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Anticonvulsant preclinical profile of CHF 3381 Dopaminergic and glutamatergic mechanisms

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Abstract

Following intraperitoneal or oral administrations, CHF 3381 ([*n*-(2-indanyl)-glycinamide hydrochloride]) protected rats against maximal electroshock (MES) test seizures. As glutamatergic pathways play a pivotal role in epilepsy, to better characterize the molecular mechanisms of action of CHF 3381, the drug effects on the binding of the excitatory amino acid antagonist [^{3}H]-MK-801 in the presence of *n*-methyl-D-aspartate (NMDA), spermidine, or the combination of both ligands, were studied. CHF 3381 inhibited the [^{3}H]-MK-801 specific binding in a noncompetitive fashion in respect to NMDA and polyamines recognition sites. CHF 3381 failed to change the kinetic characteristic of glycine B receptors labeled with [^{3}H]-glycine; in contrast, it significantly increased K_d values when the receptors were labeled with the more specific compound [^{3}H]-MDL 105,519. CHF 3381 antagonized dopamine (DA)-induced behavioral responses and inhibited, in a glycine-dependent manner, the NMDA-induced [^{3}H]-DA release from rat striatal slices, but it failed to change either the kinetic characteristics of D_1, D_2, or D_3 receptors in synaptic plasma membranes (SPM) or the [^{3}H]-DA uptake from striatal synaptosomes. Moreover, in primary cell cultures of cortical neurons, this drug exhibited glycine-independent neuroprotective effects against glutamate-induced excitotoxicity. It is concluded that this compound could have a potential use in several disease states where a pathological high level of NMDA receptor activation is thought to occur.

Keywords: Anticonvulsants; Behavior; Radioligand binding assays; Dopamine (DA); Primary cell cultures; Neurotoxicity; Rat

1. Introduction

A large body of experimental evidences suggested that excitatory amino acid (EAA) glutamate might play a pivotal role in epileptogenesis and in epilepsy (Bradford and Peterson, 1987). Among the different EAA receptor subtypes, the voltage-sensitive blockade of the *N*-methyl-D-aspartate (NMDA) receptors by Mg^{2+} was considered of primary importance in epilepsy, since the neural depolarisation in epilepsy could remove Mg^{2+} blockade and increase receptor-mediated currents in rat hippocampus (Coan and Collingridge, 1985). Convulsion induced by glutamate and aspartate in humans (Hayashy, 1952) and NMDA, kainic acid (KA), and quisqualic acid in mice (Koek and Colpaert, 1990) were blocked both by competitive and noncompetitive NMDA antagonists. In several models of epilepsy [i.e., kindling, De Sarro et al., 1985; genetic photosensitive baboon, Meldrum et al., 1983; genetic audiogenic seizures, Croucher et al., 1982; maximal electroshock (MES) seizures, Czuczwar et al., 1984], a good correlation has been found between anticonvulsant action and NMDA receptor channel blocking affinity. Several studies almost unanimously suggested that NMDA antagonists prevent electrographic seizures, triggering epileptogenesis without blocking seizures expression (Anderson et al., 1987; Stasheff et al., 1989; Bawin et al., 1991). Inconsistently, the correlation between NMDA receptor affinity and anticonvulsant effectiveness of several drugs has not always been found; in the MES seizures mode, the ratio between the toxic $dose_{50}$/MES effective $dose_{50}$ of some competitive antagonists did not correlate with their potency against NMDA-induced depolarisation (Rogawsky

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et al., 1991). In other models, NMDA antagonists did not block seizures (Koek and Colpaert, 1990).

Pharmacologic evidences showed that several ''traditional'' (GABA mimetics) anticonvulsants suppressed both basal and K^+-evoked D-aspartate release. However, although all these traditional anticonvulsants could directly or indirectly alter the activity of glutamatergic receptors, they invariably acted at concentrations above therapeutic levels, suggesting that the inhibition could not account for their primary anticonvulsant mechanism that could lie on different neurotransmitter systems.

Among them, the neurotransmitter dopamine (DA) represented a neuroactive substance capable of inhibiting the propagation of seizures activity (Trimble, 1977). Moreover, the n. caudate–putamen (n. striatum) is a key structure in containment of seizures perhaps due to its anatomical connections with cortical and limbic regions rich of NMDA-sensitive glutamate receptors and glutamatergic terminals (Walaas and Fonnum, 1979). The role of DA in epilepsy was recently reviewed in the excellent paper by Starr (1996).

In this study, pharmacological, behavioral, and biochemical experiments were undertaken in order to clarify whether DAergic mechanisms could be the major target of the CHF 3381 anticonvulsant effect. SKF-38393-induced grooming was taken as a behavioral response by D_1 receptor activation, quinpirole-induced hypermotility as a response elicited by D_2 receptor stimulation, apomorphine (APO) and methamphetamine (MA)-induced stereotypies were triggered by both D_1 and D_2 receptor activation. The drug effects on DA receptor subtypes were assayed by [^{3}H]-SCH 23390, [^{3}H]-spiroperidol, and [^{3}H]-7-OH-DPAT radioligand binding studies to D_1, D_2, and D_3 receptors, respectively, in crude synaptic plasma membranes (SPM) from rat striata. The presynaptic involvement of CHF 3381 was assessed by performing both [^{3}H]-DA reuptake studies by striatal synaptosomes and [^{3}H]-DA release from striatal slices.

The CHF 3381 effects on the kinetic characteristics of [^{3}H]-MK-801 specific binding in well-washed SPM from rat cerebral cortex were evaluated in order to clarify the drug capability to modulate glutamatergic function. Moreover, since it has been reported that glycine, acting at strychnine-insensitive receptors (glycine B receptors) greatly increased the frequency of the Na^+, Ca^{2+} channel opening at the NMDA-sensitive glutamate receptor complex (Johson and Asher, 1987) in this research the effects of CHF 3381 on glycine B recognition sites in the same SPM preparation were assessed.

Finally, we assayed the putative neuroprotective effects of CHF 3381 against glutamate-induced neurotoxicity in primary cultures of rat cortical neurones. All these results allowed us to hypothesize that the molecular mechanism of the anticonvulsant effect of CHF 3381 could lie in the inhibition of NMDA receptor function, although the exact molecular target requires better clarification.

At present, the effects of CHF 3381 on the NMDA-evoked increase in guanosine 3′,5′-cyclic monophosphate (cGMP) accumulation in neonatal rat cerebellar slices (PD8) are under investigation aiming to clarify whether in this experimental model glycine B receptors could represent the primary target of the drug action.

2. Materials and methods

Male Sprague–Dawley rats (200–225 g, Harlan-Nossan, Italy) were housed in groups of four under standard laboratory conditions of light (from 7:00 a.m. to 7:00 p.m.), temperature (22 ± 2°C), and humidity (65%) and were allowed free access to standard laboratory diet and tap water. For oral administration only, from the afternoon of the day before treatment, animals were fed a 20% glucose solution. All experimental protocols were approved by the local Bioethical Committee, while the procedures and the conditions were controlled by the University Veterinary Service.

2.1. Pharmacological studies

2.1.1. Maximal electroshock test

An electrical stimulus (150 mA; 60 Hz; ECT unit model 7801, Ugo Basile, Italy) was delivered through corneal electrodes primed with a drop of electrolyte solution. The stimulus produced a hindlimb tonic extension in 100% of control animals. The anticonvulsant activity of CHF 3381 was determined as the complete suppression of the hindlimb tonic component of the seizures. Thirty minutes before the test, at least three doses of the compound were administered intraperitoneally (3–25 mg/kg) or orally (12.5–50 mg/kg) to groups of 10–20 rats to calculate ED_{50} doses.

2.2. Behavioral studies

2.2.1. Rotarod test

Different groups of rats ($n = 8$ per group) were observed for ataxia following high doses of the drug. The day before the execution of the test, the rats were trained to maintain their equilibrium on the test apparatus (Rotarod Treadmills model 7650, Ugo Basile Italy) according to Kinnard and Carr (1957). The training consisted of three subsequent 1-min sessions on a rod rotating at 8 rpm. On the day of the test, only the animals able to maintain their equilibrium were retained for the experimental procedure. CHF 3381 or vehicle were administered orally (150–600 mg/kg) or intraperitoneally (70–240 mg/kg) to groups of eight rats 15 min before the execution of the test. The number of rats falling for three subsequent 1-min attempts were used to calculate the dose displaying 50% ataxia (TD_{50}). The therapeutic index value (TI) was calculated by dividing the TD_{50} by the ED_{50}.

2.2.2. Locomotor activity

Rat locomotor activity was evaluated by means of actometric cages ($38 \times 30 \times 25$ cm) as already described

(Dall'Olio et al., 1988). Groups of naive rats (8–12 per group), treated with different doses of CHF 3381 (1.25, 2.5, 5, 10 mg/kg ip) or saline were placed in the cages and their exploratory mobility was recorded for 60 min. Different groups of animals (8–12 per group) were habituated to the experimental cages for 1 h before receiving CHF 3381 (2.5 mg/kg ip) or saline. Thirty minutes later, the animals were administered with APO (0.25 mg/kg sc), MA (2 mg/kg ip), or quinpirole (0.3 mg/kg ip). Their locomotor activity was recorded for the following 60 min.

2.2.3. Stereotyped behavior

Groups of rats (8–16 per group), habituated to the experimental cage for 1 h, were treated with CHF 3381 (2.5 mg/kg ip) or saline 30 min before receiving APO (0.25 mg/kg sc) or quinpirole (0.3 mg/kg ip). Starting 10 min after, observers unaware of the treatments assigned every 10 min for 30 min stereotypy scores according to the following rating scale: 0 (*no change in normal behavior*), 1 (*intermittent sniffing*), 2 (*continuous sniffing*), 3 (*intermittent biting or licking*), 4 (*continuous biting or licking*).

2.2.4. SKF 38393-induced grooming behavior

This response is generally considered a nonstereotyped D_1 receptor-mediated behavior consisting of "episodes of grooming with the snout being directed vigorously into body" (Molloy and Waddington, 1984). Groups of rats (8–12 per group) were allowed to explore the experimental environment (60 min) before receiving CHF 3381 (2.5 mg/kg ip) or saline. Thirty minutes following the pharmacological treatments, the animals received the D_1 DA agonist SKF 38393 (10 mg/kg ip). Immediately following drug administrations, observers unaware of the treatments recorded the total time (min) of grooming episodes exhibited by animals for 60 min.

2.3. Biochemical studies

2.3.1. Radioligand binding studies

[^{3}H]-SCH 23390, [^{3}H]-spiroperidol and [^{3}H]-7-OH-DPAT specific bindings to D_1, D_2, and D_3 DA receptor subtypes, respectively, were assessed in crude SPM prepared from rats n. striatum according to Billard et al. (1984), Creese et al. (1975), and Wallace and Booze (1995). Briefly, groups of animals were sacrificed by cervical dislocation, the brains rapidly removed, placed into ice-cold saline, and striata were dissected, pooled, and homogenized with Polytron in ice-cold 50 mM Tris–HCl buffer, pH 7.4. Homogenates were spinned twice in the same buffer. The final pellets were resuspended in the same buffer containing NaCl 120, KCl 5, $CaCl_2$ 2, and $MgCl_2$ 1 mM. Aliquots of membrane suspensions were incubated with different concentrations of [^{3}H]-SCH 23390 (0.05–5 nM, 37°C, 30 min), [^{3}H]-spiroperidol (0.025–0.5 nM, 37°C, 15 min), or [^{3}H]-7-OH-DPAT (0.125–5 nM, 22°C, 60 min). The reaction was stopped by rapid filtration under negative pressure through Whatmann GF/C filters. The radioactivity remaining on the filters was counted by liquid scintillation spectometry using Atomlight. The specific bindings were determined between the total binding and the binding remaining in the presence of a specific displacer; 1 μM cold SCH 23390, 1 μM cold (+) butaclamol, or 10 μM cold 7-OH-DPAT, respectively, for [^{3}H]-SCH 23390, [^{3}H]-spiroperidol, and [^{3}H]-7-OH-DPAT specific bindings. For the assessment of [^{3}H]-spiroperidol specific binding to D_2 receptors, ketanserine (300 nM) was included into the incubation mixture in order to exclude the $5HT_2$ receptor component. Aliquots of CHF 3381 were added to suspensions of crude SPM to study the effect of the in vitro addition of the drug on the specific bindings.

[^{3}H]-MK-801 and [^{3}H]-glycine (or [^{3}H]-MDL 105,519) specific bindings were assessed in well-washed SPM prepared from rat frontal cortex, according to Stirling et al. (1989) and Ogita et al. (1989). Cortices were homogenized (glass–Teflon) in ice-cold 0.32 M sucrose. Homogenates were centrifuged ($1000 \times g$), with the pellets (P1 fraction) resuspended in sucrose and centrifuged again. The supernatants were pooled, centrifuged at $45{,}000 \times g$ to yield P2 pellets, which were suspended in Tris–HCl buffer (pH 7.7) and centrifuged ($45{,}000 \times g$, 40 min, 4°C). The pellets were resuspended in ice-cold water and centrifuged as previously. This final procedure was repeated three times and the pellets were frozen at −20°C for at least 18 h. On the day of the assay, pellets were thawed, resuspended in Tris–HCl (pH 7.7), and centrifuged ($45{,}000 \times g$, 40 min). The pellets were washed four times and the final suspension was incubated at room temperature for 20 min prior to centrifugation ($45{,}000 \times g$, 40 min). The final pellet was suspended in 10 volumes Tris–HCl (pH 7.7) to give a protein concentration of approximately 1 mg/ml. [^{3}H]-MK-801 and [^{3}H]-MDL 105,519 binding assays were carried out in incubation mixtures containing 5 mM Tris–HCl buffer and different concentrations of [^{3}H]-MK-801 (0.5–15 nM) or [^{3}H]-MDL 105,519 (0.5–80 nM), respectively. After incubation for 2 h at 25°C (equilibrium) for [^{3}H]-MK-801 binding, or for 30 min at room temperature for [^{3}H]-MDL 105,519 binding, the mixtures were rapidly filtered and radioactivity was determined as already described. The specific bindings were determined as the difference between the total binding and the binding left by incubating the samples in the presence of cold MK-801 (100 μM) or cold glycine (1 mM). In some experiments, for the assessment of strychnine-insensitive [^{3}H]-glycine binding (glycine B), 100 μM strychnine was included in the incubation mixture together with different [^{3}H]-glycine concentrations (0.1–50 nM, 2°C, 10 min). NMDA, spermidine, or glutamate, together with threshold or saturating concentrations of glycine, were added to the incubation tubes in the presence or in absence of CHF 3381 (100 μM) with [^{3}H]-MK-801 (5 nM). In all experiments, binding assays were carried out in triplicate with a variation of less than 10% at an interval of 15 s.

2.3.2. Determination of [^{3}H]-DA uptake by rat striatal synaptosomes

Crude synaptosomes were prepared by glass–teflon homogenization of striata in 0.32 M sucrose, 5 mM HEPES, pH 7.4 followed by centrifugation ($1000 \times g$, 10 min) to give a pellet containing nuclei and cell bodies (P1 fraction). [^{3}H]-DA uptake was assayed accordingly to Near et al. (1988) in Krebs buffer containing ascorbate (50 μM), dextrose (0.2%), EDTA (0.002%), and pargyline (10 μM). After preincubating the mixture (37°C, 20 min), aliquots of [^{3}H]-DA (20 nM) were added and incubation (37°C, 4 min) was terminated by addition of ice-cold (4 ml) buffer. The samples were filtered over Whatmann GF/C filters under negative pressure. Filters, placed in scintillation vials containing Atomlight, were kept at room temperature overnight and radioactivity was determined as already described. Nonspecific uptake was determined in the presence of the specific DA uptake blocker nomifensin (0.3 mM).

2.3.3. Determination of NMDA-induced [^{3}H]-DA release from striatal slice preparations

Groups of rats were sacrificed by decapitation and the brains were rapidly removed. The striata were rapidly excised, were rinsed in cold HEPES-buffered Krebs–Ringer solution (KRH) (NaCl 128; KCl 2.4; KH_2PO_4 1.2; HEPES 25; glucose 10; $CaCl_2$ 3.2; ascorbic acid 1 mM; pargyline 10 μM; pH 7.4) and saturated by bubbling with 95% O_2–5% CO_2. Slices (300 μm) were prepared in a sagittal plane using a McIlwain tissue chopper. Slices from each of three animals were gently separated and incubated (25 min, 37°C) in KRH in the presence of [^{3}H]-DA. At the end of the incubation period, the slices were washed twice with ice-cold KRH to remove the external radioactivity and four slices (around 1 mg of protein) were placed on a paper filter disk in a perfusion chamber. The perfusion apparatus consisted of 12 serial perfusion chambers at a low flow rate (0.5 ml/min, in the presence of nomifensin) to avoid [^{3}H]-DA uptake into the slices. The perfusing media were continuously oxygenated at 37°C and the slices were allowed to equilibrate for 50 min. After a stabilization period, two 2-min samples of superfusate were collected. [^{3}H]-DA release was evoked by NMDA or kainic acid (1 mM) in the presence or in the absence of CHF 3381 (50 μM) and/or glycine (1 mM) dissolved in KRH. At the end of the experiment, slices were removed from the apparatus and solubilized with NaOH (0.5 mM) to determine proteins and radioactivity content of tissue. The radioactivity of slices and superfusate fractions were measured by liquid scintillation counter and calculated in terms of fractional release.

2.4. Glutamate-induced neurotoxicity in primary cortical cell cultures

Primary cultures were prepared from postnatal (PD1) rats according to Alho et al. (1988). Briefly, after careful dissection from diencephalic structures and hippocampus under sterile conditions, the cerebral cortices of six rats were pooled, sliced (300 μm) in two orthogonal directions (McIlwain tissue chopper), incubated in trypsin solution (0.025%) and the minces dispersed by trituration in a solution of DNase and soybean trypsin inhibitor (0.001% and 0.05%, respectively). In this way, about a 95% pure population of neurons, assayed from immunostaining for glial fibrillary acidic protein (GFAP, Sigma), was obtained. Neurons (1.5×10^6 approximate plating density) were plated in culture dishes (NUNC), which were coated with poly-L-lysine (10 μg/ml). The cells were cultured in Eagle's Basal medium supplemented with heat-inactivated fetal calf serum (10%), KCl (25 mM), glutamine (2 mM), and gentamicin (100 μg/ml). Cultures were kept at 37°C in a humidified atmosphere (95% O_2–5% CO_2). After 4 h in vitro, nonneuronal cell division was halted by cytosine arabinoside (10 μM). On 8 days in vitro (8 DIV), after removal of the cultures medium, the cells were washed with Mg^{2+}-free Locke's buffer (NaCl 154, KCl 5.6, $NaHCO_3$ 3.6, $CaCl_2$ 2.3, glucose 5.6, HEPES 5 mM, pH 7.4), and incubated (15 min, room temperature) with (−)-glutamate (300 μM) alone or in the presence of CHF 3381 (1–100 μM) or glycine (1 mM). The treatment with glutamate was terminated by replacing the buffer with serum free Eagle's basal medium and the dishes were returned to the culture incubator for the evaluation of neurotoxicity 24 h later. Neurotoxicity was measured by a colorimetric assay with 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) according to Mosmann (1988). MTT, a pale yellow substrate that produces a dark blue formazan product when incubated with live cells, was dissolved (1 mg/ml, 37°C, 30 min) in phosphate buffered saline (PBS, 0.1 M). The MTT solution was removed and the blue precipitate (produced via cleavage of the tetrazolium ring) was dissolved in Tris–Triton (95/5%) solution (1 ml/well). Solutions were measured with a spectrophotometer (Beckman, DU 530) at a wavelength of 57 nM.

According to the dose/effect curve, the optical density measured on (−)-glutamate (300 μM) + glycine (10 μM)-treated cultures was considered to be 100% of neurotoxicity. Each point was determinated in sextuplicate, and the data are presented as the mean ± S.E.M. of three independent experiments.

In all experiments, protein contents were assessed according to Lowry et al. (1951), with bovine serum albumin (BSA) as a standard.

2.5. Statistical analysis

Results from [^{3}H]-specific bindings and from [^{3}H]-DA reuptake studies were expressed as the mean ± S.E.M. and the statistical significance was determined by Student's *t* test. In the [^{3}H]-DA release studies, the difference between means was calculated with one-way repeated-measures test ANOVA followed by Dunnett's *t* test or

Newman–Keuls. Behavioral data were analyzed following Dunnett's *t* test after ANOVA analysis. *P* values lower than .05 were considered to represent statistically significant differences. The method of Litchfield–Wilcox was used to calculate ED_{50} values (with 95% confidence intervals) for MES and rotarod.

2.6. Drugs

[^{3}H]-SCH 23390 (90 Ci/mmol), [^{3}H]-spiroperidol (15 Ci/mmol), [^{3}H]-DA (53 Ci/mmol) and Atomlight were purchased from NEN (Boston, MA); [^{3}H]-7-OH-DPAT (164 Ci/mmol), [^{3}H]-MK-801 (20 Ci/mmol) [^{3}H]-MDL 100,519 (85 Ci/mmol) and [^{3}H]-glycine (16.2 Ci/mmol) from Amersham; SCH 23390, 7-OH-DPAT, glycine, SKF 38393, ketanserin, (L)-glutamic acid and kainic acid were purchased from Tocris Neuramin (Buckhurst Hill, England); quinpirole, (+)-MK-801 hydrogenate and NMDA were purchased from Research Biochemical International (Wayland, MA); APO, BSA, (+)-butaclamol, EDTA, HEPES, MA, pargyline, strychnine, spermidine, nomifensine were purchased from Sigma (St. Louis, MO); the kind gift of CHF 3381 was from Chiesi Farmaceutici, Parma, Italy.

3. Results

3.1. Anticonvulsant activity

CHF 3381 displayed dose-dependent anticonvulsant activity in rats with ED_{50} values of 21 (13–22, 95% confident limit values) and 7.5 (5.3–11) mg/kg after oral or intraperitoneal administrations, respectively, and motor impairment in the rotarod test at the doses 299 (210–422) mg/kg and 113 (91–141) mg/kg. TI values of CHF 3381 with reference to MES test were 15 and 14 after intraperitoneal or oral administrations.

3.2. Behavioral studies

Fig. 1a shows that CHF 3381 (5 and 10 mg/kg ip) inhibited the exploratory activity of naive rats. The pretreatment of CHF 3381, given at a dose ineffective by itself on animal locomotion (2.5 mg/kg), differently affected the animals' motor activity induced by different DAergic agonists (Fig. 1b). While the hyperactivity induced either by the D_2 agonist quinpirole (0.3 mg/kg ip) or by the indirect DA agonist MA (2 mg/kg ip) was significantly inhibited by CHF

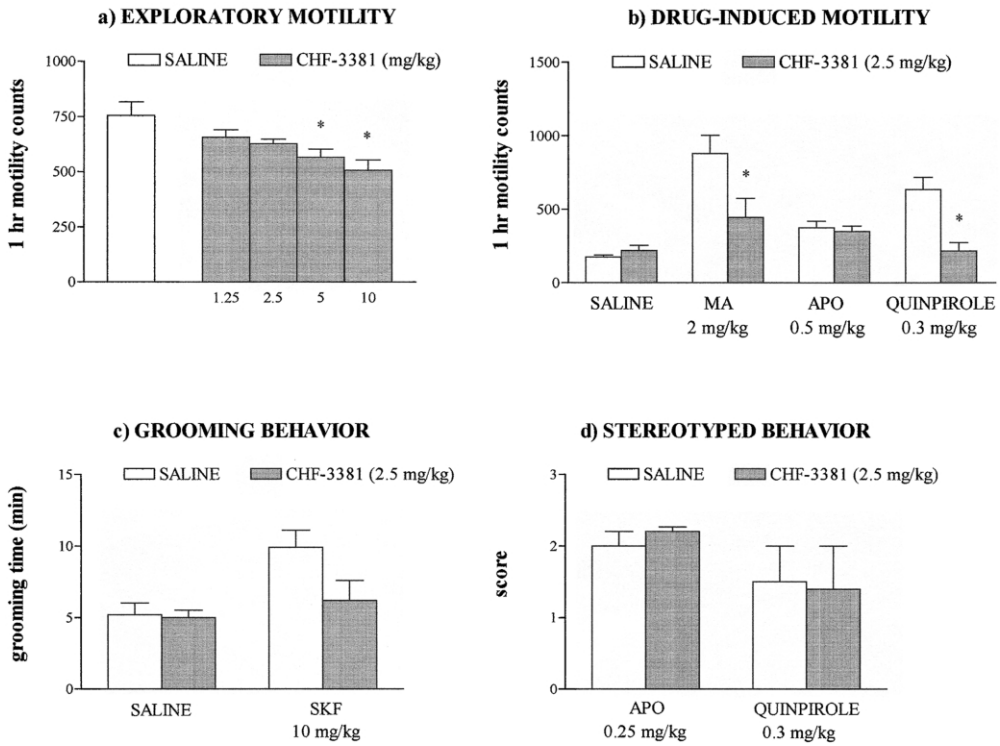

Fig. 1. Behavioral profile of CHF 3381. (a) Locomotor activity induced by different doses of CHF 3381 (1.25–10 mg/kg ip; 30 min before). Mean values ± S.E.M. of motility counts recorded for 60 min. * $P < .05$ in comparison to saline-pretreated group (Dunnett's *t* test after ANOVA). (b) Effects of CHF 3381 (2.5 mg/kg ip) on locomotor activity by methamphetamine (MA 2 mg/kg ip), apomorphine (APO 0.25 mg/kg sc), or quinpirole (0.3 mg/kg ip). Mean values ± S.E.M. of 60 min motility counts. * $P < .05$ in comparison to the respective control groups (Dunnett's *t* test after ANOVA). (c) Effect of CHF 3381 (2.5 mg/kg ip) on spontaneous and SKF 38393 (10 mg/kg ip)-induced grooming behavior. Mean values ± S.E.M. of grooming time (min). (d) Effect of CHF 3381 (2.5 mg/kg ip) on stereotyped responses induced by apomorphine (0.25 mg/kg sc) or quinpirole (0.3 mg/kg ip). Mean score ± S.E.M. of the individual average scores from three 10-min spatial observations.

3381, this drug failed to affect the hypermotility elicited by the direct D_1-D_2 DAergic agonist APO (0.25 mg/kg sc). In contrast, the same dose of CHF 3381 did not affect the stereotyped responses induced both by APO or by quinpirole (Fig. 1d). The figure (Part c) shows also that grooming induced by the specific D_1 agonist SKF 38393 (10 mg/kg ip) was decreased, although not reaching statistical significancy, by a dose of CHF 3381 (2.5 mg/kg ip), which failed to change grooming behavior in saline-treated animals.

3.3. Radioligand binding studies

3.3.1. [^{3}H]-SCH 23390, [^{3}H]-spiroperidol, and [^{3}H]-7-OH-DPAT specific bindings

In SPM prepared from rat n. striatum, [^{3}H]-SCH 23390 labeled a single population of D_1 binding sites (B_{max} = 1250 ± 66 fmol/mg prot) with good affinity (K_d = 0.4 ± 0.02 nM). [^{3}H]-Spiroperidol labeled D_2 receptors (B_{max} = 186 ± 11 fmol/mg prot, K_d = 0.17 ± 0.02 nM) and [^{3}H]-7-OH-DPAT labeled D_3 DA receptors (B_{max} = 15.3 ± 0.9 fmol/mg prot, K_d = 0.97 ± 0.1 nM) (data not shown). The in vitro addition of a concentration of CHF 3381 (10 μM), which has been detected in rat CNS after anticonvulsant effective doses, failed to change the kinetic characteristics of all subtypes of DA receptors (not shown). Consistently, the same concentration failed to modify the [^{3}H]-DA specific uptake by rat striatal synaptosomes (not shown).

3.3.2. [^{3}H]-MK-801 specific binding

Several observations have demonstrated that the [^{3}H]-MK-801 specific binding in well-washed SPM is sensitive to the state of activation of NMDA receptor. In our hands, Scatchard analysis from the saturation transformed data showed a single population of [^{3}H]-MK-801 binding sites with B_{max} = 1166 ± 48 fmol/mg prot, and apparent affinity K_d = 11.7 ± 0.6 nM (not shown). Table 1 shows that CHF 3381 (0.1–100 μM) effectively displaced the [^{3}H]-MK-801 specific binding (5 nM) with good affinity (IC_{50} = 8.1 ± 0.5μM). In conditions of (L)-glutamate (100 μM) stimulation of the NMDA receptor complex, CHF 3381 slightly inhibited [^{3}H]-MK-801 binding with different efficacies depending on threshold (0.01 μM) or saturating (30 μM) glycine stimulation.

Table 1
Effects of the in vitro addition of CHF 3381 on the [^{3}H]-MK-801 (5 nM) specific binding in SPM prepared from rat cerebral cortex, in basal conditions and in different stimulation levels of the NMDA receptor complex

	IC_{50} (μM)
No agonist added	8.1 ± 0.5
+ Glutamine (100 μM) + glycine (0.01 μM)	17.4 ± 1.3*
+ Glutamine (100 μM) + glycine (30 μM)	28.4 ± 2.6*

IC_{50} values were derived from log–logit analysis of specific binding data using 10 concentrations of CHF 3381 (0.1–100 μM).
Compounds were run in triplicate using 5 nM [^{3}H]-MK-801 (see Materials and Methods).
Values are expressed as mean ± S.E.M. for at least three separate experiments.
* $P < .05$ (Student's t test) when compared to the basal level.

Table 2
[^{3}H]-MK-801 (5 nM) specific binding (% of control) in SPM from rat cerebral cortex

		+ CHF 3381 (100 μM)
No drug added	100	19.7
NMDA (1 μM)	203.3	51.1
Glycine (30 μM)	209.3	48.4
Glycine (100 μM)	267.4	46.5
NMDA (1 μM) + glycine (30 μM)	297.6	88.4
Glutamate (100 μM)	193.3	121.8
Glutamate (0.1 μM) + glycine (0.03 μM)	116.2	27.9
Glutamate (100 μM) + glycine (100 μM)	348	127.4
Glutamate (0.1 μM) + glycine (0.03 μM) + spermidine (100 μM)	480.1	116.3

Membranes were incubated with a subsaturating concentration (5 nM) of [^{3}H]-MK-801. In these conditions, the percentage specific binding over total was more than 75%. The data are from three separate experiments replicated twice.
The mean value (± S.E.M.) for control samples (no drug added) was 272 ± 29 fmol/mg prot.

Table 2 shows that the in vitro addition of 30 or 100 μM glycine increased in a similar extent (209% and 267%, respectively) the [^{3}H]-MK-801 (5 nM) specific binding in SPM prepared from rat cerebral cortex; moreover, 1 μM NMDA increased [^{3}H]-MK-801 specific binding in a similar extent with glycine (30 μM). The table shows also that when the combination of the two drugs was added, the [^{3}H]-MK-801 specific binding increased. Glutamate (100 μM) enhanced the [^{3}H]-MK-801 specific binding with two orders of potency lesser than NMDA, however, this increase was potentiated (348%) by 100 μM glycine. Furthermore, 0.1 μM glutamate, together with subthreshold concentrations of glycine (0.03 μM), failed to increase [^{3}H]-MK-801 binding (116.2%) which, however, was fully increased (by 480%) by the concomitant addition of 100 μM spermidine. In all experimental conditions, CHF 3381 inhibited the [^{3}H]-MK-801 specific binding in a noncompetitive manner with respect to the NMDA, glycine, or spermidine recognition sites.

In well-washed SPM prepared from rat frontal cortex, the binding of [^{3}H]-glycine to strychnine-insensitive sites (glycine B) was saturable (Table 3). The in vitro addition of CHF 3381 (100 μM) did not significantly change either the relative affinity or the maximum number of glycine B recognition sites. In contrast, when glycine recognition sites were labeled with the more specific marker [^{3}H]-MDL 105,519 (0.5–20 nM, Part A), the addition of CHF 3381 decreased the affinity while B_{max} values were not affected. The table shows also (Part B) the [^{3}H]-MDL 105,519 specific binding assayed at a subsaturating ligand concentration (5 nM) and displaced by 11 glycine concentrations (0.01–10 μM). In this experimental condition, the in vitro addition of CHF 3381 decreased by six times the affinity of glycine B recognition sites while leaving unmodified the receptor number.

Table 3
Effects of in vitro addition of CHF 3381 on the kinetic characteristics of glycine B receptors in SPM prepared from rat cerebral cortex

	[^{3}H]-Glycine binding		[^{3}H]-MDL 105,519 binding			
			B_{max} (pmol/mg)		K_d (nM)	
	B_{max} (pmol/mg)	K_d (nM)	A	B	A	B
Control	3.1 ± 0.2	102 ± 16	3.0 ± 0.2	1.9 ± 0.1	4.6 ± 0.3	69 ± 3
CHF 3381 (100 μM)	2.6 ± 0.1	94 ± 14	2.7 ± 0.2	2.3 ± 0.1	6.4 ± 0.3*	430 ± 19*

Well-washed SPM prepared from rat cerebral cortex were incubated with different concentrations of [^{3}H]-glycine (0.5–50 nM, 2°C, 10 min; in the presence of strychnine 100 nM) or with:

(A) Different concentrations of [^{3}H]-MDL 105,519 (0.5–80 nM, 25°C, 30 min). Nonspecific binding, defined as that remaining in the presence of cold glycine 1 mM.

(B) A subsaturating concentration of [^{3}H]-MDL 105,519 (5 nM) displaced by 11 different concentrations (10^{-9}–10^{-3}) unlabeled glycine.

* $P < .05$ (Student's t test) when compared to control (no drug added).

3.4. [^{3}H]-DA release from striatal slices

In slices prepared from rat n. striatum, 1 mM NMDA increased the [^{3}H]-DA release (by about 300%) in Mg^{2+}-free buffer. This effect did not appear to be maximal since it was potentiated in a concentration-dependent manner by adding glycine to the superfusion buffer (not shown). Fig. 2 (Part A) shows that the NMDA-induced [^{3}H]-DA release from striatal slices was completely inhibited by the in vitro addition of CHF 3381 at a concentration (50 μM) which was unable to change the spontaneous nonstimulated [^{3}H]-DA release. Moreover, the excess of glycine (1 mM) completely reversed the CHF 3381-induced attenuation of the NMDA-evoked [^{3}H]-DA release stimulation, suggesting that the drug inhibition of the NMDA-evoked [^{3}H]-DA release may be mainly due, at least in this experimental model, by drug blockade of the glycine B site associated to the NMDA receptor/ionophore complex. The specificity of the CHF 3381 interaction with the NMDA receptor complex is shown in Fig. 2 (Part B). While kainic acid increased the [^{3}H]-DA release from striatal slices in a similar extent with NMDA, CHF 3381 failed to inhibit kainic acid-evoked release, supporting the view that the selective inhibiting effect is through the NMDA subtype receptor/ionophore complex.

3.5. Primary cell cortical cultures

The exposure of rat cortical cell cultures in vitro (8 DIV) to a high concentration of L-glutamate (300 μM, 15 min) resulted on the following day in about 48% of highly reproducible toxicity compared to that observed in control cultures. The addition of glycine concentrations (10 μM) to the glutamate exposure solution resulted in at least 20% greater cell death than in cultures incubated with glutamate alone. The addition of CHF 3381 (1–100 μM) during the full stimulation produced a significant dose-related neuro-

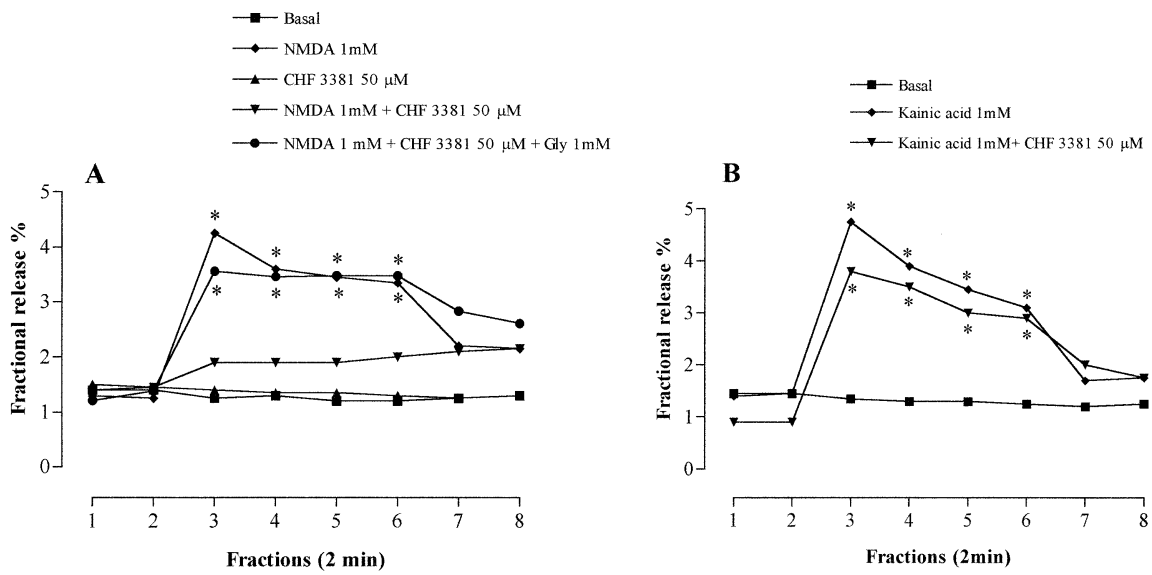

Fig. 2. Effects of CHF 3381 on the NMDA (1 mM, Part A) and kainic acid (1 mM, Part B)-induced [^{3}H]-DA release from slices prepared from rat n. striatum. The duration of drug perfusion is indicated by the horizontal bar after which KRH was perfused until the end of the experiment. The results are expressed as a percentage of fractional release. * $P < .05$ (one-way ANOVA followed by Dunnett's test) represents values significantly different from the mean value ($t = 0$ min).

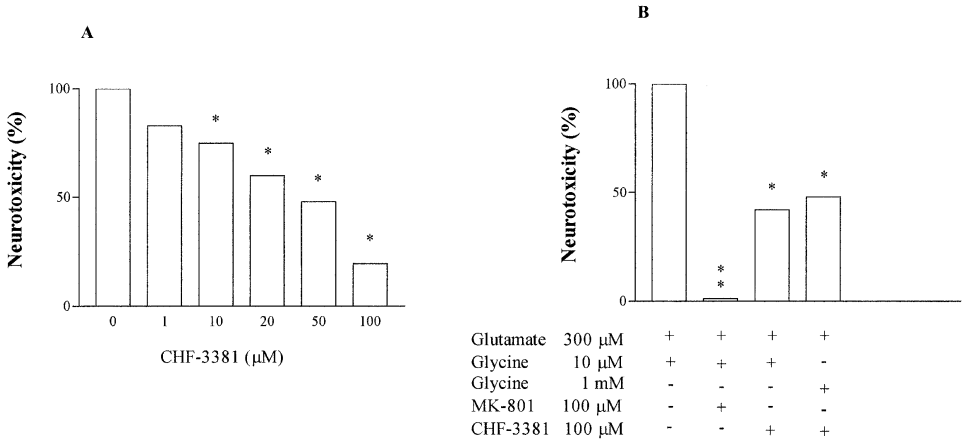

Fig. 3. (A) Dose–response curve for the protective effects of CHF 3381 against glutamate (300 μM) + glycine (10 μM)-induced neurotoxicity (as 100%) in rat cortical cell cultures. (B) Partial protective effects of CHF 3381 and lack of reversal by high glycine concentrations (1 mM) on neurotoxicity induced by (L)-glutamate in rat cortical cell cultures. The data were obtained from three independent experiments. * $P<.05$, ** $P<.01$ (Student's t test) when compound to control (glutamate 300 μM + glycine 10 μM).

protective effect (Fig. 3A) with ED_{50} value of 35 μM. Fig. 3B shows that differently from MK-801, CHF 3381 only partially prevented glutamate-evoked neurotoxicity and this protective effect was not overcome by the in vitro addition of full glycine concentrations (1 mM).

4. Discussion

The main goal of this study was to gain insight into the intimate mechanisms underlying the anticonvulsant activity of CHF 3381. After intraperitoneal and oral administrations, CHF 3381 quite potently antagonized MES-induced seizures. Comparison of ED_{50} values obtained with CHF 3381 after either administration routes is suggesting that the compound is well-adsorbed after oral administrations in rats and maintains a good anticonvulsant activity regardless of the route of administration. A variety of competitive and noncompetitive NMDA receptor antagonists have been shown to possess anticonvulsant and anxiolytic activity, however, differently from known competitive or noncompetitive antagonists, CHF 3381 impaired rotarod performance only at doses higher than those needed to demonstrate anticonvulsant activity. Both competitive (AP-5, AP-7) and noncompetitive (MK-801) antagonists induce in rats a behavioral syndrome characterized by ataxia, hyperlocomotion, and stereotypies, which are thought to result (at least partially) from an imbalance between glutamatergic and DAergic circuitries, therefore, in this study, we investigated whether CHF 3381 could modify DAergic receptors, [^{3}H]-DA uptake, and function. The behavioral profile of CHF 3381 appeared to be quite different from NMDA antagonists as in previous studies MK-801, administered at a dose which did not affect the spontaneous rat motility, did not change hypermotility induced by the DAergic agent MA (Gandolfi et al., 1992), but potentiated the responses elicited by APO (Dall'Olio et al., 1995). In contrast, the present results showed that low doses of CHF 3381 decreased both MA- and APO-induced hyperlocomotion. Invariably, either noncompetitive, competitive, as well as CHF 3381, reduced the hypermotility induced by quinpirole, whereas both MK-801 and the competitive antagonist CGP 43487, but not CHF 3381 increased both the APO-induced stereotyped response and the grooming response to the D_1 agonist SKF 38393 (Dall'Olio et al., 1996). All these observations taken together with the conflicting results that CHF 3381 inhibited some DA-induced responses and did not change either [^{3}H]-DA specific uptake or the kinetic characteristics of D_1, D_2 and D_3 receptors, suggest that this drug, having a different DAergic profile in comparison to NMDA antagonists, could be lacking of psychotomimetic side effect in humans.

The binding of [^{3}H]-MK-801 has been generally proposed to provide a model of receptor activity, in that it is reciprocally modulated by agents which functional assays defined as agonists or antagonists (Baron et al., 1990) and is positively modulated both by glycine (Reynolds et al., 1987) or polyamines (Reynolds, 1990).

In our hands, CHF 3381 displaced with a good affinity [^{3}H]-MK-801 binding (in the range of concentrations obtained in rat brain after ip administrations of anticonvulsant doses) and this value decreased upon different stimulation levels of the NMDA receptor complex suggesting a drug negative modulatory effect related to the receptor activation. CHF 3381 decreased [^{3}H]-MK-801 binding even in the presence of a maximal level of NMDA and shifted to the right of the spermidine concentration–response curve displaying noncompetitive mechanism.

CHF 3381 decreased [^{3}H]-MK-801 binding in an apparently noncompetitive way with respect to glycine, therefore, we performed more accurate experiments aiming to better clarify the role of glycine B recognition sites in the mechanism of CHF 3381 inhibition of the [^{3}H]-MK-801 binding. The two different experimental approaches have shown that CHF 3381 was able to decrease the affinity of the glycine B

recognition sites labeled with the most specific label [^{3}H]-MDL 105,519 in well-washed SPM prepared from rat cerebral cortex and this effect was mainly evident when a classic "competition" study between a subsaturating concentration of the label and different concentrations of the cold displacer was run. Moreover, the analysis of binding isotherm of [^{3}H]-MDL 105,519 specific binding exhibited a Hill slope near unity consistent with the measured binding to a population of noninteracting binding sites with uniform affinity for glycine. This coefficient was slightly modified in a nonsignificant way by the addition of CHF 3381.

We switched therefore our interest to different experimental models more feasible to elucidate functional changes of the NMDA receptor complex. Our results clearly show that CHF 3381 specifically inhibited, in a Mg^{2+}-dependent way, the NMDA- but not the kainate-induced [^{3}H]-DA release from striatal slices and this effect was completely overcome by glycine, suggesting the possible involvement of glycine B recognition sites in the drug inhibitory action of NMDA receptor function. In contrast, the partial protective effect (in comparison to MK-801) elicited by CHF 3381 on glutamate-evoked neurotoxicity in primary cell cultures failed to be reversed by full glycine concentrations, casting additional doubts on the glycine B recognition site involvement in drug action.

In conclusion, our results are indicative of a noncompetitive interaction of CHF 3381 with respect to NMDA and spermidine recognition sites and cast some doubt on the hypothesis of an action at the glycine B binding sites. At present, our attention is focused to study whether full glycine concentrations could antagonize the CHF 3381 inhibition of NMDA-induced stimulation of cGMP levels in neonatal rat cerebellar slices (our preliminary data), and whether this drug could display anticonvulsant properties towards sound-induced seizures in mice and kindling procedures in rats.

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