

Ketamine Inhibits Monoamine Transporters Expressed in Human Embryonic Kidney 293 Cells

Mitsuhiro Nishimura, M.D.,* Kohji Sato, M.D., Ph.D.,† Tomoya Okada, M.D.,‡ Ikuto Yoshiya, M.D., Ph.D.,§ Patrick Schloss, Ph.D.,|| Shoichi Shimada, M.D., Ph.D.,† Masaya Tohyama, M.D., Ph.D.#

Background: Ketamine has been characterized as having psychotomimetic and sympathomimetic effects. These symptoms have raised the possibility that ketamine affects monoaminergic neurotransmission. To elucidate the relation between ketamine and monoamine transporters, the authors constructed three cell lines that stably express the norepinephrine, dopamine, and serotonin transporters and investigated the effects of ketamine on these transporters.

Methods: Human embryonic kidney cells were transfected using the Chen-Okayama method with the human norepinephrine, rat dopamine, and rat serotonin transporter cDNA subcloned into the eukaryotic expression vector. Using cells stably expressing these transporters, the authors investigated the effects of ketamine on the uptake of these compounds and compared them with those of pentobarbital.

Results: Inhibition analysis showed that ketamine significantly inhibited the uptake of all three monoamine transporters in a dose-dependent manner. The K_i (inhibition constant) values of ketamine on the norepinephrine, dopamine, and serotonin transporters were $66.8 \mu\text{M}$, $62.9 \mu\text{M}$, and $162 \mu\text{M}$, respectively. Pentobarbital, a typical general anesthetic agent with no psychotic symptoms, did not affect the uptake of monoamines, however. Further, neither the glycine transporter 1 nor the glutamate/aspartate transporter was affected

by ketamine, indicating that ketamine preferentially inhibits monoamine transporters.

Conclusions: Ketamine inhibited monoamine transporters expressed in human embryonic kidney cells in a dose-dependent manner. This result suggests that the ketamine-induced inhibition of monoamine transporters might contribute to its psychotomimetic and sympathomimetic effects through potentiating monoaminergic neurotransmission. (Key words: Dopamine; neurotransmission; norepinephrine; pentobarbital; serotonin; uptake.)

KETAMINE is known to have psychotomimetic and sympathomimetic effects.¹ For example, recovery from anesthesia with ketamine is associated with a variety of central nervous system excitatory phenomena, including restlessness, agitation, increased motor activity, screaming, crying, and visual hallucinations.¹ These various symptoms have been explained through many different mechanisms. First, ketamine has been reported to act as a noncompetitive antagonist of the N-methyl-D-aspartate (NMDA) receptor through binding to the phencyclidine binding site, leading to the blockade of glutamatergic neurotransmission.^{2,3} In addition, many other systems, such as the opioidergic, monoaminergic systems also have been postulated to be the targets of ketamine anesthesia.^{4,5}

Enhancement of the monoaminergic transmission by ketamine has been widely reported.^{3,6-8} It has been speculated that this enhancement is related to two observations. First, the blockade of NMDA receptors suppresses GABAergic neurons, which mediate tonic inhibition of monoaminergic neurons, resulting in the increase of monoamines in the extracellular space.^{3,8} Second, ketamine directly inhibits high-affinity monoamine uptake systems.^{6,7,9}

Recently, monoamine (norepinephrine, dopamine, and serotonin) transporters have been cloned, and their pharmacologic characteristics showed that they are responsible for high-affinity monoamine uptake systems.¹⁰⁻¹⁴ The cloning of these genes enabled us to study the effects of ketamine on each monoamine trans-

* Research Fellow, Departments of Anesthesiology and of Anatomy and Neuroscience, Osaka University Medical School.

† Associate Professor, Department of Anatomy and Neuroscience, Osaka University Medical School.

‡ Research Fellow, Department of Anatomy and Neuroscience, Osaka University Medical School.

§ Professor, Department of Anesthesiology, Osaka University Medical School.

|| Associate Professor, Abteilung Neurochemie, Max-Planck-Institut.

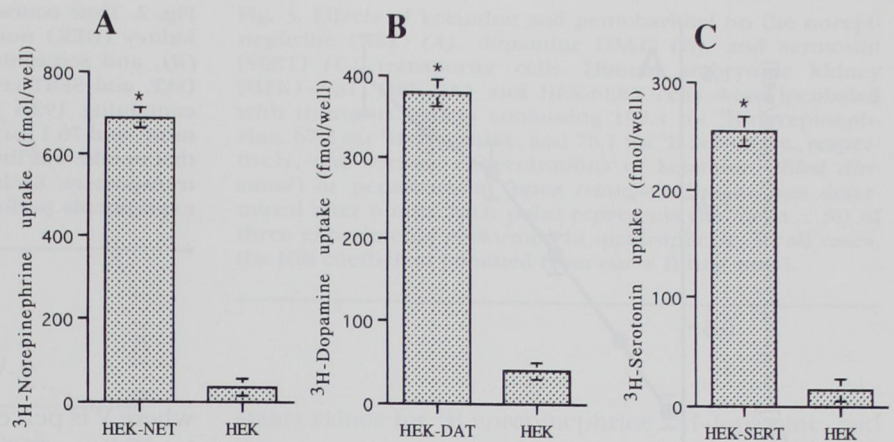
Professor, Department of Anatomy and Neuroscience, Osaka University Medical School.

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Address reprint requests to Dr. Nishimura: Department of Anesthesiology, Osaka University Medical School, 2-2 Yamadaoka Suita Osaka 565 Japan.

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Fig. 1. Ligand uptake into human embryonic kidney (HEK) norepinephrine (NET), dopamine (DAT), and serotonin (SERT) transporter cells and HEK cells (control). The cells were incubated with transport buffers containing 192.4 nM ^3H -norepinephrine, 67.2 nM ^3H -dopamine, or 76.1 nM ^3H -serotonin. Uptake was determined after 6 min. Each point represents the mean \pm SD of two experiments performed in quadruplicate. * $P < 0.01$.



porter directly. Thus, we constructed cell lines that stably express monoamine transporters and investigated the effects of ketamine on them.

Materials and Methods

Materials

DL-Ketamine and pentobarbital were purchased from Sigma Chemical Co. (St. Louis, MO) and Tokyokasei Chemical Co. (Tokyo, Japan), respectively. ^3H -Norepinephrine, ^3H -dopamine, ^3H -serotonin, ^{14}C -glycine, and ^{14}C -glutamate, with specific activities of 10.4, 31.0, 26.3, 106.8, and 261.6 Ci/mmol, respectively, were purchased from New England Nuclear (Boston, MA).

Construction of Human Embryonic Kidney Cells that Stably Express Transporters

Human embryonic kidney (HEK 293) cells were transfected using the Chen-Okayama method with the human norepinephrine transporter (NET), rat dopamine transporter (DAT), rat serotonin transporter (SERT), mouse glycine transporter 1 (GLYT1), and bovine glutamate/aspartate transporter (GLAST) complementary deoxyribonucleic acids (cDNAs) subcloned into the eukaryotic expression vectors pBK/CMV, which contain the gene for geneticin resistance (Stratagene).^{15,16} The human NET cDNA clone (pNET) was a gift from Dr. Susan Amara (Oregon Health Sciences University, OR). The rat cDNAs for the DAT and SERT were cloned by Shimada *et al.*¹¹ and Schloss and Betz,¹⁷ respectively. Twenty-four hours after the transformation, geneticin (1 mg/ml) was added into Dulbecco's Modified Eagle Medium (Gibco-Brl, NY; DMEM)/10% fetal calf serum

and selection was done for 4 weeks. Cells that developed geneticin resistance were used for further experiments. We designated these cells as HEK-NET, HEK-DAT, HEK-SERT, HEK-GLYT1, and HEK-GLAST cells.

Uptake Assay

For uptake measurements, HEK-NET, HEK-DAT, HEK-SERT, HEK-GLYT1, and HEK-GLAST cells were plated into 24-well dishes. After 48-h incubation, these cells reached confluence. At this point, the culture medium was removed and the cells were washed once with 0.2 ml transport buffer (125 mM NaCl, 2 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, pH 7.5). After washing, 0.2 ml transport buffer containing ^3H -norepinephrine, ^3H -dopamine, ^3H -serotonin, ^{14}C -glycine, or ^{14}C -glutamate was applied into each well to yield final concentrations of 192.4 nM, 67.2 nM, 76.1 nM, 1.85 μM , and 1.53 μM , respectively. After incubation at room temperature for 6 min, the solution was removed by suction and the cells were washed twice with the transport buffer and then extracted with 0.4 ml of 10% (weight/vol) sodium dodecyl sulfate. Radioactivity was determined by scintillation counting using a Beckman (Fullerton, CA) LS600011C scintillation counter. The specific ^3H -monoamine uptakes were determined by taking the difference between the uptake values measured in HEK-NET, DAT, or SERT and those in untransfected HEK cells. Three independent experiments, each done in quadruplicate, were performed. Inhibition curves were fitted (Cricket Graph III, Computer Associates International Inc., New York) to determine the IC_{50} (50% inhibitory concentration) value and the Hill coefficient, which were estimated using nonlinear iterative curve fitting to a sigmoid function.

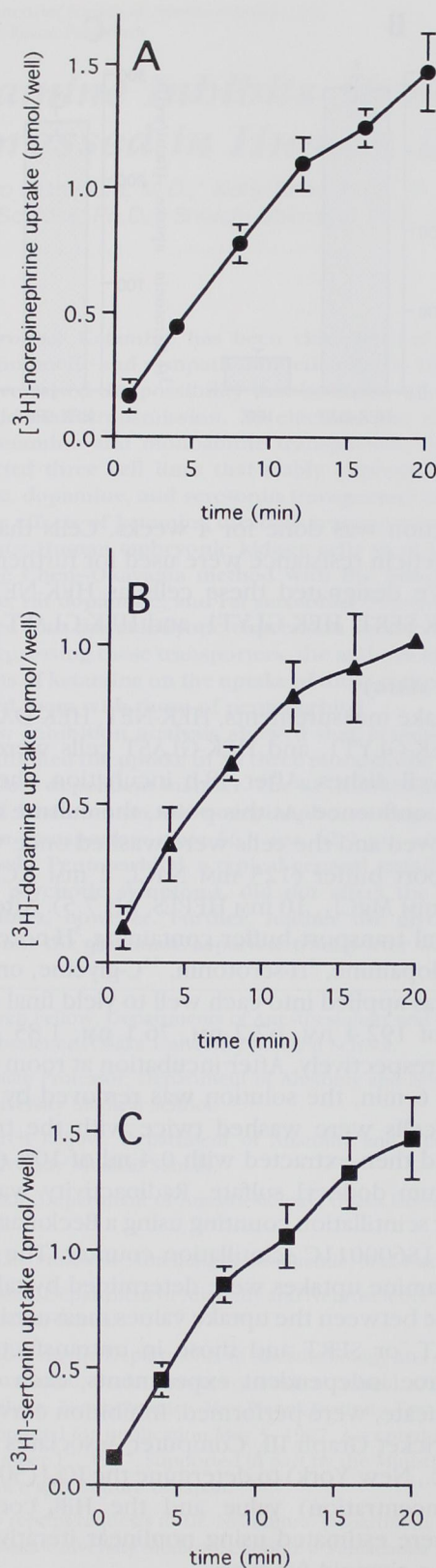


Fig. 2. Time course of ligand uptake into human embryonic kidney (HEK) norepinephrine (NET) (A), dopamine (DAT) (B), and serotonin (SERT) (C) transporter cells. HEK-NET, DAT, and SERT cells were incubated with transport buffers containing 192.4 nM [³H]-norepinephrine, 67.2 nM [³H]-dopamine, and 76.1 nM [³H]-serotonin, respectively. It is noteworthy that uptake was linear, at least until 8 min, for all monoamine transporters. Each point represents the mean \pm SD of two experiments performed in quadruplicate.

$$V = 100 \times I^h / (I^h + C^h)$$

where V is percent control of uptake, I is the IC₅₀ value, h is Hill coefficient, and C is the concentration of ketamine. Then we converted the IC₅₀ values to K_i (inhibition constant) values using the Cheng and Prusoff equation:

$$K_i = IC_{50} / (1 + [L]/K_m)$$

where [L] is the radioligand concentration of norepinephrine, dopamine, or serotonin.

Statistics

To test significantly higher uptake for HEK-NET, DAT, SERT, GLYT1, and GLAST compared with untransfected HEK cells, an unpaired *t* test was performed. A probability value <0.05 was considered statistically significant. All data are presented as mean \pm SD.

Results

Human Embryonic Kidney Cells Express Functional Monoamine Transporters

To assess the ability of HEK-NET, HEK-DAT, and HEK-SERT cells to take up the corresponding molecules, we performed uptake assay. HEK-NET, HEK-DAT, and HEK-SERT cells showed approximately 15, 14, and 23 times higher uptake ($P < 0.01$) than control HEK cells, respectively (figs. 1A–C).

To confirm the linearity of the monoamine transporter uptakes within 6 min, we investigated the time course of uptake. Figure 2 clearly shows that uptake was linear, at least until 8 min, at the same monoamine concentration as used in the uptake assay for each transporter. Therefore, in the subsequent experiments, uptake was routinely determined after incubation for 6 min at room temperature.

Competition studies with increased concentration of unlabeled substrate revealed that K_m (Michaelis con-

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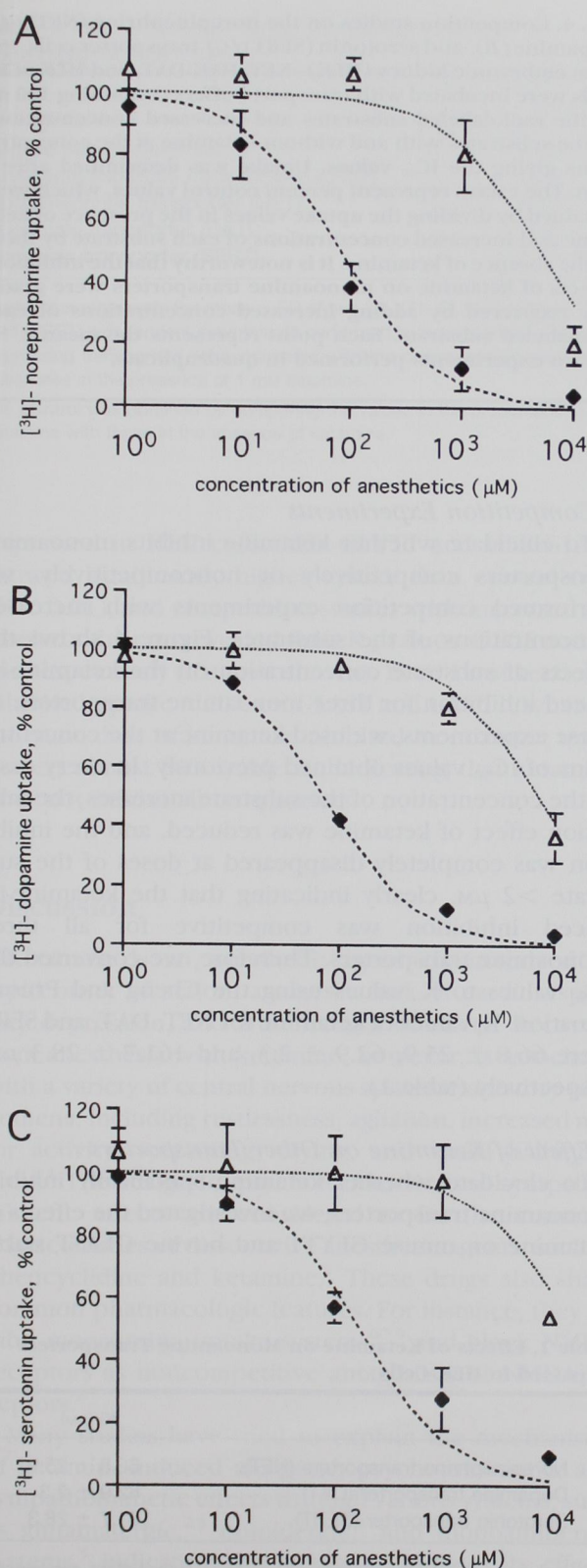


Fig. 3. Effects of ketamine and pentobarbital on the norepinephrine (NET) (A), dopamine (DAT) (B), and serotonin (SERT) (C) transporter cells. Human embryonic kidney (HEK)-NET, HEK-DAT and HEK-SERT cells were incubated with transport buffers containing 192.4 nM ³H-norepinephrine, 67.2 nM ³H-dopamine, and 76.1 nM ³H-serotonin, respectively, and various concentrations of ketamine (filled diamond) or pentobarbital (open triangle). Uptake was determined after 6 min. Each point represents the mean \pm SD of three experiments performed in quadruplicate. In all cases, the Hill coefficient obtained from curve fitting was 1.

stant) values for ³H-norepinephrine, ³H-dopamine, and ³H-serotonin were approximately 864 nM, 1,406 nM, and 1,170 nM, respectively (data not shown). These values are similar to those reported before,^{10,13,18} showing that these three cell lines express the corresponding functional transporters.

Effect of Ketamine on the Norepinephrine Transporter

To investigate the effect of ketamine on NET, we added various concentrations of ketamine into the uptake buffer (the transport buffer containing 192.4 nM ³H-norepinephrine) and performed inhibition assay using HEK-NET cells. The uptake of tracer was not inhibited at 1 μM ketamine; however, at doses >1 μM, ketamine significantly inhibited the uptake in a dose dependent manner, reaching maximum inhibition at \approx 1 mM (fig. 3A). The IC₅₀ value was 81.5 ± 31.6 μM (n = 3). In contrast, pentobarbital showed inhibition only at doses >100 μM (fig. 3A).

Effect of Ketamine on the Dopamine Transporter

Using HEK-DAT cells, we investigated the effect of ketamine on DAT. Ketamine inhibited DAT at dose >1 μM. At 1 mM, ketamine inhibited it almost completely (fig. 3B). The IC₅₀ value was 66.0 ± 2.4 μM (n = 3). Even at 1 mM, pentobarbital showed only 20% inhibition (fig. 3B).

Effect of Ketamine on the Serotonin Transporter

When compared with the aforementioned two transporters, ketamine showed weaker effect on SERT in HEK-SERT cells. Inhibition was observed at doses >10 μM, however, and maximal inhibition was reached at 10 mM (fig. 3C). The IC₅₀ value was 171.4 ± 30 μM (n = 3). Pentobarbital had almost no effect at doses <1 mM (fig. 3C).

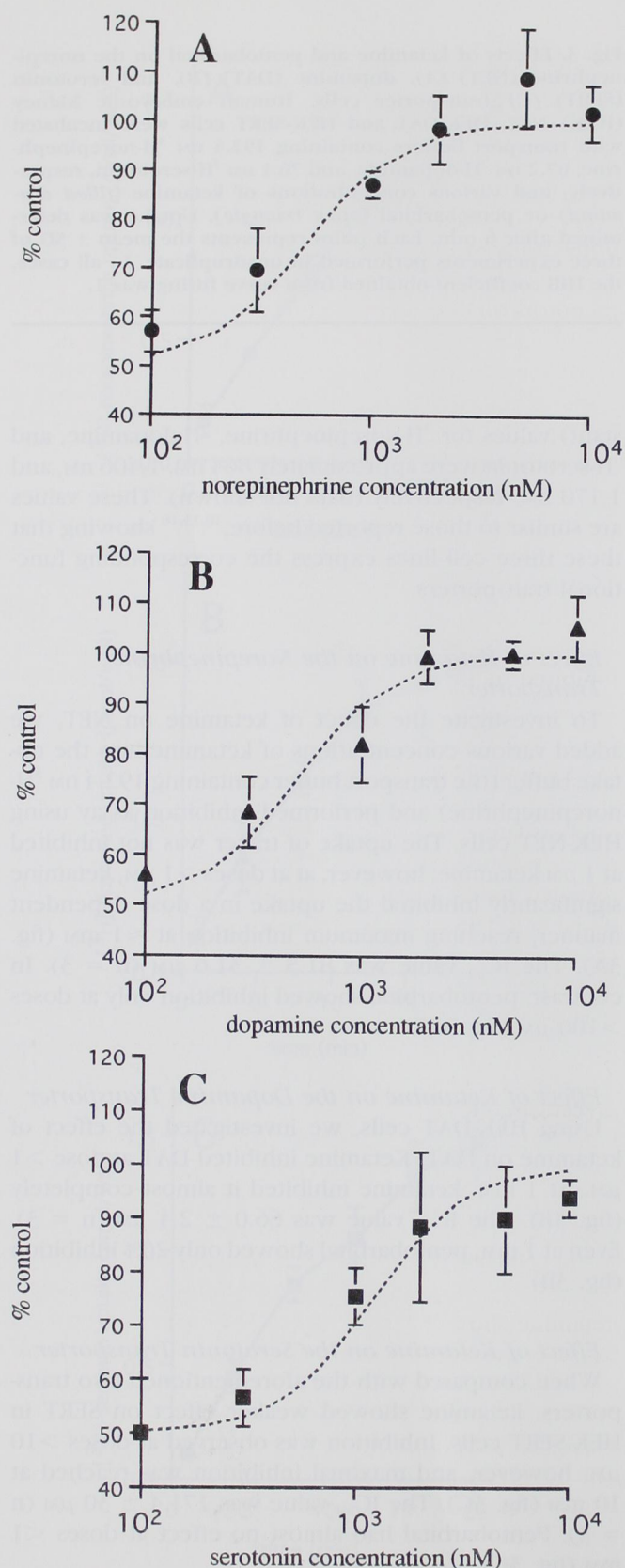


Fig. 4. Competition studies on the norepinephrine (NET) (A), dopamine (B), and serotonin (SERT) (C) transporter cells. Human embryonic kidney (HEK)-NET, HEK-DAT, and HEK-SERT cells were incubated with transport buffers containing 100 nM of the radiolabeled substrates and increased concentrations of the substrates with and without ketamine at the concentrations giving the IC_{50} values. Uptake was determined after 6 min. The y axes represent percent control values, which were obtained by dividing the uptake values in the presence of ketamine and increased concentrations of each substrate by those in the absence of ketamine. It is noteworthy that the inhibitory effects of ketamine on monoamine transporters were gradually recovered by adding increased concentrations of each nonlabeled substrate. Each point represents the mean \pm SD of two experiments performed in quadruplicate.

Competition Experiments

To elucidate whether ketamine inhibits monoamine transporters competitively or noncompetitively, we performed competition experiments with increased concentrations of the substrates. Figure 4 shows the effects of substrate concentration on the ketamine-induced inhibition for three monoamine transporters. In these experiments, we used ketamine at the concentrations of IC_{50} values obtained previously. In every case, as the concentration of the substrate increases, the inhibition effect of ketamine was reduced, and the inhibition was completely disappeared at doses of the substrate $>2 \mu M$, clearly indicating that the ketamine-induced inhibition was competitive for all three monoamine transporters. Therefore, we converted the IC_{50} values to K_i values using the Cheng and Prusoff equation. K_i values of ketamine for NET, DAT, and SERT were 66.8 ± 25.9 , 62.9 ± 2.3 , and $161.7 \pm 28.3 \mu M$, respectively (table 1).

Effect of Ketamine on Other Transporters

To elucidate whether ketamine specifically inhibits monoamine transporters, we investigated the effects of ketamine on mouse GLYT1 and bovine GLAST stably

Table 1. Effects of Ketamine on Monoamine Transporters Expressed in HEK Cells

	K_i (μM)
Norepinephrine transporters (NET)	66.8 ± 25.9
Dopamine transporter (DAT)	62.9 ± 2.3
Serotonin transporter (SERT)	161.7 ± 28.3

Each value represents the mean \pm SD of three independent experiments performed in quadruplicate.

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Table 2. Effects of 1 mM Ketamine on Transporters Expressed in HEK Cells

	% Control*
Glycine transporter 1 (GLYT1)	81.3 ± 6.9
Glutamate/aspartate transporter (GLAST)	96.4 ± 7.4
Norepinephrine transporter (NET)	20.8 ± 4.0
Dopamine transporter (DAT)	12.2 ± 2.3
Serotonin transporter (SERT)	27.7 ± 4.4

Each value represents the mean ± SD of two (GLYT1, GLAST) or three (NET, DAT, SERT) independent experiments performed in quadruplicate. HEK cells expressing transporters were incubated with the corresponding radioactive substrates in the presence of 1 mM ketamine.

* % Control was obtained by comparing the values in the presence of 1 mM ketamine with those in the absence of ketamine.

expressed in HEK cells. HEK-GLYT1 and HEK-GLAST showed significantly higher uptake than control cells ($P < 0.01$), and competition studies with increased unlabeled substrate revealed their K_m values of 805 and 70 μM , respectively (data not shown). Even 1 mM ketamine inhibited uptake $< 20\%$ on GLAST and GLYT1, whereas the same dose of ketamine inhibited monoamine transporters almost 80% (table 2), indicating that ketamine does not inhibit all transporters.

Discussion

Ketamine is an anesthetic agent characterized by rapid onset of action, analgesia, lack of cardiorespiratory depression, and wide margin of safety. Recovery from anesthesia with ketamine, however, is associated with a variety of central nervous system excitatory phenomena, including restlessness, agitation, increased motor activity, screaming, crying, and visual hallucinations.¹ The analgesic, psychotomimetic, and sympathomimetic effects are considered to be the common characteristics of dissociative anesthetic agents, such as phencyclidine and ketamine.¹ These drugs also share common pharmacologic features. For instance, they inhibit monoamine uptake systems^{6,7,9} and block NMDA receptors as noncompetitive antagonists of NMDA receptors.²

Many studies have tried to explain the mechanisms of ketamine-induced analgesic, psychotomimetic, and sympathomimetic effects through various systems, such as glutamatergic,^{2,3} opioidergic,⁴ and monoaminergic systems,⁵ indicating that ketamine induces its effects through many different mechanisms. The notion that

ketamine enhances monoaminergic systems, however, has been widely accepted.^{3,6-8}

The enhancement of monoaminergic systems has been explained through at least two mechanisms. First, the inhibition of monoamine uptake systems by ketamine results in the increase of monoamine concentrations in the synaptic cleft, leading to the enhancement of the systems.^{6,7,9} Second, ketamine inhibits the NMDA receptors on GABAergic neurons, which mediate tonic inhibition of monoaminergic neurons, resulting in the increase of monoaminergic neurotransmitter release.^{3,8} The first concept has been supported by reports showing that ketamine inhibited the uptakes of monoamines into synaptic vesicles in a dose dependent manner.^{6,7,9} In the current study, we directly showed that the recombinant monoamine transporters expressed in HEK cells were significantly inhibited by ketamine. The K_i values coincide with those previously reported,^{6,7,9} indicating that the three transporters (NET, DAT, and SERT) correspond to the high-affinity monoamine uptake systems inhibited by ketamine in the central nervous system.

The competition experiments with increasing unlabeled substrate revealed that ketamine inhibition of monoamine uptake is competitive regarding the monoamines. This suggests that monoamines and ketamine might share same binding sites on monoamine transporter proteins. Our investigation on other transporters revealed that neither GLYT1 nor GLAST was affected by 1 mM ketamine, indicating ketamine does not inhibit all transporters.

Akunne *et al.* have reported that phencyclidine has two high affinity binding sites: Site one corresponds to the NMDA receptor complex, and site two is associated with the biogenic amine reuptake complex.¹⁹ They also investigated the affinity of ketamine on site two, showing a K_i value of 59 μM . This K_i value is consistent with those obtained in the current study, indicating that site two is really associated with monoamine transporters.

The K_i values of ketamine on the phencyclidine site in the NMDA receptor complex have ranged widely from 0.6 μM –46.3 μM .¹⁹⁻²¹ These values indicate that ketamine shows relatively higher affinity to the NMDA receptor complex than to monoamine uptake systems. It has been reported that the effects of ketamine are different depending on the plasma levels. In humans, ketamine causes analgesia with plasma concentrations of 0.4–0.6 μM and anesthesia with 3–4 μM .¹ Therefore, it might be possible to speculate that low-dose ketamine mainly affects the NMDA receptor complex and high-dose ketamine acts through the NMDA receptor com-

plex and the monoamine transporters. The mechanism may be more complicated, however, because many other binding sites of ketamine have been reported. Øye *et al.* have reported ketamine affinity for its biologically important targets; ketamine interacts with the phencyclidine recognition site within the NMDA receptor ($K_i = 0.9 \mu\text{M}$ for *S*-ketamine and $2.5 \mu\text{M}$ for *R*-ketamine). At higher concentrations, *S*- and *R*-ketamine interact with opioid μ receptors ($K_i = 11$ and $28 \mu\text{M}$, respectively) and σ sites ($K_i = 131$ and $19 \mu\text{M}$, respectively).²² These values are comparable to the values for monoamine transporters in the current study. Therefore, these sites might also be activated at anesthetic concentrations of ketamine. Further investigations on each component are essential to delineate the exact mechanism of ketamine anesthesia.

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