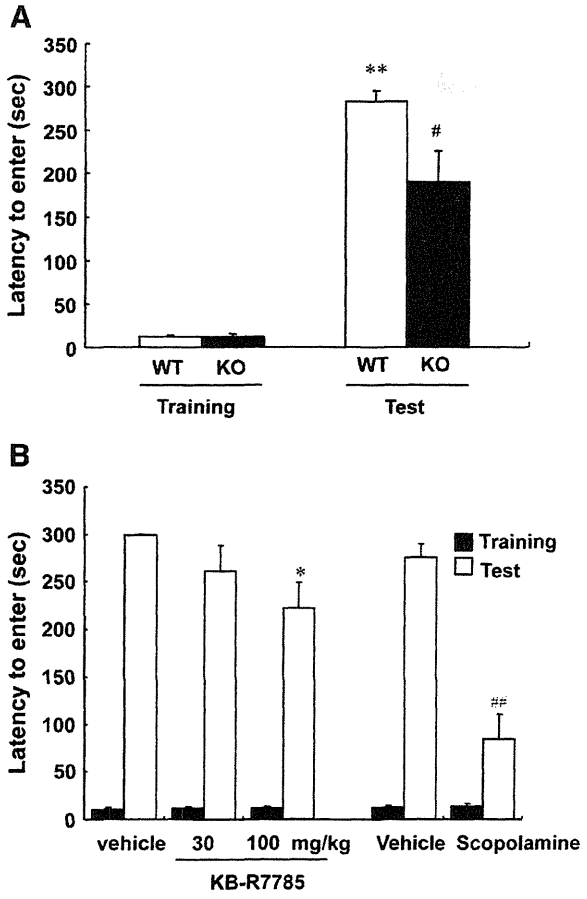


**Fig. 2 – (A) Passive avoidance test results for HB-EGF KO mice. Latency to enter dark compartment was recorded in HB-EGF WT (n=14) and KO (n=12) mice at training and test sessions. Values are means±SEM. $^{**}$ p<0.01 vs. WT in training session. $^{\#}$ p<0.05 vs. WT in test session. (B) Effect of an ADAMs inhibitor on behavior in a passive avoidance test. KB-R7785 (30 and 100 mg/kg) and vehicle (0.5% CMC) were subcutaneously administrated to mice once a day for 4 days. The passive avoidance test was conducted on the third to fourth days, 30 min after vehicle, KB-R7785, or scopolamine administration. Latency to enter a dark compartment was recorded in KB-R7785 and vehicle-treated mice during training and test sessions. Values are means±SEM. $^{*}$ p<0.05, $^{\#\#}$ p<0.01, vs. vehicle (test trial). Vehicle (n=16 or 8), KB-R7785 30 mg/kg (n=10), 100 mg/kg (n=16), and scopolamine (n=6).**

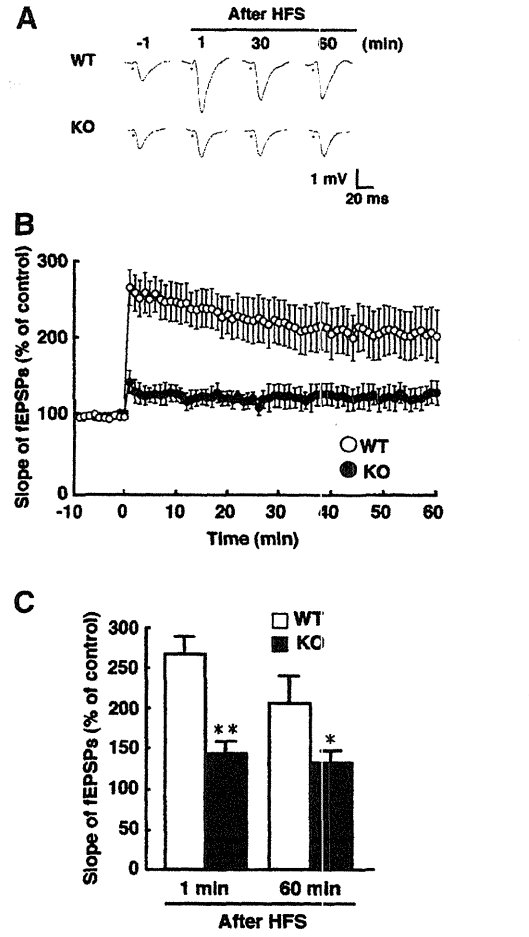


**Fig. 3 – Long-term potentiation (LTP) in HB-EGF KO mice. (A) Representative field excitatory postsynaptic potentials (fEPSPs) recorded from the CA1 region. (B) Changes in slopes of fEPSPs following high frequency stimulation (HFS) in the CA1 region from WT (n=5) and HB-EGF KO (n=5) mice. (C) Level of LTP potentiation at 1 and 60 min after HFS in the CA1 region from WT and HB-EGF KO mice. Values are means ±SEM. $^{*}$ p<0.05, $^{**}$ p<0.01 vs. WT.**

baseline, $F_{(3,12)}=18.194$, $P<0.0001$, n=5) (Figs. 3A, B, and C). These results indicate that HB-EGF is essential for hippocampal LTP in the CA1 region, and therefore HB-EGF may contribute to cognitive function and memory formation.

### 2.4. *Phosphorylation of various protein kinases in the hippocampus of HB-EGF KO mice*

Activation of synaptic proteins, such as CaMKII by a high-frequency stimulation (HFS) is essential for hippocampal LTP induction (Fukunaga et al., 1993; Silva et al., 1992). We examined intrinsic CaMKII activity in CA1 slices by western blotting before and after HFS (Figs. 4A, B, C, and D). The summary histogram or relative immunoreactivity demonstrated that basal phospho-CaMKII level was significantly decreased in HB-EGF KO mice, when compared to WT mice (72.5±4.9% of base line, $F_{(1,8)}=7.228$, $P<0.05$, n=5), without changes basal protein levels (Figs. 4 A and B). In addition, HFS increased phospho-CaMKII levels in WT and HB-EGF KO mice [WT; 153.2±8.5% of baseline, $F_{(1,8)}=18.589$, $P<0.01$, KO; 121.6±11.8% of baseline, $F_{(1,8)}=14.717$, $P<0.01$, n=5] (Figs. 4 A and B). Therefore, autophosphorylated CaMKII level after HFS tended to be lower in HB-EGF mice than in WT mice, but the difference was not statistically significant (Figs. 4 A and B). Basal levels of phosphorylated α-amino-3-hydroxy -5-methylisoxazole-4-propionic acid hydrobromide (AMPA)-type glutamate receptor subunit 1 (GluR1) (Ser-831), which is a postsynaptic CaMKII substrate (Derkach et al., 1999), was also significantly lower in HB-EGF KO mice than in WT mice [77.2±4.7% of base line, $F_{(1,7)}=9.292$, $P<0.05$, n=4 or 5] (Figs. 4 C and D). On the other hand,

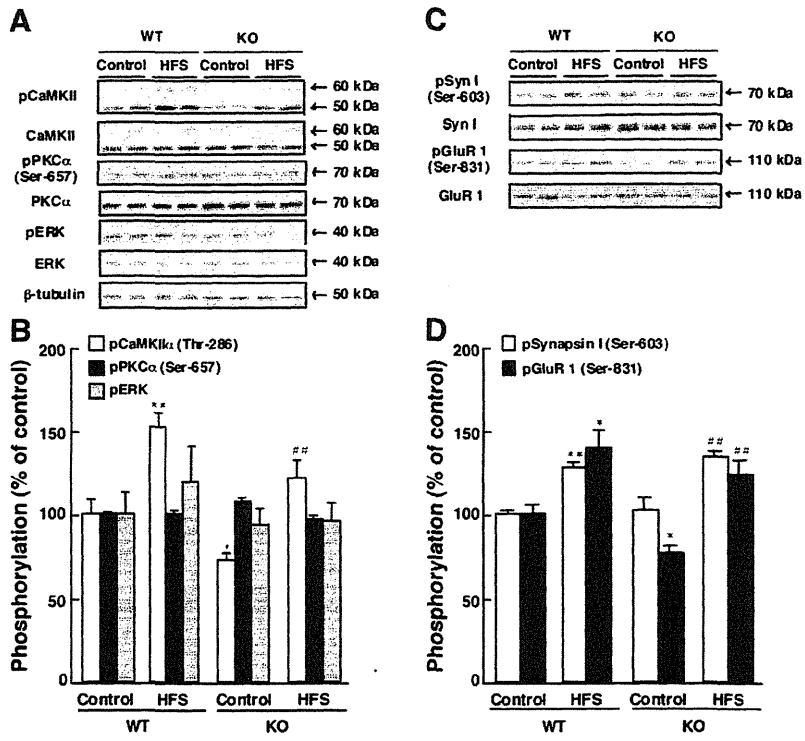


**Fig. 4 – Phosphorylation of various protein kinases in the hippocampus of HB-EGF KO mice. (A) Representative images of immunoblots using antibodies against autophosphorylated CaMKII (pCaMKII), CaMKII, phosphorylated PKCα (Ser675) (pPKCα), PKCα, phosphorylated ERK (pERK), ERK, and β-tubulin. (B) Quantitative analyses of pCaMKII, CaMKII, pPKCα, PKCα, pERK, ERK, and β-tubulin. (C) Representative images of immunoblots using antibodies against phosphorylated synapsin (pSyn 1), synapsin 1 (Syn 1), phosphorylated GluR1 (Ser831) (pGluR1), and GluR1. (D) Quantitative analyses of pSyn 1, Syn 1, pGluR1 (Ser831), and GluR1. Values are means ± SEM., WT (n = 4 or 5), KO (n = 5). $^{*}p<0.05$, $^{**}p<0.01$ vs. WT (Control), $^{\#\#}p<0.01$ vs. HB-EGF KO (Control).**

HFS increased the levels of phosphorylated GluR1 (Ser-831) and synapsin I (Ser-603) levels in WT and HB-EGF KO mice [WT pGluR1 (Ser-831); 140.0 ± 10.9% of baseline, $F_{(1,7)}=8.885$, $P<0.05$, KO pGluR1 (Ser-831); 124.3 ± 8.0% of baseline, $F_{(1,8)}=25.725$, $P<0.01$, n = 4 or 5, WT pSynapsin I (Ser-603); 128.0 ± 3.6% of baseline, $F_{(1,8)}=37.993$, $P<0.01$, and KO pSynapsin I; 134.8 ± 3.1% of baseline, $F_{(1,8)}=17.229$, $P<0.01$, n = 5] (Figs. 4 C and D). No significant changes were observed in the phosphorylation levels of signals such as PKCα, ERK, and Synapsin I in either the WT or the HB-EGF KO mice.

### 2.5. *Changes in various neurotrophic factors levels in the brain of HB-EGF KO mice*

Next, we investigated the effect of HB-EGF deletion on the production of various neurotrophic factors by measuring the levels of NGF, BDNF, NT-3, and GDNF in the several brain regions in WT and HB-EGF KO mice. In the hippocampus, protein levels of NGF and NT-3 were significantly increased, while GDNF level was decreased in HB-EGF KO mice when compared with WT mice (Fig. 5A). The levels of NGF and BDNF were significantly higher in the cortex of HB-EGF KO mice than in WT mice (Fig. 5B). BDNF level was also upregulated in the striatum of HB-EGF KO mice, compared with WT mice (Fig. 5C). Although only BDNF levels showed a tendency to increase in olfactory bulb of HB-EGF KO mice, no significant changes were observed in the levels of neurotrophic factors in either mouse group (Fig. 5D).

## 3. Discussion

Neurotrophic and growth factors play distinct roles in development and maturation of the nervous system. HB-EGF is widely distributed in neuron and neuroglia throughout the brain, and is especially enriched in hippocampus, cerebral cortex, and cerebellum (Mishima et al., 1996). This distribution predicts HB-EGF to be an important contributor to neuronal development and higher brain function. In this study, we investigated possible roles for HB-EGF in memory formation and synaptic plasticity.

We first showed that conditional HB-EGF KO mice have cognitive defects, characterized by impairment in both spatial and fear memory. These results correspond to our previous report that HB-EGF KO mice displayed deficits in short term memory in a Y-maze test and in object identification memory in a novel object recognition test (Oyagi et al., 2009). The Morris water maze test is used to investigate hippocampal-dependent learning, including acquisition of spatial and long-term memory (Denayer et al., 2008; Peters et al., 2003). In this test, HB-EGF KO mice were deficient in the probe trial (spatial memory). The HB-EGF KO mice also exhibited impairment of fear memory in

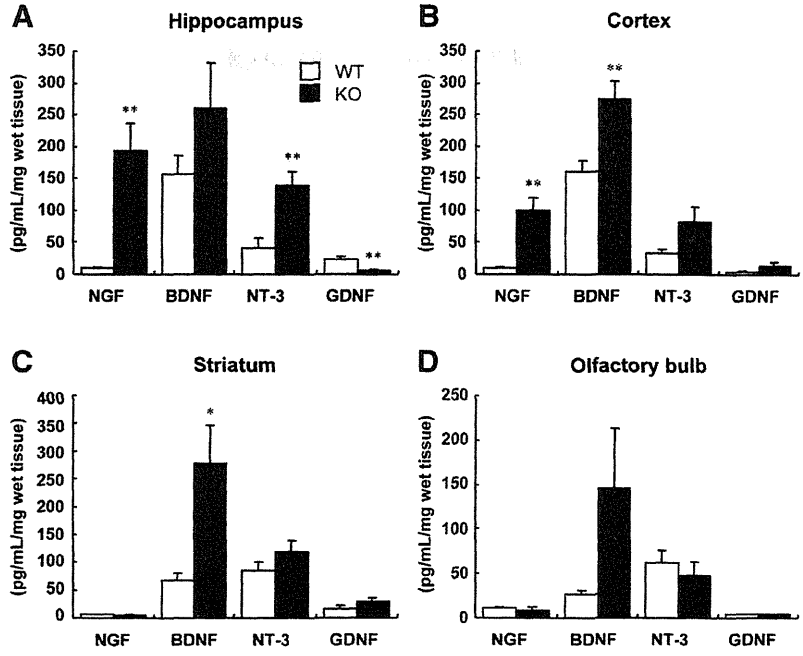


**Fig. 5 – Tissue neurotrophic factors levels in individual brain regions. Levels of several neurotrophic factors were assayed by enzyme immunoassay (EIA) in the (A) hippocampus, (B) cortex, (C) striatum, and (D) olfactory bulb of WT (n = 5 or 6) and HB-EGF KO (n = 6) mice. Values are means ± SEM. $^{*}p<0.05$, $^{**}p<0.01$ vs. WT mice. NGF: nerve growth factor, BDNF: brain-derived neurotrophic factor, NT-3: neurotrophin-3, and GDNF: glial cell line-derived neurotrophic factor.**

a test session of a passive avoidance test, which was conducted 24 h after a training session. These behavioral dysfunctions suggest that loss of HB-EGF might especially affect memory retention. On the other hand, the latency to platform of HB-EGF KO mice showed a tendency to increase, compared with WT mice, in the acquisition trial (especially at days 3–5) of Morris water maze test, but the difference was not statistically significant. Further studies will be needed to investigate the effect of HB-EGF on memory acquisition.

Long-term potentiation (LTP) at CA1 synapses in the hippocampus, a cellular model for learning and memory, is initiated by the influx of $Ca^{2+}$ through N-methyl-D-aspartate (NMDA)-type glutamate receptors. The insertion of AMPA-type glutamate receptors into the postsynaptic site and the associated morphology of dendritic spines are believed to be critical for LTP induction (Derkach et al., 2007; Matsuzaki et al., 2004; Shi et al., 1999). In the present study, hippocampal LTP induced by a high-frequency stimulation (HFS) was markedly impaired in HB-EGF KO mice. HB-EGF KO mice also showed the reduction in activity of CaMKII and phosphorylated GluR1.

CaMKII has been implicated as a key molecule in the induction of LTP (Lisman et al., 2002). Phosphorylation of AMPA receptors by CaMKII is reported to be particularly important for LTP induction (Barria et al., 1997; Derkach et al., 1999). Phosphorylation of GluR1 (Ser831) by CaMKII underlies the increase in AMPA receptor-mediated ionic conductance observed in LTP (Lledo et al., 1995; Mammen et al., 1997; Roche et al., 1996). Decreased phosphorylation of CaMKII and GluR1 observed in the hippocampus of HB-EGF KO mice suggest that CaMKII and GluR1 mediate the effects of HB-EGF on synaptic plasticity.

Neurotrophic factors and cytokines display profound neuromodulatory functions and are involved in the survival and homeostatic maintenance of the central nervous system through regulation of each other's expression. Disruption of the neurotrophin balance has been associated with pathogenesis of various neurological diseases, such as schizophrenia, amyotrophic lateral sclerosis (ALS), and Alzheimer's disease (AD) (Narisawa-Saito et al., 1996; Schulte-Herbruggen et al., 2007; Takahashi et al., 2000). Altered neurotrophic factors levels were observed in several brain regions in the HB-EGF KO mice; in particular, NGF, NT-3, or BDNF levels were upregulated in hippocampus and/or cortex of HB-EGF KO mice, compared with WT mice. Since HB-EGF itself has a neurotrophic effect, the absence of HB-EGF may secondarily alter the expression of neurotrophins. Taken together, these findings suggest that the induction of these growth factors compensates for the deficit in HB-EGF and that the imbalance of neurotrophic and growth factors might partly associate with impaired memory function and synaptic plasticity in HB-EGF KO mice.

Typically, HB-EGF is processed from its precursor protein, pro-HB-EGF, which is anchored in the plasma membrane. Pro-HB-EGF is susceptible to proteolytic cleavage, namely ectodomain shedding, and is converted to the mature secreted factor, HB-EGF (Goishi et al., 1995). Accordingly, ectodomain shedding is essential for HB-EGF exerts its biological effects and ADAMs are key enzymes in this pathway (Asakura et al., 2002; Nanba et al., 2003). The administration of an ADAMs inhibitor also impaired memory retention in the passive avoidance test.

In conclusion, the current study demonstrated that HB-EGF KO mice exhibited impairments in spatial and fear memory,

and also showed decreased LTP in hippocampal CA1 neurons. These behavioral and synaptic dysfunctions are associated with impaired activation of CaMKII and GluR1 in the hippocampus. Further studies using in vitro neuronal and non-neuronal cell cultures would provide the proof for a causal link between the behavioral/physiological findings and the molecular profile. These results suggest that HB-EGF plays a significant, but yet to be fully identified, role in synaptic plasticity and memory formation.

## 4. Experimental procedures

### 4.1. Animals

Ventral forebrain specific HB-EGF KO mice were generated using the Cre-loxP system, as described previously (Oyagi et al., 2009). All procedures relating to animal care and treatment conformed to the animal care guidelines of the Animal Experiment Committee of Gifu Pharmaceutical University. All efforts were made to minimize both suffering and the number of animals used. The animals (10–15 weeks old) were housed at 24±2 °C under a 12 h light–dark cycle (lights on from 8:00 to 20:00) and had ad libitum access to food and water. In all experiments, we used wild-type (WT) littermates as a control group for the HB-EGF KO mice.

### 4.2. Morris water maze test

A circular pool (diameter 120×height 45 cm) was filled to a depth of 30 cm with water (21–23 °C). Four equally spaced points around the edge of the pool were designated as four starting positions. The pool was placed in a dimly lit, soundproof test room with various visual cues. A hidden platform (diameter 10 cm) was set 0.5 cm below the surface of the water in a fixed position. Mice were placed in the water facing the wall and trained with 4 trials per a day for 5 days. In each trial, the starting position was changed, and the mice swam until they found the platform, or after 60 s were guided to the platform; the mice were then placed on the platform for 15 s before being picked up. Three days after the last training trial, the mice were given a probe test without the platform. In this test, mice were placed in the pool once and allowed to search for 120 s. Mean duration to the platform, and the time spent in the quadrant where the platform had been, was recorded using a video camera-based Ethovision XT system (Noldus, Wageningen, The Netherlands).

### 4.3. Passive avoidance test

Mice were tested using a two-compartment box with foot shock grid (Neuroscience, Tokyo, Japan). On habituation day, mice were placed in the lighted compartment, facing away from the dark compartment and were allowed to explore for 30 s. After 30 s, the door was raised and the mice were allowed to explore freely. When mice entered the dark compartment with all four paws, the guillotine door was closed, and the mice were immediately removed and returned to the home cage. On training day (at 24 h after habituation), mice were placed in the lighted compartment, facing away from the dark compartment and allowed to explore for 30 s. After 30 s, the guillotine door was lifted. When the mice entered the dark compartment with all four paws, the guillotine door was closed, and the latency to enter was recorded (from the time the door is lifted). Three seconds after the door was closed, a foot shock (0.25 mA, 2 s duration) was delivered. Thirty seconds after the foot shock, the mice were removed to the home cage. On test day (at 24 h after training), the mice were returned to the lighted compartment, facing away from the dark compartment. After 30 s, the guillotine door was lifted. When the mice entered the dark compartment with all four paws, the guillotine door was closed, and the latency to enter the dark compartment was recorded (from the time the door was lifted). The mice were removed and returned to the home cage. Animals who failed to enter the dark compartment within 300 s were assigned a maximum test latency sore of 300 s.

An ADAM inhibitor, KB-R7785 (30 and 100 mg/kg), was dissolved in carboxymethyl cellulose (CMC) and subcutaneously administrated to ICR mice (6 weeks old, SLC, Shizuoka, Japan) once a day for 4 days. The passive avoidance test was conducted on the third day (training trial) to fourth day (test trial), 30 min after KB-R7785 administration. Scopolamine HBr (3 mg/kg) dissolved in saline was intraperitoneally administered to mice.

### 4.4. Electrophysiology

Preparation of hippocampal slices was performed as described previously (Moriguchi et al., 2008). Briefly, the brain was rapidly removed from each ether-anesthetized male WT or HB-EGF KO mouse and the hippocampus was dissected out. Transverse hippocampal slices (400 μm thickness), prepared using a vibratome (microslicer DTK-1000, Dosaka, Kyoto, Japan), were incubated for 2 h in continuously oxygenized (95% $O_2$, 5% $CO_2$) artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 5 mM KCl, 26 mM $NaHCO_3$, 1.3 mM $MgSO_4{\cdot}7H_2O$, 1.26 mM $KH_2PO_4$, 2.4 mM $CaCl_2{\cdot}2H_2O$, and 1.8% glucose at room temperature (28 °C). After a 2 h recovery periods, a slice was transferred to an interface recording chamber and perfused at a flow rate of 2 ml/min with ACSF warmed to 34 °C. Field excitatory postsynaptic potentials (fEPSPs) were evoked by a 0.05 Hz test stimulus through a bipolar stimulating electrode placed on the Schaffer collateral/commissural pathway and recorded from the stratum radiatum of CA1, using a glass electrode filled with 3 M NaCl. A single-electrode amplifier (CEZ-3100, Nihon Kohden, Tokyo, Japan) was used to record the responses, and the maximal value of the initial fEPSP slope was collected and averaged every 1 min (3 traces) using an A/D converter (PowerLab 200, AD Instruments, Castle Hill, Australia) and a personal computer. After a stable baseline was obtained, high frequency stimulation (HFS) of 100 Hz and 1 s duration was applied twice with a 10 s interval and test stimuli were continued for the indicated periods.

### 4.5. Western blotting

Hippocampal CA1 slices were homogenized in a buffer (70 μl) containing 50 mM Tris HCl (pH 7.4), 0.5% Triton X-100, 4 mM EGTA, 10 mM EDTA, 1 mM $Na_3VO_4$, 40 mM sodium pyrophosphate, 50 mM NaF, 100 nM calyculin A, 50 μg/ml leupeptin,

25 g/ml pepstatin A, 50 μg/ml trypsin inhibitor, and 1 mM DTT. After the removal of insoluble materials by centrifugation (15,000 rpm for 10 min), the samples were subjected to immunoblotting as previously described (Moriguchi et al., 2009). After determining protein concentration in supernatants using Bradford's solution, samples were boiled for 3 min in Laemmli sample buffer. Samples containing equivalent amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to an immobilon polyvinylidene difluoride membrane for 2 h at 70 V. After blocking with 50 mM Tris–HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.5, containing 2.5% bovine serum albumin for 1 h at room temperature, membranes were incubated overnight at 4 °C with anti-phospho CaMKII, [1:5000, (Fukunaga et al., 1988)], anti-CaMKII, [1:5000, (Fukunaga et al., 1988)], anti-phospho-synapsin I (site 3) (1:2000, Chemicon, CA, USA), anti-synapsin 1 [1:2000, (Fukunaga et al., 1992)], anti-phospho-GluR1 (Ser831) (1:1000, Upstate, MA, USA), anti-GluR1 (1:1000, Chemicon), anti-phospho-ERK (1:1000, Cell Signaling Technology, MA, USA), anti-ERK (1:1000, Cell Signaling Technology), anti-phospho-PKCα (1:2000, Upstate), and anti-PKCα (1:2000, Upstate) antibodies. Bound antibodies were visualized using the enhanced chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK) and analyzed semiquantitatively using the NIH Image software.

### 4.6. *Enzyme immunoassay (EIA)*

Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and glial-cell line derived-neurotrophic factor (GDNF) levels were measured with an EIA with a minor modification (Nitta et al., 1999a; Nitta et al., 1999b; Nitta et al., 2004; Niwa et al., 2007). HB-EGF KO and WT mice (12 weeks old) were used in this study. Homogenate buffer [0.1 mol/l Tris–HCl (pH 7.4) containing 1 mol/l sodium chloride (NaCl), 2% bovine serum albumin, 2 mmol/l ethylenediamine-N,N,N′,N′-tetraacetic acid (EDTA), and 0.2% sodium nitride ($Na_3N$)] was added to the brain tissue at a ratio of 1 g wet weight/19 ml of buffer, pulse-sonicated for 100 s, and centrifuged at 100,000 g for 30 min. The supernatant was collected and used for the EIA. Multiwell plates (Falcon 3910; Becton Dickinson and Co., NJ, USA) were incubated with 5 ml of each primary antibody (NGF; MAB256, BDNF; MAB648, NT-3; MAB267, GDNF; and MAB212, R & D Systems, Minneapolis, USA) in 0.1 M Tris–HCl buffer (pH 9.0) (10 mg/ml) per well for 12 h, washed with washing buffer (0.1 M Tris–HCl buffer, pH 7.4, containing 0.4 M NaCl, 0.02% $Na_3N$ 0.1% BSA, and 1 mM $MgCl_2$), and then blocked with washing buffer containing 1% (w/v) skim milk. Tissue extract or each protein standard (30 μl, R & D Systems) in washing buffer was then added to each antibody-coated well; and incubation was carried out for 5 h at 25 °C. After three washes with washing buffer, 30 μl of biotinylated anti-NGF, BDNF, NT-3, and GDNF-antibodies (BAF 256, BAF648, BAF267, and BAF212, respectively; 10 ng/ml, R&D Systems) in washing buffer was added to each well; and the plate was incubated for 12 to 18 h at 4 °C. The biotinylated secondary antibodies were reacted with avidin-conjugated β-galactosidase (Boehringer Mannheim GmbH, Mannheim, Germany) for 1 h. After a thorough washing with washing buffer, enzyme activity retained in each well was measured by incubation with the fluorogenic substrate; 4-methylumbelliferyl-β-D-galactoside (100 μM) in washing buffer. The intensity of fluorescence was monitored with 360 nm excitation and 448 nm emission. The detection limit of the EIA was as low as 5 pg/ml. The recovery of each protein (61.8 pg/ml) exogenously added into the homogenizing buffer following disruption of the rat hippocampus was about 80%. The value of protein content thus obtained was used without correction.

### 4.7. *Statistical analysis*

All data were expressed as the mean±SEM. Statistical significance was evaluated by Student's t-test, Steel test, Mann–Whitney *U*-test, and one-way or two-way ANOVA test followed by a post hoc Tukey or Scheffe's tests. A *p*-value of $<0.05$ was considered to be statistically significant.

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# 依存症の現状

## アルコールおよび向精神薬の乱用について

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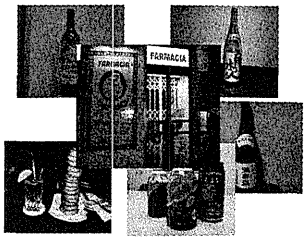


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## 1 はじめに

「依存症」とは，乱用薬物の摂取の自己コントロール不可能な状態を意味する．乱用とは，“医学的常識を故意に逸脱した用途あるいは用法のもとに薬物を大量に摂取する行為(WHOの提唱)”とされ，薬物や嗜好品を社会的・健康的許容から逸脱して摂取することをいう．依存性薬物で最も人体に害を与えるのは，覚せい剤・大麻によるものである．覚せい剤は「使う・持つ・創る」すべてが犯罪である．麻薬と覚せい剤については，医療用の麻薬性鎮痛剤の使用のことも含めて，本誌46巻9月号(2010)で特集され，多方面からの提言があった．本稿では，法規制されていないが依存性を持つ化学物質であるアルコールや，処方薬の生体への影響および依存の現状について紹介する．

## 2 アルコール

アルコールは百薬の長と表現することもあるくらい，日常生活の中でのリラックス効果があり，コミュニケーションツールでもある．飲酒は，仕事や学業に差し障りがなく，周囲に迷惑をかけたり健康を害さない範囲であれば，本人や周囲にとっても大きな問題にはならない．一方で，習慣性のあるアルコールの飲用は，糖尿病，高血圧症のような生活習慣病や肝炎と結び付く健康上のデメリットがある．

和田ら[1]の報告では，過去1年間に1回でもお酒を飲んだことがあることを示す生涯経験率が94.6%と報告されていることから，成人のほぼ全員が一度はお酒を口にしていると考えられる．飲酒の機会としては，食事，団らん，冠婚葬祭，付き合いが挙げられ，ほぼ毎日飲酒する割合は，各年代を平均して男性は32.5%にのぼった．一方で，禁酒を実行または考えたことがある人が生涯経験者の中で男性35.2%，女性19.1%と高い割合を示した．[1]その理由として，「健康上の不調を感じたから」「健康上の不調は感じないが可能性が心配になったから」という理由が挙げられていることからも，飲酒のリスクを意識していることがうかがえる．

アルコールがどのようなメカニズムで生体に作用するかについては，多くの研究がなされている(表1)．エタノール分子は，G protein-activated inwardly rectifying potassium channel(GIRK)を活性化させることで膜電位を興奮させることや，グルタミン酸が結合する興奮性のチャネルである*N*-methyl-D-aspartate receptor channelに作用することが分かっている．[2,3] 鎮痛作用については，モルヒネの受容体の1つである$\mu$受容体への作用も関係していることが分かっている．[2,4] GIRKに結合したエタノール分子は，モルヒネがオピオイド受容体と結合した場合と同じメカニズムで，依存性を持つ可能性がある．[4] エタノールは他の生体内リガンドと比較すると，多数の作用点を持っているため完全な生理作用メカニズムの解明は難しいかもしれない．

## 3 向精神薬

精神・心の働きに作用する化学物質を向精神薬という．広義では，覚せい剤，麻薬，アルコール，タバコなども向精神作用を持つが，ここでは処方薬，すなわち，我が国において医薬品として承認されているものを向精神薬として扱う．向精神薬に限らず，すべての医薬品は，承認までに非臨床および臨床試験を経て，審査がなされて市販されるに至って

表1 エタノールが影響することが報告されている生体構成分子

| | 機能 | エタノールの作用 | 参考文献 |
|---|---|---|---|
| **受容体・チャンネル** | | | |
| nAChR | 神経伝達促進 | 調整 | 7 |
| ATP-R | 神経伝達促進 | 抑制 | 8 |
| BK | 膜の過分極 | 活性化 | 9 |
| $GABA_A$-R | 神経伝達抑制 | 活性化(鎮静) | 10 |
| GIRk | 膜の過分極 | 活性化 | 2 |
| Gly-R | 神経伝達抑制 | 活性化 | 11 |
| 5-$HT_3$-R | 神経伝達促進 | 活性化 | 12 |
| L-type $Ca^{2+}$ channel | 神経伝達促進 | 抑制 | 13 |
| NMDA-R | 神経伝達促進・シナプス可塑性 | 抑制 | 3 |
| $\mu$-R | 鎮痛 | 活性化(鎮痛作用) | 4 |
| P2X4-R | 神経伝達物質遊離・シナプス可塑性 | 抑制 | 14 |
| VDCC | 神経伝達物質遊離 | 抑制 | 15 |
| **酵素** | | | |
| Fyn | チロシンキナーゼ | 増加 | 16 |
| PKC$\gamma$ | プロテインキナーゼ | 減少 | 17 |
| PKC$\varepsilon$ | プロテインキナーゼ | 増加 | 18 |
| PKA | プロテインキナーゼ | 減少 | 19, 20 |

いる．適正な使用の範囲であれば，薬物摂取の不利益はメリット(疾病の治療効果)がデメリット(依存を含む好まれない作用)を上回ることから医薬品として承認されている．睡眠薬や抗不安薬では，“眠ることができるようになる”“不安を感じずに気分よく暮らせる”がそれぞれメリットであるが，対して，依存性を持ち適用症状が現れなくとも服用せざるを得ない状態になることがデメリットである．

向精神薬の過量投与が問題になっている.[5] 患者自身が向精神薬を過剰量内服する時は，現実逃避・快感を目的とする場合が多い．過量内服を繰り返すと，記憶がなくなったり意識を失ったりするようになる．服用をやめようとすると，禁断症状(おう気・おう吐，頻脈，発汗，血圧・体温上昇などの身体症状と，不眠，不安や手の震え，幻覚などの神経・精神症状)が出る．この過量投与を問題視し，警戒をしている精神科医，薬剤師や看護師も少なくない．しかし，患者(乱用者)が複数の病院をまわり，その都度処方を受けた場合は，1病院からの処方せんは常用量であっても，結果としてその何倍もの薬を手に入れることができる(ドクターショッピング)．「患者が，睡眠薬や抗うつ薬の商品名を指定してきた時は，処方しないようにしている」という考えを持つ精神科医師もいる．患者の病状の訴えに応じて，睡眠薬や抗うつ剤の処方が重ねられた結果，類似の薬を一緒に服用し続けてしまっている場合もある．このような場合は，何かのきっかけ(救急外来に運ばれる等)で主治医以外の医師の診察を受けるまで判明しないこともある．このような不適切な薬品の使用については，現在の医療システムでは阻止するのに限界がある．保険請求の折にチェックする，またはお薬手帳の必須化などが解決には有効であると考えられる．

## 4 ベンゾジアゼピン系薬物

和田らの薬物使用に関する全国民調査によると,[1] 調査時点前1年間で睡眠薬を使用したことのある者の割合は2007年のピーク時には7.7%になっている．また睡眠薬を週3回以上使用している人の割合も，2.7%にのぼる．睡眠薬は，特別な人が服用するものというイメージは低くなり，「必要な時には心配せずに使っている」としている人が多い．

現在使用されている睡眠薬の多くは，ベンゾジアゼピン系である．ベンゾジアゼピンは，ベンゼン環，ジアゼピン環およびアリール環から構成され，脳内の抑制系の神経伝達物質GABA受容体に作用することにより，睡眠作用や抗不安作用を有するこ

とが知られている．ベンゾジアゼピン系の睡眠薬や抗不安薬が市販される以前に使用されていたバルビツール酸系の睡眠薬の副作用を補うものとして開発された．

1960年代に塩野義製薬がニトラゼパムを商品名ベンザリンとして市販したのを最初として，ベンゾジアゼピン系薬物が我が国で使用されるようになった．ベンゾジアゼピンは，抗てんかん，抗けいれん剤，静脈麻酔薬として使用されるが，不安症，抗うつ薬や睡眠薬としての適応があり，作用時間の長短で使い分けがなされている．ベンゾジアゼピン系の睡眠薬が登場する前に使用されていたバルビツール酸系睡眠薬と比較して，依存性は少ないとされているが，反して，最近，これら抗不安薬や睡眠薬を継続的に使用することが大きな社会問題となっている．ハルシオンをはじめ一部では，ネットや暴力団を介して非常な高値で売買されることも報道されており，薬の適性使用に加えて社会の治安に対しても問題となっている．

尾崎らが，継続的に調査している「全国の精神科医療施設における薬物関連精神疾患の実態調査」は，薬物乱用の現状を的確に把握することができ，継続的に行われているものとしては，我が国では唯一のものである．毎年の報告書の中で，睡眠薬や抗不安薬等による精神疾患患者数の移り変わりを表2にまとめた．睡眠薬についての常用者数は，トリアゾラムやフルニトラゼパムが多いことが分かる．トリアゾラムは，ハルシオンの商品名で10年以上前から販売され，当初は“ハルシオンの朝”という広告があったほど，翌朝に眠気やだるさが残らず，即効性がある睡眠導入剤として，寝付きの悪さを訴えている患者には吉報であったと考えられる．

このように患者自身が効果をすぐに実感できる薬物は，服用の継続を望まれることが多く，継続使用から依存へと推移する可能性を持つ．トリアゾラムは薬物代謝酵素CYP3A4で代謝されるため，抗真菌剤のイトラコナゾールやマクロライド系の抗生物質やシメチジンとの併用が禁忌であることから，他の疾患の治療に支障が出る場合がある．また，中途覚醒時の記憶障害も添付文書には記載されている．中途覚醒を訴える患者に処方されることが多い，中間型睡眠薬のフルニトラゼパム(商品名；ロヒプノール)による薬物関連精神病報告例も多い．2000年の調査では，ブロムワレリル尿素製剤やフェノバルビタールの合剤であるベゲタミン-AまたはB服用での症例報告もあったが，直近の2009年の調査では減少している．ブロムワレリル尿素系はバルビツール酸系より依存性が低いとされ，1960年代から多用されてきた．OTC薬のウットはブロムワレリル尿素を含有し，フワフワ感(ラリる)を求めて購入・服用し，依存に陥っている．このように，処方せんなしに購入できるOTC薬についても今後の課題である．

抗不安薬については2000年の調査では，エチゾラム(デパス)とアルプラゾラム(コンスタンチン・ソラナックス)を原因とする患者が多かったが，最近ではエチゾラムによる症例が目立って多くなっている．エチゾラムは，チエノベンゾジアゼピン系薬物であり，我が国では1984年から使用されている．ベンゾジアゼピン系に比べて薬理効果が強く，さらに持続時間が6時間以内と短い．このように“キレのよい薬”は，患者も効果を実感しやすい．エチゾラムは，不安症，心身症および不眠に加えて，頸椎症，腰痛症，筋収縮性頭痛における筋緊張にも適用されていることから，整形外科や内科等の精神科以外の診療科でも処方されることが多く，精神科医が関与していない処方例も多いと考えられる．このようなことも考え併せると，患者自身が自覚せずに依存に陥っているケースや他人が気付かない程度の事例も含めると，膨大な潜在患者がいると考えられる．

向精神薬については依存性に加えて，車を運転している時の注意力や認知力が低下することも報告されている．抗うつ薬や抗不安薬を服用した健常ボランティアに模擬運転装置における試験を行ったところ，車線を維持する指標においてアミノトリプチンが技能低下をもたらすことが報告されている．[6] 社会生活を営みながら向精神薬を服用しなければならない状況を考えると，医師は常にこれらの薬が注意力を低下する可能性を考え併せて処方し，患者も服用しなければならない．このような観点からも，向精神薬の服用を治療上必要なく続けることは避けね

表2 向精神薬使用に関連する精神疾患患者の原因薬物の推移

一般名の○○塩の部分は省略した．例えば，ロキソプロフェンナトリウム塩はロキソプロフェンと記述している．

| | 一般名 | 代表的な商品名等，その他特記事項 | 報告症例数 調査年度(年) | | | |
|---|---|---|---|---|---|---|
| | | | 2000 | 2004 | 2006 | 2008 |
| 睡眠薬 | トリアゾラム | ハルシオン | 46 | 25 | 19 | 15 |
| | フルニトラゼパム | ロヒプノール，サイレース | 40 | 12 | 28 | 9 |
| | ブロムワレリル尿素製剤 | ブロバリン | 31 | 19 | 12 | 5 |
| | ベゲタミン | AとBがあり，いずれも塩酸プロマジン，塩酸プロメタジン，フェノバルビタールの合剤 | 15 | 2 | 6 | 3 |
| | ブロチゾラム | レンドルミン | 13 | 3 | 8 | 2 |
| | ニトラゼパム | ベンザリン | 13 | 5 | 10 | 2 |
| | バルビタール類 | | 9 | − | 7 | − |
| | エスタゾラム | ユーロジン | 5 | 1 | 1 | 1 |
| | メタカロン | ハイミナール | 2 | − | − | − |
| | ゾピクロン | アモバン | 2 | 2 | 7 | 1 |
| | ゾルピデム | マイスリー | − | 3 | 10 | 4 |
| | ニメタゼパム | エリミン | − | 1 | 2 | − |
| | ペントバルビタール | ラボナ | − | 1 | − | − |
| | リルマザホン | リスミー | − | 1 | − | − |
| | ジフェンヒドラミン | ドリエル(OTC) | − | 1 | − | − |
| 抗不安薬 | エチゾラム | デパス | 24 | 7 | 25 | 11 |
| | アルプラゾラム | ソラナックス，コンスタン | 18 | 4 | 5 | 1 |
| | ジアゼパム | セルシン | 13 | 4 | 3 | 2 |
| | ブロマゼパム | レキソタン，セニラン | 6 | 2 | 5 | 2 |
| | ロラゼパム | ワイパックス | 3 | 1 | 1 | 2 |
| | クロチアゼパム | リーゼ | 2 | − | − | − |
| | クロキサゾラム | セパゾン | − | − | 2 | 2 |
| | その他BZ系 | | 6 | − | − | − |
| | その他 | | 3 | − | − | − |
| 鎮痛薬 | ペンタゾシン | ペンタジン | 7 | − | 5 | 4 |
| | ロキソプロフェン | ロキソニン(スイッチOTC 2011年発売) | 3 | − | − | − |
| | ブプレノルフィン | レペタン | 2 | − | − | − |
| | モルヒネ | | 1 | − | − | − |
| 鎮痛薬(OTC) | セデス | アセトアミノフェンを主成分とし，OTCに多くの種類が有り | 32 | 2 | 3 | 6 |
| | ナロン | OTCに多くの種類が有り | 6 | 6 | 4 | − |
| | バファリン | アセチルサリチル酸を主成分とし，OTCに多くの種類が有り | 5 | 5 | − | − |
| | パブロン | OTCに多くの種類が有り | − | 3 | − | − |
| | イブ | OTCに多くの種類が有り | − | 1 | − | − |
| | ノーシン | アセトアミノフェン，エテンザミド，カフェインを含み，OTCに多くの種類が有り | − | 1 | − | − |
| | その他市販薬 | | 8 | − | − | − |
| | その他(NSAID) | | 5 | − | − | − |

本データは，国立精神神経センター精神保健研究所薬物依存研究部・尾崎　茂らによる，厚生労働科学研究費補助金(医薬品・医療機器等レギュラトリーサイエンス総合研究事業)分担報告書　全国の精神科医療施設における薬物関連精神疾患の実態調査(同研究所Webサイト http://www.ncnp.go.jp/nimh/yakubutsu/drug-top/paper.htm にて公表されている)の2000〜2008年版を集計し，まとめたものである．精神科病床を有する全国の1,600前後の医療機関にアンケートを依頼し，調査依頼後の2か月のアルコール以外での精神作用物質使用に関連した精神疾患患者が，入院または外来で診察を受けた症例を対象としている．

ばならない．向精神薬の中でも“治療上の必要性がなくなる”という状態のタイミングの諮り方が難しい抗不安薬と睡眠薬は，知らず知らずに依存状態になり常用する可能性もある．そのような患者が車や精密機器の運転をした時に注意力が散漫になり，大きな事故につながる可能性もある．また今までに起

こった事故の中にも，このように向精神薬を服用していた例があったかもしれず，防止策の検討が必要である．

## 5 今後の対策

薬物依存に関連する基礎研究は，日本では海外諸国と比較して研究者人口が少なく，研究資金でも潤沢とはいえない．筆者が分担研究者として加わっている厚生労働科学研究費補助金・医薬品・医療機器等レギュラトリーサイエンス総合研究事業「乱用薬物による薬物依存の発症メカニズム・予防・診断及び治療法についての研究」(代表研究者・名城大学薬学部教授・鍋島俊隆)では基礎と臨床の研究者が両輪となり，薬物依存のメカニズムから治療薬の開発，さらには再使用の診断尺度の研究を精力的かつ幅広く行っている．この研究グループに属する研究者が扱っているのは，主に覚せい剤，麻薬および違法ドラックであり，アルコールや向精神薬については，それほど活発な研究がなされていない．

その原因の1つとして，アルコールや薬は，元来は嗜好と病気の治療を目的としており，連続的に飲む，または服用することは全く乱用でなく，依存と結び付かないことに起因していると考えられる．また筆者の薬物依存の基礎研究の経験からの感想として，覚せい剤依存の動物モデルと比較して，アルコールやベンゾジアゼピンの依存形成をマウスやラットで行うことが難しいことも研究の推進の妨げになっていると感じている．

嗜好品であるアルコールが，嗜好の範囲を超えた時の短期的または長期的な生体への影響を明確にし，それぞれの場合への対策が必要と考えられる．我々は向精神薬やOTC薬から期待していない作用が発現して依存症状や薬物由来精神病に陥っているという現状を認識し，薬の影の部分を少なくする努力が必要と考えられる．

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# Association Analysis of the Adenosine A1 Receptor Gene Polymorphisms in Patients with Methamphetamine Dependence/Psychosis

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**Abstract:** Several lines of evidence suggest that the dopaminergic nervous system contributes to methamphetamine (METH) dependence, and there is increasing evidence of antagonistic interactions between dopamine and adenosine receptors in METH abusers. We therefore hypothesized that variations in the A1 adenosine receptor (*ADORA1*) gene modify genetic susceptibility to METH dependence/psychosis. In this study, we identified 7 single nucleotide polymorphisms (SNPs) in exons and exon-intron boundaries of the *ADORA1* gene in a Japanese population. A total of 171 patients and 229 controls were used for an association analysis between these SNPs and METH dependence/psychosis. No significant differences were observed in either the genotypic or allelic frequencies between METH dependent/psychotic patients and controls. A global test of differentiation among samples based on haplotype frequencies showed no significant association. In the clinical feature analyses, no significant associations were observed among latency of psychosis, prognosis of psychosis, and spontaneous relapse. These results suggest that the *ADORA1* gene variants may make little or no contribution to vulnerability to METH dependence/psychosis.

**Keywords:** Single nucleotide polymorphism, SNP, variation, human, Japanese, MAP, abuse, dopamine.

## INTRODUCTION

Methamphetamine (METH) is a psychomotor stimulant with high liability for abuse, and METH abuse has become a very serious social problem in Japan [1]. Chronic METH abusers have been shown to have persistent dopaminergic deficits [2, 3]. Amphetamines are thought to produce their stimulant effects mainly *via* the dopaminergic system [4, 5], although other systems may also be involved. Dopamine D1 and D2 receptors form heterodimeric complexes with adenosine A1 and A2a receptors respectly, which modulate their responsiveness [6-9], suggesting that responses to amphetamines may also depend on adenosinergic function.

Several lines of evidence suggest that adenosine A1 receptors play a role in inhibiting the effects of METH. Adenosine receptor antagonists potentiate the effects of lower METH doses and substitute for the discriminative stimulus effects of METH [10, 11]. Adenosine receptor agonists protect against METH-induced neurotoxicity, and amphetamine-induced stereotypy and locomotor activity, and reduce the acquisition of conditioned place preference induced by amphetamine [12-15]. These results suggest that adenosine A1 receptors play important roles in the expression of METH-induced neurotoxicities and behaviors.

To date, however, there has been no association analysis between A1 adenosine receptor (*ADORA1*) gene variants and drug addiction. The purpose of this study was (1) to identify novel sequence variants in all coding exons as well as exon-intron boundaries of the *ADORA1* gene in Japanese, and (2) to investigate whether these polymorphisms and/or haplotypes were associated with METH dependence/psychosis.

## MATERIALS AND METHODS

### Subjects

One-hundred seventy-one unrelated patients with METH dependence/psychosis (138 males and 33 females; mean age 37.5±12.0 years) meeting ICD-10-DCR criteria (F15.2 and F15.5) were used as case subjects; they were outpatients or inpatients of psychiatric hospitals. The 229 control subjects (119 males and 110 females; mean age 41.2±12.3 years) were mostly medical staff members who had neither per-

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sonal nor familial history of drug dependence or psychotic disorders, as verified by a clinical interview. All subjects were Japanese, born and living in the northern Kyushu, Setouchi, Chukyo, Tokai, and Kanto regions. This study was approved by the ethical committees of each institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA), and all subjects provided written informed consent for the use of their DNA samples for this research [16]. After informed consent was obtained, blood samples were drawn and genomic DNA was extracted by the phenol/chloroform method.

### Defining Variants of the *ADORA1* Gene

Initially, DNA samples from 16 METH dependent/psychotic patients were used to identify nucleotide variants within the *ADORA1* gene (GenBank accession no. AC105940). Exon numbers were based on the report by Ren and colleagues [17]. Exons 1A, 1B, 2, 3 and exon-intron boundaries were amplified by polymerase chain reaction (PCR) using a thermal cycler (Astec, Fukuoka, Japan), and the products were sequenced in both directions using BigDye terminators (Applied Biosystems, Foster City, CA) by an ABI Genetic analyzer 3100 (Applied Biosystems). The primer sequences used in this study are shown in Table **1**.

Genotyping of IVS1A+182 (rs56298433) was performed by PCR amplification using 2F-2R primers followed by restriction enzyme *Nla* III digestion. Genotyping of Exon2+363 (rs10920568) was performed by PCR amplification using 4F-4R primers followed by sequencing with the same primers. IVS2+35826 (rs5780149) was performed by PCR amplification using 5F-9R primers followed by sequencing with 5F and 5R primers. Genotyping of Exon3+937 (rs6427994), Exon3+987 (rs41264025), and Exon3+1064 (rs16851030) was performed by PCR amplification using 5F-9R primers followed by sequencing with 7F and 7R primers.

### Patient Subgroups

For the clinical category analysis, the patients were divided into two subgroups by three different clinical features. (A) Latency of psychosis from first METH intake: less than 3 years or more than 3 years. The course of METH psychosis varied among patients, with some patients showing psychosis sooner after the first METH intake, as previously reported [16, 18]. Because the median latency was 3 years, this time point was used as the cutoff in defining the two groups. (B) Duration of psychosis after the last METH intake: transient (<1 month) or prolonged (≧1 month). Some patients showed continuous psychotic symptoms even after METH discontinuation, as previously reported [16, 18]. Patients with the transient type showed a reduction of psychotic symptoms within one month after the discontinuation of METH consumption and the beginning of treatment with neuroleptics. Patients with the prolonged type showed a psychotic symptoms continued for more than one month even after the discontinuation of METH consumption and the beginning of neuroleptic treatment. (C) Spontaneous relapse: present or not. It has been well documented that once METH psychosis has developed, patients in the remission phase are liable to spontaneous relapse without reconsumption of METH [16, 18].

### Statistical Analysis

The Hardy-Weinberg equilibrium of genotypic frequencies in each SNP was tested by the chi-square test. The level of statistical significance was set at $\alpha$= 0.05. The allelic and genotypic frequencies of the patient and control groups were compared using the chi-square test. Haplotype frequencies were calculated by the Arlequin program available from http://anthropologie.unige.ch/arlequin [19]. Locus by locus linkage disequilibrium (LD) was evaluated by D' and $r^2$, which were calculated by the haplotype frequencies using the appropriate formula in the Excel program. A global test of differentiation among samples based on haplotype frequencies was also performed by the Arlequin program.

## RESULTS

### Analysis of the *ADORA1* Gene Variants

To identify polymorphisms in the *ADORA1* gene, exons 1A, 1B, 2, and 3, and exon-intron boundaries were analyzed using genomic DNA from Japanese METH dependent/psychotic subjects. Seven SNPs were identified (Table **2**). Five out of seven of these SNPs were previously reported by Deckert [20]. In the two SNPs, the frequencies of the minor

**Table 1. Primers Used in this Study**

| Exon | Forward | | Reverse | |
|---|---|---|---|---|
| Exon1A | 1F: | TGG ACT GGA TGC CTT ATG GCT TAG | 1R: | GGC GCA GGA GCT GAG TGA CAA TCG |
| | 2F: | TCT CAC CCA GTA TCA CTT CCT TTG | 2R: | ATC ACA TGG TAC GGC AGA GAC TCA |
| Exon1B | 3F: | AAT AGG GAG AAA CGC CCC AGC CTT | 3R: | AAG CAC CTG TGT GGT CAG GGA AGC |
| Exon2 | 4F: | GGT AGG AGC TGC ATG TGA CAA GTG | 4R: | GCA GAG TGA GGA CTG GAG CAC GAT |
| Exon3 | 5F: | GGC TGT CAT GAA GCA ATG ATG AGA | 5R: | CCA GCG ACT TGG CGA TCT TCA GCT |
| | 6F: | TCT ACC TGG AGG TCT TCT ACC TAA | 6R: | CCC TGA AGC TCT GGA CTG CTC ATG |
| | 7F: | GTG GTC CCT CCA CTA GGA GTT AAC | 7R: | ACA GGT AAT TAC ACT CCA AGG CTC |
| | 8F: | CTG ATA TTT GCT GGA GTG CTG GCT | 8R: | ACA CCT GCA ACA GAG CTT CCA AAG |
| | 9F: | CCT TGC TGT CAT GTG AAT CCC TCA | 9R: | CAA GAG GAA GAT GCC AAT GGG AGA |

alleles differed between our patients and those of Deckert. In the Exon2+363 (rs10920568) SNP, the G allele was present in 15.5% of our Japanese controls (Table **3**) and 36.9% of the German controls [20]. In the Exon3+1064 (rs16851030) SNP, the T allele was present in 35.8% of our Japanese controls and 1.2% of the German controls [20]. These differences were suggested to be related to the difference in ethnicity between the two cohorts. One SNP, Exon2+363 (rs10920568), was a synonymous mutation (Ala to Ala) (Table **2**). All the other SNPs were located either in the introns or an untranslated region in the exon 3. Two SNPs (Exon3+937 (rs6427994) and Exon3+1454 (rs11315020)) were in linkage disequilibrium (LD) in the sense that the genotypic patterns of the 16 samples examined were the same, representing Exon3+937 (rs6427994) for these two SNPs. IVS1A+182 (rs56298433), Exon2+363 (rs10920568), IVS2+35826 (rs5780149), Exon3+937 (rs6427994), Exon3+987 (rs41264025), and Exon3+1064 (rs16851030) were chosen for further analysis.

**Table 2.** ***ADORA1*** **Gene Variants Found in the Japanese Population**

| Location | Variants | rs# | SNP Name | Function |
|---|---|---|---|---|
| IVS1A+182 | G/T | rs56298433 | | intron |
| Exon2+363 | T/G | rs10920568 | 805T/G | synonymous (Ala->Ala) |
| IVS2+35826 | T4/T5 | rs5780149 | | intron |
| Exon3+937 | A/C | rs6427994 | 1777C/A | untranslated |
| Exon3+987 | C/T | rs41264025 | 1827C/T | untranslated |
| Exon3+1064 | C/T | rs16851030 | 1904C/T | untranslated |
| Exon3+1454 | T/del | rs11315020 | 2294insT | untranslated |

The nucleotide sequence of the *ADORA1* gene was refereneed to the NCBI nucleotide database under accession number AC105940. Exon numbers were based on the report by Ren and colleagues [17]. The column labelled rs# shows SNP numbers from the NCBI SNP database. The data in the column labelled SNP name are from the report by Deckert [20].

**Table 3. Genotypic and Allelic Distribution of the *ADORA1* Gene SNPs in the METH Subjects and the Controls**

| SNP | Group | N | Genotype (%) | | | P | Allele (%) | | P |
|---|---|---|---|---|---|---|---|---|---|
| IVS1A+182 (rs56298433) | | | G | G/T | T | | G | T | |
| | Control | 224 | 222 (99.1%) | 2 (0.9%) | 0 (0.0%) | 0.961 | 446 (99.6%) | 2 (0.4%) | 0.823 |
| | METH | 168 | 166 (98.8%) | 2 (1.2%) | 0 (0.0%) | | 334 (99.4%) | 2 (0.6%) | |
| Exon2+363 (rs10920568) | | | T | T/G | G | | T | G | |
| | Control | 229 | 162 (70.7%) | 63 (27.5%) | 4 (1.7%) | 0.333 | 387 (84.5%) | 71 (15.5%) | 0.233 |
| | METH | 171 | 132 (77.2%) | 36 (21.1%) | 3 (1.8%) | | 300 (87.7%) | 42 (12.3%) | |
| IVS2+35826 (rs5780149) | | | T4 | T4/T5 | T5 | | T4 | T5 | |
| | Control | 229 | 150 (65.5%) | 69 (30.1%) | 10 (4.4%) | 0.887 | 369 (80.6%) | 89 (19.4%) | 0.708 |
| | METH | 171 | 108 (63.2%) | 55 (32.2%) | 8 (4.7%) | | 271 (79.2%) | 71 (20.8%) | |
| Exon3+937 (rs6427994) | | | A | A/C | C | | A | C | |
| | Control | 229 | 2 (0.9%) | 46 (20.1%) | 181 (79.0%) | 0.248 | 50 (10.9%) | 408 (89.1%) | 0.222 |
| | METH | 171 | 5 (2.9%) | 38 (22.2%) | 128 (74.9%) | | 48 (14.0%) | 294 (86.0%) | |
| Exon3+987 (rs41264025) | | | C | C/T | T | | C | T | |
| | Control | 229 | 215 (93.9%) | 14 (6.1%) | 0 (0.0%) | 0.937 | 444 (96.9%) | 14 (3.1%) | 0.888 |
| | METH | 171 | 162 (94.7%) | 9 (5.3%) | 0 (0.0%) | | 333 (97.4%) | 9 (2.6%) | |
| Exon3+1064 (rs16851030) | | | C | C/T | T | | C | T | |
| | Control | 229 | 89 (38.9%) | 116 (50.7%) | 24 (10.5%) | 0.071 | 294 (64.2%) | 164 (35.8%) | 0.572 |
| | METH | 171 | 80 (46.8%) | 67 (39.2%) | 24 (14.0%) | | 227 (66.4%) | 115 (33.6%) | |

N: number of samples.
P: Significance values between the METH subjects and the controls.

### Relationship Between the *ADORA1* Gene SNPs and METH Dependence/Psychosis

Association analyses between these SNPs in the *ADORA1* gene and METH dependence/psychosis were performed using DNA samples from 171 METH dependent/psychotic subjects and 214 control subjects (Table **3**). Among them, the genotypes of five control samples and three METH samples could not be determined at IVS1A+182 (rs56298433). The genotypic frequencies in these SNPs were within the Hardy-Weinberg expectations. No significant differences of the genotypic and allelic distributions of these SNPs in these samples were observed. As the minor allele frequencies of two SNPs, IVS1A+182 (rs56298433) and Exon3+987 (rs41264025), were less than 5%, another four SNPs, Exon 2+363 (rs10920568), IVS2+35826 (rs5780149), Exon3+937 (rs6427994), and Exon3+1064 (rs16851030), were used for further analyses.

A global test of differentiation among samples based on haplotype frequencies was performed using the Arlequin program, but no significant association with METH dependence/psychosis was observed (P=0.590). Haplotype frequencies were estimated by the Arlequin program, and locus by locus LD was calculated by using the appropriate formula in the Excel program. Most of the SNPs in exon 2 and exon 3 were in LD, suggesting that the locus from exon 2 to exon 3 was in a LD block (Table **4**).

Subcategory analyses were conducted on the clinical parameters (latency of psychosis, prognosis of psychosis, and spontaneous relapse) (Table **5**). Significant differences were observed in the shorter latency of psychosis (P=0.025) at Exon3+937 (rs6427994). However, this significance disappeared after Bonferroni correction by the sub-group numbers, two ($P < 0.025$).

## DISCUSSION

We analyzed the *ADORA1* gene variations in a Japanese population and found seven SNPs in exons and exon-intron boundaries. However, no significant associations were

**Table 4. Linkage Disequilibrium Mapping of the *ADORA1* Gene**

| | Exon2+363 (rs10920568) | IVS2+35826 (rs5780149) | Exon3+937 (rs6427994) | Exon3+1064 (rs16851030) | |
|---|---|---|---|---|---|
| **Exon2+363** | | 0.807 | 0.729 | 0.374 | |
| **IVS2+35826** | 0.029 | | 1.000 | 0.676 | D' |
| **Exon3+937** | 0.012 | 0.030 | | 1.000 | |
| **Exon3+1064** | 0.014 | 0.061 | 0.068 | | |
| | | $r^2$ | | | |

D' and $r^2$ values for Controls are shown in the upper right and lower left, respectively.

**Table 5. Genotypic Distribution of the *ADORA1* Gene SNPs in Subcategorized METH Subjects**

| | | SNP | Exon2+363 (rs10920568) | | | | IVS2+35826 (rs5780149) | | | | Exon3+937 (rs6427994) | | | | Exon3+1064 (rs16851030) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Genotype | T | T/G | G | | T4 | T4/T5 | T5 | | A | A/C | C | | C | C/T | T | |
| Group | | N | | | | P | | | | P | | | | P | | | | P |
| Control | | 229 | 162 | 63 | 4 | | 150 | 69 | 10 | | 2 | 46 | 181 | | 89 | 116 | 24 | |
| METH | Latency of Psychosis | | | | | | | | | | | | | | | | | |
| | <3 years | 67 | 48 | 16 | 3 | 0.387 | 46 | 17 | 4 | 0.684 | 4 | 10 | 53 | 0.025 | 30 | 26 | 11 | 0.173 |
| | ≧3 years | 71 | 56 | 15 | 0 | 0.275 | 40 | 29 | 2 | 0.229 | 0 | 22 | 49 | 0.124 | 35 | 28 | 8 | 0.237 |
| | Prognosis of Psychosis | | | | | | | | | | | | | | | | | |
| | Transient (<1 month) | 91 | 70 | 19 | 2 | 0.465 | 59 | 29 | 3 | 0.883 | 3 | 22 | 66 | 0.190 | 42 | 37 | 12 | 0.269 |
| | Prolonged (≧1 month) | 56 | 41 | 14 | 1 | 0.932 | 33 | 20 | 3 | 0.654 | 1 | 11 | 44 | 0.835 | 27 | 21 | 8 | 0.205 |
| | Spontaneous Relapse | | | | | | | | | | | | | | | | | |
| | Not present | 104 | 81 | 22 | 1 | 0.381 | 64 | 34 | 6 | 0.733 | 4 | 25 | 75 | 0.107 | 52 | 39 | 13 | 0.081 |
| | Present | 60 | 45 | 13 | 2 | 0.519 | 39 | 19 | 2 | 0.923 | 1 | 11 | 48 | 0.831 | 25 | 24 | 11 | 0.163 |

N: number of samples.
P: Significance values between the METH subjects and the controls.

observed between these SNPs and METH dependence/psychosis in the genotypic, allelic, haplotypic or clinically subcategorized analyses.

This is the first association analysis between *ADORA1* gene variants and drug addiction. We failed to find associations between the *ADORA1* gene SNPs and METH dependence/psychosis. While the significant difference (P=0.025) in the shorter latency of psychosis at Exon3+937 (rs6427994) disappeared after Bonferroni correction, this may have been due to the sample size, and thus further analysis with a larger sample is warranted.

The variants we found were one synonymous SNP, two intron SNPs and four exon SNPs in the untranslated region. These SNPs are unlikely to affect receptor function because they are not non-synonymous SNPs or promoter SNPs. Because several animal studies have suggested a modulatory role of adenosine receptors for dopamine systems, it remains possible that another region in the *ADORA1* gene, such as a promoter region or intron regions, contributes to the alteration of *ADORA1* gene function.

Although a few association analyses of the *ADORA1* gene and psychiatric diseases have been performed, no significant association has been reported between *ADORA1* variants and bipolar affective disorder or panic disorder [20, 21]. As caffeine is a nonselective adenosine receptor antagonist, the association between the psychoactive effects of caffeine and gene variants of adenosine receptors have also been studied. However, the anxiogenic response to an acute dose of caffeine in healthy, infrequent caffeine users was not associated with *ADORA1* gene polymorphism [22]. Interindividual variation in the anxiety response to amphetamine has also been studied in healthy volunteers, but no association was observed with *ADORA1* gene variants [23]. These results suggest that the *ADORA1* gene variations have little effect on psychiatric symptoms and/or personality traits.

In conclusion, our data suggest that the *ADORA1* gene variants may not play a major role in the development of METH dependence/psychosis.

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# Methylone and Monoamine Transporters: Correlation with Toxicity

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**Abstract:** Methylone (2-methylamino-1-[3,4-methylenedioxyphenyl]propane-1-one) is a synthetic hallucinogenic amphetamine analog, like MDMA (3,4-methylenedioxy- methamphetamine), considered to act on monoaminergic systems. However, the psychopharmacological profile of its cytotoxicity as a consequence of monoaminergic deficits remains unclear. We examined here the effects of methylone on the transporters for dopamine (DAT), norepinephrine (NET), and serotonin (SERT), using a heterologous expression system in CHO cells, in association with its cytotoxicity. Methylone inhibited the activities of DAT, NET, and SERT, but not GABA transporter-1 (GAT1), in a concentration-dependent fashion with a rank order of NET > DAT > SERT. Methylone was less effective at inhibiting DAT and NET, but more effective against SERT, than was methamphetamine. Methylone alone was not toxic to cells except at high concentrations, but in combination with methamphetamine had a synergistic effect in CHO cells expressing the monoamine transporters but not in control CHO cells or cells expressing GAT1. The ability of methylone to inhibit monoamine transporter function, probably by acting as a transportable substrate, underlies the synergistic effect of methylone and methamphetamine.

**Keywords:** Methylone, neurotransmitter transporter, uptake, cocaine, methamphetamine, MDMA.

## INTRODUCTION

Various illegal and/or restricted "designer drugs" have been created by modifying amphetamines. Methylone (2-methylamino-1-[3,4-methylenedioxy- phenyl]propane-1-one) is one such synthetic hallucinogenic amphetamine analogue, which resembles MDMA (3,4-methylenedioxymethamphetamine) but differs structurally by the presence of a ketone at the benzylic position [1, 2]. This compound has been newly placed under legal control as a drug of abuse in Japan [3].

Because of its structural similarity to MDMA, methylone is thought to act on monoaminergic systems. A behavioral study by Dal Cason *et al.* [4] found that methylone substituted for MDMA in rats trained to discriminate MDMA from saline. In studies of its pharmacology in vitro methylone was threefold less potent than MDMA at inhibiting platelet serotonin transporter (SERT) and as potent as MDMA in inhibiting transporters for dopamine (DAT) and norepinephrine (NET), but only weakly inhibited the vesicular monoamine transporter [5, 6]. However, there have been few pharmacological investigations of methylone in animal models, or studies about its mechanism of action.

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In addition, the cytotoxicity of MDMA is considered a consequence of monoaminergic deficits through the drug's effects on the plasmalemmmal and vesicular monoamine transporters [7, 8]. Again, methylone may resemble MDMA in cytotoxic profiles. However, Nakagawa *et al.* [9] reported that methylone did not exhibit cytotoxic effects on isolated rat hepatocytes in contrast to MDMA and its analogues. The possibility can not be excluded that methylone is cytotoxic in some circumstances, since Nagai *et al.* [6] observed that like MDMA, it reduced mitochondrial membrane potential.

Recently, we examined the effects of 5-methoxy-N, N-diisopropyltryptamine (5-MeO-DIPT), known as Foxy, on monoamine neurotransmitter transporters, including DAT, NET and SERT, using a heterologous expression system in COS-7 cells and rat brain synaptosomes, in association with its cytotoxicity [10]. In the present study, we used the same strategy to evaluate the relationship between the effects of methylone and methamphetamine on monoamine transporters and cell toxicity.

The results indicated an ability of methylone to inhibit monoamine transporter function, and cause damage synergistcally with methamphetamine in cells heterologously expressing monoamine transporters, suggesting that the transport of these drugs underlies their cytotoxicity.

## MATERIALS AND METHODS

### Materials

Methylone was synthesized at the Division of Pharmacognosy, Phytochemistry and Narcotics, National Institute of

Health Sciences (Tokyo, Japan). Its structure and purity were confirmed by melting point (degradation, 225°C), TLC, GC-MS and $^1$H-NMR analyses [11]. Other chemicals used were purchased from commercial sources. [$^3$H]Dopamine (DA) (1.29 TBq/mmol), [$^3$H]serotonin (5-HT) (1.04 TBq/mmol), and [$^3$H]GABA (1.2 TBq/mmol) were obtained from PerkinElmer Life Science, Inc. (Boston, MA, USA), and [$^3$H] norepinephrine (NE) (1.18 TBq/mmol) from GE healthcare Bioscience, Inc. (Buckinghamshire, UK).

### Cell Culture and Expression

Chinese hamster ovary (CHO) cells were cultured at 37°C under 5 % $CO_2$ / 95 % air in Minimum Essential Medium-alpha (α-MEM) supplemented with 10 % fetal calf serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml fungisone.

For the preparation of cell lines stably expressing transporters, CHO cells at subconfluence were transfected with cDNA of rat DAT (rDAT), NET (rNET), or SERT (rSERT), or mouse GABA transporter-1 (mGAT1) using FuGENE6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's directions. The cells were then diluted sequentially, seeded in 96-well plates, and selected using G418. The cell lines were confirmed to stably express the transporters based on the uptake of each tritium-labeled ligand, and designated CHO/rDAT, CHO/rNET, CHO/rSERT and CHO/mGAT1, respectively.

### Uptake and Release Assay

The uptake assay using radio-labeled ligands was performed, as described previously [10, 12]. Cells were washed three times with an oxygenated Krebs Ringer HEPES-buffered solution (KRH; 125 mM NaCl, 5.2 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 5 mM glucose, and 20 mM HEPES, pH 7.3) and incubated for 10 min at 37°C with 10 nM of [$^3$H]DA or other radio-labeled ligand. Nonspecific uptake was determined in mock-transfected cells and also in each plate in the presence of 100 μM cocaine for monoamine and 1 mM nipecotic acid for GABA. Data were analyzed using Eadie-Hofstee plots with Prism 5 (GraphPad Software, Inc., San Diego, CA). Statistical analyses were performed using the unpaired Student's t-test.

Reverse transport (release) was analyzed, as described [10]. Cells loaded with [$^3$H]substrate were incubated with or without the drug under investigation at 37°C for 2 min, and separated from the incubation solution. The radioactivity retained in the cells and also in the separated solution was measured by liquid scintillation counting. Statistical tests were performed using an analysis of variance (Kruskal-Wallis test) with pairwise comparisons using Dunn's multiple comparison test.

### Cell Toxicity Assay

The amount of lactate dehydrogenase (LDH) released into the culture medium was measured for the evaluation of methylone's toxicity, as described previously [12] with some modifications. Briefly, cells cultured in 96-well culture plates were washed and incubated without phenol red in α-MEM supplemented with 1% BSA and different concentrations of methylone and/or methamphetamine for 24 h. The amount of LDH released into the medium was measured colorimetrically (Wako, Tokyo, Japan). Statistical analyses were performed using the Kruskal-Wallis test and Dunn's multiple comparison test.

## RESULTS

### Effects of Methylone on the Uptake of Substrate in CHO Cells Stably Expressing Monoamine and GABA Transporters

The effect of methylone on the transport of monoamines was examined in CHO cells stably expressing the rat monoamine transporters, rDAT, rNET and rSERT, in comparison with that on the mouse GABA transporter, mGAT1. Simultaneous incubation with [$^3$H]DA, [$^3$H]NE, or [$^3$H]5HT and methylone caused a decrease in the uptake of [$^3$H]substrate in a concentration-dependent fashion, although the effects differed between transporters in contrast to the effects of methamphetamine (Fig. **1**). The rank order of the transporters in terms of the potency with which they were inhibited by methylone was NET > DAT >> SERT (Fig. **1** and Table **1**). Methylone inhibited SERT more, DAT and NETless, than methamphetamine. However, it had no effect on GAT transport activity at concentrations up to 1 mM (Fig. **1**), while nipecotic acid, an inhibitor of neuronal GABA transporter such as GAT1, inhibited [$^3$H]GABA uptake in a concentration-dependent manner (data not shown).

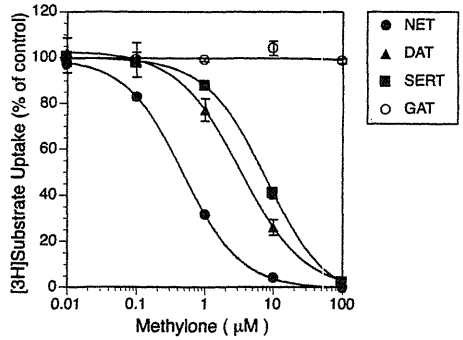


**Fig. (1). Effects of methylone on the uptake of substrates in CHO cells stably expressing monoamine and GABA transporters.** Cells were incubated with [$^3$H]substrates at 10 nM in the absence or presence of methylone at various concentrations. Specific uptake was expressed as a percentage of the control, and values represent the mean ± SEM, n=3. Control uptake in the absence of drugs was 4715 ± 182, 4961 ± 170, 13964 ± 1135, and 798 ± 114 dpm/well for DAT, NET, SERT and GAT, respectively.

**Table 1. Effects of Methylone and Methamphetamine on the Uptake of Monoamines in CHO Cells Stably Expressing rat DAT, NET and SERT**

| Transporter | IC50 (μM) | |
|---|---|---|
| | Methylone | Methamphetamine |
| DAT | 2.84 ± 0.36 | 0.65 ± 0.06 |
| NET | 0.48 ± 0.03 | 0.16 ± 0.00 |
| SERT | 8.42 ± 1.01 | 27.62 ± 2.87 |

Values represent the mean ± SEM for three experiments each performed in triplicate.

Next, we analyzed the effect of methylone on uptake kinetically. Table **2** summarizes the effects on [$^3$H]substrates in comparison with those of methamphetamine. Methylone increased the $K_m$ value without changing the $V_{max}$ for the uptake of [$^3$H]NE, indicating competitive inhibition similar to metamphetamine (Table **2**). On the other hand, it showed uncompetitive inhibition of [$^3$H]DA and [$^3$H]5-HT, tending to decrease the $V_{max}$ while increasing the $K_m$, as methamphetamine did.

### Effects of Methylone and Methamphetamine on the Reverse Transport of [$^3$H]substrates

To further characterize the effects of methylone on the monoamine transporters, we examined its influence on the reverse transport of [$^3$H]substrates through DAT, NET and SERT in comparison with methamphetamine, since unlike cocaine, methamphetamine induces the release of monoamines *via* a reversal of transport [13]. Methylone elicited the release of [$^3$H]DA, [$^3$H]NE and [$^3$H]5-HT from the cells expressing rDAT, rNET and rSERT, respectively, similar to methamphetamine (Fig. **2**). In addition, the combination of methylone and methamphetamine did not cause a further increase in the release of [$^3$H]substrates.

### Cytotoxicity Elicited by Methylone and/or Methamphetamine

Initially, we examined the effect of methylone and methamphetamine on cell viability using a MTT-based WST-1 assay previously applied to Foxy [10]. However, we found no changes with methylone and/or methamphetamine at any concentrations tested (data not shown). Therefore, we used a LDH release assay to evaluate methylone's toxicity.

**Table 2. Changes in the Transport Kinetics Induced by Methylone and Methamphetamine in CHO Cells Stably Expressing the Monoamine Transporters**

| | $K_m$ (μM) | $V_{max}$ (fmol/μg protein/min) |
|---|---|---|
| DAT | | |
| Control | 1.73 ± 0.55 | 34.12 ± 7.64 |
| Methylone 5μM | 2.33 ± 0.67 | 26.68 ± 8.70 |
| Methamphetamine 1μM | 2.06 ± 0.83 | 24.18 ± 9.89 |
| NET | | |
| Control | 0.25 ± 0.03 | 3.63 ± 0.31 |
| Methylone 0.5μM | 0.58 ± 0.10* | 4.54 ± 1.01 |
| Methamphetamine 0.2μM | 0.71 ± 0.20 | 4.69 ± 0.49 |
| SERT | | |
| Control | 0.27 ± 0.04 | 40.68 ± 7.23 |
| Methylone 10μM | 1.12 ± 0.25* | 36.95 ± 5.68 |
| Methamphetamine 30μM | 0.86 ± 0.21* | 31.41 ± 4.63 |

Values represent the mean ± SEM for three experiments each performed in triplicate. *P<0.05 vs control.

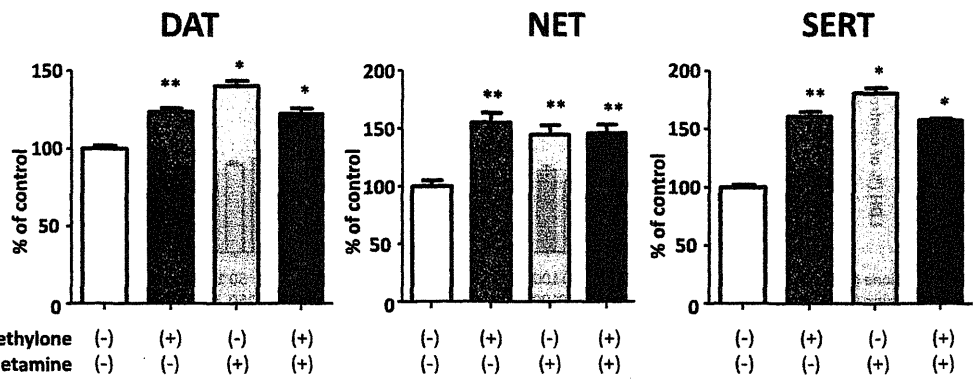


**Fig. (2). Effects of methylone and methamphetamine on the reverse transport of [$^3$H]substrates through the monoamine transporters.** CHO cells stably expressing rDAT, rNET, or rSERT were preloaded with a [$^3$H]substrate at 20 nM for 30 min. They were then washed with KRH buffer, and incubated for 2min in fresh medium containing 100 μM methylone and/or 100 μM methamphetamine. Values represent the mean ± SEM, n=8-11, and are expressed as a percentage of the control (without drugs). *P< 0.05, **P< 0.01 vs control.