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Norketamine, the main metabolite of ketamine, is a non-competitive NMDA receptor antagonist in the rat cortex and spinal cord

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Abstract

The enantiomers of the potent non-competitive NMDA receptor antagonist ketamine and its major metabolite, norketamine were evaluated as NMDA receptor antagonists using the rat cortical wedge preparation and the neonatal rat spinal cord preparation, respectively, for electrophysiological studies and [^{3}H](*RS*)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine ([^{3}H]MK-801) in homogenate binding experiments. In agreement with earlier studies (*S*)-ketamine (K_i 0.3 μM) was found to possess a 5 times higher affinity for the NMDA receptor complex than (*R*)-ketamine (K_i 1.4 μM). (*S*)-Norketamine (K_i 1.7 μM) had approximately an 8 times higher affinity than (*R*)-norketamine (K_i 13 μM) in the inhibition of [^{3}H]MK-801 binding. All compounds inhibited responses to NMDA in the rat cortical wedge preparation and the hemisected neonatal rat spinal cord, being approximately four times more potent in the cortex than in the spinal cord except for (*R*)-norketamine being only twice as potent. In light of the clinically obtained concentrations of norketamine after oral administration of ketamine, these data strongly suggest that (*S*)-norketamine may contribute significantly to the clinical activity of (*S*)-ketamine, especially when given orally. © 1997 Elsevier Science B.V.

Keywords: NMDA receptor antagonist, non-competitive; Electrophysiology; Cortex, rat; Spinal cord, rat; Enantiomer

1. Introduction

Excitatory amino acid receptors are thought to be implicated in several physiological and pathophysiological processes (Greenamyre et al., 1988; Lodge, 1988; Monaghan et al., 1989; Carlsson and Carlsson, 1990; Meldrum et al., 1991; Wheal and Thomson, 1991; Krogsgaard-Larsen and Hansen, 1992). It has been suggested that excitatory amino acid receptors in general and the (NMDA) receptor subtypes in particular, are involved in the phenomenon termed wind-up (Dickenson and Sullivan, 1987; Thomson et al., 1990). Experiments using the competitive NMDA receptor antagonist (*R*)-2-amino-5-phosphonopentanoic acid (AP5) or the non-competitive NMDA receptor antagonist (*RS*)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (MK-801) have suggested that blockade of NMDA receptors can completely abolish wind-up (Woolf and Thomson, 1991; Zeman and Lodge, 1992; Coderre et al., 1993). Ketamine, a well characterized non-competitive NMDA receptor antagonist (Martin and Lodge, 1985; Church and Lodge, 1990), has been used clinically as an analgesic and anaesthetic for the last 30 years (Reich and Silvay, 1989). In sub-anaesthetic doses the analgesic effects of ketamine is thought to be mediated primarily via a blockade of the NMDA receptors, although κ-opioid receptor agonists have been suggested to be a contributing factor, too (Husveith et al., 1995).

An earlier pharmacokinetic study (Grant et al., 1981) has shown that plasma concentrations of ketamine following an i.m. injection of 0.5 mg per kg are in the range (approximately 1 μM), where, based on receptor binding affinities (Vincent et al., 1979; Zukin and Zukin, 1979; Husveith et al., 1995) a substantial non-competitive NMDA receptor blockade may be expected. In these experiments, the plasma concentration of the first metabolite, norketamine, was approximately one third of that of ketamine corresponding to 0.4 μM. In comparison, following an oral dose of 0.5 mg per kg of ketamine, the plasma concentration of norketamine was markedly higher than

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the ketamine concentration and in the same range as ketamine following an i.m. dose. However, analgesic effects and the duration of analgesia were similar when the two application forms were compared. These data, therefore, strongly suggest that norketamine under some conditions (Bushnell and Craig, 1995) may contribute to the pharmacological profile of ketamine, especially following oral administration.

The present study characterises the enantiomers of ketamine and norketamine, MK-801 and phencyclidine in [^{3}H]MK-801 binding (Ebert et al., 1991) and in electrophysiological test systems using the rat cortical wedge (Harrison and Simmonds, 1985; Wheatley, 1986) and the neonatal rat spinal cord preparations (Birch et al., 1988; Zeman and Lodge, 1992).

2. Materials and methods

2.1. [^{3}H]MK-801 binding

[^{3}H]MK-801 binding was used as an assay for determining non-competitive NMDA receptor antagonist binding affinities. Dissection of rat brain regions was performed as described by Glowinski and Iversen (1966). Tissue preparation was performed as described by Ransom and Stec (1988). In short: tissue originating from the cortex, hippocampus and striatum was homogenized (500 rpm) in 10 volumes (w/v) of ice-cold 0.32 M sucrose by 8 strokes of a glass–teflon homogenizer. The homogenate was centrifuged at $1000 \times g$ for 10 min and the supernatant was then centrifuged at $20\,000 \times g$ for 20 min at 4°C to yield a P_2-pellet. This pellet was then re-suspended in 20 volumes of ice-cold distilled water and homogenized (100 rpm) with a glass–teflon homogenizer. The homogenate was centrifuged at $8000 \times g$ for 20 min at 4°C. The supernatant and buffy coat was decanted and re-centrifuged at $48\,000 \times g$ for 20 min at 4°C. The pellet was resuspended in 20 volumes of ice-cold distilled water and centrifuged at $48\,000 \times g$ for 20 min at 4°C. The last step was repeated once and the pellet was frozen at −20°C for at least 18 h.

On the day of the assay, the membrane pellet was thawed at room temperature for 45 min, suspended in 75 volumes of 5 mM Tris–HCl buffer (pH 7.4 at 25°C) using a sonicator (2×10 s) and centrifuged for 20 min ($48\,000 \times g$) at 4°C. This step was repeated four times. The resulting membrane suspension is referred to as well-washed membranes. The binding assay was performed following a previously published procedure (Ebert et al., 1991) slightly different from that of Ransom and Stec (1988). Well-washed membranes were re-suspended in 5 mM Tris–HCl buffer (pH 7.4) with a tissue/buffer ratio of 100 corresponding to approximately 200–300 μg protein/vial. Binding experiments were carried out at 25°C and under maximum stimulation, i.e. a final concentration of 30 μM (*S*)-glutamate and 1 μM glycine. [^{3}H]MK-801 was used at a concentration of approximately 5 nM. Non-specifically bound [^{3}H]MK-801 was determined using 100 μM *N*-(1-(2-thienyl)cyclohexyl)piperidine (TCP). Following incubation for 4 h, binding was determined by filtration through Whatman GF/B filters (presoaked for at least 2 h in 0.1% polyethylenimine solution) using a Brandell M-48R cell harvester and 3×2 ml ice-cold buffer as washing medium.

Analysis of the displacement curves was carried out as previously described (Ebert et al., 1991) using an equation describing interaction with one receptor population: %Inhibition $= 100 \times [\text{inhibitor}]/([\text{inhibitor}] \times IC_{50})$. The determined IC_{50} value was converted to a K_i value using the equation of Cheng and Prusoff (1973): $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of [^{3}H]MK-801 (5 nM) and K_d is the affinity of [^{3}H]MK-801 (1.9 nM) in the present assay system (Ebert et al., 1991).

2.2. The rat cortical wedge preparation

The rat cortical wedge preparation is a preparation especially suitable for the evaluation of ligands interacting with excitatory amino acid receptors. Wedges (500 μm thick) of rat brain containing cerebral cortex and corpus callosum were placed with the corpus callosum part between two layers of nappy liner and constantly superfused with a Mg^{2+}- and Ca^{2+}-free oxygenated Krebs buffer solution at room temperature. The cortex part was likewise placed between layers of absorbent fiber and superfused with a Mg^{2+}-free oxygenated Krebs buffer solution. The two parts were electrically insulated with a grease gap. Ag/AgCl electrodes were placed in contact with the nappy liner on each side of the grease gap and the potential difference between the electrodes recorded on a chart recorder. Standard and test compounds were dissolved in Mg^{2+}-free oxygenated Krebs buffer solution and applied to the cortex part of the wedges. 10 μM NMDA was applied for 90 s for determination of depolarising effect. An interval of 12 to 15 min between two applications was used. For measurement of antagonist effects, the compound was constantly superfused during the experiment. Antagonist application was continued until two consecutive responses to 10 μM NMDA were similar. This procedure is a modified version of that earlier described (Harrison and Simmonds, 1985; Wheatley, 1986).

Dose–response curves for the antagonists were obtained the following way: application of 10 μM NMDA was repeated with an interval of 12–15 min until responses to two subsequent applications were of similar magnitude. Once stabilized the response remained stable for 3 to 4 h. The obtained value was used as a control value. The antagonist was then applied for 12–15 min and 10 μM NMDA was applied in the presence of the antagonist. This procedure was continued (usually five to eight times) until two subsequent applications of 10 μM NMDA produced similar responses. %Response was calculated as the ratio

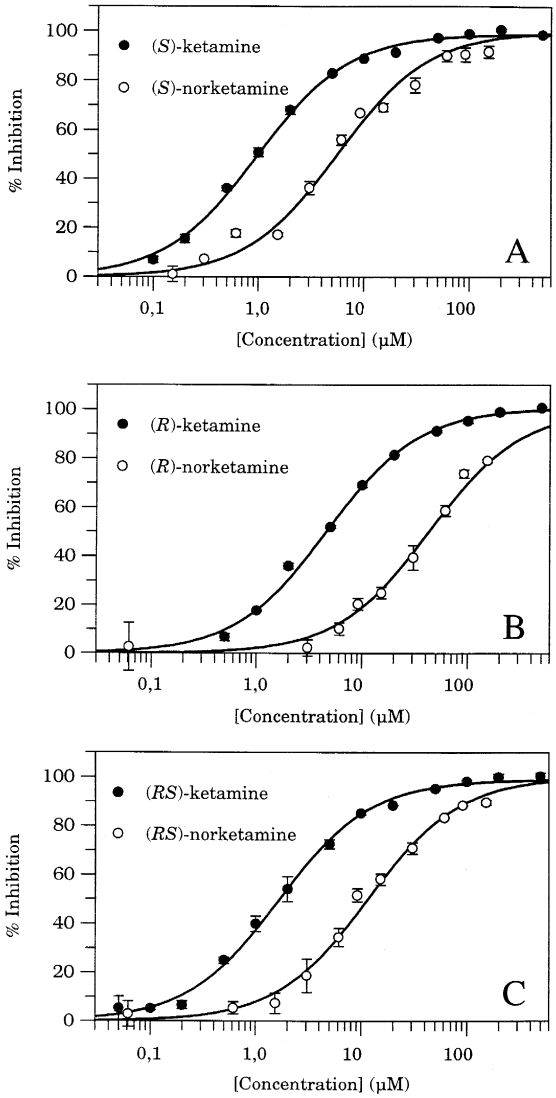

Fig. 1. Displacement of [^{3}H]MK801 in rat brain homogenates for racemates and enantiomers of ketamine and norketamine, respectively. (A) (*S*)-enantiomers; (B) (*R*)-enantiomers; (C) racemates. Values are mean ± S.E.M. of at least three individual experiments.

of the responses to 10 μM NMDA in the presence of antagonist and prior to the antagonist, respectively. Due to a very slow recovery of the agonist response after antagonist treatment, each cortical slice was used to test only one concentration of antagonist. Each data point presented in Fig. 2 is the mean value ± SEM of at least four individual experiments. IC_{50} values were determined by analyses of the inhibition curves using the equation: Effect = $100 \times (1 - ([\text{Inhibitor}]^n / ([\text{Inhibitor}]^n + IC_{50}^n))$, where [Inhibitor] is the concentration of the inhibitor and *n* is the Hill slope of the curve. The IC_{50} values are the result of curve fitting to the mean value using GraFit 3.0 (Erithacus software, Staines, UK).

2.3. The neonatal rat spinal cord preparation

Following published procedures (Birch et al., 1988; Zeman and Lodge, 1992), 2–7 day-old Sprague–Dawley or Wistar rats were decapitated and the spinal cords exposed from the ventral side by removal of overlying internal organs and vertebral bodies. By gentle lifting at the rostral end, while the ventral and dorsal roots were cut, the cords were freed and placed in Krebs solution in a Petri dish. Any remaining roots were trimmed off and a midline hemisection made. The hemicord was placed with the caudal part between two layers of nappy liner and constantly superfused with a Mg^{2+}- and Ca^{2+}-free oxygenated Krebs buffer solution at room temperature. The rostral part was likewise placed between layers of absorbent fibre and superfused with a magnesium-free oxygenated Krebs buffer solution. The two parts were electrically insulated with a grease gap. Ag/AgCl electrodes were placed in contact with the nappy liner on each side of the grease gap and the potential difference between the electrodes recorded on a chart recorder. Standard and test compounds were dissolved in Mg^{2+}-free oxygenated Krebs buffer solution and applied to the caudal part of the wedges.

Experiments and calculations were carried out as described in Section 2.2.

3. Results

3.1. Binding

All compounds displaced [^{3}H]MK-801 binding with Hill slopes not significantly different from one (Fig. 1). Displacement curves were therefore analysed assuming

Table 1
Pharmacological profile of ketamine and norketamine enantiomers

Compound	[^{3}H]MK-801 binding	Electrophysiology in vitro	
	K_i values ± S.E.M. (μM)	IC_{50} (μM)	
		Cortex	Spinal cord
(*RS*)-Ketamine	0.53 ± 0.078		
(*R*)-Ketamine	1.4 ± 0.1	3.0 (1.4)	14 (1.4)
(*S*)-Ketamine	0.30 ± 0.013	0.9 (1.4)	4.0 (1.4)
(*RS*)-Norketamine	3.6 ± 0.49		
(*R*)-Norketamine	13 ± 1.8	39 (1.4)	75 (1.4)
(*S*)-Norketamine	1.7 ± 0.050	3.0 (0.8)	15 (1.4)
MK-801	0.0019 ± 0.0010	0.09 (1.0)	
PCP	0.060 ± 0.013	0.4 (0.9)	

Values are mean values of least four individual experiments.
Binding: K_i values ± S.E.M. of at least three individual experiments. IC_{50} values were determined by analyses of the inhibition curves: %Inhibition = $100 \times [\text{Inhibitor}] / ([\text{Inhibitor}] + IC_{50})$, where [Inhibitor] is the concentration of the inhibitor. K_i values were calculated using the equation by Cheng and Prusoff (1973): $K_i = IC_{50} / (1 + L / K_d)$, where *L* is the concentration of [^{3}H]MK-801 (5 nM) and K_d is the affinity of [^{3}H]MK-801 (1.9 nM) (Ebert et al., 1991).
Electrophysiology: IC_{50} values were determined by analyses mean values for each data point of the inhibition curves using the equation: Effect = $100 \times (1 - ([\text{Inhibitor}]^n / ([\text{Inhibitor}]^n + IC_{50}^n))$, where [Inhibitor] is the concentration of the inhibitor and *n* is the Hill slope of the curve (values in parentheses). The IC_{50} values are the result of curve fitting to the mean value using GraFit 3.0 (Erithacus software, Staines, UK).

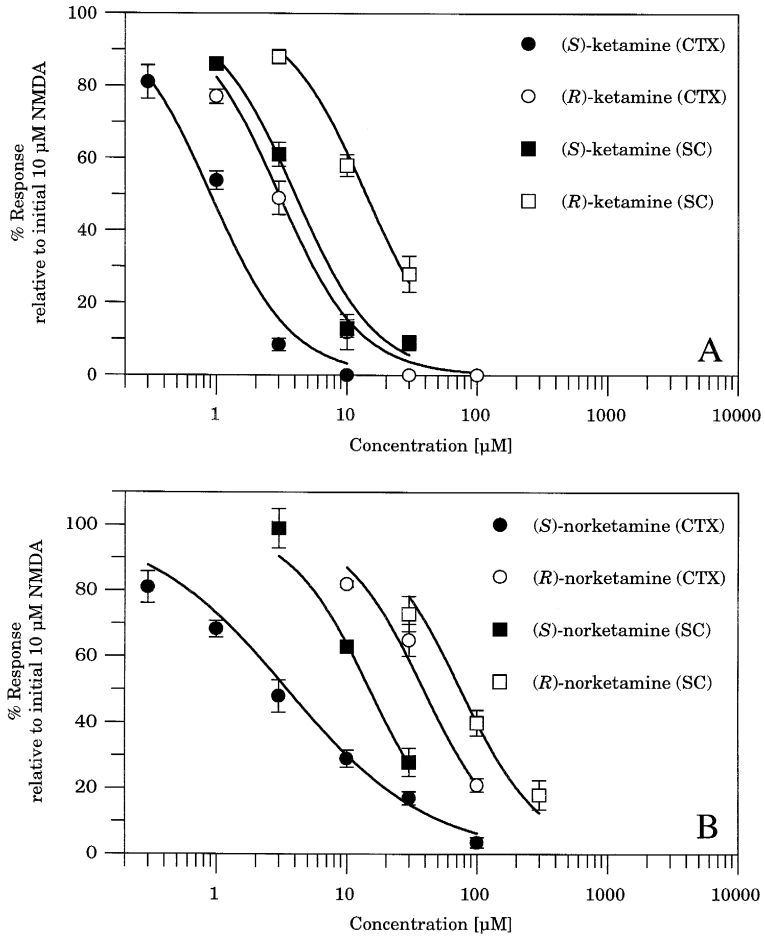

Fig. 2. Inhibition of responses to 10 μM NMDA in the rat cortical wedge preparation (CTX) and in the neonatal rat spinal cord preparation (SC) after repeated co-applications by enantiomers of ketamine (A) and norketamine (B). Values are mean ± S.E.M. of at least three individual experiments.

interaction with one homogenous receptor population. In agreement with earlier findings, (*S*)-ketamine was approximately five times more potent than (*R*)-ketamine as an inhibitor of [^{3}H]MK-801 binding. Fig. 1 illustrates the displacement curves for (*RS*)-, (*R*)- and (*S*)-ketamine, respectively. A similar rank order of potency was seen with norketamine. Thus, (*S*)-norketamine was approximately 8 times more potent than (*R*)-norketamine and equipotent with (*R*)-ketamine (Table 1).

3.2. Electrophysiology

Binding affinities measured in [^{3}H]MK-801 binding are reflected in the rat cortical wedge preparation. The inhibition curve for (*S*)-ketamine versus the response to 10 μM NMDA showed that 50% of the NMDA response was blocked at a concentration of 1.1 μM, a value which is 4 times higher than the K_i value obtained in the binding assay. The IC_{50} value for (*R*)-ketamine was found to be approximately 3 μM. The Hill slope of the inhibition curves was 1.4, indicating a non-competitive interaction between the ketamine enantiomers and the NMDA receptor. (*S*)-Norketamine was, with an IC_{50} value of 3 μM, equipotent with (*R*)-ketamine. However, the Hill slope for the (*S*)-norketamine inhibition curve was 0.8 as compared to 1.4 for the other compounds. (*R*)-Norketamine inhibited the NMDA response with an IC_{50} value of 45 μM.

The concentration dependent inhibition of responses to 10 μM NMDA by MK-801 and PCP in the cortical wedge preparation was subsequently characterised. As shown in Table 1, MK-801 and PCP were approximately ten and four times, respectively, more potent than (*S*)-ketamine in the cortical wedge preparation, with Hill slope of the curves close to 1.

In the hemisected neonatal rat spinal cord all the compounds were approximately five times weaker than in the rat cortical wedge preparation, except for (*R*)-norketamine, which was two times weaker Table 1 and Fig. 2).

4. Discussion

The widely used anaesthetic ketamine has during the last 30 years been given parenterally (Reich and Silvay, 1989). When ketamine is given i.v. or i.m. very high peak concentrations of ketamine are measured, whereas the

main metabolite, norketamine, is detected in low and nearly constant concentrations during a 7 h period following the administration (Grant et al., 1981). Thus, the clinical effects of ketamine has been ascribed to the parent compound, whereas the metabolite has been considered inactive. However, following p.o. administration, where ketamine due to a very high first pass hepatic metabolism (Reich and Silvay, 1989; Kharasch and Labroo, 1992) only appears at low concentrations in the blood, the analgesic effect remains practically similar to that obtained after an i.m. injection (Grant et al., 1981). It has therefore been suggested that norketamine may have some analgesic properties (Grant et al., 1981; Bushnell and Craig, 1995). [^{3}H]MK-801 binding and electrophysiological experiments in the rat cortical wedge preparation and the hemisected neonatal rat spinal cord preparation clearly supports this idea (Table 1 and Figs. 1 and 2). (*S*)-Norketamine is approximately five times weaker than (*S*)-ketamine in both binding assay and functional experiments. Therefore following p.o. administration of ketamine, (*S*)-norketamine is present in the blood at sufficiently high micromolar concentrations to account for the analgesic activity of ketamine. Quite interesting all compounds seem to display some degree of selectivity between cortex and spinal cord (Table 1). In agreement with earlier results for methadone and ketobemidone (Ebert et al., 1995), the ketamine and norketamine enantiomers have lower affinity for the spinal cord NMDA receptor than the cortical NMDA receptor.

The Hill slope of (*S*)-norketamine in the cortical preparation is 0.8 as compared to 1.4 for the ketamine enantiomers and (*R*)-norketamine in the cortex and spinal cord. This difference in the Hill slope might be the result of interaction with other receptor systems as, for example, the μ- or κ-opioid receptor or the muscarinic receptor. In order to rule out these possibilities we characterized (*RS*)-ketamine and (*RS*)-norketamine with respect to opioid receptor affinities. [^{3}H](D-Ala^2, *N*-Me-Phe^4, Gly-ol^5)enkephalin (DAMGO), [^{3}H](D-Ala^2, D-Leu^5)enkephalin (DADL) and [^{3}H](+)-(5a,7a,8b)-*N*-methyl-*N*[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzenacetamide (U69593) binding (as described by Kristensen et al., 1994) was used for measuring the affinities for μ-, ∂- and κ-receptors, respectively; IC_{50} values were well above 100 μM, which was the largest concentration used (data not shown). We further tested the muscarinic antagonists pirenzepine, quinuclidylbenzilate and atropin as antagonists of NMDA responses in the rat cortical wedge preparation. None of these compounds at 1 mM concentrations showed any significant effects on the responses to NMDA (data not shown) indicating that muscarinic antagonists in the rat cortical wedge preparation does not indirectly interact with the NMDA receptor system. The lower Hill slope of (*S*)-norketamine in the rat cortical wedge preparation can therefore not be explained based on these experiments. However, as the inhibition curves for MK-801 and PCP in cortex have Hill slopes close to 1, this indicates, in agreement with earlier experiments (Harrison and Simmonds, 1985), that even highly selective non competitive NMDA receptor antagonists may appear like competitive antagonists in the rat cortical wedge preparation.

Only a few studies on the pharmacokinetics of (*S*)-ketamine and (*S*)-norketamine following p.o., i.m. or i.v. administration have been published (Eide et al., 1995; Qureshi et al., 1995; Malinowsky et al., 1996), therefore, it is still unclear if sufficient concentrations of (*S*)-norketamine can be obtained following p.o. administration (Grant et al., 1981). However, based on the earlier report on the analgesic activity of (*RS*)-ketamine following p.o. administration this is very likely.

We therefore conclude that the main metabolite of (*S*)-ketamine, (*S*)-norketamine, acts as a weak non-competitive NMDA receptor antagonist in the rat cortex and spinal cord. Following oral administration of (*RS*)-ketamine, (*S*)-norketamine will be present in human plasma at sufficiently high concentrations to account for some of the observed analgesic activity. Clinical studies are now under way to investigate whether orally administrated (*S*)-norketamine may have fewer side effects than (*S*)-ketamine.

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