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Enantiomers of HA-966 (3-amino-1-hydroxypyrrolid-2-one) exhibit distinct central nervous system effects: (+)-HA-966 is a selective glycine/*N*-methyl-D-aspartate receptor antagonist, but (−)-HA-966 is a potent γ-butyrolactone-like sedative

(glycine modulatory site/radioligand binding/electrophysiology/anticonvulsant)

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ABSTRACT **The antagonist effect of (±)-3-amino-1-hydroxypyrrolid-2-one (HA-966) at the *N*-methyl-D-aspartate (NMDA) receptor occurs through a selective interaction with the glycine modulatory site within the receptor complex. When the enantiomers of (±)-HA-966 were resolved, the (*R*)-(+)-enantiomer was found to be a selective glycine/NMDA receptor antagonist, a property that accounts for its anticonvulsant activity *in vivo*. In contrast, the (*S*)-(−)-enantiomer was only weakly active as an NMDA-receptor antagonist, but nevertheless it possessed a marked sedative and muscle relaxant action *in vivo*. In radioligand binding experiments, (+)-HA-966 inhibited strychnine-insensitive [^{3}H]glycine binding to rat cerebral cortex synaptic membranes with an IC_{50} of 12.5 μM, whereas (−)-HA-966 had an IC_{50} value of 339 μM. In electrophysiological experiments, (+)-HA-966 selectively antagonized NMDA receptor responses in rat cortical slices, whereas the (−)-enantiomer was much weaker. On cultured cortical neurones (+)-HA-966 inhibited glycine-potentiated NMDA responses with an IC_{50} = 13 μM compared with (−)-HA-966, which has an IC_{50} = 708 μM. In agreement with findings with racemic HA-966, even high concentrations of (+)-HA-966 did not completely inhibit NMDA responses, suggesting that (+)-HA-966 is a low-efficacy partial agonist. (+)-HA-966 produced parallel shifts to the right of the glycine concentration curve for potentiation of NMDA responses, resulting in an estimated pK_b = 5.6. In mice, (+)-HA-966 antagonized sound and *N*-methyl-DL-aspartic acid (NMDLA)-induced seizures with ED_{50} values of 52.6 mg/kg of body weight (i.p.) and 900 mg/kg (i.v.), respectively. The coadministration of D-serine dose-dependently (10–100 μg into the cerebral ventricles per mouse) antagonized the anticonvulsant effect of a submaximal dose of (+)-HA-966 (100 μg administered directly into the cerebral ventricles) against NMDLA-induced seizures. The sedative/ataxic effect of racemic HA-966 was mainly attributable to the (−)-enantiomer, which was >25-fold more potent than the (+)-enantiomer. It is suggested that, as in the case of the sedative γ-butyrolactone, disruption of striatal dopaminergic mechanisms may be responsible for this action.**

Receptors for excitatory amino acid neurotransmitters have been subdivided into three major populations on the basis of their selective recognition of *N*-methyl-D-aspartic (NMDA), kainic, and quisqualic acids, respectively. The pharmacology of the NMDA receptor is currently best understood, and antagonists with high affinity and selectivity for the NMDA recognition site or its associated ion channel have been described. In experimental animals, such agents have anticonvulsant, neuroprotective, and anxiolytic actions (1).

Electrophysiological studies have shown that in cultured cortical neurones, glycine is able to potentiate NMDA receptor-mediated responses via a strychnine-insensitive mechanism (2). Glycine is also able to enhance the specific binding of radioligands interacting with the ion channel of the NMDA receptor complex (3–5). Despite the high concentrations of glycine in body fluids, this glycine modulatory site may not be maximally stimulated *in vivo* (6, 7), and selective glycine receptor antagonists such as 7-chlorokynurenic acid are effective anticonvulsants and protect against NMDA receptor-induced excitotoxicity (7, 8).

HA-966 (3-amino-1-hydroxypyrrolid-2-one) has long been known as an NMDA-receptor antagonist (9, 10), an action that has been shown recently to occur through selective antagonism of the glycine modulatory site (11–14). However, unlike 7-chlorokynurenic acid, which may be a full antagonist (15), HA-966 appears to be a low-efficacy partial agonist at the glycine site (12–14). HA-966 has been shown previously to possess anticonvulsant, anti-tremor, and sedative actions in rodents and causes a marked elevation of dopamine concentration in the striatum (16, 22). Since it is a racemic compound, we recently have resolved HA-966 (18) and now present evidence that glycine/NMDA receptor antagonism and anticonvulsant activity resides in the (*R*)-(+)-enantiomer. In contrast, the (*S*)-(−)-enantiomer is much less active at this site but produces a marked sedative action and disrupts striatal dopamine metabolism in a manner analogous to γ-butyrolactone.

MATERIALS AND METHODS

Materials. Racemic HA-966 was synthesized by the method of Smrt *et al.* (17). The individual enantiomers (*R*)-(+)-HA-966 and (*S*)-(−)-HA-966 were prepared from D- and L-methionine, respectively, as described (18). *N*-Methyl-DL-aspartic acid (NMDLA) and D-serine were purchased from Sigma. All other drugs and reagents were obtained from commercial sources.

Animals. Male Sprague–Dawley rats (200–300 g), Swiss–Webster (18–20 g), and DBA/2 (21–23 days old; 7–9 g) mice were obtained from Bantin and Kingman (Hull, U.K.). An-

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Abbreviations: NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDLA, *N*-methyl-DL-aspartic acid; i.c.v., intracerebroventricularally.

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imals were housed in groups of five under a 12-hr light/dark cycle (lights on at 7:00 a.m.) with food and water ad libitum.

Radioligand Binding Studies. [^{3}H]Glycine binding experiments were performed with crude synaptic membranes from rat cerebral cortex as described (12). The radioligand concentration in the assay was 50 nM, and 1 mM unlabeled glycine was used to determine nonspecific binding.

Rat Cortical Slice Recordings. Excitatory amino acid-induced depolarizations of rat cortical tissue were recorded by using a greased-gap technique previously reported in detail (14) and based on that originally described by Harrison and Simmonds (19). Whole-cell patch-clamp recordings from rat cortical neurones in primary cell culture were performed as described (14, 15).

Anticonvulsant Studies. Swiss–Webster mice were injected s.c. with NMDLA (500 mg/kg of body weight) and observed for the following 30 min. Test drugs were administered i.v. via a tail vein or directly into the cerebral ventricles (7) 15 min before NMDLA. Animals not exhibiting tonic seizures during the following 30-min observation period were considered protected. The anticonvulsant activity against audiogenic seizures was examined following i.p. administration of test drugs in DBA/2 mice 15 min before exposure to sound (125 decibels, 14 kHz). The number of mice not showing a tonic seizure during the 30-s exposure to sound were considered protected. The ED_{50} dose (dose protecting 50% of mice) was calculated by probit analysis.

Rotarod Performance. The sedative/ataxic effects of HA-966 and its enantiomers were examined in Swiss–Webster and DBA/2 mice by using a rotarod apparatus. Groups of animals were trained to remain on a revolving rotarod (15 revolutions per min) for 2 min. The test compounds were administered i.v. in Swiss–Webster and i.p. in DBA/2 mice. The latency to fall off the rotarod was determined 15 min after treatment. Animals not falling within 2 min were given a maximum score of 120 s.

Measurement of Striatal Dopamine Content. Thirty minutes after i.v. administration of vehicle (10 ml/kg of body weight), racemic HA-966 (50 mg/kg), (+)-HA-966 (30 mg/kg), or (−)-HA-966 (30 mg/kg), mice were killed by stunning and decapitation, and the brains were removed. A 2-mm slice of tissue was taken at the level of the olfactory tubercules, and a sample of the striatum was removed by using a steel punch. Tissue samples were immediately frozen on dry ice and then kept at −70°C until required for analysis of dopamine concentration by high-performance liquid chromatography as described (20).

RESULTS

[^{3}H]Glycine Binding. HA-966 and its enantiomers were tested as inhibitors of the strychnine-insensitive binding of [^{3}H]glycine to crude synaptic membranes (P_2 fraction) from rat cerebral cortex. The activity resided principally in the (+)-enantiomer, IC_{50} values [geometric mean (−SEM, + SEM; n = 3–5)] being (+)-HA-966 = 12.5 (10.2, 15.4) μM, (−)-HA-966 = 339 (282, 406) μM, and (±)-HA-966 = 27.2 (23.9, 30.5) μM.

Electrophysiological Studies. On rat cortical slices, (+)-HA-966 selectively antagonized depolarizing responses to NMDA. At a concentration of 100 μM, (+)-HA-966 produced a 3.0 ± 0.2 (mean ± SEM; n = 5)-fold shift to the right of the NMDA concentration–response curve (Fig. 1) with little effect on quisqualate receptor-activated responses (concentration–response ratio = 1.08 ± 0.15; n = 5). The NMDA receptor antagonist effects of (+)-HA-966 were almost fully reversed by addition of D-serine (100 μM). At the same concentration, (−)-HA-966 had much less effect, producing only a 1.2 ± 0.01 (n = 3)-fold shift to the right of the NMDA concentration–response curve. When examined at 300 μM,

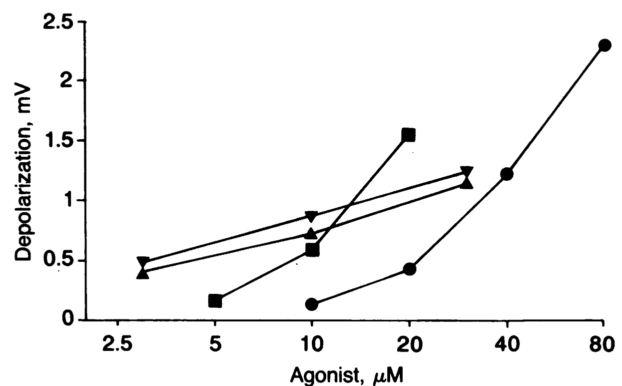

FIG. 1. Antagonist effect of (+)-HA-966 on NMDA but not quisqualate-induced depolarizations of a rat cortical slice. Concentration–response curves were constructed for NMDA and quisqualate in the absence (■ and ▲, respectively) and presence (● and ▼, respectively) of (+)-HA-966 at 100 μM. HA-966 produced a parallel shift to the right of the NMDA concentration–response curve with a corresponding logarithmic concentration ratio of 0.40 (calculated at the 1.3-mV level). There was no antagonism of the quisqualate responses by (+)-HA-966, which in this case were slightly potentiated.

(−)-HA-966 produced a slightly greater depression of NMDA responses, but there was also a concomitant reduction in responses evoked by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA): the concentration-ratios produced by (−)-HA-966 against NMDA and AMPA were 1.5 ± 0.09 (n = 3) and 1.8 ± 0.11 (n = 3), respectively. Following a washout period of 1 hr, there was no evidence of recovery, and the effect probably reflected deterioration of the slices.

The NMDA antagonist effects of (+)-HA-966 are in agreement with those previously reported (14) for the racemic mixture, which produced a 1.6-fold shift at a concentration of 100 μM and a 3-fold shift at a concentration of 250 μM. To obtain a direct comparison of the glycine antagonist potency of the two enantiomers of HA-966, they were examined for their ability to block the glycine potentiation of NMDA responses on rat cortical neurones in tissue culture. (+)-HA-966 produced a concentration-dependent inhibition of glycine (0.3 μM)-potentiated NMDA (30 μM) currents in

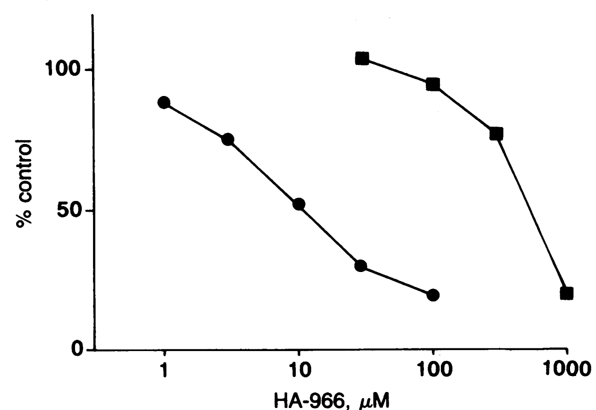

FIG. 2. Comparison of the glycine antagonist properties of the (+)- and (−)-enantiomers of HA-966. Membrane currents were recorded from cultured neurones by using the whole-cell variant of the patch-clamp technique (holding potential = −60 mV). The figure shows the effects of increasing concentrations of (+)-HA-966 (●) and (−)-HA-966 (■) on membrane currents evoked in a single rat cultured cortical neurone by 5-s applications of NMDA (30 μM)/glycine (300 nM). Comparison of IC_{50} values revealed that (+)-HA-966 was >50-fold more potent than the (−)-enantiomer. Each point is expressed as a percentage of control NMDA/glycine responses obtained in the absence of antagonists.

voltage-clamped neurones (Fig. 2) with an IC_{50} [geometric mean (−SEM, +SEM; n = 3)] of 13 (12, 15) μM. The IC_{50} of the (−)-enantiomer was 708 (617, 813) μM (n = 3), making it weaker by a factor of 54 than (+)-HA-966 (Fig. 2). Like the racemic mixture of HA-966, the (+)-enantiomer never produced a complete block of the NMDA-induced current, even at high concentrations (3 mM), indicating that (+)-HA-966 is probably a low-efficacy partial agonist at the glycine modulatory site (14).

The potency of (+)-HA-966 as a glycine antagonist was also determined by measuring its ability to shift to the right the glycine concentration–response curve for potentiation of NMDA responses. Partial glycine concentration–response curves, over the linear part of the full glycine concentration–response curve, were constructed and then repeated in the presence of 100 μM (+)-HA-966 (Fig. 3). This resulted in a 48 ± 11 (n = 4)-fold shift to the right of the glycine concentration–response curve, giving an estimated pK_b value for (+)-HA-966 of 5.6 ± 0.08.

Seizures Induced by NMDLA. Seizures induced in mice by NMDLA (500 mg/kg, s.c.) were dose-dependently antagonized by racemic HA-966, although high doses of the compound were required (ED_{50} = 1820.9 mg/kg, i.v.; Fig. 4 *Upper*). This anticonvulsant activity clearly resided in the (+)-enantiomer of the compound because (*i*) (+)-HA-966 was about twice as potent (ED_{50} = 892.7 mg/kg) as racemic HA-966, and (*ii*) the (−)-enantiomer, even at a dose of 2000 mg/kg, was completely without effect (Fig. 4 *Upper*). When given directly into the cerebral ventricles, (+)-HA-966 (10–200 μg per mouse) again dose-dependently antagonized NMDLA-induced seizures (Fig. 5 *Left*). To investigate the involvement of the glycine/NMDA receptor in the anticonvulsant action of (+)-HA-966, the glycine receptor agonist, D-serine (10–100 μg) was administered intracerebroventricularally (i.c.v.) concomitantly with a submaximal dose of (+)-HA-966 (100 μg per mouse): the increase in latency to convulse induced by (+)-HA-966 was dose-dependently antagonized by D-serine (Fig. 5 *Right*).

Audiogenic Seizures. (±)-HA-966 was considerably more potent at preventing audiogenic seizures in DBA/2 mice (Fig. 4 *Lower*; ED_{50} = 9.6 mg/kg, i.p.) than seizures induced by NMDLA. However, (−)-HA-966 was the more potent enantiomer against audiogenic seizures with an ED_{50} of 4.7 mg/kg (i.p.) compared with a value of 52.6 mg/kg (i.p.) for the (+)-enantiomer (Fig. 4 *Lower*).

Rotarod Performance. (±)-HA-966 impaired the performance of both Swiss–Webster and DBA/2 mice on the rotarod at doses close to those antagonizing sound-induced seizures (Fig. 6). (−)-HA-966 was again the most potent enantiomer, with animals falling from the rotarod at doses as low as 5 mg/kg compared with a minimum effective dose of 250 mg/kg for the (+)-enantiomer (Fig. 6).

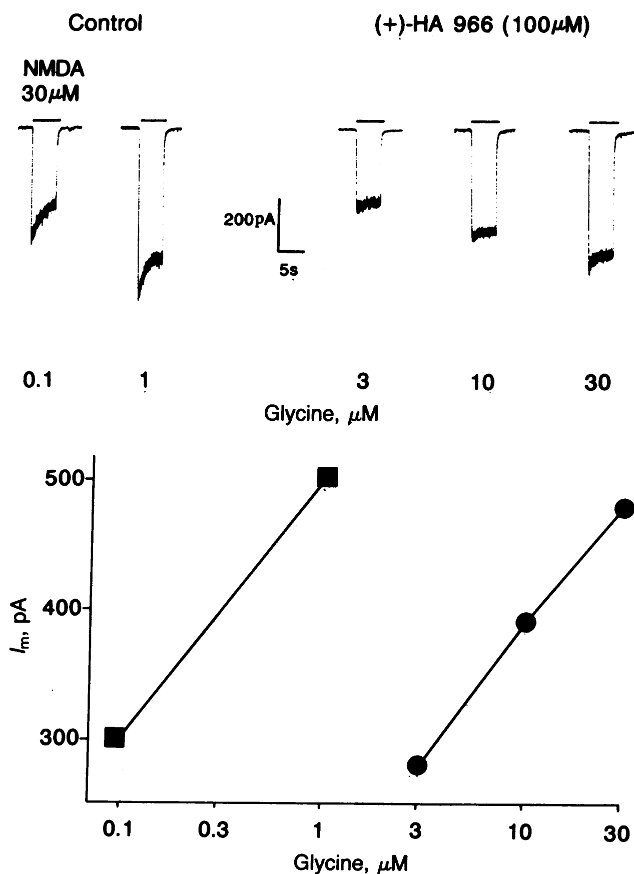

FIG. 3. Competitive antagonist effect of (+)-HA-966 on the potentiation of NMDA responses by glycine. (*Upper*) Whole-cell currents recorded from a single patch-clamped (holding potential = −60 mV) rat cortical neurone showing the concentration-related potentiating effect of glycine and its antagonism by (+)-HA-966 (100 μM). Responses were evoked by 5-s applications of 30 μM NMDA separated by 20-s intervals. The trace has been broken for clarity. (*Lower*) Graphical representation of responses shown in A. Membrane current (I_m) is plotted as a function of glycine concentration.

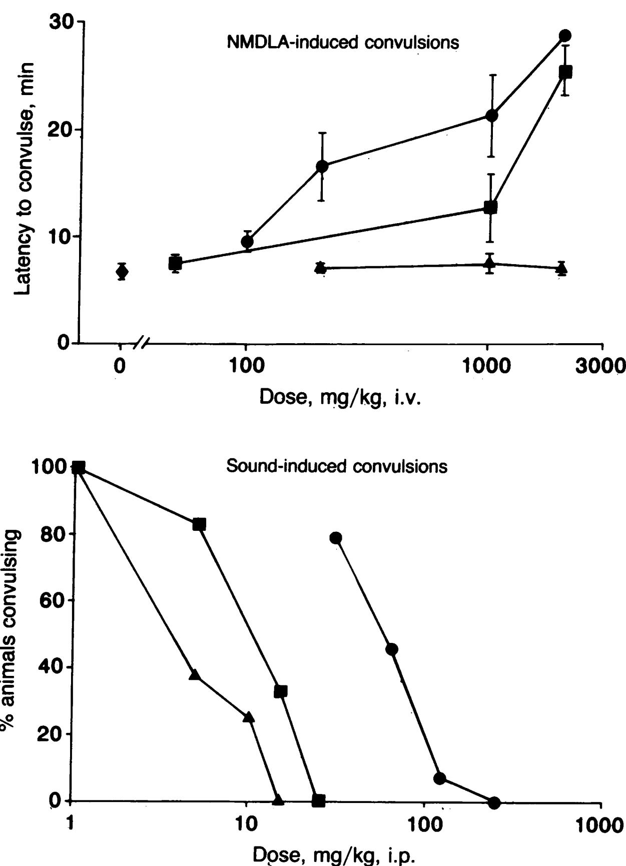

FIG. 4. Comparison of the anticonvulsant effects of the enantiomers of HA-966 on NMDLA-induced seizures in Swiss–Webster mice (*Upper*) and audiogenic seizures in DBA/2 mice (*Lower*). At least four doses of each compound were examined in groups of eight mice. The test compounds were given i.v. 15 min before the s.c. administration of NMDLA (500 mg/kg) or i.p. 15 min before exposure to sound (125 decibels, 14 kHz) for 30 s. Mice not exhibiting a tonic seizure within 30 min of injection of NMDLA or during the 30-s exposure to sound were considered protected. Results are shown as the mean seizure latencies ± SEM or as the percentage of animals protected. ■, (±)-HA-966; ●, (+)-HA-966; ▲, (−)-HA-966.

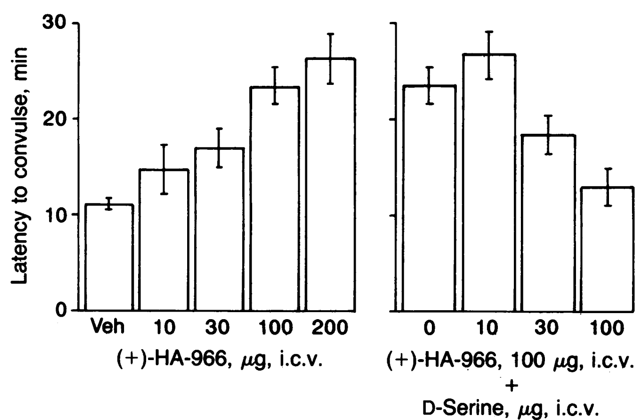

FIG. 5. The anticonvulsant activity of (+)-HA-966 in Swiss-Webster mice against NMDLA-induced seizures following direct administration into the cerebral ventricles (*Left*) and antagonism of this protective effect by D-serine (*Right*). In *Left*, (+)-HA-966 was given i.c.v., and in *Right* D-serine was coadministered i.c.v. with (+)-HA-966 (100 μg per mouse) 15 min before NMDLA (500 mg/kg, s.c.), and the latency to the tonic extension of the forepaws was noted. For further details see the legend to Fig. 4. Results are shown as the mean seizure latencies ± SEM of at least eight mice per group. Veh, vehicle.

Measurement of Striatal Dopamine and 5-Hydroxytryptamine (Serotonin) Concentration. One previously reported effect of racemic HA-966 administration is an elevation of the concentration of dopamine and its metabolites in brain (16, 22). Like the sedative/ataxic effects, this property was found to reside in the (−)-enantiomer. Thus, a dose of 30 mg/kg (−)-HA-966 gave a similar elevation of striatal dopamine concentration as 50 mg/kg of the racemate (155% compared with 144% of control, respectively) while (+)-HA-966 at 30 mg/kg was without effect (Table 1). Striatal 5-hydroxytryptamine concentration was not significantly affected by racemic HA-966 or its (+)- or (−)-enantiomers (Table 1).

DISCUSSION

The radioligand binding and electrophysiological results presented here indicate that HA-966 interacts stereoselectively with the glycine modulatory site on the NMDA receptor complex, (+)-HA-966 being 30- to 50-fold more active than the (−)-enantiomer. Previous studies have shown that racemic HA-966 is virtually inactive as an inhibitor of radioligand binding to NMDA, quisqualate, or kainate recognition sites or of [^{3}H]strychnine binding to the glycine inhibitory receptor (14). In the present experiments, activity in the [^{3}H]glycine binding assay was found to reside principally in the (+)-enantiomer of HA-966, which was 30-fold more potent than the (−)-enantiomer. Furthermore, the electrophysiological studies show that the ability to antagonize NMDA responses

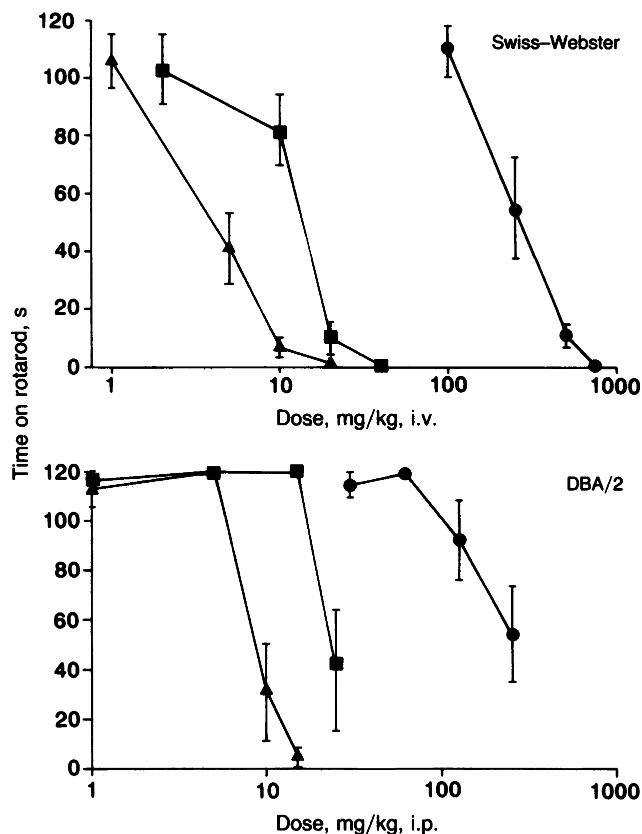

FIG. 6. Comparison of the effects of the enantiomers of HA-966 on rotarod performance in Swiss-Webster mice (*Upper*) and DBA/2 mice (*Lower*). Mice were trained to remain on a revolving rotarod for 2 min. Four doses of each enantiomer of HA-966 were administered i.v. in Swiss-Webster mice or i.p. in DBA/2 mice 15 min before determining the latency to fall off the rotarod. Results are shown as the mean latency ± SEM of at least eight mice per group. ■, (±)-HA-966; ●, (+)-HA-966; ▲, (−)-HA-966.

was also a property of the (+)-enantiomer in both cortical slices and cortical neurones in culture and that this antagonism was reversed by glycine and by the glycine receptor agonist, D-serine. The absolute stereochemistry of (+)-HA-966 was shown to be *R* (18), in common with other ligands (e.g., serine and cycloserine) for the glycine site.

Previously it has been reported that racemic HA-966 is not able to fully antagonize responses to NMDA in the rat cortical slice preparation (13, 14). In the present study, the more potent (+)-enantiomer, even at high concentrations, also failed to antagonize completely NMDA-induced responses. These findings differ from those reported for the selective glycine receptor antagonist 7-chlorokynurenic acid,

Table 1. Effects of the enantiomers of HA-966 on mouse striatal dopamine and 5-hydroxytryptamine (5-HT) concentration

Treatment	Dose, mg/kg, i.v.	Striatal concentration, μg/g (% control)	
		Dopamine	5-HT
Experiment 1			
Vehicle		8.7 ± 0.36	0.53 ± 0.04
(±)-HA-966	50.0	12.5 ± 0.25* (144%)	0.59 ± 0.03 (112%)
Experiment 2			
Vehicle		10.3 ± 0.54	0.81 ± 0.07
(+)-HA-966	30.0	8.9 ± 0.31 (86%)	0.75 ± 0.05 (92%)
(−)-HA-966	30.0	16.0 ± 0.78* (155%)	0.96 ± 0.07 (119%)

Values are represented as mean ± SEM of seven or eight mice per group.
*$P < 0.05$ compared with appropriate vehicle-treated group (analysis of variance followed by Tukey test).

which has been shown to completely antagonize the basal and glycine-induced potentiation of NMDA responses in the same preparation (15). Since agonist occupation of the glycine modulatory site is an absolute requirement for activation of the NMDA receptor, at least in *Xenopus* oocytes (21), 7-chlorokynurenic acid is more likely to be a full antagonist than an inverse agonist of the glycine modulatory site. Accordingly, (+)-HA-966 therefore should be considered a low-efficacy partial agonist rather than a full antagonist. When administered intravenously (+)-HA-966 dose-dependently antagonized seizures induced in the mouse by NMDLA, although the doses required were high (ED_{50} = 893 mg/kg). Consistent with an involvement of the glycine modulatory receptor, the anticonvulsant action of centrally administered (+)-HA-966, like that of kynurenic acid (7), was dose-dependently reversed by coadministration of D-serine. In contrast to the blockade of NMDLA-induced seizures, those induced by sound in DBA/2 mice were most potently antagonized by (−)-HA-966, which was 2-fold more potent than the racemate and 11-fold more active than the (+)-enantiomer. However, at anticonvulsant doses the (−)-enantiomer also caused marked sedation/ataxia, and if sensory processes were also impaired, such behavioral depression might falsely indicate an anticonvulsant effect. This is supported by the lack of activity of (−)-HA-966 for antagonism of NMDLA-induced seizures, even at doses 200-fold higher than those active in the DBA/2 mouse. In contrast, (+)-HA-966 produced sedation/ataxia only at doses 5-fold higher than those required to block audiogenic seizures.

Previous work has shown that racemic HA-966 causes sedation/ataxia in animals (16) and causes an accumulation of dopamine in the striatum (16, 22). The present results indicate that much of the behavioral depression is attributable to the (−)-enantiomer, (+)-HA-966 being about 1/50th as potent at impairing rotarod performance. Similarly, the (−)-enantiomer appears to be responsible for the effects on striatal dopamine content. Interestingly, the structurally related compounds γ-hydroxybutyrate and γ-butyrolactone also induce sedation and inhibit dopamine release from striatal neurones (17, 23, 24). The accumulation of dopamine and the sedative effects induced by (−)-HA-966 are thus probably due to a selective interruption of the flow of nerve impulses in the dopaminergic nigrostriatal tract. The exact mechanism by which such compounds exert their effects on dopamine neurones is not yet understood, but in view of the inactivity of (+)-HA-966, the glycine/NMDA receptor complex is unlikely to be involved.

In conclusion, the antagonist effect of racemic HA-966 at the NMDA receptor is due to the (+)-enantiomer, whereas the (−)-isomer is responsible for the potent sedative and ataxic action. The (+)-enantiomer is a systemically active anticonvulsant that selectively acts through the glycine modulatory site on the NMDA receptor and therefore may prove to be a useful pharmacological tool for the investigation of this site *in vivo*. Racemic HA-966 also has been reported to possess neuroprotective actions (8), and this potential for the (+)-enantiomer needs assessment. It remains to be established whether (+)-HA-966 has advantages over other NMDA receptor antagonists, such as dizocilpine (MK-801), that cause motor impairment and induce phencyclidine-like behaviors in animals at doses similar to those required for anticonvulsant and neuroprotective effects (25–28).

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