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Nonpsychotropic cannabinoid acts as a functional *N*-methyl-D-aspartate receptor blocker

(neurotoxicity/cannabinoid/seizure/glutamate receptors)

JEFFERY J. FEIGENBAUM*, FELIX BERGMANN*, SAUL A. RICHMOND*, RAPHAEL MECHOULAM*, VARDA NADLER†, YOEL KLOOG†, AND MORDECHAI SOKOLOVSKY†‡

*The Brettler Medical Research Center, Faculty of Medicine, Hebrew University, Jerusalem 91120, Israel; and †Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel

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ABSTRACT **Binding studies using the enantiomers of the synthetic cannabinoid 7-hydroxy-Δ^6-tetrahydrocannabinol 1,1-dimethylheptyl homolog in preparations of rat brain cortical membranes reveal that the (+)-(3*S*,4*S*) enantiomer HU-211 blocks *N*-methyl-D-aspartate (NMDA) receptors in a stereospecific manner and that the interaction occurs at binding sites distinct from those of other noncompetitive NMDA antagonists or of glutamate and glycine. Moreover, HU-211 induces stereotypy and locomotor hyperactivity in mice and tachycardia in rat, effects typically caused by NMDA receptor antagonists. HU-211 is also a potent blocker of NMDA-induced tremor, seizures, and lethality in mice. This compound may therefore prove useful as a nonpsychoactive drug that protects against NMDA-receptor-mediated neurotoxicity.**

The enantiomers of the synthetic cannabinoid 7-hydroxy-Δ^6-tetrahydrocannabinol 1,1-dimethylheptyl homolog have recently been described (1, 2). The (−)-(3*R*,4*R*) enantiomer, code-named HU-210 (Fig. 1), is a highly potent cannabimimetic compound (≈80 times more active than δ-1-tetrahydrocannabinol, the active component of hashish); the (+)-(3*S*,4*S*) enantiomer (code-named HU-211) (Fig. 1) is inactive as a cannabimimetic, even at doses several thousand times higher than the ED_{50} of HU-210 as assayed in a number of tests (2). In characterizing the drug profile of the nonpsychotropic (+) enantiomer HU-211, we noted a striking similarity between its pharmacological, autonomic, and behavioral effects and those of noncompetitive *N*-methyl-D-aspartate (NMDA) antagonists over a wide range of activities (including stereotypy, locomotor hyperactivity, and tachycardia). This similarity suggested that HU-211 might be functionally active as an NMDA-receptor antagonist. To explore the action of HU-211 and its interaction with specific receptors in the brain, we conducted both pharmacological experiments and binding studies.

MATERIALS AND METHODS

^{3}H-labeled *N*-[1-(2-thienyl)-cyclohexyl]piperidine ([^{3}H]TCP) (40 Ci/mmol; 1 Ci = 37 GBq; >98% pure) was purchased from the Israel Nuclear Center (Negev, Israel). L-Glutamate and glycine were from Sigma; D-(−)-2-amino-5-phosphonovaleric acid (AP-5) and NMDA were from Cambridge Research Biochemicals (Harston, U.K.). HU-211 and HU-210 were synthesized as described (1). Both enantiomers melt at 140–141°C and have identical IR and NMR spectra. HU-211 has a rotation of $[\alpha]_D = +227°$ in $CHCl_3$; HU-210 has a rotation of $[\alpha]_D = -226°$ in $CHCl_3$. HU-211 was dissolved in ethanol/Emulphur 620 (GAF, Tel-Aviv); double-distilled water was added. The ratio (by volume) in the final solution was 1:1:18.

HU−211 HU−210

FIG. 1. Chemical structures of HU-210 and HU-211.

Stereotypy was measured according to the stereotypy-rating scale of Feigenbaum *et al.* (3). This 11-point scale ranges from 0.0 to 5.0 (in half-number steps). It takes into account stereotyped behavior such as grooming, rearing, scratching, licking, tail-biting, etc. The highest rating, 5.0, indicates constant licking and biting, with the animals not distractable by noise. All animals were habituated for 45 min before HU-211 (or vehicle) administration and tested 60 min after injection. Locomotor hyperactivity was measured as body displacement over 7-cm squares, movement from one square to the next constituting a score of 1. This movement was independently assessed by three trained observers.

Heart rate measured over 75 min was determined by the standard transducer-amplifier-recorder technique, and respiratory frequency was visually observed by three trained independent observers with a very high meter-rater correlation. Tremor and seizure were monitored on a platform mounted on four spring-coils. Any vibration of the platform was transduced into an electrical input to an amplifer and then to an oscilloscope and recorder. The onset, nature, and duration of the tremor and seizure were also visually observed by three independent observers for 1 hr continuously and 6 hr intermittently (HU-211-treated animals only).

Six mice or rats were used in each of the above experiments (including controls).

In experiments designed to explore the anti-NMDA effects of HU-211, the latter was injected 75 min before NMDA administration. All injections were administered s.c. in volumes of 10 cc/kg of body weight. Sabra mice were injected with NMDA at 200 mg/kg and C57BL mice with NMDA at 100 mg/kg, as the higher dose killed these animals shortly after administration.

Student's *t* test was used for statistical analysis of locomotor activity and tachycardia. Because the stereotypy gra-

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Abbreviations: NMDA, *N*-methyl-D-aspartate; [^{3}H]TCP, ^{3}H-labeled *N*-[1-(2-thienyl)-cyclohexyl]piperidine; AP-5, D-(−)-2-amino-5-phosphonovaleric acid; MK-801, [(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate].

‡To whom reprint requests should be addressed.

dient consists of discrete noncontinuous scores, a nonparametric test (the Mann Whitney U) was used.

Binding of [^{3}H]TCP to well-washed membranes (4) was determined under the following conditions: membranes (80 μg of protein) were incubated at 25°C in 200 μl of 20 mM Hepes buffer (pH 7.4) containing 5 nM [^{3}H]TCP (total binding) or 5 nM [^{3}H]TCP and 100 μM phencyclidine (nonspecific binding); the reactions were terminated at the indicated times by rapid filtration over polyethyleneimine-treated GF/C filters (4, 5). The filters were counted in scintillation liquid (Hydroluma; Lumac, Schaesberg, The Netherlands).

Dissociation of [^{3}H]TCP–receptor complexes were measured by the isotopic dilution technique; unlabeled phenylcyclidine (100 μM) was added to the preformed complexes (18 nM [^{3}H]TCP, 24 hr, 25°C), and the reaction was terminated at t_0 or at the times indicated in the figure. Basal dissociation reactions were initiated by the addition of 100 μM unlabeled phencyclidine with or without 10 μM HU-211. For the induced dissociation reactions 1 μM glutamate and 1 μM glycine were also added.

RESULTS AND DISCUSSION

HU-211 induces stereotypy and locomotor hyperactivity in mice (25 mg/kg; s.c.) and mild tachycardia in rats (2.5 mg/kg; s.c.) (Tables 1 and 2). These properties are consistent with those of noncompetitive antagonists of the NMDA subclass of glutamate receptors (6–8), thus suggesting that HU-211 might function as an NMDA-receptor antagonist. This possibility was explored by examining the activity of HU-211 in protecting against the tremorogenic, convulsive, and lethal effects of NMDA in mice. Such effects are counteracted by virtually all NMDA antagonists (9).

Sabra mice (a local heterogeneous strain) were pretreated with either vehicle alone (control) or HU-211 (1.25 or 2.50 mg/kg; s.c.), followed 75 min later by NMDA (200 mg/kg; s.c.). As shown in Table 3, control and HU-211-pretreated animals were significantly different in the latency from NMDA injection to first tremor and first seizure and in duration of survival. Similar experiments were done with C57BL mice, in which the NMDA dose was decreased (100 mg/kg; s.c.). Counteraction by HU-211 of the NMDA-induced effects was more pronounced in the latter strain (which is more sensitive than Sabra mice to NMDA effects). In the control series of both strains, which received NMDA without HU-211, all animals died <10 min after seizure onset. In contrast, two of the five Sabra mice and six of the seven C57BL mice pretreated with HU-211 exhibited no tremors or seizures and stayed alive for >4 days after NMDA administration (Table 3). Preliminary results indicate that the anti-NMDA effects of HU-211 in C57BL mice persisted for >24 hr. This was demonstrated by absence of tremor and seizure and the continuing survival seen after readministration of NMDA (100 mg/kg) 1 day later to the six surviving C57BL mice. Further experiments on the activity of NMDA were conducted on C57BL mice with the enantiomer of HU-211—namely, HU-210—by using doses of HU-210 ranging from 0.00025 mg/kg to 1.6 mg/kg (six animals at the highest dose). Although sedation was seen for all doses of HU-210 from 0.025 mg/kg upward (ranging from mild to very severe), we observed no significant effect by any HU-210 dose on the actions (tremor, seizure, and death) of NMDA. We also administered HU-211 (2.5 mg/kg) with HU-210 (0.0032 mg/kg) to C57BL mice followed by NMDA (100 mg/kg). The results were similar to those seen with HU-211 alone. These results eliminate the possibility that the activities induced by HU-211 are due to its contamination with HU-210.

Table 2. Effect of HU-211 on induction of tachycardia in Sabra rats

	Heart rate, beats per min	
	0 min	75 min
Vehicle	170 ± 2.0	171 ± 1.3
HU-211	169 ± 2.5	186 ± 2.5*

HU-211 dose was 2.5 mg/kg injected s.c. Values are means ± SEM.
*$P < 0.001$.

Because both competitive and noncompetitive NMDA antagonists characteristically displayed anticonvulsant activity, we also examined the effect of HU-211 on convulsions induced by picrotoxin in C57BL mice. Animals were pretreated (s.c.) with either vehicle (controls, $n = 7$) or HU-211 (1.0–2.5 mg/kg; $n = 8$), 1.25 hr before picrotoxin administration (12.5 mg/kg, s.c.). Pretreatment with HU-211 resulted in a 2-fold increase in seizure latency (9.0 ± 0.5 min as compared with 4.8 ± 0.2 min in controls), a 4-fold decrease in the average duration of convulsions per animal (1.1 ± 0.2 min as compared with 4.0 ± 1.1 in controls), and a 2-fold increase in the interval from picrotoxin injection to death, (28.3 ± 2.6 min as compared with 13.1 ± 1.3 min in controls). Anticonvulsants such as pentobarbital (40 mg/kg) and diazepam (50 mg/kg) did not prevent the convulsions or death caused by NMDA (100 mg/kg). Taken together, these data provide evidence that HU-211 is, indeed, an NMDA antagonist; moreover, because convulsion is a centrally mediated phenomenon, the drug appears to exhibit a high degree of central penetration.

To identify the site of action of HU-211, we conducted a series of binding assays using well-washed rat brain cortical membranes (4, 5, 10) and a potent noncompetitive blocker of the NMDA receptor, [^{3}H]TCP (4, 11–13). In initial experiments with 100 μM HU-211 the equilibrium binding of 5 nM [^{3}H]TCP was not inhibited, suggesting that the behavioral effects of HU-211 are not exerted through the noncompetitive blocker site at the NMDA-receptor ion channel. Equilibrium binding experiments with 2–100 nM [^{3}H]TCP in the presence of 1 μM glutamate and 1 μM glycine confirmed this suggestion: 10 μM HU-211 did not alter the maximum binding

Table 1. Effects of HU-211 on induction stereotypy and locomotor activity in Sabra mice

	Time*, min				
	0 (0–15)	15 (15–30)	30 (30–45)	45 (45–60)	60 (60–75)
			Stereotypy gradient		
Vehicle	0.6 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	0.3 ± 0.2	0.5 ± 0.3
HU-211	0.4 ± 0.1	1.3 ± 0.2†	2.2 ± 0.1‡	0.7 ± 0.2	0.6 ± 0.3
			Locomotor activity		
Vehicle	21.0 ± 0.6	20.0 ± 6.0	17.0 ± 10.0	6.0 ± 4.0	5.0 ± 3.0
HU-211	32.0 ± 6.0	45.0 ± 2.0‡	37.0 ± 4.0‡	28.0 ± 13.0†	17.0 ± 7.0

HU-211 dose was 25 mg/ml injected s.c. Values are means ± SEM.
*Time durations for locomotor activity are expressed in parentheses.
†$P < 0.01$; ‡$P < 0.001$.

Table 3. Effects of NMDA: Inhibition by HU-211

Mouse strain	HU-211 dose, mg/kg	*N*	Time from NMDA injection to first event, min	*P*
		Tremorogenic effect		
Sabra	0	6	2.6 ± 0.4	
	2.5	5	8.2 ± 1.3*	<0.001
C57BL	0	6	3.4 ± 0.4	
	1.25	5	12.0 ± 2.6*	<0.001
	2.50	7	—†	
		Convulsive effect		
Sabra	0	6	5.2 ± 1.6	
	2.5	5	18.0 ± 7.0*	<0.001
C57BL	0	6	6.5 ± 0.6	
	1.25	5	13.2 ± 2.6	<0.001
	2.50	7	—†	
		Lethal effect		
Sabra	0	6	9.3 ± 1.4	
	2.5	5	20.2 ± 7.0*	<0.001
C57BL	0	6	7.7 ± 0.8	
	1.25	5	18.6 ± 4.2	<0.001
	2.50	7	—†	

NMDA doses were 200 mg/kg in the Sabra mouse strain and 100 mg/kg in the C57BL strain. Values are means ± SEM.

*Two of five animals pretreated with HU-211 did not exhibit tremor or seizure and stayed alive for >4 days; the number indicated in the table relates only to the remaining three animals that exhibited tremor, convulsed, and died.

†Six of seven animals stayed alive for >4 days and did not exhibit tremor or seizure. Readministration of NMDA (100 mg/kg) to these animals 24 hr after the first administration failed to induce tremor, seizure, or death, and they lived for at least 4 more days.

capacity of [^{3}H]TCP (values recorded were 3.4 and 3.5 pmol/mg of protein for controls and HU-211, respectively) and had no effect on the dissociation constant (K_d) for [^{3}H]TCP (27 nM for control and 26 nM for HU-211).

Kinetic experiments were then done to determine whether HU-211 exerts its behavioral effects by acting directly at the glutamate- (as reviewed in refs. 9 and 14) or the glycine- (13, 15–17) binding sites of the NMDA receptor. The use of this approach in previous studies, aimed at demonstrating the noncompetitive nature of [^{3}H]TCP and [^{3}H]MK-801 binding to the NMDA receptor (4, 5, 18), showed that these compounds preferentially bind to the activated state of the receptor ion channel (19–21) and that glutamate and glycine accelerate the association rates of noncompetitive blockers to the receptor and their dissociation from it without altering their equilibrium binding (4, 5). Results of the kinetic experiments with HU-211 are summarized in Fig. 2. As shown, addition of 10 μM HU-211 resulted in only a small decrease in the association rate of [^{3}H]TCP binding to the NMDA receptor without altering the level of its equilibrium binding; in the presence of 1 μM glutamate and 1 μM glycine, however, addition of 10 μM HU-211 markedly decreased the association rate (Fig. 2*A*). Fig. 2 *B* and *C* shows that addition of 10 μM HU-211 also decreased the dissociation rate of [^{3}H]TCP from the NMDA receptor, both without and with 1 μM glutamate and 1 μM glycine, but much more strongly in their presence.

The kinetic data thus show that HU-211 acts functionally as an NMDA antagonist in the [^{3}H]TCP binding assay. Like the competitive NMDA antagonist AP-5 (14), HU-211 reduces the glutamate/glycine potentiation of [^{3}H]TCP binding (4). However, unlike AP-5, which strongly inhibits [^{3}H]TCP binding even in the absence of glutamate and glycine (i.e., basal binding), HU-211 appears to be a much more active blocker of [^{3}H]TCP binding when glutamate and glycine are present (i.e., induced binding) (Fig. 2 *A* and *C*). This finding, as well as the marked structural differences between AP-5 and the cannabinoid, led us to consider the possibility that the latter does not act at the glutamate or at the glycine site. Indeed, we found that HU-211 (10 and 50 μM) reduced the efficacy of glutamate- or glycine-induced [^{3}H]TCP binding, but HU-211 did not alter their apparent affinities (Fig. 3). Taken together, our results suggest that HU-211 exerts at least some of its behavioral effects in animals by acting at a specific site of the NMDA receptor that is distinct from the binding sites for TCP, glutamate, or glycine.

Several lines of evidence suggest that the effects of HU-211 on [^{3}H]TCP binding to the NMDA receptor are, indeed, exerted via a specific binding site rather than through nonspecific perturbations of the membrane structure: (*i*) the effect on the initial rate of [^{3}H]TCP binding is dose-dependent (Fig. 4), with an IC_{50} value of 6–10 μM (IC_{50} is the dose producing 50% inhibition of glutamate- or glycine-induced

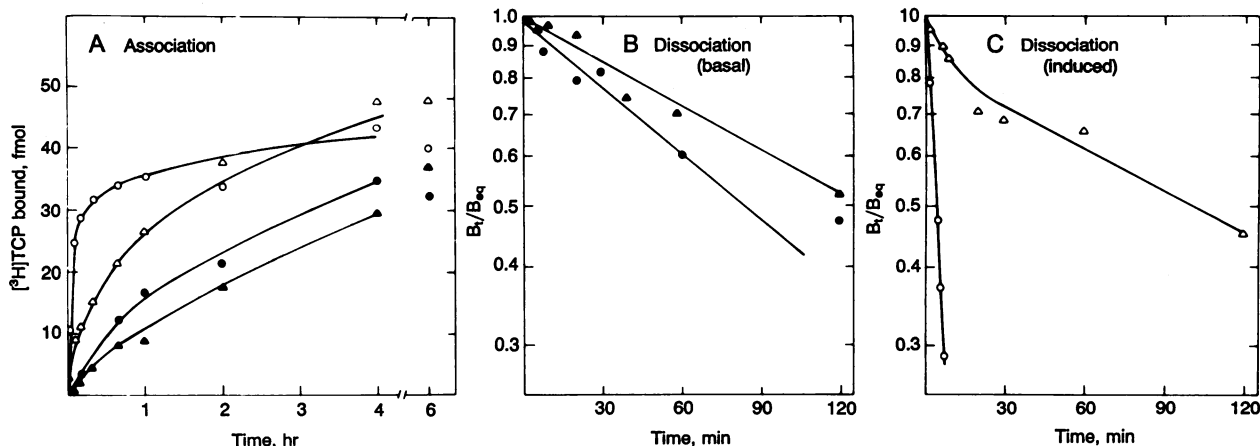

FIG. 2. HU-211 reduces the rate of binding of [^{3}H]TCP to the NMDA-receptor channel and the dissociation rate of [^{3}H]TCP–receptor complexes. (*A*) Time course of basal (without added agonist) and induced (with 1 μM L-glutamate and 1 μM glycine) [^{3}H]TCP binding to the NMDA receptor of rat brain cortical membranes. Data represent the basal binding determined without (●) and with (▲) 10 μM HU-211 and the induced binding determined without (○) and with (△) 10 μM HU-211. Mean values (triplicates) of the specific binding of [^{3}H]TCP (total minus nonspecific binding) are plotted as a function of incubation time. The data shown are from one of three experiments that gave similar results. (*B*–*C*) First-order plots of the basal (*B*) and the induced (*C*) dissociation of [^{3}H]TCP–receptor complexes. Data represent the basal (without added agonist) dissociation rates determined without (●) and with (▲) 10 μM HU-211; the induced (with 1 μM L-glutamate and 1 μM glycine) dissociation rates were determined without (○) and with (△) 10 μM HU-211. B_{eq}, amount of [^{3}H]TCP bound at t_0. B_t, amount of [^{3}H]TCP bound at time t. Data shown are from one of three experiments, each performed in triplicate, yielding similar results.

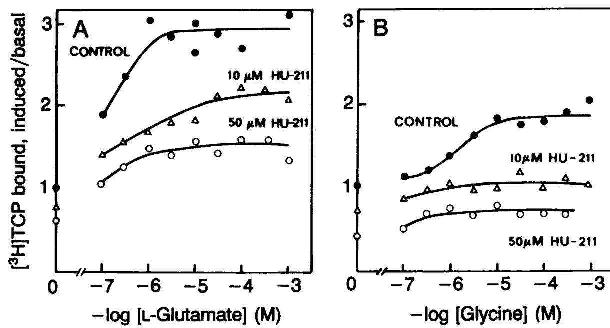

FIG. 3. Noncompetitive inhibition of glutamate- or glycine-induced [^{3}H]TCP binding by HU-211. Induced over basal [^{3}H]TCP-binding (corresponding to the ratio of binding in the presence of glutamate or glycine to binding in their absence) was determined with 5 nM [^{3}H]TCP at 25°C for 10 min. Binding of [^{3}H]TCP to well-washed rat cortical membranes was determined in triplicate, without (basal) and with various concentrations of L-glutamate (*A*) or glycine (*B*) with and without HU-211, as indicated. Data are from one of two experiments yielding similar results.

binding); (*ii*) HU-211 is a far more potent inhibitor of the induced [^{3}H]TCP binding to the NMDA receptor than its (−) enantiomer HU-210 (Fig. 4), thus clearly pointing to stereospecific interaction between HU-211 and the NMDA receptor; and (*iii*) the IC_{50} value for HU-211, determined under the same conditions but using phencyclidine/NMDA receptors solubilized with sodium cholate (10), was also 10 μM, indicating that the observed effects of HU-211 are not attributable to nonspecific perturbations of the membrane.

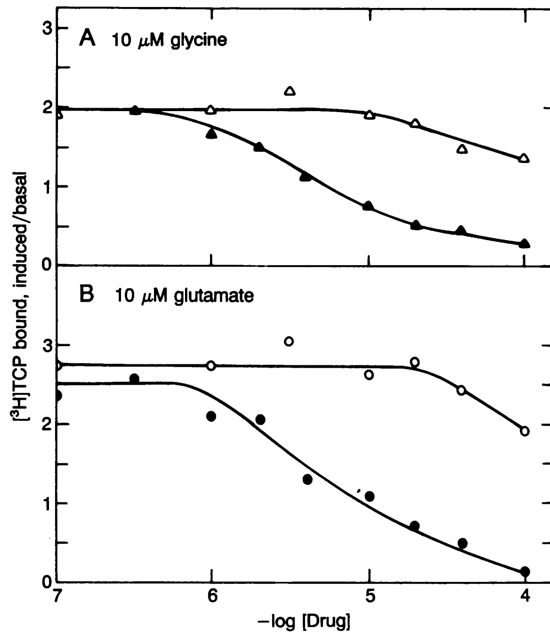

FIG. 4. HU-211 reduces the rate of binding of [^{3}H]TCP to the NMDA receptor in a stereoselective manner. Data represent the concentration-dependent decrease of [^{3}H]TCP binding by HU-211 (▲, ●) and by its enantiomer HU-210 (△, ○). Binding of [^{3}H]TCP (5 nM) was determined as described for Fig. 2*A*, either without (basal) or with (induced) 10 μM glutamate (*A*) or 1 μM glycine (*B*). Reactions were terminated after 10 min. Data are expressed as induced over basal binding as a function of cannabinoid concentrations. Basal binding was 188 fmol/mg of protein. Data are from one of two experiments yielding similar results.

Recent evidence has indicated that brain damage induced by ischemia as well as by hypoglycemia is mediated, *inter alia*, by NMDA receptors (for review, see refs. 9, 20, and 21). In light of the absence of psychotropic or other untoward side effects seen after HU-211 administration at doses that cause blocking of NMDA effects, this drug merits serious consideration as a possible treatment against NMDA-receptor-mediated neuropathologies, including epilepsy, Huntington disease, and neuronal necrosis from cerebral ischemia.

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