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Neurochemistry International 62 (2013) 258–264

Contents lists available at SciVerse ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Ketamine prevents seizures and reverses changes in muscarinic receptor induced by bicuculline in rats

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ARTICLE INFO

Article history:
Received 19 July 2012
Received in revised form 27 November 2012
Accepted 12 December 2012
Available online 29 December 2012

Keywords:
Ketamine
Bicuculline
Experimental epilepsy
Muscarinic receptors
Seizures

ABSTRACT

The cholinergic system has been implicated in several experimental epilepsy models. In a previous study bicuculline (BIC), known to antagonize GABA-A postsynaptic receptor subtype, was administered to rats at subconvulsant (1 mg/kg) and convulsant (7.5 mg/kg) doses and quinuclidinyl benzilate ([^{3}H]-QNB) binding to CNS membranes was determined. It was observed that ligand binding to cerebellum increases while it decreases in the case of hippocampus. Saturation binding curves showed that changes were due to the modification of receptor affinity for the ligand without alteration of binding site number. The purpose of this study was to assay muscarinic receptors employing other BIC dose (5 mg/kg), which induces seizures and allows the analysis of a postseizure stage as well. To study further muscarinic receptor involvement in BIC induced seizures, KET was also employed since it is a well known anticonvulsant in some experimental models. The administration of BIC at 5 mg/kg to rats produced a similar pattern of changes in [^{3}H]-QNB binding to those recorded with 1.0 and 7.5 mg/kg doses. Here again, changes were observed in receptor binding affinity without alteration in binding site number for cerebellum or hippocampus membranes. Pretreatment with KET (40 mg/kg) prevented BIC seizures and reverted [^{3}H]-QNB binding changes induced by BIC administration. The single administration of KET invariably resulted in [^{3}H]-QNB binding decrease to either cerebellar or hippocampal membranes. KET added *in vitro* decreased ligand binding likewise. Results of combined treatment with KET plus BIC are hardly attributable to the single reversion of BIC effect since KET alone invariably decreased ligand binding. It is suggested that besides alteration of cholinergic muscarinic receptor other(s) neurotransmitter system(s) may well also be involved.

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1. Introduction

Experimental evidence indicates a relationship between epileptogenicity and the alteration of some neurotransmitter systems. Changes in levels of GABA, glutamate and noradrenaline, as well as in their receptor or enzymes, lead to appearance of seizure activity (Roberts, 1986).

Acetylcholine is an essential neurotransmitter/neuromodulator in several experimental epilepsy disorders and its ability to regulate neuronal excitability in neocortex is well known (Wasterlain et al., 1986). Seizures may be induced by direct stimulation of the central cholinergic system, such as the administration of acetylcholine itself, its enhancers or analogues, as well as acetylcholinesterase inhibitors, and may be abolished by certain cholinergic antagonists (Emson, 1978).

GABA is the major central inhibitory neurotransmitter, and it is not surprising, therefore, that it has been involved in epileptic activity genesis (Ribak, 1991; Meldrum, 1994; Meldrum, 1995; Wasterlain and Chen, 2008). The biosynthesis of GABA requires the activity of glutamic acid decarboxylase (GAD), a cytosolic enzyme that is found in neurons where GABA is a neurotransmitter (Olsen and Betz, 2006). Decreased GABA receptors in hippocampus are associated with behavioral deficit in experimental epilepsy (Mathew et al., 2011).

The development of several models of experimental epilepsy in animals have helped in the study of action mechanisms of epileptic drugs as well as to disclose the specific brain areas capable to induce convulsions (Rubio et al., 2010).

Convulsant 3-mercaptopropionic acid, a GAD inhibitor (Sprince et al., 1969), alters not only GABA synthesis (Rodríguez de Lores Arnaiz et al., 1972, 1973) but also the binding of muscarinic antagonist quinuclidinyl benzilate ([^{3}H]-QNB) to CNS membranes, suggesting the involvement of muscarinic receptor in seizure activity (Schneider et al., 1992; Schneider and Rodríguez de Lores Arnaiz, 1997, 2000).

After administration to rats of bicuculline (BIC), a GABA-A receptor antagonist (Wasterlain and Chen, 2008), at subconvulsant (1 mg/kg) and convulsant (7.5 mg/kg) doses, it was observed that

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0197-0186/$ - see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.neuint.2012.12.013

[^{3}H]-QNB binding to membranes increases in cerebellum but decreases in hippocampus (Schneider and Rodríguez de Lores Arnaiz, 2006).

The purpose of this study was to assay muscarinic receptors employing other BIC dose (5 mg/kg), which induces seizures and allows the analysis of a postseizure stage. To study further muscarinic receptor involvement in BIC induced seizures, anesthetic KET was also employed since it is a well known anticonvulsant in some experimental models (Velisková et al., 1990; Fujikawa, 1995). Results obtained indicated significant changes in [^{3}H]-QNB binding to cerebellar and hippocampal membranes after administration of BIC and KET, either separately or in combined treatment.

2. Methods

2.1. Animals and drug administration

Adult young male Wistar rats (30–45 days old) weighing 100–150 g were used. Animals caged in groups of five were housed at constant temperature (20–23 °C) and maintained at least one week in a 12 h light–dark cycle (from 9.00 a.m. to 9.00 p.m.) with free access to food and water. Rats were injected intraperitoneally (i.p.) between 9.30 and 11.30 a.m.

All studies described for animal experimentation from NIH/USA were conducted in accordance with the Guide for Care and Use of Laboratory Animals provided by the National Institutes of Health, U.S.A. and the Committee on Animal Experimentation (CICUAL), Facultad de Medicina, Universidad de Buenos Aires.

2.2. Drugs

Bicuculline (BIC), ketamine (KET) and atropine sulfate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. L-[^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) was from Du Pont Corp. New England Nuclear, Boston, MA, U.S.A, specific activity 14,443 GBq/mmol. BIC was dissolved in 0.1 N HCl and brought to pH 5 with 0.1 N NaOH and immediately injected. KET was dissolved in redistilled water.

2.3. Treatments and experimental groups

The following experimental groups were used: control, BIC seizure, BIC postseizure, 5, 20 and 40 mg/kg KET with or without BIC. Every experimental condition was repeated 3–6 times. Uninjected rats (naive) were used to serve as controls because vehicle solution administration produce no change in [^{3}H]-QNB binding to CNS membranes (data not shown).

2.3.1. BIC treatment

BIC was injected i.p. to groups of five rats at the dose of 5 mg/kg, which produced generalized tonic–clonic seizures between 1.3–2.0 min after injection. Animals were decapitated during seizure (seizure stage) or at 16 min after injection (postseizure stage).

2.3.2. KET + BIC administration

Groups of five rats were injected i.p. with 5, 20 and 40 mg/kg KET; thirty minutes later animals received i.p. 5 mg/kg BIC. Each combined treatment was repeated 3 times. Animals receiving KET + BIC were decapitated 30 min after BIC administration.

2.3.3. KET administration

Groups of five rats were injected i.p. with 5, 20 and 40 mg/kg KET and decapitated 30 min later. These treatments failed to produce behavioral changes. Each treatment was repeated 4 times.

2.4. Membrane preparations

In each experiment, for BIC treated rats, cerebellum, hippocampus, cerebral cortex and striatum from five animals each time were harvested and separately pooled. For KET + BIC, and KET treated rats, as well as for *in vitro* assays, cerebellum and hippocampus from five animals each time were harvested and separately pooled. Tissues were rapidly homogenized at 10% w/v, except for cerebral cortex at 4% w/v, in 0.32 M sucrose neutralized with Tris base solution (0.4 mM Tris final concentration) in a Teflon glass Potter–Elvehjem homogenizer with 0.25 mm clearance.

Homogenates were centrifuged at 900×*g* for 10 min and pellets discarded; resulting supernatants were diluted with 0.16 M sucrose to achieve a final concentration of 0.25 M sucrose, centrifuged at 100,000×*g* for 30 min. Membrane pellets were stored at −70 °C and use within 40 days after preparation without change in ligand binding.

2.5. [^{3}H]-QNB binding assay

[^{3}H]-QNB binding was determined according to the method described by Yamamura and Snyder (Yamamura and Snyder, 1974) with slight modifications. Membrane pellets were resuspended and later diluted in 50 mM sodium, potassium phosphate buffer (pH 7.4) to reach 0.1 mg protein/ml concentration. Triplicate membrane aliquots were incubated (2 ml final volume) at 30 °C in the presence of 0.5 nM L-[^{3}H]-QNB with or without 5 μM atropine sulfate. When indicated, 1×10^{-7}–1×10^{-3} M KET was included. Incubation proceeded for 60 min, because [^{3}H]-QNB binding reached equilibrium after 45 min incubation (Schneider and Rodríguez de Lores Arnaiz, 2006).

After incubation, 3 ml of ice-cold sodium, potassium phosphate buffer were added and samples vacuum-filtered through Whatman GF/B glass disks. Filters were washed twice with 3 ml of ice-cold buffer, placed in plastic vials and dried overnight at 70 °C. To each vial, 3 ml of ml of OptiPhase "Hisafe" were added and radioactivity quantified in a liquid scintillation counter.

Specific binding was calculated as the difference between the binding in the absence and presence of 5 μM atropine sulfate, and represented *ca* 90% of total binding.

Results averaged were obtained with different membranes isolated from tissue pooled (from 5 rats each group). The whole experiment (administration of drugs, tissue harvesting, membrane preparation and binding assay in triplicate) was carried out in 3 or 7 different occasions.

For saturation studies, cerebellar and hippocampal membranes were incubated in the presence of [^{3}H]-QNB ranging from 0.125 to 2.000 nM concentration and processed as described above.

To study *in vitro* KET effect samples of cerebellar and hippocampal membranes from control rats were incubated at 30 °C for 30 min in the presence of 1×10^{-7} M, 1×10^{-5} M, 1×10^{-4} M and 1×10^{-3} M KET and processed for [^{3}H]-QNB binding.

Protein was assayed according to Lowry et al. (Lowry et al., 1951) using bovine serum albumin as standard.

2.6. Data analysis

Data are presented as mean values. To determine statistical significance of difference *versus* control, Student's *t* test was employed. For comparison of different groups *inter se* statistical significance of differences was assessed by one way ANOVA followed by Newman Keuls Multiple Comparisons Test. Significance levels were set at $P < 0.05$.

For saturation assays, non-linear regression of the data were processed using EBDA program (G.A. Mc Pherson 1983 V 2.0). Scat-

chard transformation of the data was employed to show whether more than one receptor population was operative.

3. Results

3.1. Effect of BIC administration

Intraperitoneal rat injection of 5 mg/kg BIC resulted in the development of generalized tonic–clonic seizures as recorded by visual observation. After 90–120 s latency, all rats suddenly ran amok (during 10–30 s) and interrupt its normal exploratory or grooming behavior, briefly crawl on the bottom of the cage. Then, animals stop with its head propped on its forelimbs, staying motionless (1–10 s), regained tonus (30–45 s) and ended with a four-limb clonic phase (90–150 s).

Present experiments were performed with animals decapitated at the onset of seizure (seizure stage) or at 14–16 min after seizure period (postseizure stage).

[^{3}H]-QNB binding to cerebellar membranes was approximately 0.30 pmol/mg protein, a value which increased 19% and 22% at seizure and postseizure stages, respectively. Binding to hippocampal membranes was 1.54 pmol/mg protein and resulted decreased 12% and 21% at seizure and postseizure stages, respectively. In striatal membranes [^{3}H]-QNB binding was 2.04 pmol/mg protein and enhanced 11% and 8% at seizure and postseizure stages, respectively. In cerebral cortical membranes, binding was 1.17 pmol/mg protein and remained unaltered by BIC administration (Table 1).

3.2. Saturation studies

In order to determine whether binding changes observed in cerebellum and hippocampus were due to modifications in affinity and/or site number, [^{3}H]-QNB binding was studied at variable ligand concentrations. Saturation values were attained with 0.50–1.00 nM ligand in membranes obtained either from control rats or from rats injected with 5 mg/kg BIC (Figs. 1 and 2).

Scatchard analysis of [^{3}H]-QNB binding data recorded at equilibrium in membranes obtained from rats at seizure and postseizure stages after BIC administration were carried out. In cerebellum, significant decreases of 35–38% for K_d value were observed. At variance, hippocampus K_d value increased 49% and 21% at seizure and postseizure, respectively. No change in receptor site number was recorded in either area. Hill number was close to unity and remained unaltered in both cerebellar and hippocampal membranes (Table 2).

Equilibrium binding data for [^{3}H]-QNB obtained at several ligand concentrations were studied by computer analysis to disclose whether just a single or two population sites were operative; it was observed that the best fit indicated a single population site.

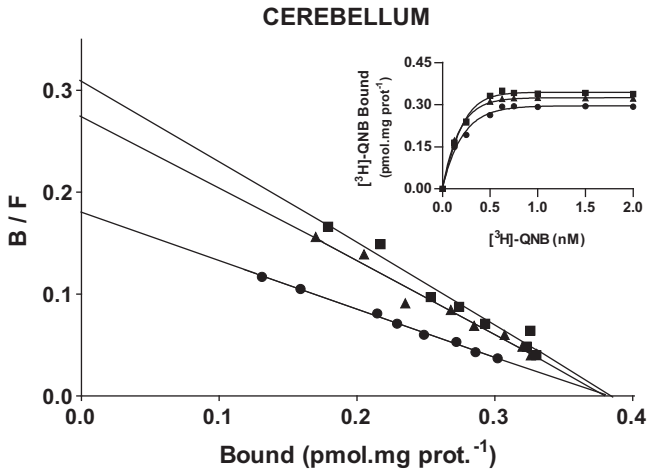

Fig. 1. Scatchard plots for [^{3}H]-QNB binding to rat cerebellar membranes at equilibrium after administration of 5.0 mg/kg BIC. Results are from a single experiment representative of a set of three, each performed in duplicate. Inset, saturation curves. (●) Control; (▲) BIC seizure; (■) BIC postseizure.

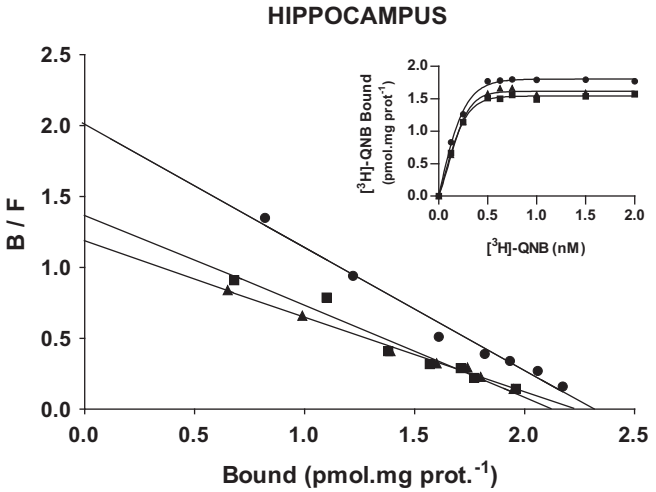

Fig. 2. Scatchard plots for [^{3}H]-QNB binding to rat hippocampal membranes at equilibrium after administration of 5.0 mg/kg BIC. Results are from a single experiment representative of a set of three, each performed in duplicate. Inset, saturation curves. (●) Control; (▲) BIC seizure; (■) BIC postseizure.

3.3. Effect of KET plus BIC administration

Combined treatments with BIC and KET were carried out. Administration of 40 mg/kg KET prevented epileptic seizures induced by 5 mg/kg BIC in all injected animals. After injection of 5 and 20 mg/kg KET, followed by 5 mg/kg BIC, some of the animals

Table 1
[^{3}H]-QNB binding to CNS membranes after the administration of 5 mg/kg BIC.

Area	[^{3}H]-QNB binding Condition							
	Control, pmol mg protein^{-1}		Seizure, pmol mg protein^{-1}		Δ (%)	Postseizure, pmol mg protein^{-1}		Δ (%)
Cerebellum	0.30 ± 0.03	(7)	0.36 ± 0.03*	(7)	+19	0.37 ± 0.03*	(7)	+22
Hippocampus	1.54 ± 0.13	(5)	1.35 ± 0.08*	(5)	−12	1.22 ± 0.16*	(5)	−21
Striatum	2.04 ± 0.03	(4)	2.27 ± 0.06*	(4)	+11	2.20 ± 0.05*	(4)	+8
Cerebral cortex	1.17 ± 0.03	(3)	1.07 ± 0.06	(3)	−8	1.18 ± 0.07	(3)	–

Values are means ± S.D. Figures in parentheses denote number of separate experiments performed in triplicate.
* $P < 0.05$ *versus* control, by Student's *t* test.

Table 2
[^{3}H]-QNB binding constants in cerebellar and hippocampal membranes after the administration of 5 mg/kg BIC.

Area	Condition	K_d (pM)	Δ (%)	B_{max} (pmol mg protein^{-1})	N_H
Cerebellum	Control	204.0 ± 6.5		0.37 ± 0.02	1.00 ± 0.01
	Seizure	133.0 ± 4.6	−35	0.37 ± 0.02	1.02 ± 0.05
	Postseizure	127.0 ± 4.0	−38	0.38 ± 0.02	0.99 ± 0.04
Hippocampus	Control	126.1 ± 10.1		2.38 ± 0.11	1.02 ± 0.05
	Seizure	188.6 ± 0.8	+49	2.26 ± 0.04	0.97 ± 0.05
	Postseizure	152.3 ± 1.3	+21	2.21 ± 0.13	1.05 ± 0.04

For each experiment, cerebellum and hippocampus from five rats were pooled, membranes separated and [^{3}H]-QNB binding performed. Data from three experiments were processed to calculate constants. Results (mean values ± S.E.M.) were analyzed by one way ANOVA followed by Newman–Keuls Multiple Comparison Test.
Cerebellum: Control *versus* seizure $P < 0.001$; Control *versus* postseizure $P < 0.001$; Seizure *versus* postseizure $P > 0.05$. Hippocampus: Control *versus* seizure $P < 0.001$; Control *versus* postseizure $P < 0.01$; Seizure *versus* postseizure $P < 0.001$.

exhibited minimal seizures characterized by head and forelimb tonic muscle contractions, with postural control preservation.

Administration of KET at 5, 20 and 40 mg/kg dose, followed by 5 mg/kg BIC, resulted in 15%, 28% and 37% binding decrease in cerebellum membranes, respectively. All differences were statistically significant *versus* control and *inter se*, showing a dose-dependent effect (Fig. 3A). These treatments resulted in 27%, 35% and 45% binding increase in hippocampal membranes. Differences *versus* control were all statistically significant but were dose-dependent only between 5 and 40 mg/kg KET doses (Fig. 3B).

3.4. Effect of KET administration

In order to test whether KET had an effect *per se* on binding, additional experiments were performed. Visual observation of rats after administration of ketamine at doses up to 40 mg/kg indicated that animal behavior did not differed from those of control animals.

It was observed that ligand binding to cerebellar and hippocampal membranes remained unaltered after 5 mg/kg KET, whereas a decrease in binding to cerebellar membranes which attained approximately 18% and 25% after 20 mg/kg and 40 mg/kg KET, respectively. In general, differences between groups were not statistically significant, except for 40 mg/kg KET dose *versus* control ($P < 0.05$) (Fig. 4A). In hippocampal membranes, the single administration of 20 mg and 40 mg/kg KET decreased 26% and 32% binding, respectively. Differences between doses were only statistically significant between 5 mg/kg and 20 mg/kg doses, indicating a partial dose-dependent effect (Fig. 4B). No changes *versus* control were recorded after the single administration of 5 mg/kg KET for either area.

3.5. In vitro KET effect

To analyze potential effect of KET *in vitro*, samples of cerebellar and hippocampal membranes from control rats were incubated at 30 °C for 30 min in the presence of 1×10^{-7} M, 1×10^{-5} M, 1×10^{-4} M and 1×10^{-3} M KET and processed for [^{3}H]-QNB binding. It was observed that addition of KET at 1×10^{-7} M and 1×10^{-5} M concentration failed to alter ligand binding whereas at 1×10^{-4} M and 1×10^{-3} M concentrations decreased ligand binding 36% and 67% for cerebellum and 32% and 60% for hippocampus membranes, both respectively. Differences between effective doses were dose-dependent (Fig. 5A and B).

4. Discussion

[^{3}H]-QNB binding to CNS membranes was determined after i.p. administration to rats of 5 mg/kg convulsant BIC. Significant increases and decreases in ligand binding were recorded for cerebellum and hippocampus, respectively, whereas minor or no changes were observed in the case of striatum or cerebral cortex. Ligand binding alteration correlated with receptor affinity modification. Pretreatment with KET (40 mg/kg) prevented BIC seizures and reverted [^{3}H]-QNB binding changes induced by BIC administration. The single administration of KET resulted in [^{3}H]-QNB binding decrease either to cerebellar or to hippocampal. Differences between doses were only statistically significant between 5 mg/kg and 20 mg/kg doses, indicating a partial dose-dependent effect. KET added *in vitro* decreased ligand binding likewise.

Administration to rats of BIC at 5 mg/kg dose induced seizures and also allowed the analysis of a postseizure stage. [^{3}H]-QNB binding to cerebellum and hippocampus membranes were significantly increased and decreased by this treatment, respectively. Kinetic studies carried out at equilibrium disclosed changes in ligand receptor affinity both in cerebellum and hippocampus, without alteration in receptor number sites. Hill number was close to unity and remained unaltered in both cerebellar and hippocampal membranes. These findings are in line with those already recorded after BIC administration at 1 mg/kg (subconvulsant) and 7.5 mg/kg (convulsant) doses (Schneider and Rodríguez de Lores Arnaiz, 2006).

KET is neuroprotective when administered after the onset of epilepticus status (Fujikawa, 1995; Martin and Kapur, 2008; Hsieh et al., 2010), is able to control prolonged status epilepticus (Borris et al., 2000; Cunha et al., 2009) and reduces lethality and generalized tonic–clonic seizures induced by lidocaine (Guler et al., 2005). Synergistic action of diazepam and KET in terminating status epilepticus was demonstrated, suggesting that KET-diazepam combination might be a clinically useful therapeutic option for the treatment of refractory status epilepticus (Martin and Kapur, 2008).

In order to prevent BIC seizure, KET at 5, 20 and 40 mg/kg doses was administered. Results obtained indicated that KET is also effective to prevent seizure induced to rats by 5 mg/kg BIC.

The effect of BIC administration on [^{3}H]-QNB binding was modified by pretreatment with KET. Therefore, KET not only prevented seizure development but also reversed the action of BIC on ligand binding, suggesting an interaction at central muscarinic receptor.

Present results indicated that the single administration of KET resulted in [^{3}H]-QNB binding decrease to cerebellar and to hippocampal membranes. These findings may suggest that the treatment interferes with further interaction of QNB to membranes isolated from central nervous system. Taking into consideration that QNB is an antagonist muscarinic ligand, the postulation that KET behaves as an antagonist at muscarinic receptor level seems tenable. In support, this drug inhibits the response to cholinergic agonist acetyl-beta-methylcholine. The IC50 for this effect is 5.7 μM, a value within the clinically relevant KET concentration range (Durieux, 1995). Most interesting, KET *in vitro* concentrations here required to decrease ligand binding to cerebellum and hippocampus membranes are not far from this value (Fig. 5). Besides, it is

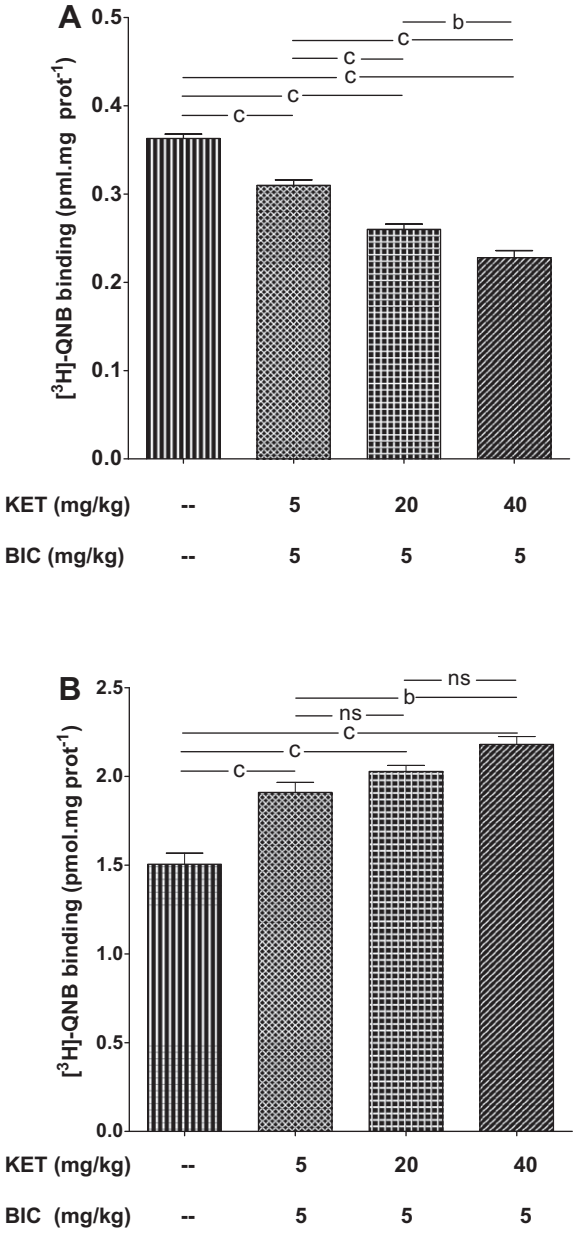

Fig. 3. [^{3}H]-QNB binding to rat brain membranes after combined treatment with BIC and KET. Rats were injected i.p. with 5, 20 or 40 mg/kg KET and 30 min later, with 5 mg/kg BIC. Thirty minutes after BIC, animals were decapitated, tissue harvested and processed for binding assay. Results are expressed as pmoles [^{3}H]-QNB bound per mg membrane protein and are mean values (±S.E.M) from 4 separate experiments. (A) cerebellar membranes and (B) hippocampal membranes. $^{b}P < 0.01$; $^{c}P < 0.001$; $^{ns}P > 0.05$ by one way ANOVA followed by Newman–Keuls Multiple Comparison Test.

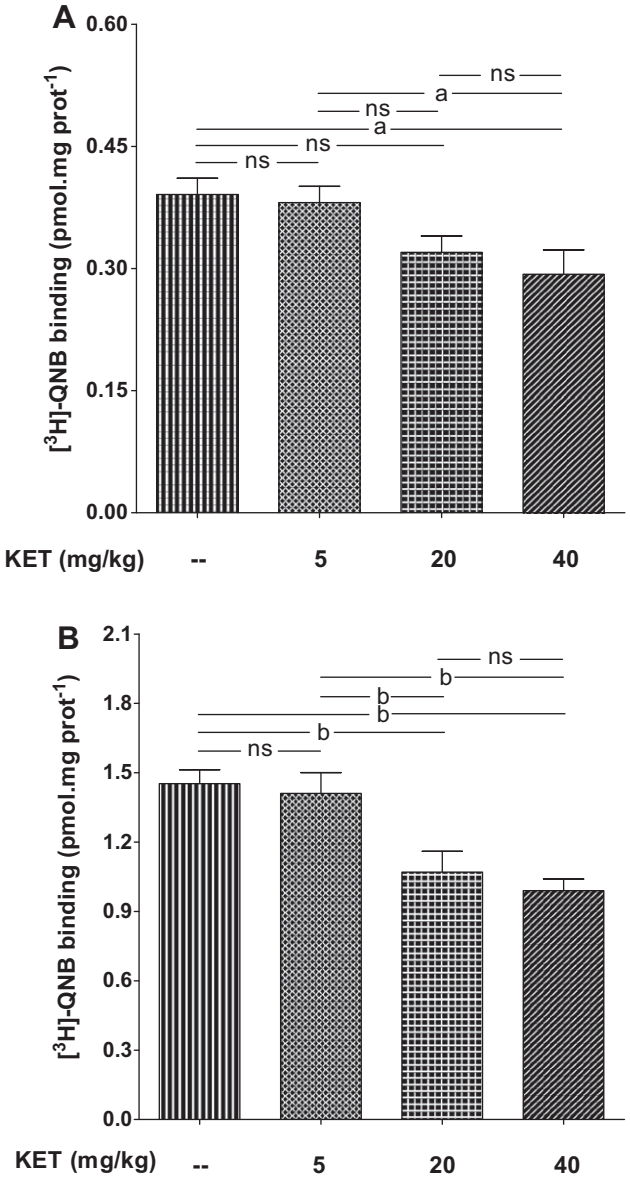

Fig. 4. [^{3}H]-QNB binding to rat brain membranes after administration of KET. Rats were injected i.p. with 5, 20 or 40 mg/kg KET and 30 min later animals were decapitated, tissue harvested and processed for binding assay. Results are expressed as pmoles [^{3}H]-QNB bound per mg membrane protein and are mean values (± S.E.M) from 4 separate experiments. (A) cerebellar membranes and (B) hippocampal membranes. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{ns}P > 0.05$ by one way ANOVA followed by Newman–Keuls Multiple Comparison Test.

known that KET inhibits muscarinic signaling and the suggestion that this effect may well explain some of the anticholinergic clinical effects of KET recorded both at central and peripheral level has been advanced (Durieux, 1995).

KET produces "dissociative anesthesia" and amnesia. Its side effects include saliva, tears, bronchial secretions, as well as spontaneous movement with increase of muscular tone (Ivani et al., 2003; Evers et al., 2006). These effects may well be explained through an interaction with muscarinic cholinergic receptors at peripheral nervous system. In this regard, our results point to a relationship between KET and the cholinergic system. With respect to KET effects on seizure activity, it may enhance or decrease seizure (Modica et al., 1990).

Present results which show that KET modifies [^{3}H]-QNB binding to CNS membranes may provide a demonstration that this agent is able to alter muscarinic receptor at CNS.

The single administration of KET invariably decreased binding to cerebellum and hippocampus membranes, perhaps acting as a muscarinic receptor antagonist. However, this hypothesis is hardly tenable on analyzing hippocampus data when KET was combined with BIC. Indirect effects are most likely occurring, since diverse actions at other neurotransmitter systems were described for KET.

It is known that at CNS diverse neurotransmitter systems are affected by KET as mentioned below. KET is an arilciclohexilamine, congener of phencyclidine, which acts as an antagonist for glutamate NMDA receptor for its binding to receptor phencyclidine site and exerts potent anesthetic effect (Evers et al., 2006). It should be recalled that NMDA receptor is involved in nociceptive modula-

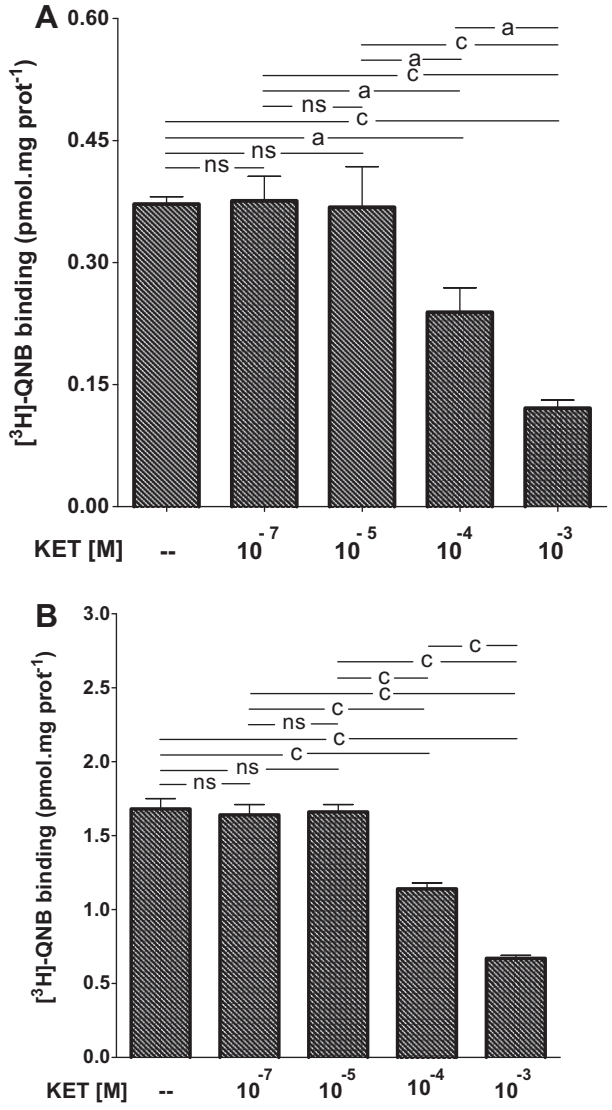

Fig. 5. [^{3}H]-QNB binding to rat brain membranes in the presence of KET. Cerebellum and hippocampus from control rats were harvested and processed for binding assay with or without KET. Results are expressed as pmol [^{3}H]-QNB bound per mg membrane protein and are mean values (±S.E.M) from 4 separate experiments. (A) cerebellar membranes, and (B) hippocampal membranes. $^{a}P < 0.05$; $^{c}P < 0.001$; $^{ns}P > 0.05$ by one way ANOVA followed by Newman–Keuls Multiple Comparison Test.

tion, in the wind-up phenomenon, in peripheral receptive field expansion, in hyperalgesia and neuronal plasticity (Ivani et al., 2003). The effects of KET on the electroencephalogram and in the open field in rats of a genetic model of generalized absence epilepsy were studied. The pattern of changes recorded suggested that KET action cannot be explained by a simple blockade of the NMDA receptor (Midzyanovskaya et al., 2004).

Other neurotransmitter systems including sigma receptor, GABA-A–benzodiazepine-chloride channel complex and GABA-B receptors seem involved in KET protective effect against maximal electroshock test (Manocha et al., 2001). A relationship between KET and GABA system is operative, since some of its effects (i.e. the enhancement in brain blood flow and intracranial pressure) are attenuated by administration of benzodiazepines (Belopavlovic and Buchthal, 1982), indicating the involvement of GABA-A receptor (Evers et al., 2006). Such relationship may also involve the cholinergic systems, since KET reduces striatal GABA release induced by cholinergic over stimulation (Grasshoff et al., 2005).

At present it is not possible to assess whether KET actions after its administration are direct or in-direct ones. However, *in vitro* addition of ketamine on assaying ligand [^{3}H]-QNB binding in control CNS membranes, a direct interaction most likely takes place. Results showed dose-dependent, statistically significant decreases in ligand binding to cerebellum and hippocampus membranes (Fig. 5A and B). This finding suggested a direct interaction of ketamine at the cholinergic muscarinic receptor.

One of the reasons for the medical interest to deep in KET action mechanism is due to its effectiveness in patients who have been refractory to standard analgesic medication regimens (Bell et al., 2003; Annetta et al., 2005). However, it is not largely employed as a general anesthetic in humans due to its undesirable psychic actions, adverse events, neurobiological and behavioral effects even at subanesthetic doses (Rowland, 2005).

It was observed that both muscimol and diazepam, respectively agonists for GABA-A and benzodiazepine receptors, potentiate KET-induced anesthesia. Neither benzodiazepine receptor antagonist flumazenil nor GAD inhibitor allylglycine affect anesthetic activity of KET, suggesting that KET exerts GABA-A receptor agonist properties (Irifune et al., 2000).

KET prevented tonic–clonic seizures induced by bicuculline. This result entirely agree with that of Velisková et al. (1990) who also employed Wistar rats injected with 40 mg/kg KET 30 min before bicuculline. At variance, it has been reported that KET prevents tonic but not clonic seizures (Irifune et al., 2000). This discrepancy is attributable to differences in the methodology used.

Because BIC is an antagonist for GABA-A receptor (Wasterlain and Chen, 2008), protection of BIC seizure by pretreatment with KET here described may be explained as an interaction at GABA-A receptor. Reversion of BIC effect on ligand binding seems to indicate an interaction at cholinergic muscarinic receptor likewise.

It seems that KET may have a particular role in the management of neurophatic and/or pain syndromes in patients that are poorly responsive to opioids. Most interestingly, haloperidol or benzodiazepines are effective in minimizing KET psychomimetic side effects (Fitzgibbon and Viola, 2005). This indicates that KET mechanism of action is not restricted to NMDA receptor and to GABA system, involving the dopaminergic system likewise.

Together with MDMA (3,4-methylenedioxymethamphetamine), flunitrazepam and GHB (γ-HO butyrate), KET is one of the four club drugs which are more commonly used at nightclubs, music festivals, raves and dance parties to enhance social intimacy and sensory stimulation. Severe drug reactions can occur even with small doses of club drugs. CNS stimulation or depression may occur, as well as hyperthermia, hypertension, rhabdomyolisis and serotonine syndrome (Gahlinger, 2004). Although short-term physiological toxicity and adverse behavioral effects of club drugs GHB, KET, MDMA and Rohypnol (flunitrazepam) were determined (Gable, 2004), the knowledge about the mechanism of action is not entirely understood. Since the club drugs are frequently used, both for human health and for prevention and treatment of drug addiction, a better knowledge of basic mechanisms involved in club drugs is desirable. Our results showing an interaction between KET and muscarinic receptor may provide additional information to elucidate its mechanism of action at CNS.

5. Conclusions

The administration of convulsant BIC altered [^{3}H]-QNB binding to cerebellum and hippocampus membranes. The changes implied alteration in muscarinic receptor affinity. KET prevented the development of seizure and reverted ligand binding changes produced by BIC. In a general sense, findings observed after either BIC alone or KET plus BIC treatments were area-dependent. Results of com-

bined treatment are not in fact only attributable to reversion of BIC effect since KET alone invariable decreased ligand binding. It is suggested that combined effects on GABA-A and muscarinic receptors are operative.

Acknowledgments

G.R. de L.A. is chief investigator from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). This study was supported by grants from CONICET and Universidad de Buenos Aires, Argentina.

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