine-induced increases in cGMP levels (Fig. 2).

In order to define the locus of action of BMY-14802-mediated increases in basal cGMP and to define BMY-14802-mediated reversal of D-serine responses, BMY-14802 was administered



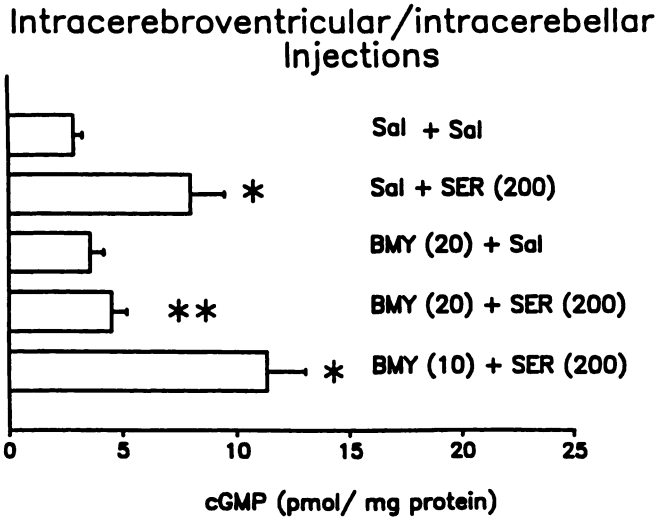


Fig. 3. Determination of locus of antagonism of D-serine response by BMY-14802. For ICB injections (*upper*), BMY-14802 (*BMY*) and D-serine (*SER*) were coinjected at doses (μg/mouse) indicated in parentheses, and the animals were sacrificed 10 min after injection. For ICB/ICV experiments (*lower*), mice received saline (*Sal*, ICV) plus saline (ICB), saline (ICV) plus D-serine (*SER*, ICB), BMY-14802 (*BMY*, ICV) plus saline (ICB), or BMY-14802 (ICV) plus D-serine (ICB). ICV and ICB treatments were, respectively, given 20 and 10 min before sacrifice. $^{*}p < 0.05$ versus control group (Dunnett's test); $^{**}p < 0.05$ versus saline plus D-serine group (Neuman-Keuls' test).

with or without D-serine given centrally. Direct ICB injections of BMY-14802 increased cGMP levels and co-administration of BMY-14802 (20 μg) and D-serine (200 μg) did not result in the antagonism of the D-serine-mediated response (Fig. 3, *upper*). However, the ICB D-serine response was significantly attenuated by ICV pretreatment with 20 μg, but not by 10 μg, of BMY-14802 (Fig. 3, *lower*). In contrast, CPP, at a dose that did not alter basal levels (0.5 μg), completely reversed the D-serine-induced increases in cGMP following direct ICB injection [cGMP levels (pmol/mg of protein; mean ± SE) were saline, 2.03 ± 0.3 ($n = 8$); D-serine (200 μg), 11.9 ± 2.2 ($n = 8$); and CPP (0.5 μg) plus D-serine (200 μg), 3.0 ± 0.3 ($n = 8$)].

In a separate experiment, BMY-14802 (50 mg/kg, intraperitoneally) did not reverse the quisqualate-induced increases in cGMP (Fig. 4).

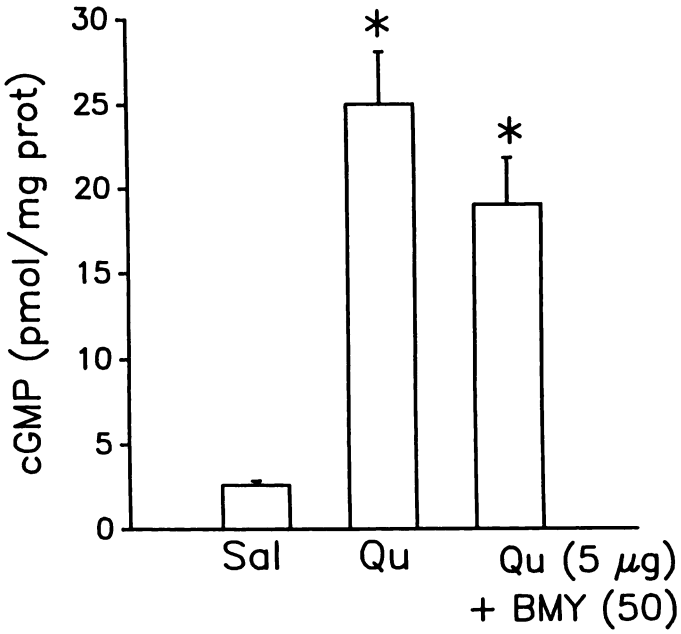


Fig. 4. BMY-14802 (*BMY*) does not reverse quisqualate (*Qu*)-induced increases in cGMP levels. Mice received BMY-14802 (50 mg/kg, intraperitoneally) 30 min and quisqualate (5 μg, ICB injection) 10 min before sacrifice. $^{*}p < 0.05$ versus control group (Dunnett's test).

Discussion

A number of pharmacological studies have indicated that cerebellar cGMP levels represent an index of ongoing neuronal activity mediated by the NMDA/PCP/glycine/ion channel complex (10, 12, 13). The ongoing neuronal activity can be enhanced by glutamate receptor agonists, such as NMDA, glutamate, kainate, and quisqualate (10, 11, 13), and by the NMDA receptor-associated glycine receptor agonists glycine and D-serine and it is decreased by PCP receptor agonists, such as PCP and dizocilipine (13). The ongoing neuronal activity can also be increased by harmaline, a specific activator of the climbing fibers, the activation of which results in the release of glutamate and/or aspartate, the endogenous transmitter in this pathway (12). The harmaline-induced increases in cGMP were shown to be mediated through the NMDA/PCP/glycine/ion channel complex by demonstration of the reversal by PCP agonists PCP and tiletamine, by competitive NMDA antagonists CPP and 2-amino-7-phosphonoheptanoate, and by the glycine antagonist HA-966 (10, 12–14). D-Serine, a glycine agonist acting through glycine recognition sites of the NMDA receptor complex, also increases cerebellar cGMP levels, as evidenced by the reversal of the D-serine-induced increases by both competitive and noncompetitive NMDA antagonists, by the glycine antagonist HA-966, and by a partial glycine agonist, D-cycloserine (10). We have earlier demonstrated that D-serine-induced increases do not involve the release of an endogenous ligand (10).

The antagonism by BMY-14802 of the harmaline and the D-serine responses suggests a functional modulation of the NMDA receptor complex by σ ligands. To our knowledge, this represents the first direct neurochemical evidence for a σ li-

gand-mediated functional modulation of an event mediated through the NMDA receptor complex *in vivo*.

The increases in basal cGMP levels following the administration of BMY-14802 indicate its ability to increase the ongoing neuronal activity through the NMDA receptor complex. In contrast, the competitive NMDA antagonist CPP attenuated both the basal activity and the harmaline response. However, the abilities of BMY-14802 to attenuate both the harmaline-induced and D-serine-induced increases in cGMP indicate that this novel σ ligand can attenuate the excessive neuronal activity mediated through various recognition sites of the NMDA/glycine/PCP/ion channel complex. In contrast, the lack of reversal of quisqualate-induced increases in cGMP by BMY-14802 indicates that this modulation is specific to NMDA-type receptors.

The increases in basal cGMP levels following both ICB and parenteral administration of BMY-14802 indicate a central locus of action for this response. Similarly, ICV BMY-14802 pretreatment significantly attenuated the ICB D-serine response, indicating that the site of action of such antagonism is still central but not peripheral. However, BMY-14802 (20 μg) did not antagonize the D-serine (200 μg) response following coinjection into the cerebellum, suggesting a possible locus of action outside this region. Higher doses of BMY-14802 could not be employed, due to the acidity of the resulting solutions, to unequivocally address this issue. These results suggest that the modulation of NMDA receptor-mediated events by BMY-14802 is functional, not localized at the receptor level, but with a locus of action outside the cerebellum. Such a transsynaptic modulation is exemplified in previous studies that revealed that haloperidol, a dopaminergic antagonist, decreases cerebellar cGMP levels by acting via the striatal efferents that regulate the mossy fiber input into the cerebellum. These conclusions are supported by a decrease in cGMP levels only after intrastriatal but not after ICB injection of haloperidol (16). BMY-14802 might possibly be modulating the NMDA receptor complex activity in the cerebellum by such a transsynaptic pathway. Such a mechanism is also consistent with pharmacological evidence favoring the dissociation of σ receptors from the NMDA/PCP receptors (17). However, it remains to be seen whether an endogenous substance might be mediating such a functional interaction.

Receptor binding studies have characterized BMY-14802 as having minimal, if any, interaction with PCP and dopamine receptors (8), thus supporting a σ receptor role in the functional modulation. The contribution of dopaminergic effects of BMY-14802 to its ability to modulate cGMP levels is minimal, if any, because dopamine receptor antagonism *in vivo* results in a decrease in basal levels of cGMP (18), not an increase as seen with BMY-14802. Furthermore, the active metabolite of BMY-14802 resulting from the oxidation of the alcohol moiety, BMY-14786, is devoid of dopaminergic receptor affinity (19). Several PCP ligands result in the production of 180° preservation in a four-arm radial maze. However, BMY-14802 is inactive in this *in vivo* assay (20). The inability to produce 180° preservation in a four-arm radial maze and the lack of attenuation of basal cGMP levels, properties characteristic of PCP agonists, rule out the possibility of a "PCP-like" metabolite being generated *in vivo*. Furthermore, the observed ED_{50} of 46 mg/kg for harmaline reversal in mice is well within the range of doses required to offer neuroprotection against the nitrogen anoxia-induced lethality in rats and against the NMDA-induced lethality in mice (25–100 mg/kg) (9) and is also within the ED_{50} doses required to elicit conditioned avoidance responding and to antagonize the apomorphine-induced climbing and sterotype (25, 35, and 60 mg/kg, respectively) (8), tests predictive of its antipsychotic potential, indicating a functional correlation for this response. These data together suggest the selective involvement of σ receptors in the functional modulation of NMDA receptor-mediated events by BMY-14802.

The actions of several competitive and noncompetitive NMDA antagonists to decrease the basal ongoing neuronal activity limit their potential therapeutic utility as novel antiischemic agents with minimal side effects (13). In contrast, the ability of BMY-14802 to attenuate increased neuronal activity but not the ongoing basal tone suggest that σ ligands such as BMY-14802 may offer better therapies for stroke, ischemia, and other neurodegenerative disorders.

In summary, the present investigation has demonstrated a functional modulation of an event mediated by the NMDA receptor complex *in vivo* by BMY-14802, possibly through a central transsynaptic mechanism.

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