J Anesth (1995) 9:271–275

Journal of
Anesthesia

Dose-dependent effects of repeated ketamine administration on muscarinic acetylcholine receptors in the mouse forebrain

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Abstract: To study the effects of repeated ketamine administration (0: saline, 12.5, 25, and 50 mg·kg^{-1} every 3 days for a total of five times, subcutaneously) on the central muscarinic acetylcholine receptors (mAchRs), receptor binding assays of mAchR were carried out in the forebrain of mice, using [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) as a ligand. We also examined whether repeated ketamine administration could modify the sensitivity to scopolamine (0.5 mg·kg^{-1}) (a muscarinic antagonist). Repeated ketamine administration produced a significant increase in the receptor density values (Bmax) for [^{3}H]QNB (1520 ± 51 fmol·mg protein^{-1} for the control group, 1650 ± 43 for the 12.5 mg·kg^{-1} group, 1966 ± 70 for the 25 mg·kg^{-1} group, and 2064 ± 125 for the 50 mg·kg^{-1} group) ($P < 0.05$, when the 25 mg·kg^{-1} and 50 mg·kg^{-1} groups were compared to the control group) without any change in apparent affinity. Repeated ketamine reduced scopolamine-induced hyperlocomotion at 50 mg·kg^{-1} ($P < 0.05$). We conclude that repeated ketamine administration produces up-regulation of mAchRs, which is probably associated with the altered Ach transmission of the central nervous system.

Key words: Ketamine, Muscarinic acetylcholine receptors, Forebrain, Up-regulation, Behavior sensitivity to scopolamine

Introduction

Ketamine, a dissociative anesthetic, is a useful anesthetic agent characterized by rapid onset of action, strong analgesic properties and a wide margin of safety. However, it is known to produce psychostimulant actions such as hyperlocomotion or memory impairment by activation of dopaminergic transmission in rodents [1,2]. Recently, much attention has been focused on the interaction between the acetylcholine (Ach) and the dopaminergic systems in the striatum [3,4]. Dopamine is known to inhibit the release of Ach through presynaptic muscarinic acetylcholine receptors (mAchRs). In addition, the anesthetic action of ketamine is believed to be antagonism of the *N*-methyl-D-aspartate (NMDA) receptors. Several classes of the NMDA antagonists are known to reduce Ach release [5]. Ketamine may alter the regulatory mechanisms of mAchRs by inhibiting presynaptic Ach release, especially if ketamine is chronically or repeatedly administered.

We have previously reported that repeated ketamine administration produced an up-regulation of mAchRs in the forebrain, and that no notable changes were observed in any other lesions studied (the brainstem and the cerebellum) [6]. Therefore, in this study of the dose-dependent effects of repeated ketamine administration on the mAchRs, a quantitative investigation of mAchR in the mouse forebrain was carried out using [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB), a specific muscarinic antagonist, as a labeled ligand for receptor binding assays.

Scopolamine increases locomotor activity due to the activation of the dopaminergic system through the antagonistic action on the central muscarinic receptors [3,7]. Therefore, we also examined whether repeated ketamine administration could modify the sensitivity to scopolamine using a behavioral pharmacological technique.

Materials and methods

This experimental protocol was approved by the Animal Care and Use Committee of Gunma University School of Medicine.

Animals

Five-week-old male ddY mice (Japan Laboratory Animals, Tokyo), weighing 26–30 g, were used. The animals

Address correspondence to: S. Hitomi
Received for publication on September 26, 1994; accepted on February 21, 1995

were housed in groups of 10 in aluminum cages and were given free access to a solid diet (MF, Oriental Yeast, Tokyo Japan) and tap water. The breeding room was controlled to maintain a light-dark cycle with a light period between 6:00 and 18:00. The temperature was kept constant at 23° ± 2°C. The animals were injected with either saline or ketamine (12.5, 25, or 50 mg·kg^{-1}) subcutaneously. This treatment was repeated every 3 days for a total of five times. Ketamine was dissolved in physiological saline, and the administration volumes of ketamine and saline were fixed at 0.1 ml·10 g body weight^{-1}. In the case of the saturation binding assays, this drug treatment was performed between 11:00 and 12:00 using 10 mice for each group. The behavioral experiments were carried out between 9:00 and 15:00 using 20 mice for each group. Four groups of 10 mice each were separately housed throughout the study.

Chemicals

[^{3}H]QNB (specific activity 1217 GBq·mmol^{-1}) was obtained from Du Pont/NEN Research Products (Boston, MA, USA). Ketamine HCl, atropine sulfate, and scopolamine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The protein assay kits were obtained from Bio-Rad Laboratories (Richmond, CA, USA).

Experimental procedures for the [^{3}H]QNB binding assays

Mice were sacrificed by decapitation 24 h after the final administration of saline or ketamine. The forebrain was quickly dissected and homogenized in 10 volumes of ice-cold 0.32 M sucrose using a Potter-Elvehjem glass homogenizer fitted with a teflon pestle. After centrifugation for 10 min at 900 × g, the pellet was discarded and the supernatant was centrifuged with the same volume of ice-cold 0.32 M sucrose for 20 min at 11 500 × g. The pellet was rinsed and homogenized, and then centrifuged for 20 min at 11 500 × g with ice-cold 50 mM potassium phosphate buffer (pH 7.4). The procedure was repeated, and the pellet was washed twice with ice-cold 50 mM potassium phosphate buffer (pH 7.4). The final membrane pellet was stored at −80°C until the time of the assay.

Protein concentrations were determined according to the method of Bradford, using bovine plasma γ-globulin as a standard [8].

Saturation binding of [^{3}H]QNB was carried out as previously described [6]. Membrane fractions (50 μg of protein) were mixed with 2 ml of 50 mM potassium phosphate buffer (pH 7.4), containing various concentrations of muscarinic ligands. After incubation at 30°C for 1 h, the samples were filtered through Whatman GF/C filters (Maidspone, Ingland), and immediately washed three times with 3 ml of ice-cold 50 mM potassium phosphate buffer (pH 7.4). The filters were placed in plastic minivials, dried, and 3 ml of the scintillation cocktail Reaflor was added. The radioactivity was measured using an Aloca 650 liquid scintillation counter (Pittsburgh, PA, USA). Specific binding was defined as the difference between binding in the absence and in the presence of 1 μM atropine.

The data was subjected to a computer-assisted nonlinear regression analysis (Delta Graph Pro 3 Delta Point, CA, USA). Values of receptor density (Bmax) and affinity (Kd) were obtained by Scatchard analysis of the binding data [9].

Behavioral pharmacological study

The ambulatory activity of each mouse was measured using a tilting-type ambulometer (AMB, O'Hara, Tokyo). Each mouse was placed in a Plexiglas activity cage and was allowed 30 min to adapt to the cage, and then the ambulatory activity was measured for 1.5 h after the administration of ketamine (0: saline, 12.5, 25, and 50 mg·kg^{-1}) at 3-day intervals for a total of five times. One day after the fifth administration of ketamine or saline, each mouse was challenged with a subcutaneous administration of scopolamine (0.5 mg·kg^{-1}), and the ambulatory activity was then monitored for 1.5 h in each mouse. The basal ambulatory activity was measured for 1.5 h before the scopolamine challenge.

Statistical analysis

The data are expressed as mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by group comparisons using Scheffe's F-test. For analysis of the locomotor activities produced by repeated ketamine administration, a repeated measures ANOVA was used. A P value less than 0.05 was considered significant (Statview II, Version 4.0, Adacus, CA, USA).

Results

Saturation binding assays

Repeated ketamine administration increased the Bmax values in a dose-dependent way The Bmax values (fmol·mg protein^{-1} ± SEM) were as follows: 1520 ± 51 for the control group, 1650 ± 43 for the 12.5 mg·kg^{-1} group, 1966 ± 70 for the 25 mg·kg^{-1} group, and 2064 ± 125 for the 50 mg·kg^{-1} group. Repeated ketamine administration produced significant increases in the Bmax values at 25 mg·kg^{-1} or 50 mg·kg^{-1} compared to the control group ($P < 0.05$, by Scheffe's F-test). There were no

significant changes in Kd values (defined as the reciprocal of the dissociation constant) (Table 1).

Behavioral pharmacological study

As described elsewhere [10], ketamine increased the mouse's ambulatory activity in a dose-dependent way with ataxia (defined as staggering gait but a normal righting reflex) [$F(3, 304) = 75.2$, $P < 0.0001$). The repeated administration of ketamine enhanced its ambulation-increasing action [$F(4, 304) = 30.5$, $P < 0.001$]. The ambulation-increasing effects persisted for approximately 40–50 min. In addition, there was a significant interaction between dose and administration [$F(12, 304) = 4.3$, $P < 0.001$]. One day after the administration of ketamine, the mouse's ambulatory activity returned to the control value (Table 2). Administration of a scopolamine challenge ($0.5\,mg \cdot kg^{-1}$) increased the mouse's ambulation activity by approximately sevenfold. Repeated ketamine administration reduced this scopolamine-induced hyperlocomotion in a dose-dependent way [$F(3, 76) = 8.0$, $P = 0.001$] (Table 2). In addition, individual comparisons by Scheffe's F-test revealed a significant reduction in scopolamine-induced hyperlocomotion at $50\,mg \cdot kg^{-1}$ of ketamine ($P < 0.05$).

Discussion

We previously described how repeated ketamine administration produced a consecutive up-regulation of mAchRs only in the forebrain [6]. In the present study, we have demonstrated a dose-dependent effect of repeated ketamine administration on the mAchRs. Drug-induced up-regulation of mAchR such as by antidepressants or antimuscarinic drugs is found in the cerebral cortex, but not in either the brainstem or the cerebellum [3,6,11]. Therefore, we focused our study only on the forebrain in the present study.

QNB is known to be a specific muscarinic antagonist common to all muscarinic subtypes. No significant differences were observed in the Kd values for [^{3}H]QNB binding. These results were in accordance with our previous administration-time relationship study, in which the fourth and the fifth repeated administration of ketamine ($25\,mg \cdot kg^{-1}$) increase the binding sites of [^{3}H]QNB [6]. These results suggest that the binding characteristics of mAchRs are similar between the control and the ketamine groups, although the total number of [^{3}H]QNB binding sites were markedly increased at 25 and $50\,mg \cdot kg^{-1}$ (Table 1).

Ketamine is a well-known noncompetitive antagonist of NMDA receptors [12–15], which is believed to be closely linked to ketamine's anesthetic action. Ketamine has also direct and/or indirect effects on the other neurotransmitters, such as the dopaminergic [1], noradrenergic [1], serotonergic [16], cholinergic [6,17], and GABA-ergic systems [18]. It has been widely accepted that transmitter receptors "cross-talk" with each other and interact with other receptors via several second messenger systems [19–21]. The most likely reasons for this up-regulation are due to the neurotransmitter receptors cross-talk mechanisms via numerous second messenger cascades [22,23]. Our recent study has showed that ketamine impaired learning in a passive avoidance task caused by a stimulation of dopamine D2 receptors in mice [2]. Irifune et al. have also demonstrated that ketamine has an indirect dopaminergic agonistic action [1]. The dopaminergic and NMDA systems have pivotal roles in the modulation of the presynaptic Ach release in the striatum [4,5]. In fact ketamine is known to inhibit Ach release in the striatum of rodents through the dopaminergic activating action and/or the antagonistic action on the NMDA receptors [5]. Presyn-

Table 1. Effects of repeated ketamine administration on receptor density (Bmax) and affinity (Kd) values for [^{3}H]QNB in the forebrain

Dose of ketamine ($mg \cdot kg^{-1}$)	Bmax ($fmol \cdot mg^{-1} \cdot prot^{-1}$)	Kd (pM)
0 (saline)	1520 ± 51	16.1 ± 0.7
12.5	1650 ± 43	16.2 ± 1.0
25	1966 ± 70*	16.9 ± 0.5
50	2064 ± 125*	16.6 ± 0.6

Values are the means ± SEM of 8 independent Scatchard plots.
*$P < 0.05$ vs. saline-treated group (Scheffe's F-test).

Table 2. Effects of scopolamine challenge ($0.5\,mg \cdot kg^{-1}$) on the ambulatory activity after repeated ketamine administration

Dose of ketamine ($mg \cdot kg^{-1}$)	Ambulatory activities (counts /1.5 h)		%increase of activity
	Saline	Scopolamine	
0 (saline)	152 ± 23	1017 ± 87	705 ± 61
12.5	166 ± 31	1228.3 ± 111	743 ± 77
25	144 ± 19	814 ± 100	564 ± 69
50	144 ± 26	610 ± 72*	423 ± 50*

Each value indicates mean ± SEM.
*$P < 0.05$ vs. saline-treated group (Scheffe's F-test).

aptic inhibition of the Ach release is known to produce up-regulation of mAchRs in vivo and in vitro [19]. In addition, recent reports have shown that several classes of the competitive and noncompetitive NMDA antagonists maintain their actions relatively long [24]. Therefore, it is strongly suggested that up-regulation of mAchRs observed in this study was responsible for the dopaminergic activating action and/or the NMDA receptor antagonist action of ketamine.

Muscarinic acetylcholine receptors are genetically classified into five subtypes, but are pharmacologically classified into three subtypes, i.e., M1, M2, and M3 [25–27]. These three subtypes have a distinct localization, and are linked to distinct second messenger systems. Although the aim of the present study was not to determine subtype-specific changes in mAchRs, the abundance of M1-receptors in the forebrain (more than 80% of total number of mAchRs) [6,21,28] implies that the up-regulation of mAchRs in the forebrain caused by repeated ketamine administration might be associated with increases in the M1-receptors. Further study is necessary to clarify the subtype-specific changes of mAchRs and the resultant changes in the second messenger cascade systems.

Both ketamine and scopolamine increase the ambulatory activity of rodents via activation of the dopaminergic systems in the nucleus accumbens [3,7]. However, they are known to activate the dopaminergic systems by different mechanisms. In addition, the effects of repeated administration of these drugs are different. As shown in Fig. 1, repeated ketamine administration enhanced the locomotion-increasing effects, while repeated scopolamine administration is known to reduce the locomotion-increasing effects due to up-regulation of mAchRs [7]. Although it is unclear that ketamine-induced hyperlocomotion is responsible for NMDA antagonism, scopolamine potentiates dopamine release in the nucleus accumbens, presumably by inhibiting the presynaptic muscarinic heteroreceptors, which modulate the release of neurotransmitters other than Ach, for example dopamine [4,7]. Therefore, when the number of mAchRs is increased in the forebrain which contains the nucleus accumbens, the scopolamine-induced hyperlocomotion should be reduced. This hypothesis is consistent with the results obtained in the present study; that is, repeated ketamine administration reduced the scopolamine-produced hyperlocomotion in a dose-related way, and this change was significant at the highest dose of ketamine ($50\,mg \cdot kg^{-1}$) (Table 2). These results suggest that repeated ketamine administration was associated with the altered Ach transmission in the central nervous system (Table 2). The fact that up-regulation by $25\,mg \cdot kg^{-1}$ did not result in a significant change in the behavioral effect of scopolamine could be due to the difference in the sensitivities of the experiment. In addition, several classes of NMDA receptor antagonists,

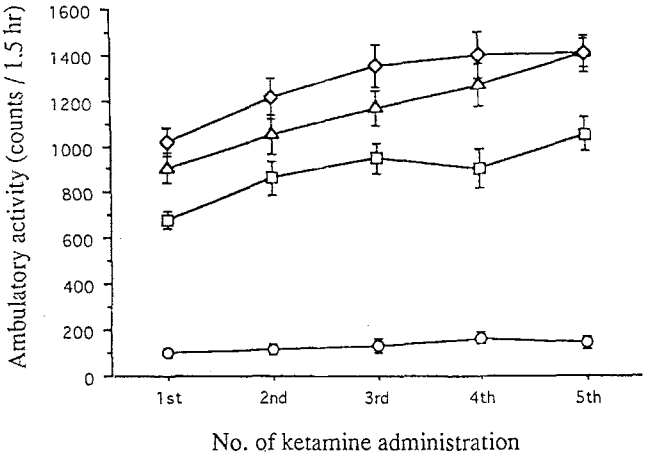

Fig. 1. Effects of repeated ketamine administration on ambulatory activity. *Open circles,* saline; *open squares,* $12.5\,mg \cdot kg^{-1}$; *open triangles,* $25\,mg \cdot kg^{-1}$; *open diamonds,* $50\,mg \cdot kg^{-1}$. Values are the mean ambulatory activities with SEM. Twenty mice were used for each group

including ketamine, are known to be toxic to the cholinergic and GABA-nergic neurons in the forebrain [29]. Therefore, the cholinergic neurons may affect Ach transmission after repeated ketamine administration.

In conclusion, repeated ketamine produced an up-regulation of mAchR in a dose-dependent way in the forebrain. This up-regulation was associated with altered Ach transmission in the central nervous system. These phenomena may be adaptive changes of mAchRs and may not always reflect the deleterious side effects during repeated ketamine administration. However, several classes of NMDA antagonists, including ketamine, are known to produce morphological damage in neurons in the cerebral cortex, but this can be prevented by diazepam and barbiturates [29]. Because of the NMDA receptor antagonism of ketamine, recent reports indicate that ketamine may be useful for the prevention of anoxic damage from stroke in humans [18]. Ketamine may be used as an agent for clinical neuro-resuscitation or for anesthesia in neurosurgery. In addition, it is used as a repeat anesthetic agent in several clinical conditions, for example, in children undergoing radiotherapeutic procedures, and for anesthesia in difficult circumstances, such as patients with burns [30,31]. In these clinical situations, ketamine should be used in combination with other agents, such as barbiturates and/or benzodiazepines.

Acknowledgments. The authors are very grateful to Prof. Tatsushi Fujita (Department of Anesthesiology and Reanimatology, Gunma University School of Medicine) for his encouragement and advices. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture (no. 05671248).

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