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Short communication

Binding properties of [^{3}H]gacyclidine in the rat central nervous system

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Abstract

Gacyclidine (1-[1-(2-thienyl)-2-methylcyclohexyl]piperidine), the racemate of (+)-and (−)-GK11, exhibits potent neuroprotective properties due to its antagonism at the NMDA receptor. In its tritiated form, gacyclidine showed a binding distribution similar to that of NMDA receptors in the rat brain. With membrane preparations, the (−)-enantiomer of gacyclidine exhibited an affinity similar to that of MK-801 (dizocilpine, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine) in the low nanomolar range, while the (+)-enantiomer was about 10 times less potent. Gacyclidine affinity was lower in the cerebellum than in the forebrain or the spinal cord. In this latter region and in the cerebellum, two binding sites were evidenced, one of which was a low-affinity site insensitive to MK-801. In all regions, PRE-084 (2-(4-morpholino)ethyl-1-phenylcyclohexane-1-carboxylate), a σ receptor ligand, had no effect on [^{3}H]gacyclidine binding.

Keywords: NMDA receptor; Phencyclidine receptor; Gacyclidine; Gacyclidine enantiomer; Neuroprotection

1. Introduction

Gacyclidine ((±)-1-[1-(2-thienyl)-2-methylcyclohexyl]-piperidine, (±)-GK11), a 1-[1-(2-thienyl)cyclohexyl]-piperidine (TCP) derivative, is a compound which exhibits in vitro (Michaud et al., 1994) and in vivo (Lallement et al., 1997; Feldblum et al., 1998) potent neuroprotective properties. It protects cultured neurones against glutamate-induced toxicity through NMDA receptors. Preliminary experiments have shown that it is a potent inhibitor of [^{3}H]TCP binding at the NMDA ionic channel (Michaud et al., 1994). When administered in vivo, gacyclidine does not exhibit deleterious neuronal toxicity as observed with dizocilpine (MK-801, (+)-5 methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine) (Privat, unpublished observation, Olney et al., 1991). This compound is now in a phase II–III clinical trial and is indicated for the treatment of traumatic brain and spinal cord injuries. In contrast to most NMDA receptor antagonists previously investigated in clinical trials, no deleterious side effects have precluded the development of gacyclidine. In order to determine whether only the NMDA receptor antagonist properties of gacyclidine are involved in its neuroprotective properties, gacyclidine was tritiated and its binding parameters were characterised in the rat central nervous system.

2. Materials and methods

2.1. Chemicals

Gacyclidine, (+)-(1*R*-2*S*)-1-[1-(2-thienyl)-2-methylcyclohexyl]piperidine ((+)-GK11) and (−)-(1*S*-2*R*)-1-[1-(2-thienyl)-2-methylcyclohexyl]piperidine ((−)-GK11) were obtained from Beaufour-Ipsen (Les Ulis, France). MK-801was purchased from RBI (Sigma–Aldrich, France). [^{3}H]gacyclidine (55 Ci/mmol) was obtained from Amersham, (Les Ulis, France, custom synthesis). All other chemicals were obtained from commercial sources at the highest purity available.

2.2. Binding assay

Membrane homogenates from the telencephalon, cerebellum and spinal cord were prepared as previously described (Vignon et al., 1986). Binding experiments were performed on fresh homogenates in a 5 mM Tris/HEPES, pH 7.7 buffer at a 0.5–1.0 mg/ml protein concentration.

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Incubations were terminated by rapid filtration over GF/B (Whatman) glass fibre filters pre-soaked in 0.1% polyethyleneimine using a Brandel cell harvester. The filters were then rinsed three times with 5 ml of a 100 mM NaCl, 10 mM Tris–HCl, pH 7.7 buffer and the radioactivity retained was counted in 3.5 ml ACS II (Amersham) with an Excel 1410 (LKB) liquid scintillation spectrophotometer. The non-specific binding was determined in parallel incubations in the presence of 100 μM unlabelled TCP.

2.3. Autoradiography

Male adult Wistar rats (200–250 g) were killed by i.p. administration of a lethal dose (120 mg/kg) of pentobarbital. The central nervous system was rapidly removed and frozen in isopentane at −40°C and then stored at −80°C. Tissues from three to five animals were sectioned at −20°C with a freezing microtome at a thickness of 14 μm. Slices were mounted on gelatine (2%)- and alun chrome (0.1%)-coated slides. Slices from three different animals were fixed on each slide. The slides were rinsed for 30 min with a 10 μM glutamate, 10 μM glycine, 5 mM Tris/HEPES, pH 7.7 buffer and then incubated for 120 min with the same buffer in the presence of 5 nM (forebrain) or 20 nM (cerebellum and spinal cord) [^{3}H]gacyclidine. CGS-19755 (*cis*-4-(phophonomethyl)-piperidine-2-carboxylic acid, 100 μM) was then added and the incubation was continued for another 30 min. Slides were then rinsed six times for 30 s in a 10 μM CGS-19755, 5 mM Tris/HEPES, pH 7.7 buffer and dried under a stream of cold air. Non-specific binding was determined in parallel incubations in the presence of 100 μM unlabelled TCP. Slides were then exposed for 3 weeks at room temperature in light-tight cassettes on ^{3}H-hyperfilm (Amersham) with ^{3}H-microscales standards (Amersham). The films were developed with Kodak D19, fixed and dried.

2.4. Data analysis

In each experiment, values are the means of triplicates and each experiment was performed at least three times. The data from kinetic and competition experiments were analysed by a nonlinear regression method (Marquardt–Levenberg algorithm) using the SigmaPlot® 4 software (Jandel) according to a single- or a two-site model. Experimental results were submitted to an analysis of variance (F value). The Residual mean squares of the fits were then compared by the Fisher's bilateral test. The two-site model was preferred when the Fisher's test was considered significant ($P < 0.05$).

Autoradiographic data were analysed using the Samba (Alcatel) software image analyser system. Mean densitometry from at least 27 fields (three slides carrying three slices) was determined via ^{3}H microscales standards. Results are expressed in fmol/mg of tissue.

3. Results

3.1. Autoradiography

Binding sites were the most abundant in telencephalic regions (Fig. 1A). The white matter was almost devoid of specific binding (Fig. 1A,C,D). The non-specific binding was very low (Fig. 1B). [^{3}H]Gacyclidine binding was 1.5 times higher in the hippocampus (346 fmol/mg of tissue) than in the cerebral cortex (215 fmol/mg of tissue). It was moderate in the striatum (133 fmol/mg of tissue), the lateral septum (172 fmol/mg of tissue) and the posterior thalamus (159 fmol/mg of tissue). Binding was low in the hypothalamus (38 fmol/mg of tissue), the mesencephalon (29 fmol/mg of tissue) and the medial thalamus (54 fmol/mg of tissue) (Fig. 1A). In the cerebellum and the spinal cord the labelling was low, even at the highest [^{3}H]gacyclidine concentration (20 nM). In the cerebellum (Fig. 1C), the granular layer (67 fmol/mg of tissue) was slightly more labelled than the molecular layer (52 fmol/mg of tissue). In the spinal cord (Fig. 1D), the labelling was very similar at the cervical, thoracic and lumbar levels. The outer dorsal horn was the most labelled (70 fmol/mg of tissue) and the labelling of the ventral horn was 1.3 times lower (50 fmol/mg of tissue).

3.2. Binding experiments

In preliminary experiments the effects of the positive effectors of NMDA receptors (glutamate, glycine and spermine, alone or in combination) were tested. No stimulatory effect on radioligand binding was detected, thus indicating that their concentrations were sufficient in the incubation medium. Conversely on washed membrane preparations, glutamate and glycine stimulated [^{3}H]gacyclidine binding. This stimulatory effect was different according to the homogenate used (not shown).

Kinetic studies have shown that [^{3}H]gacyclidine association and dissociation processes were far slower than those of other NMDA receptor antagonists like TCP or MK-801. With 2 nM [^{3}H]gacyclidine, equilibrium was reached in 6 to 7 h at 25°C. In all three regions, a two-site model of interaction better described the radioligand association. Dissociation processes were also very slow, with half-times ranging from 1.5 to 4 h. On forebrain homogenates the dissociation was best described by a two-site model of interaction (not shown).

The potencies of gacyclidine and its enantiomers to inhibit the binding of [^{3}H]gacyclidine were compared to those of MK-801 (NMDA receptor ligand) and PRE-084 (2-(4-morpholino)ethyl-1-phenylcyclohexane-1-carboxylate, a σ receptor ligand). The data from these experiments are summarised in Table 1. They showed that in forebrain and cerebellum homogenates single-site interactions were more probable. In contrast, in spinal cord homogenates, a

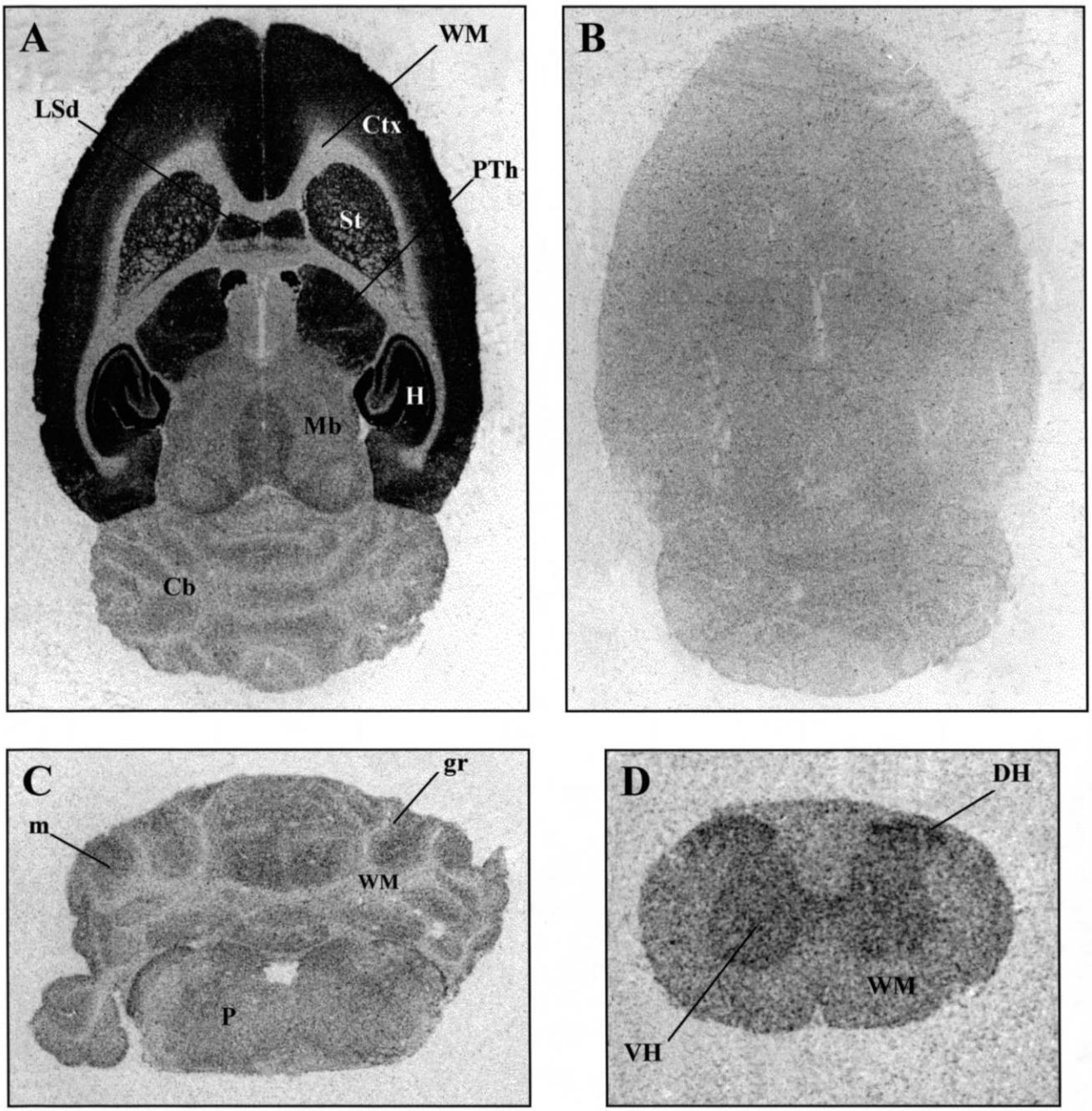

Fig. 1. Regional distribution of [^{3}H]gacyclidine binding in the rat brain (5 nM in the forebrain and 20 nM in the cerebellum and the spinal cord). (A) Horizontal section, total binding; (B) horizontal section, non-specific binding; (C) cerebellum; (D) spinal cord at the cervical level. Abbreviations: Ctx: Cortex; H: Hippocampus; St: Striatum; LSd: Lateral Septum (dorsal part); PTh: Ventroposteior thalamus; Mb: Midbrain; Cb: cerebellum; gr: granular layer; m: molecular layer; DH: Dorsal Horn; VH: Ventral Horn; WM: White matter.

two-site model of interaction was more likely. Whatever the region, the rank order of potency of the unlabelled drugs was similar for the high-affinity (spinal cord) or the single- (forebrain and cerebellum) site. (−)-GK11 was as potent as MK-801. (+)-GK11 had an affinity about 10 times lower than that of the (−)-enantiomer. Surprisingly, gacyclidine exhibited an affinity close to that of (+)-GK11. These results also confirmed, that as previously described with [^{3}H]TCP (Hamon et al., 1999), affinities determined in the cerebellum are lower than those evidenced in the forebrain or the spinal cord.

In the cerebellum MK-801 inhibited only a part of [^{3}H]gacyclidine binding, thus suggesting that in this region two different binding sites could be detected. In spinal cord homogenates, high-affinity sites were in the same proportion as that determined for MK-801 in the cerebel-

Table 1
Inhibition of [^{3}H]gacyclidine binding by unlabelled drugs in the different CNS regions. Crude homogenate preparations were incubated in the presence of increasing concentration of the test compounds with [^{3}H]gacyclidine for 6 h in the forebrain, 7 h in the cerebellum and 5 h in the spinal cord. The mean of at least three independent experiments was analysed according to a single or a two-site model. When the two-site model was statistically more probable, the proportion of high- and low-affinity sites is expressed as $\%_1$ and $\%_2$ and the corresponding K_i (expressed in nM) values as K_{i1} and K_{i2}, otherwise only K_i is indicated.

	Forebrain [^{3}H]gacyclidine (2 nM)				Cerebellum [^{3}H]gacyclidine (2 nM)				Spinal cord [^{3}H]gacyclidine (2.5 nM)			
	$\%_1$	K_{i1} (nM)	$\%_2$	K_{i2} (nM)	$\%_1$	K_{i1} (nM)	$\%_2$	K_{i2} (nM)	$\%_1$	K_{i1} (nM)	$\%_2$	K_{i2} (nM)
(−)-GK11	102.9	3.6	–	–	99.7	12.1	–	–	83.0	2.9	17.8	2100
(+)-GK11	101.2	30.9	–	–	99.1	104.9	–	–	72.7[a]	10.8[a]	30.9[a]	415[a]
gacyclidine	101.1	34.2	–	–	99.3	81.5	–	–	97.5	29.1	–	
MK-801	102.3	3.0	–	–	75.8	7.7	21.3	> 10 μM	72.5	2.3	22.5	> 10 μM
PRE-084	–	> 10 μM	–	–	–	> 10 μM	–	–	–	> 10 μM	–	–

[a] $P < 0.1$ with Fisher's bilateral test.

lum. The affinity of gacyclidine enantiomers for the low-affinity binding site was low, but the (+)-enantiomer was almost five times more potent than the (−)-enantiomer.

Whatever the region, the σ receptor ligand PRE-084 had no inhibitory effect, even at the highest concentration (10 μM).

4. Discussion

This study demonstrates that gacyclidine binding properties are largely accounted for by its interaction with NMDA receptors: (i) the autoradiographic experiments disclosed a pattern of labelling similar to that described for agonists and antagonists of the NMDA receptors, [^{3}H]Glutamate (Monaghan and Cotman, 1985), [^{3}H]CPP ([^{3}H]3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid, Kito et al., 1990), [^{3}H]TCP (Vignon et al., 1986), [^{3}H]MK-801 (Subramaniam and McGonigle, 1991), and with the localisation of NR_1 and $NR_{2A/2B}$ subunits (Petralia et al., 1994a,b); (ii) the NMDA receptor ligand MK-801 exhibited a high affinity in all regions. As previously observed with [^{3}H]TCP or [^{3}H]MK-801 (Hamon et al., 1999; Vignon et al., 1986), NMDA receptors have different pharmacological properties depending on the region: the affinities determined with cerebellum homogenates were about three times lower than those measured with forebrain or spinal cord homogenates. Although NMDA receptor subunits are abundantly expressed in the cerebellum (Petralia et al., 1994a,b), this is likely due to the major expression in this region of the NR_{2C} subunit, which is less sensitive to MK-801 blockade than the NR_{2A} or NR_{2B} subunits (Yamakura et al., 1993). This likely explains the low labelling intensity detected in the cerebellum and the pons.

Compared to the other non-competitive NMDA receptor antagonists, gacyclidine exhibited some differences. Low-affinity sites, insensitive to MK-801, were evidenced in the cerebellum and the spinal cord. The binding site distribution in the cerebellum revealed a similar labelling of the molecular and granular layers. Slower association and dissociation characterised its binding and several hours were required to attain equilibrium.

Low-affinity sites have already been reported for [^{3}H]TCP and some investigators have suggested that they may be σ receptors (Hamon et al., 1999). PRE-084, a σ receptor ligand, had no inhibitory effect on [^{3}H]gacyclidine binding even at high concentrations. Therefore, gacyclidine low-affinity sites are probably different from the σ receptor. Low-affinity sites were detected in the spinal cord, but they are probably present in the cerebellum since a portion of the binding was insensitive to MK-801. This limited pharmacological study, however, did not permit their precise characterisation, which will be investigated later.

In the cerebellum, the molecular layer was labelled as intensely as the granular layer, which contains high levels of NMDA receptors whereas the molecular layer is devoid of functional receptor (Cull-Candy et al., 1998). Thus, the labelling pattern suggests that, in this region, low-affinity sites might be located in the molecular layer.

The (−)-gacyclidine enantiomer, (−)-GK11, was as potent as MK-801 at NMDA receptors. It was about 10 times more potent than (+)-GK11 with telencephalon and cerebellum homogenates and three times with spinal cord homogenates. This is well correlated with their respective neuroprotective effects on neuronal cells (Michaud et al., 1994). Interestingly, the stereoselectivity between the two enantiomers was higher than when it was determined with [^{3}H]TCP (Michaud et al., 1994). This might suggest that the binding site within the channel is different depending on the ligand used. It must be noticed that at the low-affinity sites in the spinal cord the stereoselectivity was reversed, with (+)-GK11 being about 5 times more potent than (−)-GK11.

The different affinities of gacyclidine enantiomers did not allow us to perform a Scatchard analysis of its interaction. Owing to the presence of low-affinity sites, a four-site model would have been required but such an analysis cannot be statistically significant. Thus, the precise characterisation of gacyclidine binding sites can only be achieved with tritiated enantiomers. The (−)-enantiomer would be a

useful tool for the study of NMDA receptors, whereas the (+)-enantiomer would be more suitable to study low-affinity sites.

In conclusion, these results suggest that the neuroprotective properties of gacyclidine are essentially due to its binding at NMDA receptors, but its interaction with low-affinity sites may exert a positive contribution. In fact, at the same dose, in vivo, gacyclidine and its enantiomers present very similar effects (Feldblum et al., 1998). This suggests that the interaction with NMDA receptors is not the sole explanation for the neuroprotective efficacy of gacyclidine.

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