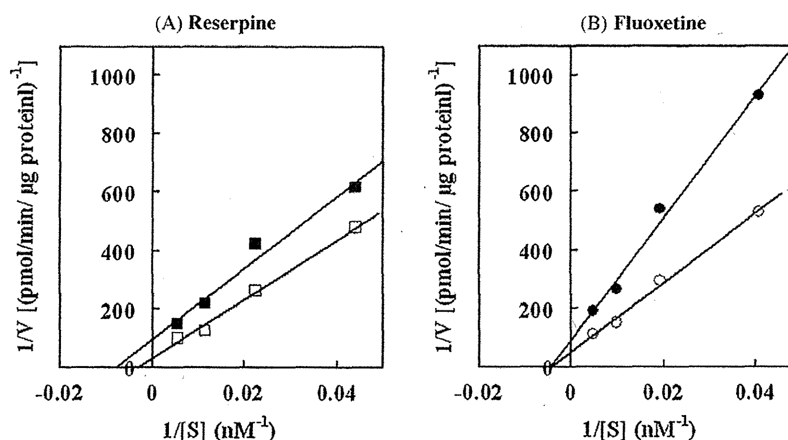

Fig. 1. Comparison of effects of fluoxetine and reserpine on [^{3}H]dopamine uptake into synaptic vesicles. A representative plot of Lineweaver–Burk analysis of initial uptake velocity of [^{3}H]dopamine in the presence and absence of reserpine (A) or fluoxetine (B). In (A), 50 nM reserpine (■) and control (□). In (B), 2.5 µM fluoxetine (●) and control (○).

Moreover, we observed that the positive control drug methamphetamine inhibited VMAT2 activity, and the negative control drugs cocaine and phencyclidine did not. Using these synaptic vesicles, three SSRIs were demonstrated to inhibit VMAT2 activity *in vitro*, and the potencies of inhibition by the SSRIs were in a range of concentrations similar to methamphetamine. Fluoxetine is reportedly unevenly distributed in rat subcellular fractions. 40% is recovered in P_1 (nuclei) and P_2 (mitochondria and synaptosomes) fractions and 10% in P_3 (microsomes) and soluble fractions, regardless of the dose and route of administration (2.5–5 mg/kg, i.v., 10–20 mg/kg, p.o.) [3], suggesting that SSRIs are transported into the synapses.

Paroxetine and MDMA show almost identical IC_{50} values for vesicular uptake of dopamine *in vitro* [1]. Our present results are consistent with previous data with paroxetine. Additionally, Gobbi et al. [10,11] reported that 10 µM fluoxetine evoked [^{3}H]serotonin release from preloaded synaptosomes, suggesting that fluoxetine may interact with serotonin storage vesicles. SSRIs would be expected to get into monoaminergic neurons and potentially interact with VMAT2 in monoaminergic neurons, but this has yet to be demonstrated directly. Our present results provide a clear explanation for fluoxetine-induced loss of vesicular serotonin.

Fluoxetine also produces a concentration-dependent inhibition of the two-pore domain potassium (2-PK) channel, TREK-1 [17]. TREK-1-deficient (Kcnk2$^{-/-}$) mice exhibit behavior similar to that of wildtype animals treated with SSRIs such as fluoxetine [15]. Not only fluoxetine, but also paroxetine and fluvoxamine, inhibits TREK-1 at concentrations similar to those for VMAT2 [15] (Table 1). Henry et al. [14] showed that brain paroxetine concentrations in patients with unipolar depression who were taking 20 mg/day were shown to be approximately 2–14 µM in a fluorine magnetic resonance spectroscopy study. Interestingly, Bolo et al. [2] reported that brain concentrations of fluvoxamine and fluoxetine (12 or 13 µM) are 10-fold higher than corresponding blood levels (1.12 or 1.73 µM, respectively). Therefore, these SSRIs at clinically relevant brain concentrations possibly inhibit VMAT2 and TREK-1 activity. These results suggest the need to consider these factors in the use of fluoxetine and other SSRIs.

From the kinetic analyses, fluoxetine was found to be a noncompetitive inhibitor of VMAT2. On the other hand, reserpine inhibited VMAT2 activity in an uncompetitive manner. Reserpine potently inhibits VMAT2 and defines a high-affinity monoamine uptake recognition site and a low-affinity monoamine binding site that may release amines into the vesicle lumen [6]. However, Merickel et al. [18] showed that substrate recognition can be separated from coupling as the driving force. They showed that mutation of D33N of VMAT2 eliminated transport activity but retained [^{3}H]reserpine binding activity and coupling to the proton electrochemical gradient. Considering that the reserpine binding site and substrate recognition site on VMAT2 are different, our results showing that reserpine uncompetitively inhibited substrate transport does not necessarily conflict with the previous report. The site of action of fluoxetine, however, may be different from that of reserpine, reflected by a K_m value of VMAT2 activity that was not affected by the presence of fluoxetine, suggesting that fluoxetine cannot bind to the substrate recognition sites of VMAT2. The precise mechanism and site of action by which SSRIs alter VMAT2 function remain unclear, but the effects of the SSRIs, all weak amines, could be through dissipation of the pH gradient and/or membrane potential that is required for VMAT2 function.

VMAT2 heterozygous mice have been shown to exhibit enhanced immobility in the tail suspension test (a depressive-like phenotype) [9]. Such an effect, however, may be more attributable to compensatory changes in serotonin and/or dopamine function, particularly early in development, than to the reduction in VMAT2 levels per se. By contrast, elevated levels of VMAT2 have been reported in the brains of bipolar disorder patients [18,28], and increased VMAT2 density (B_{max}) in the platelets of untreated patients with major depressive disorder compared with healthy control subjects has been reported [29]. Considering the reduced VMAT2 activity in a mouse depressive-like phenotype and increased VMAT2 levels in patients with depression, further studies on VMAT2 inhibition by SSRIs in clinical use are needed.

Haloperidol potently inhibited vesicular [^{3}H]dopamine uptake in the present study. This effect of haloperidol was not likely mediated via D_2 receptors because the selective D_2 receptor antagonist sultopride showed no effect on vesicular [^{3}H]dopamine uptake *in vitro*. Haloperidol has been shown to affect pH gradient [19,20,22], which is considered to regulate monoamine uptake into synaptic vesicles [5]. Haloperidol displaces [^{3}H]reserpine from up to 75% of its binding sites [21], and reserpine binding to VMAT2 is sensitive to pH gradient/membrane potential, which could explain displacement of reserpine binding to synaptic vesicles by haloperidol.

In conclusion, the present study showed that SSRIs, including fluoxetine, paroxetine, and fluvoxamine, inhibit VMAT2 activity *in vitro*. For future studies focused on the development of new antidepressants, investigation of VMAT2 inhibition by SSRIs in clinical use may be required.

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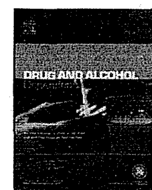

Application of the Relapse Risk Scale to alcohol-dependent individuals in Japan: Comparison with stimulant abusers☆

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ABSTRACT

Objective: To develop and validate the Alcohol Relapse Risk Scale (ARRS) for Japanese alcohol-dependent individuals and to compare the features of relapse risk for alcohol-dependent individuals with those for stimulant abusers.
Methods: The ARRS is a multidimensional self-rating scale consisting of 32 items based on the Stimulant Relapse Risk Scale (SRRS). Two hundred eighteen inpatients and outpatients with a history of alcohol dependence (181 males and 36 females) were recruited, provided informed consent, and were administered the ARRS. The Visual Analog Scale (VAS) for alcohol craving, current state of drinking, and data on relapse within 1 month after the rating were used for validation.
Results: Exploratory factor analysis highlighted five factors: stimulus-induced vulnerability (SV), emotionality problems (EP), compulsivity for alcohol (CA), lack of negative expectancy for alcohol (NE), and positive expectancy for alcohol (PE). Cronbach's α coefficient for each of the subscales ranged from .55 to .90 and was .90 for the total ARRS, indicating their adequate internal consistency. SV, EP, CA, PE, and total ARRS were significantly correlated with the VAS and current drinking state, supporting their concurrent validity. SV and total ARRS were significantly correlated with relapse, suggesting that the ARRS is useful for predicting relapse risk in alcohol-dependent individuals, similar to the SRRS for stimulant abusers. Compared with stimulant abusers, alcohol-dependent individuals tended to express their desires related to relapse more honestly on the scales.
Conclusions: The ARRS has multidimensional psychometric properties that are useful for assessing the various aspects of alcohol relapse risk.

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1. Introduction

In 2003, approximately 800,000 adults out of the Japanese general population of 120 million presented with alcohol dependence, making this group one of the largest among the various mental disorders (Osaki et al., 2005). A serious problem with the treatment of alcohol-dependent individuals is the very low rate of complete abstinence (about 20%; Noda et al., 2001).

Some clinical studies have examined psychosocial factors related to relapse in individuals with alcohol dependence. Relapse-promoting factors include anxiety (Lucht et al., 2002), craving (Gordon et al., 2006), negative mood, childhood sexual abuse (Walitzer and Dearing, 2006), and psychological distress (Sander and Jux, 2006). Some researchers have placed emphasis on relapse-inhibiting factors such as self-efficacy, social support, coping (Brown et al., 1995), other-efficacy beliefs (Demmel et al., 2006), spirituality (Gordon et al., 2006), peer support group attendance, and continuing care program involvement (Miller et al., 1999).

☆ English and Japanese versions of the Alcohol Relapse Risk Scale (ARRS) can be found by accessing the online version of this paper at http://dx.doi.org by entering doi:10.1016/j.drugalcdep.2008.10.021.

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Zywiak et al. (2006) developed the "Relapse Questionnaire" and examined its inner multiple construction of relapse-onset factors of alcohol dependence with a follow-up study. This study revealed three factors: Negative Affect/Family Influences, Craving/Cues, and Social Pressure. These factors appear to cover most of the psychosocial factors related to alcohol relapse. This questionnaire, however, is intended for relapsed patients, not for the prediction of relapse risk. Additionally, difficulty with administering the questionnaire remains problematic because it is part of a research interview.

No multidimensional scale that measures relapse risk for alcohol dependence currently exists in Japan. To further advance the development of medicines and programs for the prevention of relapse, scales for the appropriate assessment of relapse risk are necessary. We previously developed a 48-item multidimensional scale for the measurement of relapse risk for Japanese patients with stimulant dependence (i.e., the Stimulant Relapse Risk Scale, SRRS; Ogai et al., 2007) based on the Marijuana Craving Questionnaire (Heishman et al., 2001). The reliability and validity of this scale were demonstrated by analyzing 100 inpatients and outpatients with a history of stimulant abuse in Japan. Exploratory factor analysis revealed five subscales: "anxiety and intention to use drug" (AI), "emotionality problems" (EP), "compulsivity for drug use" (CD), "positive expectancy and lack of control over drug" (PL), and "lack of negative expectancy for drug use" (NE). AI, PL, NE, and total SRRS scores were significantly and positively related to relapse within 3 and 6 months. Shaffer et al. (2004) proposed a common etiology for addiction to stimulants, alcohol, and other drugs. Relapse risk may be similar between stimulant abusers and alcohol-dependent individuals.

In the present study, we developed a multidimensional scale, the Alcohol Relapse Risk Scale (ARRS), based on the SRRS. Forty-eight items in the ARRS reflect a variety of relapse risk factors, such as intention, compulsivity, expectancy for alcohol, and emotional problems. We administered the ARRS to 218 inpatients and outpatients with a history of alcohol dependence in Japan and examined its inner structure, reliability, and validity. Moreover, certain relapse risk factors appear to be common between stimulants and alcohol; we therefore compared the relapse risk for alcohol-dependent individuals with that for stimulant abusers.

2. Methods

2.1. Participants

A total of 218 patients (29 inpatients, 182 outpatients, and 7 unknown patients) with a history of alcohol dependence participated in the study (Table 1). They were recruited for ongoing research studies at Nakajo Daini Hospital, Tokamachi ($n = 68$), National Center of Neurology and Psychiatry, Musashi Hospital, Kodaira ($n = 63$), Tokyo Metropolitan Matsuzawa Hospital, Tokyo ($n = 34$), Urabe Mental Health Clinic, Tokyo ($n = 30$), and Hirakawa Hospital, Hachioji ($n = 20$). All of these treatment facilities specialized in the treatment of alcohol dependence. The participants comprised 181 males and 36 females, ranging in age from 28 to 81 years (mean = 53.6, S.D. = 11.06).

Table 1
Characteristics of the ARRS participants.

Items	Values
Number of participants	218
Age ($M \pm$ S.D.)	53.6 ± 11.5
Gender (% female)	16.6
Treatment state (*N*)	
Inpatients	29
Outpatients	182
Unknown	7
Participants with follow-up (%)	56.9
Current drinking/not drinking (*N*)	55/163
Relapse/no relapse within 1 month (*N*)	31/93
VAS (current, 0–10; $M \pm$ S.D.)	2.07 ± 2.51
VAS (past 2 weeks, 0–10; $M \pm$ S.D.)	2.47 ± 3.20
CES-D (0–60; $M \pm$ S.D.)	16.82 ± 11.06
GHQ-12 (0–12; $M \pm$ S.D.)	2.32 ± 3.63

N: number of participants, *M*: mean, S.D.: standard deviation.

The five eligibility criteria were the following: at least 20 years of age, a history of alcohol dependence, diagnosed as alcohol-dependent on the basis of the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition (DSM-IV; American Psychiatric Association, 1994), an inpatient or outpatient at a Japanese mental hospital, and the ability to understand Japanese. The study was approved by the Institutional Review Board of each facility. After an explanation of the research by a psychiatrist or a psychologist, each participant provided informed written consent and completed the ARRS, the Visual Analog Scale (VAS) for alcohol craving, the Center for Epidemiological Studies Depression Scale Japanese version (CES-D) (Shima et al., 1985), the 12-item General Health Questionnaire Japanese version (GHQ-12) (Daibo and Nakagawa, 1985), and a questionnaire on alcohol experience and demographics. One hundred twenty-four participants also answered a follow-up questionnaire about their drinking state within 1 month after the rating.

2.2. Development of the Alcohol Relapse Risk Scale

The ARRS was developed on the basis of 48 preliminary items from the Stimulants Relapse Risk Scale (SRRS). For all items, "use the drug" was replaced by "drink alcohol." Two items related to illegal activities were replaced by behaviors related to alcohol drinking (i.e., "Even though I know I will be arrested, I would use the drug" was replaced with "Even though I know I will lose my family and/or job, I would drink alcohol"; "I want to obtain the drug even by working illegally" was replaced with "I want to drink alcohol even if it deteriorates my health").

Forty-three of the preliminary items comprised the five initial factors of alcohol relapse: (i) compulsivity (C), (ii) negative expectancy for alcohol (N), (iii) clear intention of alcohol drinking (I), (iv) positive expectancy for alcohol (P), and (v) emotional problems (E). The remaining five preliminary items were provided to measure the lack of insight into one's own mental condition (i.e., denial; e.g., "I am sure that I will not drink alcohol in the future").

Each item was rated on a three-point Likert-type scale with a score ranging from 1 to 3 based on the participant's level of agreement with each statement. The anchors were "Strongly Disagree and Disagree," "Neither Agree nor Disagree," and "Strongly Agree and Agree." A three-point scale was used to reflect patient feedback, indicating the difficulty in answering a five-point scale. The following written instruction was given: "Please describe your state during the past week. For each statement below, please circle one answer that best describes you."

2.3. Measurements of concurrent validity

To evaluate the concurrent validity of the ARRS, the VAS was administered to the participants to measure their subjective desires for alcohol. The VAS was composed of two questions: "Please rate your current state of craving" and "Please rate your strongest craving for alcohol in the past 2 weeks." Participants answered each question by placing a vertical mark on a 100-mm horizontal line, labeled "not at all" on the left and "extremely" on the right end. The current state of drinking (i.e., drinking or not drinking) was also asked.

Participants also answered the CES-D and GHQ-12 that measured their emotional problems. These scales were used to examine the concurrent validity of the factor "emotionality problems" in the ARRS.

2.4. Measurements of predictive validity

To evaluate relapse risk, relapse within 1 month after the ARRS rating was investigated. Relapse was operationally defined as "consumption of any alcohol after the ARRS rating" and was determined from the patients' self-reports and/or their psychiatrists. Of 124 participants whose information was available, 16 were drinking at baseline and follow-up, 15 were abstinent at baseline but drinking at follow-up, 8 were drinking at baseline but not at follow-up, and 85 were abstinent throughout. Thirty-one participants who were drinking at follow-up were considered relapsed patients.

2.5. Questionnaire on alcohol experience and demographic factors

The participants were also asked to complete a short questionnaire to determine their age and gender, the day the questionnaire was completed, and the principal type of alcohol they were drinking (or had consumed). The questions also asked the date on which the patient had last consumed alcohol, the period of abstinence, the number of years they had consumed alcohol, other problems apart from alcohol, perceived stress, perceived social support, and the availability of social support for their problems.

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Table 2
Promax rotated factor pattern for the 43-item ARRS.

	Factor				
	1	2	3	4	5
Factor 1: Stimulus-induced vulnerability (SV)					
(C 35) If alcohol is placed in front of me, I would drink it.	**.855**	−.240	.083	.078	.023
(I 9) It would be difficult for me to refuse if someone placed alcohol in front of me.	**.849**	−.063	−.155	−.007	.056
(C 24) If someone held alcohol under my nose, I would not be able to refuse it.	**.838**	.047	−.017	−.066	−.018
(I 27) I would drink alcohol if my friends offered it to me on a street.	**.823**	.059	−.043	−.096	−.037
(I 42) I might drink alcohol at a party or a gathering.	**.661**	.084	−.074	−.051	.142
(C 29) I would drink alcohol if I am alone.	**.580**	−.118	.187	.156	.083
(I 46) I will drink alcohol in near future.	**.536**	.095	−.079	−.051	.185
(C 47) I want to drink alcohol even if it deteriorates my health.	**.522**	−.038	.226	.067	.004
(C 32) If my friend gave me alcohol, I would drink it even in the hospital.	**.479**	−.056	.272	.027	−.133
Factor 2: Emotionality problems (EP)					
(E 22) I feel lonely.	−.046	**.823**	−.114	−.019	−.015
(E 15) I am not motivated to do anything.	.112	**.733**	.119	−.290	−.236
(E 28) I am anxious about my future.	.225	**.565**	−.175	.319	−.031
(E 33) I cannot control my feeling.	.149	**.554**	−.029	.020	−.054
(E 7) I am annoyed by words from others.	−.150	**.486**	.112	−.049	.052
(E 10) I am irritated.	−.297	**.476**	.354	.009	.198
(E 34) I have significant job-related problems.	.107	**.442**	.060	.293	−.165
(E 25) I feel bored.	.149	**.404**	.079	−.236	.102
Factor 3: Compulsivity for alcohol (CA)					
(C 44) I want alcohol even if I have to steal.	.098	.029	**.702**	−.036	−.137
(C 13) I would do almost anything in order to drink alcohol.	−.109	.044	**.534**	.047	.128
(C 40) I would do anything to get money for alcohol.	.132	−.013	**.502**	.001	.047
Factor 4: Lack of negative expectancy for alcohol (NE)					
(N 30) If I drink alcohol, I think it would badly influence my job (reverse-coded).	.103	.047	−.079	**.798**	−.143
(N 23) I would not be able to control myself if I drink alcohol (reverse-coded).	−.141	.118	.145	**.541**	−.146
(N 12) If I drink a small amount of alcohol, I would not be able to stop drinking (reverse-coded).	−.001	−.129	.282	**.464**	.198
(N 39) I would feel restless if I drank alcohol (reverse-coded).	−.213	.013	.103	**.441**	.156
Factor 5: Positive expectancy for alcohol (PE)					
(P 43) If I drink alcohol, I will feel everything is going well.	.169	−.197	.039	−.025	**.780**
(P 45) If I drink alcohol, I will feel invigorated.	.181	.085	.016	−.005	**.541**
(P 26) Alcohol would save me from feeling lonely.	.186	.252	−.003	.011	**.410**
Ambiguous items					
(I 38) If I had a large sum of money, I would want to buy alcohol.	**.564**	−.089	.443	−**.107**	−.001
Other items					
(I 11) I am dying to drink alcohol.	.370	.094	.320	−.201	.103
(C 48) Even though I know I will lose my family or job, I would drink alcohol.	.341	.169	.158	.168	−.044
(E 3) The feeling I used to have while drinking alcohol sometimes comes back.	.297	.124	.092	.123	.103
(E 5) I feel a constant need to put something in my mouth.	.266	.219	−.140	−.040	.068
(E 36) I feel tired due to impatience.	−.092	.395	.227	.224	.111
(E 31) I occasionally have nightmares.	−.034	.328	.107	.008	.200
(N 20) I am afraid of withdrawal due to alcohol dependence (reverse-coded).	−.058	.326	.050	.124	.015
(E 8) I am anxious about relapse.	.163	.161	.312	−.040	−.037
(E 14) I feel easier than before (reverse-coded).	.085	−.124	−.332	.391	.123
(E 1) I want to find a job or need to improve my work environment.	.256	.158	−.025	.357	
(N 2) I need to make the most of my friend's (and AA's) support (reverse-coded).	.001	.000	−.175	.296	.089
(N 18) Thinking about my family, I can no longer drink alcohol (reverse-coded).	−.216	−.075	.055	.257	−.020
(P 16) I recall the relief from feeling blue from the time I was drinking alcohol.	−.027	.282	−.076	.102	.379
(I 4) There are times I want to drink alcohol.	.305	.119	−.130	−.063	.328
(P 41) If I drink alcohol, I would be less nervous.	.229	.300	.003	.028	.311

E: emotionality problems; C: compulsivity; I: clear intention of alcohol use; P: positive expectancy for alcohol; N: negative expectancy for alcohol in terms of the initial five concepts. Numbers followed by single parentheses indicate the order in the ARRS. Values higher than 0.4 are in bold.

2.6. *Statistical analysis*

Raw scores for the negatively worded items (items 2, 6, 12, 14, 17, 18, 19, 20, 21, 23, 30, 37) were reversed to make these items positively correlated with other items. The inner structure of the 43-item ARRS without the items that assessed insight into the patient's mental condition was examined by exploratory factor analysis using a principal factor method with promax rotation to detect simple structure. Exploratory factor analysis was used instead of confirmatory factor analysis because the inner structure of the ARRS was expected to be different from that of the SRRS. The items that assessed insight into mental condition were excluded from factor analysis of the relapse risk structure because they were added to the questionnaire to distinguish patients who are "in denial." Factors were extracted on the basis of their eigenvalues (>1) and the scree plot. Only the items loading higher than 0.4 were retained in the analyses, and all items cross-loading at higher than 0.4 were removed. The reliability of the extracted factor scales was checked by calculating Cronbach's α value. Concurrent and predictive validity of the subscales and inter-subscale correlations were analyzed by calculating Pearson's product-moment correlation coefficient. With regard to predictive validity, logistic regression analysis was also used to examine whether the ARRS subscales as independent variables predict relapse as a dependent variable. All subscales of the ARRS were analyzed at the same time. For analysis of current state of drinking and relapse, "drinking" and "relapse" were coded as 1, and "not drinking" and "no relapse" were coded as 0.

Additionally, the function of the five items that assessed insight into mental condition was examined. Relationships among insight into mental condition, relapse, and the period of abstinence were analyzed by Fisher's exact test. Median split (i.e., median of the five items' average scores = 2.0; period of abstinence = 150) was used to divide the variables into two groups. Data on relapse and the period of abstinence for 66 participants were used for the analysis.

All analyses were performed with the Statistical Package for the Social Sciences (SPSS) version 12.0 for Windows.

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Table 3
Cronbach's α for each subscale of the ARRS and correlation of the ARRS against current drinking, VAS, CES-D, GHQ-12, and relapse.

	Cronbach's α	Correlation					
		Current drinking state	VAS (current craving)	VAS (craving in the past 2 weeks)	CES-D	GHQ-12	Relapse (1 month)
ARRS subscale [number of items]							
Stimulus-induced vulnerability (SV) [9]	.897	.497**	.472**	.604**	.306**	.495**	.268**
Emotionality problems (EP) [8]	.794	.197**	.255**	.386**	.537**	.578**	.131
Compulsivity for alcohol (CA) [3]	.730	.196**	.212**	.350**	.319**	.219	.004
Lack of negative expectancy for alcohol (NE) [4]	.785	.165*	.011	−.003	−.158*	−.316**	.169
Positive expectancy for alcohol (PE) [3]	.545	.341**	.272**	.480**	.328**	.435**	.178
Total ARRS [27]	.864	.410**	.357**	.541**	.445**	.394**	.215*

Note: Reliability was calculated according to Cronbach's alpha. Concurrent validity was calculated according to correlation of ARRS against current drinking, VAS, CES-D, and GHQ-12. Predictive validity was calculated according to correlation of ARRS against relapse.
* $p<.05$.
** $p<.01$.

3. Results

3.1. Factor analysis

Exploratory factor analysis of the ARRS scores for 218 participants revealed five factors with eigenvalues of 9.38, 4.60, 2.55, 2.33, and 1.96. These factors accounted for 47.54% of the overall variance (26.60%, 8.10%, 4.84%, 4.40%, and 3.62% for factors 1–5, respectively). Cronbach's α values for factors 1, 2, 3, 4, 5, and all items were .89, .79, .73, .78, .54, and .86, respectively. The factors were subsequently rotated using the promax method. Of the original 43 items, 27 were retained and 16 were discarded. The factor structure after the promax rotation is shown in Table 2. Cronbach's α values for each subscale and the total ARRS (all 27 extracted items) are shown in Table 3.

The first factor had significant loadings for nine items, including all seven items that reflect stimulus-induced vulnerability (e.g., "If alcohol is placed in front of me, I would drink it" and "It would be difficult for me to refuse if someone placed alcohol in front of me"). The two remaining items reflected intention and desire to drink alcohol (e.g., "I will drink alcohol in the near future" and "I want to drink alcohol even if it deteriorates my health"). The first factor, therefore, was labeled "stimulus-induced vulnerability" (SV).

Eight items loaded exclusively on the second factor. All of these items reflected emotional problems related to alcohol consumption (e.g., "I feel lonely" and "I am not motivated to do anything"). This factor, therefore, was labeled "emotionality problems" (EP).

The third factor had significant loadings for three items, all of which reflecting compulsivity for alcohol (e.g., "I want alcohol even if I have to steal" and "I would do almost anything to drink alcohol"). This factor, therefore, was labeled "compulsivity for alcohol" (CA).

The fourth factor comprised four items that had originally been classified as negative expectancy for alcohol drinking (e.g., "If I drink alcohol, it would badly influence my job" and "I would not be able to control myself if I drink alcohol"). This factor, therefore, was labeled "lack of negative expectancy for alcohol drinking" (NE).

The fifth factor comprised three items that reflected positive expectancy about alcohol drinking (e.g., "If I drink alcohol, I will feel everything is going well" and "If I drink alcohol, I will feel invigorated"). The fifth factor, therefore, was labeled "positive expectancy for alcohol" (PE). Although the internal consistency of this factor was insufficient, its items were retained in the scale because positive expectancy is a significant factor of relapse risk in stimulant abusers (Ogai et al., 2007).

Additionally, we analyzed the function of the five items for assessing insight into mental condition. Cronbach's α values for these items were .68. The association between insight into mental condition and relapse was not significant regardless of the duration of abstinence.

3.2. Basic statistics of the ARRS and inter-subscale correlations

Table 4 presents means, standard deviations, and inter-correlations of the five ARRS factor scales (subscales). No significant correlations were found between "stimulus-induced vulnerability" (Factor 1) and "lack of negative expectancy for drug use" (Factor 4). The other subscales exhibited low to moderate positive inter-correlations.

3.3. Concurrent validity of the ARRS

Correlation coefficients between the ARRS scores (i.e., subscale scores and total score for the 27 items) and the variables that were measured to examine the concurrent validity were calculated (Table 3). The current state of drinking was significantly and positively correlated with the scores for stimulus-induced vulnerability, emotionality problems, compulsivity for alcohol, positive

Table 4
Mean and S.D. of the ARRS and inter-subscale correlations.

	Mean (S.D.)	SV	EP	CA	NE	PE
ARRS subscale (range: 1–3)						
Stimulus-induced vulnerability (SV)	1.53 (0.53)	–	.446**	.404**	−.076	.560**
Emotionality problems (EP)	1.80 (0.53)		–	.362**	−.318**	.507**
Compulsivity for alcohol (CA)	1.20 (0.38)			–	−.276**	.375**
Lack of negative expectancy for alcohol (NE)	1.94 (0.59)				–	−.250**
Positive expectancy for alcohol (PE)	1.54 (0.61)					–
Total ARRS (range: 1–3)	1.62 (0.34)					

S.D.: standard deviation.
** $p<.01$.

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Table 5
Logistic regression analysis of each ARRS subscale as independent variable and relapse within 1 month as dependent variable.

	Coefficient *B* (SE)	Wald statistic (d.f.)	*p*-Value	Odds ratio	95% Confidence interval
ARRS subscale					
Stimulus-induced vulnerability (SV)	.994 (.565)	3.095 (1)	.079	2.703	.893–8.186
Emotionality problems (EP)	.392 (.549)	.508 (1)	.476	1.479	.504–4.342
Compulsivity for alcohol (CA)	−.351 (.691)	.258 (1)	.612	.704	.182–2.729
Lack of negative expectancy for alcohol (NE)	1.008 (.461)	4.771 (1)	.029	2.740	1.109–6.769
Positive expectancy for alcohol (PE)	.574 (.499)	1.323 (1)	.250	1.77	5.668–4.721

Note: Predictive validity was also calculated according to odds ratio of ARRS against relapse. 118 data was used for analysis.

expectancy for alcohol drinking, and total ARRS. The current state of drinking was significantly and negatively correlated with lack of negative expectancy. The two VAS scores for alcohol craving ("current craving" and "craving in the past two weeks") were also significantly and positively correlated with the scores for stimulus-induced vulnerability, emotionality problems, compulsivity for alcohol, positive expectancy, and total ARRS. Additionally, the CES-D and GHQ-12 scores were significantly and positively correlated with the scores for all subscales, with the exception of compulsivity for alcohol in the GHQ-12 and total ARRS.

3.4. Predictive validity of the ARRS

Table 3 also presents correlations between the ARRS scores and relapse within 1 month. Relapse was significantly and positively correlated with stimulus-induced vulnerability and total ARRS. Craving in the past 2 weeks (measured by VAS; $r = 0.317$) and the period of abstinence ($r = -0.252$) were significantly correlated with relapse. A significant and positive relationship was found between lack of negative expectancy in the ARRS and participants' compliance at follow-up ($r = 0.199$; 127 participants approved, 75 refused, and 20 were not asked). Logistic regression analysis (Table 5) revealed that lack of negative expectancy significantly and positively predicted relapse (odds ratio = 2.740, $p < .05$), and stimulus-induced vulnerability showed a tendency toward positively predicting relapse (odds ratio = 2.703, $p = .079$).

3.5. Gender differences and differences between inpatients and outpatients

The relationship between ARRS scores and current state of drinking and the relationship between ARRS scores and relapse were differentiated by treatment state (i.e., inpatient *vs.* outpatient) and gender (i.e., male *vs.* female) (Table 6). Among outpatients, a significant positive correlation was observed between lack of negative expectancy and current state of drinking. For inpatients, in contrast, the current state of drinking was significantly and negatively correlated with lack of negative expectancy.

For males, stimulus-induced vulnerability, positive expectancy, and total ARRS were significantly and positively correlated with relapse. For females, lack of negative expectancy was significantly and positively correlated with relapse.

4. Discussion

The present study developed the ARRS to assess relapse risk for Japanese alcohol-dependent individuals and statistically examined its inner structure, reliability, and validity. Five factors were found, and their internal consistency, concurrent validity, and predictive validity were revealed. Notably, part of the ARRS was related to relapse, implying its potential application for relapse prediction. Our findings demonstrated that the ARRS has multidimensional psychometric properties. Thus, the ARRS may be useful for assessing various aspects of relapse risk in alcohol-dependent individuals, similar to the SRRS for stimulant abusers.

Some similarities were found in the multidimensional structures of the ARRS and the SRRS; emotionality problems (Factor 2), compulsivity for alcohol (Factor 3), lack of negative expectancy (Factor 4), and positive expectancy (Factor 5) were similar to "emotionality problems," "compulsivity for drug," "lack of negative expectancy," and "positive expectancy" of the SRRS subscales, respectively. Factor 2 revealed negative emotional states (e.g., anxiety and negative mood) that have been shown previously to be related to relapse in alcohol-dependent individuals (Lucht et al., 2002; Walitzer and Dearing, 2006). Factor 3 was considered to reflect craving based on "obsessive compulsive theory" (Anton, 2000). Factor 4 and Factor 5 were considered to reflect craving based on "expectancy theory" (Jones et al., 2001). Positive expectancy for substance increases the risk of relapse, whereas negative expectancy for substance (understanding the harmful effects of the substance) decreases the risk. The above four factors are risk factors that may trigger relapse in alcohol-dependent individuals, as well as in stimulant abusers.

Differences were also found between the ARRS and the SRRS. "Stimulus-induced vulnerability" (Factor 1) in the ARRS and "anxiety and intention to drug use" in the SRRS, both of which relating to relapse, have differences in content. This may reflect the fact that alcohol-dependent individuals often encounter environmental stimuli related to alcohol because it is not illegal and is commonly

Table 6
Correlation of the ARRS against relapse by treatment state and gender.

	Correlation with current state of drinking				Correlation with relapse	
	Inpatient (*N* = 29)	Outpatient (*N* = 182)	Male (*N* = 183)	Female (*N* = 35)	Male (*N* = 97)	Female (*N* = 27)
ARRS subscale						
Stimulus-induced vulnerability (SV)	.615**	.447**	.543**	.201	.344**	.075
Emotionality problems (EP)	.656**	.082	.312**	−.378*	.185	−.005
Compulsivity for alcohol (CA)	.565**	.094	.229**	.032	.037	−.087
Lack of negative expectancy for alcohol (NE)	−.399**	.289**	.156*	.267	.076	.503**
Positive expectancy for alcohol (PE)	.664**	.262**	.435**	−.157	.280**	−.149
Total ARRS	.690**	.341**	.512**	−.031	.357**	−.046

* $p < .05$.
** $p < .01$.

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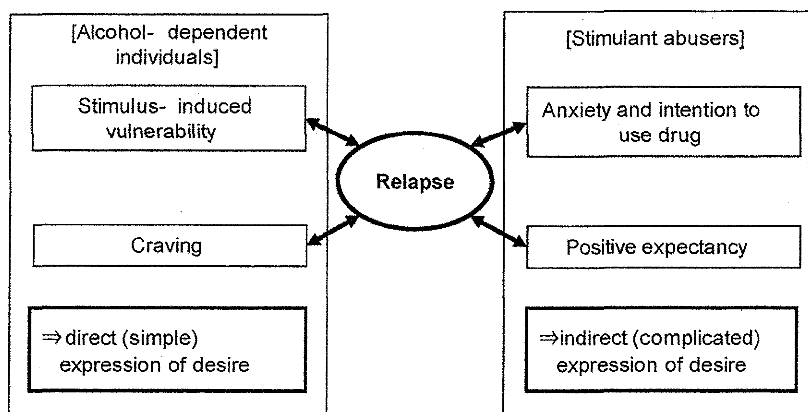

Fig. 1. Differences in the factors related to relapse between alcohol-dependent individuals and stimulant abusers. In alcohol-dependent individuals, stimulus-induced vulnerability in the ARRS and craving measured by the VAS were significantly related to relapse within 1 month. Anxiety, intention, and positive expectancy in the SRRS in stimulant abusers were significantly related to relapse within 3 months. These results indicate that alcohol-dependent individuals express signs of relapse more directly, such as craving for alcohol, and appear to be easily influenced by the environment. Stimulant abusers, in contrast, express signs of relapse indirectly, such as anxiety and expectancy, which are inner feelings.

consumed. In fact, social pressure is one of the relapse risks for alcohol-dependent individuals (Zywiak et al., 2006).

With regard to concurrent validity, the scores for total ARRS and all subscales except lack of negative expectancy were significantly correlated with the current state of drinking and the VAS scores. Specifically, the coefficients for stimulus-induced vulnerability and positive expectancy for alcohol were more than 0.4. These results suggest that stimulus-induced vulnerability and positive expectancy are important factors that govern the severity of alcohol dependence and craving related to the subjective desire for alcohol, although the difference in time frames of the ARRS (past 1 week) and the VAS (the current and past 2 weeks) must be considered.

With regard to predictive validity, the scores for stimulus-induced vulnerability and total ARRS were significantly correlated with relapse within 1 month, suggesting that these scores predict relapse risk. This was supported by the nearly significant prediction shown in the logistic regression analysis. The correlation between the scores for stimulus-induced vulnerability and relapse supports the hypothesis of a prior study of more than 900 individuals in which relapse was found to be triggered by social pressure such as temptation from alcohol-drinking friends (Zywiak et al., 2006). Particularly in Japanese collectivistic culture, refusing an offer to drink alcohol at a party is difficult. According to Hendershot et al. (2005), alcohol use is influenced by cultural background. Thus, the influence of culture on stimulus-induced vulnerability (e.g. "It would be difficult for me to refuse if someone placed alcohol in front of me") may be a more prevalent risk factor in Japan.

Interestingly, the logistic regression analysis showed that lack of negative expectancy significantly predicted relapse. This result suggested that lack of understanding of negative effects of alcohol drinking was an important factor leading to relapse. This result was also consistent with a report in which relapsed alcohol-dependent individuals and their families reported that "reduced cognitive vigilance" was the most common relapse sign (Malhotra et al., 1999). The differences between correlation analysis (Table 3) and logistic regression analysis (Table 5) relating Factor 4 with relapse indicate that some indirect effects of Factor 4 on relapse via other factors (e.g., positive expectancy) counterbalanced the direct effect of Factor 4 in the correlation score.

Although preliminary, stimulus-induced vulnerability and lack of negative expectancy in the ARRS and craving measured by the VAS among alcohol-dependent individuals were significantly related to relapse within 1 month. In stimulant abusers, in contrast, anxiety, intention, positive expectancy, and lack of negative expectancy were significantly related to relapse within 3 months. These results indicate that alcohol-dependent individuals express signs of relapse more directly, such as craving for alcohol, and appear to be easily influenced by the environment. In contrast, stimulant abusers express signs of relapse indirectly through inner feelings such as anxiety and expectancy (Fig. 1).

Other subscales (e.g., emotionality problems, compulsivity for alcohol, and positive expectancy) were not related to relapse. However, the significant correlations of these subscales with stimulus-induced vulnerability, lack of negative expectancy, and craving measured by the VAS suggest that these factors may have an indirect effect on relapse. Additionally, internal consistency of positive expectancy was low.

The relationships among the ARRS, current state of drinking, and relapse were influenced by treatment state and gender. Among inpatients, higher negative expectancy was associated with the risk of current drinking. By contrast, lower negative expectancy in outpatients was associated with the risk of current drinking. These results may reflect the fact that inpatients check into hospitals because they are more aware of the risk of drinking than outpatients. With regard to gender, higher vulnerability and positive expectancy were related to higher risk of relapse in males. By contrast, lower negative expectancy in females was related to high risk of relapse. These results were consistent with a report showing that alcohol-dependent males had a more positive affect during the week before relapse than females (McKay et al., 1996). The above results suggest the necessity of gender-specific intervention.

One possible limitation to the present study was the sampling procedure. The participants were not recruited randomly but were limited only to inpatients or outpatients who gave informed consent and whose doctors recognized their ability to answer the questionnaire. Therefore, the data of this study were not obtained from alcohol-dependent individuals as a whole, including dropout patients and non-patients, but rather only from cooperative patients with a relatively low severity of alcohol dependence. Additionally, the relatively low availability of relapse data (124/218) may have influenced the assessment of the ARRS's predictive validity. Conducting follow-up surveys for dropout cases and recruiting participants from other facilities and programs, including Alcoholics Anonymous, are necessary to gain a better understanding of relapse risk in alcohol-dependent individuals. Another limitation of the present study was the relatively low sample size. A sample of 218 participants was rather small to sufficiently support the factor analysis of 43 items. In the present

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study, the number of participants per item was 5.07. Because of the wide range of sample size recommended for factor analysis of five participants per item (Gorsuch, 1983) to ten participants per item (Everitt, 1975), further study of the ARRS with larger samples will reveal the detailed features of the ARRS factor structure.

The low to moderate correlation between relapse within 1 month and the ARRS subscales may indicate a limitation in the clinical utility of this scale. However, in the present study, the ARRS and the VAS similarly predicted relapse within 1 month. One month reflects the highest level of the hazard function and is the most clinically relevant. The ARRS will likely predict greater levels of variance than the VAS with longer follow-up periods because alcohol craving wanes as the period of abstinence increases (Tavares et al., 2005). Furthermore, the ARRS is anticipated to complement the use of the VAS because the ARRS assesses a wider range of constructs.

Although the results for the items assessing insight into mental condition were not significant, these items should remain to distinguish patients who are "in denial." The expressions of some items (e.g., "I would be fine without alcohol") posed difficulty in distinguishing dishonest from honest responses because recovering honest patients answer "agree" to these items. Changing the expressions of these items in the future is necessary.

The present results suggest that the ARRS is an effective tool with which psychiatrists, psychologists, social workers, and alcohol-dependent individuals themselves could assess the level of relapse risk, similar to the SRRS for stimulant abusers, although the predictive validity of the instrument is preliminary. The ARRS and SRRS may also contribute to the assessment of craving-inhibitory effects of pharmacotherapies and treatment programs. To improve the usefulness of these scales, further studies of at least the following are necessary: (i) cross-validity using other alcohol-dependent individuals with confirmatory factor analysis, (ii) modification of the ARRS for a better prediction of relapse, and (iii) utilization of the SRRS and ARRS as communication tools among facilities that treat stimulant abusers and alcohol-dependent individuals, such as hospitals (or other treatment facilities), legal facilities (e.g., prisons or probation offices), and research institutes.

Conflict of interest

All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the on-line version, at doi:10.1016/j.drugalcdep.2008.10.021.

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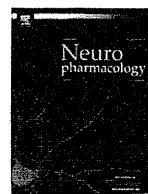

Inhibitory effects of the antiepileptic drug ethosuximide on G protein-activated inwardly rectifying K^+ channels

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Platelet aggregation

ABSTRACT

Antiepileptic drugs protect against seizures by modulating neuronal excitability. Ethosuximide is selectively used for the treatment of absence epilepsy, and has also been shown to have the potential for treating several other neuropsychiatric disorders in addition to several antiepileptic drugs. Although ethosuximide inhibits T-type Ca^{2+}, noninactivating Na^+, and Ca^{2+}-activated K^+ channels, the molecular mechanisms underlying the effects of ethosuximide have not yet been sufficiently clarified. G protein-activated inwardly rectifying K^+ channels (GIRK, or Kir3) play an important role in regulating neuronal excitability, heart rate and platelet aggregation. In the present study, the effects of various antiepileptic drugs on GIRK channels were examined first by using the *Xenopus* oocyte expression assay. Ethosuximide at clinically relevant concentrations inhibited GIRK channels expressed in *Xenopus* oocytes. The inhibition was concentration-dependent, but voltage-independent, and time-independent during each voltage pulse. However, the other antiepileptic drugs tested: phenytoin, valproic acid, carbamazepine, phenobarbital, gabapentin, topiramate and zonisamide, had no significant effects on GIRK channels even at toxic concentrations. In contrast, Kir1.1 and Kir2.1 channels were insensitive to all of the drugs tested. Ethosuximide also attenuated ethanol-induced GIRK currents. These inhibitory effects of ethosuximide were not observed when ethosuximide was applied intracellularly. In granule cells of cerebellar slices, ethosuximide inhibited GTPγS-activated GIRK currents. Moreover, ADP- and epinephrine-induced platelet aggregation was inhibited by ethosuximide, but not by charybdotoxin, a platelet Ca^{2+}-activated K^+ channel blocker. These results suggest that the inhibitory effects of ethosuximide on GIRK channels may affect some of brain, heart and platelet functions.

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1. Introduction

Antiepileptic drugs protect against seizures by modulating neuronal excitability through effects on Na^+ and Ca^{2+} channels, and GABAergic and glutamatergic neurotransmission (McNamara, 2001). Among antiepileptic drugs, ethosuximide is used mainly for its selective effect on absence (petit mal) epilepsy, which is characterized by paroxysmal three cycle per second spike-and-wave activity associated with lapses of consciousness, but not for the treatment of other generalized epilepsies (McNamara, 2001). Partial inhibition of T-type Ca^{2+} channels by ethosuximide at therapeutically relevant concentrations has been thought to play an important role in its unique antiseizure action (McNamara, 2001; Huguenard, 2002). Furthermore, ethosuximide has been shown to inhibit noninactivating Na^+ and Ca^{2+}-activated K^+ channels (Crunelli and Leresche, 2002), and the inhibition of these channels might also be relevant. Several antiepileptic drugs are commonly prescribed for the treatment of nonepileptic conditions, including neuropathic pain, mood disorders and various neuromuscular syndromes (Rogawski and Löscher, 2004). Ethosuximide has also been shown to have neuroprotective (Rekling, 2003), antinociceptive (Shannon et al., 2005) and life-span-extending effects (Evason et al., 2005), and has the potential for treating several neuropsychiatric disorders, including Parkinson's disease (Gomez-Mancilla et al., 1992), methamphetamine-induced stereotyped behavior (Jadhav et al., 1981), and alcohol dependence (Kaneto

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et al., 1986). However, the molecular and cellular mechanisms underlying the effects of antiepileptic drugs have not yet been sufficiently clarified.

G protein-activated inwardly rectifying K^+ (GIRK) channels (also known as Kir3 channels) have been shown to play an important role in regulating neuronal excitability and synaptic transmission, heart rate, and platelet aggregation (Lüscher et al., 1997; Signorini et al., 1997; Kovoor et al., 2001; Shankar et al., 2004). Four GIRK channel subunits have been identified in mammals (Kubo et al., 1993b; Krapivinsky et al., 1995; Lesage et al., 1995). Neuronal GIRK channels are predominantly heteromultimers composed of GIRK1 and GIRK2 subunits in most brain regions or homomultimers composed of GIRK2 subunits in the substantia nigra (Lesage et al., 1995; Karschin et al., 1996; Liao et al., 1996), whereas atrial GIRK channels are heteromultimers composed of GIRK1 and GIRK4 subunits (Krapivinsky et al., 1995). GIRK channels are activated by various G_i protein-coupled receptors (G_iPCRs), such as M_2 muscarinic, α_2 adrenergic, μ-opioid, D_2 dopaminergic, GABA type B ($GABA_B$), and $P2Y_{12}$ ADP receptors through the direct action of G protein βγ-subunits released from G_i family proteins (Dascal, 1997; Kobayashi and Ikeda, 2006). In addition, ethanol activates GIRK channels independently of G protein signaling pathways (Kobayashi et al., 1999; Lewohl et al., 1999). GIRK channel modulation may affect various functions of brain and peripheral tissues. In the present study, we investigated the effects of various antiepileptic drugs on GIRK channels by using the *Xenopus* oocyte expression assay. GIRK channels were significantly inhibited by ethosuximide, but not by the other antiepileptic drugs tested. The pharmacological effects of ethosuximide on GIRK channels were further examined by using granule cells in cerebellar slices and human platelets.

2. Materials and methods

2.1. Drugs and animals

All of the antiepileptic drugs tested: ethosuximide, phenytoin, valproic acid sodium salt, carbamazepine, phenobarbital, gabapentin, topiramate and zonisamide sodium salt, were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethosuximide, valproic acid and gabapentin were dissolved in distilled water or experimental solutions, and the other drugs were dissolved in dimethyl sulfoxide. The stock solution of each compound was stored at −30 °C until use. Ethanol was purchased from Wako Pure Chemical Industries (Osaka, Japan). Each compound was added to the perfusion solution in appropriate amounts immediately before the experiments. For platelet aggregation experiments, ADP, epinephrine, charybdotoxin, apyrase (grade VII) and human fibrinogen were obtained from Sigma–Aldrich.

All procedures for the care and treatment of animals were carried out in accordance with the National Institutes of Health guidelines and were approved by our Institutional Animal Care and Use Committee.

2.2. Oocyte electrophysiology

Plasmids containing the entire coding sequences for the mouse GIRK1, GIRK2, and GIRK4 channel subunits were obtained previously (Kobayashi et al., 1995, 1999). cDNAs for rat Kir1.1 in pSPORT (Ho et al., 1993) and mouse Kir2.1 in pcDNA1 (Kubo et al., 1993a) were provided generously by Dr. Steven C. Hebert (Yale University, New Haven, CT, USA) and Dr. Lily Y. Jan (University of California, San Francisco, San Francisco, CA, USA), respectively. These plasmids were linearized by digestion with the appropriate enzyme as described previously (Ho et al., 1993; Kubo et al., 1993a; Kobayashi et al., 2006). The specific mRNAs were synthesized *in vitro* by using the mMESSAGE mMACHINE™ *In Vitro* Transcription Kit (Ambion, Austin, TX, USA).

Xenopus oocytes (Stages V and VI) were isolated from adult female *Xenopus laevis* frogs (Copacetic, Soma, Aomori, Japan) that were anesthetized by immersion in water containing 0.15% tricaine (Sigma–Aldrich) as described previously (Kobayashi et al., 2006). Oocytes were injected with mRNA for GIRK1/GIRK2 or GIRK1/GIRK4 combinations (each 0.4 ng), GIRK2 (5 ng), Kir1.1 (5 ng), or Kir2.1 (0.5 ng). Oocytes were incubated at 19 °C in Barth's solution after treatment with 0.8 mg/ml collagenase for 1 h and manually defolliculated. Whole-cell currents of the oocytes were recorded from 2 to 8 days after injection with a conventional two-electrode voltage clamp (Kobayashi et al., 1999, 2006). The membrane potential was held at −70 mV, unless otherwise specified. Microelectrodes were filled with 3 M KCl. The oocytes were placed in a narrow chamber and superfused continuously with a high-potassium (hK) solution (96 mM KCl, 2 mM NaCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 5 mM HEPES, pH 7.4 with KOH) or a K^+-free high sodium (ND98) solution (98 mM NaCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 5 mM HEPES, pH 7.4 with NaOH). In the hK solution used for the *Xenopus* oocyte expression assay, the K^+ equilibrium potential was close to 0 mV, and the inward K^+ current flow through the Kir channels was observed at negative holding potentials. For examining the effect of intracellular ethosuximide, 23 nl of 1 M ethosuximide dissolved in distilled water was injected into an oocyte using a Nanoliter injector (World Precision Instruments, Sarasota, FL, USA) as described previously (Kobayashi et al., 2006), and the oocyte currents were then continuously recorded for approximately 30–40 min. As the volume of the *Xenopus* oocyte was approximately 1 μl, the intracellular concentration of ethosuximide was presumed to be approximately 22.5 mM. For analysis of concentration–response relationships, data were fitted to a standard logistic equation using KaleidaGraph (Synergy Software, Reading, PA, USA). The concentration of a drug that produces 50% of the maximal effect for that drug (EC_{50}), concentrations required to inhibit control currents by 25% or 50% (IC_{25} and IC_{50}, respectively) and Hill coefficients (n_H) were obtained from the concentration–response relationships.

2.3. Brain slice electrophysiology

Parasagittal cerebellum slices (200 μm thick) were prepared from postnatal day 22 (P22) to P33 C57BL/6 mice using a microslicer (DTK-1000; Dosaka EM, Kyoto, Japan). The slices were maintained at room temperature after an initial 1 h incubation in standard artificial cerebrospinal fluid until needed for recording (Lüscher et al., 1997). Whole-cell patch-clamp recordings were made from granule cells identified with an upright microscope (Axioskop; Carl Zeiss, Oberkochen, Germany) using an EPC-8 amplifier (HEKA Instruments, Lambrecht, Germany). Slices were continuously superfused with an extracellular solution containing 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose and 0.1 mM picrotoxin, a $GABA_A$ receptor antagonist (Wako Pure Chemical Industries), bubbled with 95% O_2 and 5% CO_2. During recordings, the external solution was maintained at 32 °C with a solution in-line heater (SH-27B; Warner Instruments, Hamden, CT, USA). Patch pipettes had a resistance of 6–8 MΩ in the intracellular pipette solution containing 122.5 mM K-gluconate, 17.5 mM KCl, 10 mM HEPES, 0.2 mM EGTA, 8 mM NaCl and 2 mM Mg^{2+}-ATP (pH 7.2, adjusted with KOH). Either 200 μM Na-GTP or 200 μM Li_4-guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) (Sigma–Aldrich), a nonhydrolyzable G protein activator, was added to the intracellular pipette solution. Granule cells were voltage-clamped at −70 mV, and GTPγS-activated inwardly rectifying currents were elicited by shifting membrane voltage from −120 mV to −20 mV after transient depolarization to 0 mV. The transient depolarization was applied to block generation of voltage-dependent sodium spikes. The effects of ethosuximide or Ba^{2+} on GTPγS-activated currents were tested by bath-application. Signals were filtered at 3 kHz, digitized at 10 kHz (Digidata 1320A; Molecular Devices, Sunnyvale, CA, USA) and analyzed by pCLAMP8 software (Molecular Devices).

2.4. Preparation of platelets and platelet aggregation

Whole blood was collected from three healthy consenting human volunteers into tubes containing one-tenth volume of 3.8% sodium citrate. Platelet-rich plasma was obtained by centrifugation at 180 × *g* for 10 min at room temperature. Then platelet-rich plasma was mixed with 15% volume of acid citrate dextrose (2.5 g sodium citrate, 1.5 g citric acid and 2 g glucose in 100 ml deionized water), and the mixture was centrifuged at 800 × *g* for 10 min. The platelet pellet was suspended in Ca^{2+}-free Tyrode buffer (138 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, 5 mM glucose and 10 mM HEPES, adjusted to pH 7.4) containing 0.01 U/ml apyrase. Platelets were counted using a Sysmex F-820 counter (Sysmex, Kobe, Hyogo, Japan) and adjusted to 3×10^8 cells/ml. Human fibrinogen (final 1 mg/ml) was exogenously added to all samples.

Platelet aggregation responses (0.2 ml samples) were determined by measuring an increase in light transmission using an NBS Hema Tracer aggregometer (Niko Bioscience, Tokyo, Japan) with stirring at 1000 rpm at 37 °C. Platelets were stimulated with 2 μM ADP or 1 μM epinephrine. Compounds tested were added 5 min prior to the addition of ADP or epinephrine. The baseline was set by using Tyrode buffer as blank.

2.5. Statistical analysis

The values obtained are expressed as the mean ± S.E.M., and *n* indicates the number of oocytes and neurons tested. Statistical analysis of differences between groups was performed using paired *t*-test, one-way ANOVA or two-way ANOVA followed by Tukey–Kramer *post hoc* test. A probability of 0.05 was taken as the level of statistical significance.

3. Results

3.1. Effects of antiepileptic drugs on GIRK channels

In *Xenopus* oocytes injected with GIRK1 and GIRK2 mRNAs, basal GIRK currents, which depend on free G protein βγ-subunits present

in the oocytes because of the inherent activity of G proteins (Dascal, 1997), were observed at a holding potential of −70 mV in an hK solution containing 96 mM K^+ (Fig. 1A). Extracellular application of ethosuximide (750 μM and 3 mM) reversibly reduced the basal GIRK currents (Fig. 1A). The current responses to an additional 3 mM ethosuximide during the application of 3 mM Ba^{2+}, which blocks Kir channels, were not significant (0.8 ± 0.6 nA: less than 0.5% of the Ba^{2+}-sensitive current components, $n = 4$). The 3 mM Ba^{2+}-sensitive current components (1324.9 ± 197.9 nA, $n = 10$) are considered to correspond to the magnitudes of GIRK currents in oocytes expressing GIRK channels (Kobayashi et al., 1999). Ethosuximide produced no significant response in a K^+-free ND98 solution containing 98 mM Na^+ instead of the hK solution ($n = 4$, data not shown), suggesting that the ethosuximide-sensitive current components show K^+ selectivity. However, in oocytes injected with mRNA for Kir1.1, an ATP-regulated Kir channel (Ho et al., 1993), or Kir2.1, a constitutively active Kir channel (Kubo et al., 1993a), 10 mM ethosuximide had no significant effect on the inward currents through the channels in the hK solution (less than 2% inhibition of the 3 mM Ba^{2+}-sensitive current components, $n \geq 4$, Fig. 1). In uninjected oocytes, 30 mM ethosuximide or 3 mM Ba^{2+} produced no significant response (2.3 ± 1.3 nA, $n = 4$; and 4.6 ± 1.2 nA, $n = 5$, respectively) in comparison with those in oocytes injected with GIRK mRNA (Fig. 1A), suggesting no effect of ethosuximide and Ba^{2+} on intrinsic oocyte channels. In addition, application of distilled water, the solvent vehicle, at the highest concentration (0.3%), had no significant effect in oocytes injected with GIRK1 and GIRK2 mRNAs ($n = 4$; data not shown). Similarly, in oocytes injected with either GIRK1 and GIRK4 mRNAs or GIRK2 mRNA, ethosuximide inhibited basal GIRK currents ($n = 7$).

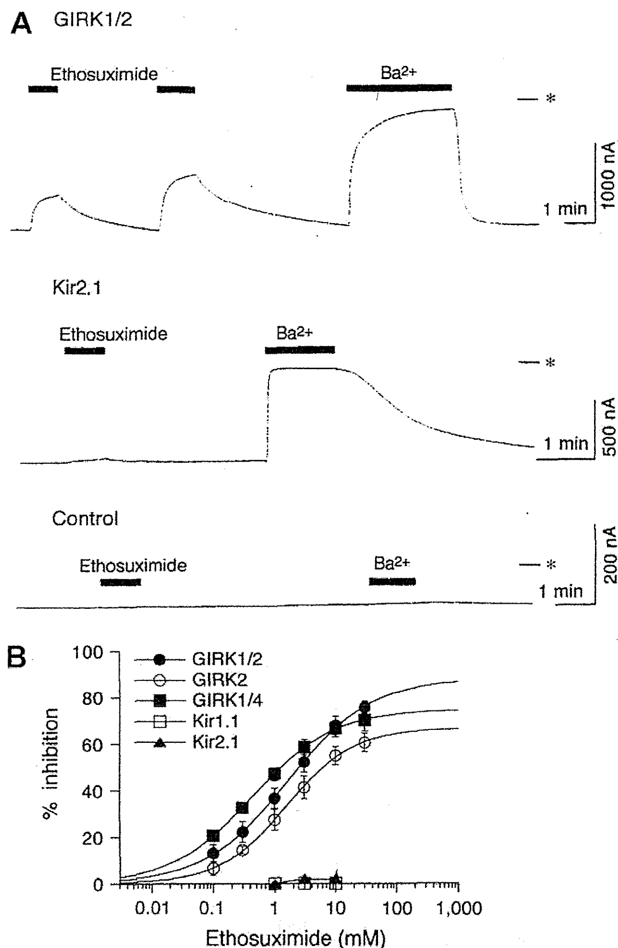

Fig. 1. Inhibitory effect of ethosuximide on GIRK channels expressed in *Xenopus* oocytes. (A) Top, in an oocyte injected with GIRK1 and GIRK2 mRNAs, current responses to 750 μM and 3 mM ethosuximide and to 3 mM Ba^{2+} are shown. Middle, in an oocyte injected with Kir2.1 mRNA, current responses to 10 mM ethosuximide and to 3 mM Ba^{2+} are shown. Bottom, in an uninjected oocyte, no significant current responses to 30 mM ethosuximide or 3 mM Ba^{2+} are shown. Current responses were measured at a membrane potential of −70 mV in an hK solution containing 96 mM K^+. Asterisks show the zero current level. Bars show the duration of application. (B) Concentration–response relationships for the inhibitory effects of ethosuximide on GIRK1/2, GIRK2 and GIRK1/4 channels. The magnitudes of inhibition of Kir currents by ethosuximide were compared to the 3 mM Ba^{2+}-sensitive current components in oocytes expressing Kir1.1 (422.5 ± 47.5 nA, $n = 4$), Kir2.1 (512.0 ± 95.5 nA, $n = 5$), GIRK1/2 (1324.9 ± 197.9 nA, $n = 10$), GIRK2 (386.0 ± 145.8 nA, $n = 7$) or GIRK1/4 (1429.4 ± 212.1 nA, $n = 7$) channels. Each point and error bar represent the mean and S.E.M. of the percentage responses. There was a significant interaction between the channel effect and the effect of ethosuximide ($P < 0.05$, two-way ANOVA). The effects of 300 μM–3 mM ethosuximide on GIRK1/4 channels were significantly higher than those on GIRK2 channels ($P < 0.05$, Tukey–Kramer *post hoc* test).

In contrast, carbamazepine slightly inhibited basal GIRK currents in oocytes expressing GIRK channels (3.6 ± 0.6, 16.0 ± 1.4 and 15.8 ± 1.7% inhibition of the 3 mM Ba^{2+}-sensitive current components for GIRK1/2, and 5.1 ± 1.3, 10.9 ± 0.9 and 16.6 ± 2.3% inhibition of those for GIRK1/4, at 100, 300 and 1000 μM, respectively; $n \geq 4$). Phenobarbital (1 mM) and zonisamide (1 mM) inhibited basal GIRK currents to a lesser extent than carbamazepine (7.7 ± 1.7 and 9.9 ± 1.5% inhibition for GIRK1/2, and 10.7 ± 1.0 and 6.5 ± 1.0% inhibition for GIRK1/4, respectively; $n \geq 4$). Phenytoin (300 μM), valproic acid (3 mM), gabapentin (500 μM), and topiramate (300 μM) had little or no effects on basal GIRK currents (less than 5% inhibition; $n \geq 4$). In oocytes expressing Kir1.1 or Kir2.1 channels, each of these antiepileptic drugs at the high concentrations tested produced no significant responses (data not shown; $n \geq 4$). In addition, since the basal GIRK currents were not significantly affected by switching to the hK external solution containing valproic acid sodium salt (3 mmol/l), it is unlikely that the inhibitory effects of ethosuximide were caused by hyperosmotic change in the external solution containing ethosuximide. Therefore, the results suggest that among eight antiepileptic drugs, only ethosuximide significantly inhibited GIRK channels, whereas Kir1.1 and Kir2.1 channels were insensitive to all of the drugs tested. Furthermore, the Ba^{2+}-sensitive current components in oocytes injected with mRNAs for GIRK1/GIRK2 or GIRK1/GIRK4 combinations (1324.9 ± 197.9 nA, $n = 10$ and 1429.4 ± 212.1 nA, $n = 7$, respectively) were very significantly larger than those in oocytes injected with the same small amount of a single GIRK mRNA (less than 30 nA, $n = 7$, respectively). The present observations in oocytes injected with mRNAs for GIRK combinations indicate that ethosuximide predominantly inhibited GIRK1/2 and GIRK1/4 heteromultimeric channels.

3.2. Characteristics of GIRK channel inhibition by ethosuximide

The inhibitory effects of ethosuximide on GIRK1/2, GIRK2 and GIRK1/4 channels were concentration-dependent with distinctive potency and effectiveness (Fig. 1B). Table 1 shows the EC_{50} and n_H values obtained from the concentration–response relationships, and percentage inhibition of the GIRK currents by ethosuximide at the highest concentrations tested. In addition, as ethosuximide could not fully block the GIRK currents even at high concentrations, the IC_{25} and IC_{50} values were also calculated to further compare the effects of ethosuximide (Table 1). The rank order of inhibition of GIRK channels by ethosuximide was GIRK1/4 ≥ GIRK1/2 > GIRK2.

Instantaneous GIRK1/4 currents elicited by the voltage step to −80 mV from a holding potential of 0 mV were diminished in the presence of 3 mM ethosuximide applied for 3 min (Fig. 2A). The percentage inhibition of the steady-state GIRK current at the end of the voltage step by ethosuximide was not significantly different

Table 1
Inhibitory effects of ethosuximide on GIRK channels

	GIRK1/2	GIRK2	GIRK1/4
EC_{50}	1717 ± 333	3502 ± 1145	415 ± 36
IC_{25}	354 ± 78	1176 ± 357	175 ± 32
IC_{50}	3047 ± 113	7306 ± 2486	1574 ± 369
% max	75.6 ± 2.7	60.5 ± 4.0	70.1 ± 4.8
(mM; *n*)	(30; 10)	(30; 7)	(30; 7)
n_H	0.71 ± 0.04	0.79 ± 0.06	0.70 ± 0.03

Mean ± S.E.M. of the concentration of ethosuximide that produces 50% of the maximal effect (EC_{50}) and the concentrations required to reduce basal GIRK currents by 25% and 50% (IC_{25} and IC_{50}, respectively) are shown in μM. The values of % max indicate the mean ± S.E.M. percentage inhibition of basal GIRK currents by ethosuximide at the highest concentrations tested. The highest concentration tested (mM) and the number of oocytes tested (*n*) are indicated in parentheses, respectively. The n_H values indicate the mean ± S.E.M. of Hill coefficients.

from that of the instantaneous current (paired *t*-test, $P > 0.05$, $n = 10$, −40, −60, −80 and −100 mV, respectively). These results suggest that the channels were inhibited by ethosuximide primarily at the holding potential of 0 mV and time-independently during each voltage pulse. Like 3 mM Ba^{2+}-sensitive currents corresponding to basal GIRK currents, 3 mM ethosuximide-sensitive currents in oocytes expressing GIRK channels increased with negative membrane potentials, and the current–voltage relationships showed strong inward rectification (Fig. 2B), indicating a characteristic of GIRK currents. The percentage inhibition of GIRK currents by 3 mM ethosuximide showed no significant difference across voltages between −100 and −40 mV (Fig. 2C), suggesting voltage-independent inhibition of GIRK channels by ethosuximide. Similar results were obtained for GIRK1/2 channels ($n = 9$; data not shown). The results suggest that ethosuximide inhibits brain-type GIRK1/2 channels and cardiac-type GIRK1/4 channels in a similar manner.

At physiological pH, the antiepileptic drug ethosuximide (pK_a 9.28) exists in a nonionized form. Seizures reduce extracellular pH in the brain (Ziemann et al., 2008). We examined whether changes in extracellular pH would affect the inhibition by ethosuximide of brain-type GIRK1/2 channels expressed in *Xenopus* oocytes. In oocytes expressing GIRK1/2 channels, the percentage inhibition by ethosuximide was not significantly affected by extracellular conditions of pH 6, 7.4 and 9.3 (no significant pH × ethosuximide interaction, $P > 0.05$, two-way ANOVA; $P > 0.05$ at each concentrations, Tukey–Kramer *post hoc* test; Fig. 3). The results suggest that the degree of GIRK inhibition by ethosuximide may be similar even under pathological pH conditions.

Ethanol affects many brain functions, resulting in intoxication, abuse, and dependence (Kobayashi et al., 1999). GIRK channels are activated by ethanol independent of G protein signaling pathways (Kobayashi et al., 1999; Lewohl et al., 1999). In oocytes injected with GIRK1 and GIRK2 mRNAs, the GIRK currents induced by 100 mM ethanol were reversibly attenuated in the presence of ethosuximide ($IC_{25} = 542.0 \pm 106.9$ μM; $IC_{50} = 3.86 \pm 0.86$ mM; $n_H = 0.81 \pm 0.09$; $n = 10$; Fig. 4A and B). In addition, 100 mM ethanol-induced GIRK currents were not significantly affected by intracellularly applied ethosuximide (110.4 ± 4.0% of untreated control current, $P > 0.05$, paired *t*-test, $n = 6$, Fig. 4C). In oocytes expressing brain-type GIRK1/2 channels, basal K^+ currents were not significantly affected by intracellularly applied ethosuximide (89.7 ± 4.8% of untreated control current, $P > 0.05$, paired *t*-test, $n = 6$). The results, together with the reversible GIRK inhibition by extracellularly applied ethosuximide (Figs. 1A and 4A), suggest that extracellular ethosuximide may inhibit GIRK channels activated by ethanol.

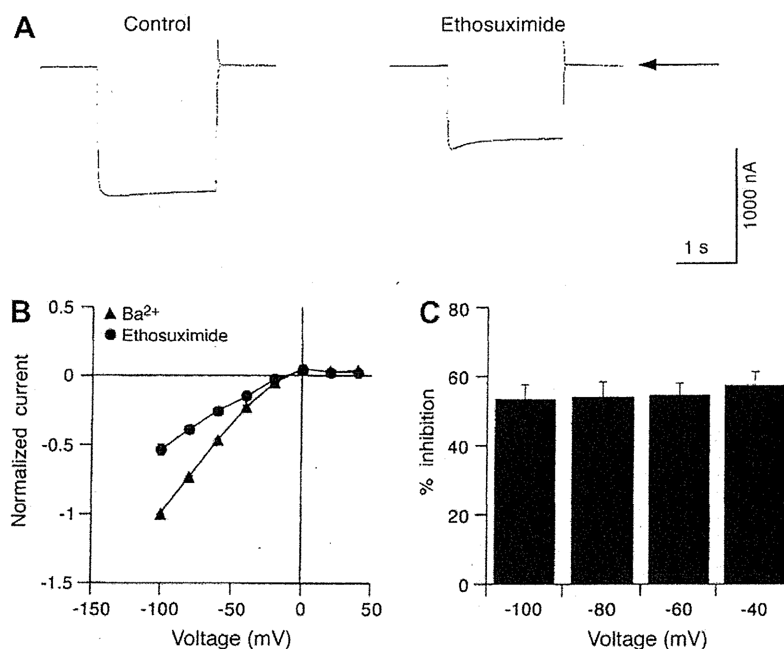

Fig. 2. Characteristics of the inhibitory effects of ethosuximide on GIRK currents. (A) Representative GIRK currents elicited by a voltage step to −80 mV for 2 s from a holding potential of 0 mV in the absence or presence of 3 mM ethosuximide in a *Xenopus* oocyte injected with GIRK1 and GIRK4 mRNAs. Current responses were recorded in an hK solution containing 96 mM K^+. Arrow indicates the zero current level. (B) Current–voltage relationships of 3 mM Ba^{2+}-sensitive currents and 3 mM ethosuximide-sensitive currents in oocytes expressing GIRK1/4 channels. Current responses were normalized to the 3 mM Ba^{2+}-sensitive current component measured at a membrane potential of −100 mV. The Ba^{2+}-sensitive current components were 1267.0 ± 181.3 nA ($n = 10$). (C) Percentage inhibition of GIRK1/4 channels by ethosuximide over the voltage range of −100 to −40 mV. The magnitudes of inhibition of GIRK currents by ethosuximide at the end of the voltage pulses were compared with the 3 mM Ba^{2+}-sensitive current components. There was no significant interaction between the ethosuximide effect and the membrane potential effect ($P > 0.05$, one-way ANOVA; $P > 0.05$ across the voltages, Tukey–Kramer *post hoc* test; $n = 10$). All values are the mean and S.E.M.

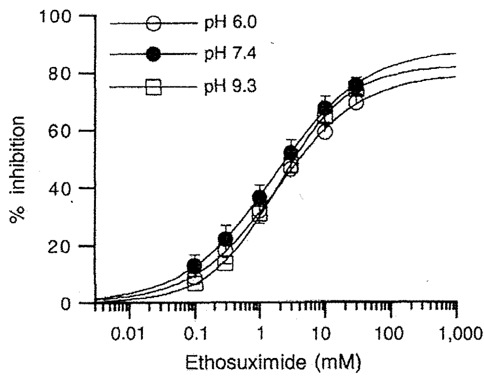

Fig. 3. Concentration–response relationships for inhibition of GIRK channels by ethosuximide at different pH values. The magnitudes of inhibition of GIRK currents by ethosuximide were compared with the 3 mM Ba^{2+}-sensitive current components in oocytes expressing GIRK1/2 channels (1046.0 ± 192.6 nA at pH 6.0, $n = 7$; 1324.9 ± 197.9 nA at pH 7.4, $n = 10$; 1208.0 ± 126.3 nA at pH 9.3, $n = 5$). Current responses were measured at a membrane potential of −70 mV in an hK solution. Each point and error bar represent the mean and S.E.M. of the percentage responses.

3.3. Inhibition of neuronal GIRK channels by ethosuximide

The effects of ethosuximide on neuronal GIRK channels were subsequently examined in granule cells in acute cerebellar slices of mouse brain. GIRK currents activated by GTPγS, a nonhydrolyzable GTP analog, included in the intracellular solution, were studied using whole-cell voltage clamp techniques as reported previously (Slesinger et al., 1997). Hyperpolarization of GTPγS-treated granule cells to −120 mV from a holding potential of −70 mV elicited large inward K^+ currents (Fig. 5A). In cerebellar granule cells, GTPγS in the patch pipettes caused a steady-state level of the inward currents, as reported previously (Slesinger et al., 1997). In contrast, the same membrane voltage shift for granule cells treated with GTP instead of GTPγS produced much less current, which was reduced slightly by 500 μM Ba^{2+} (Fig. 5A). This result suggests that the small Ba^{2+}-sensitive currents might consist of constitutively active GIRK channels with low open probability as reported in CA1 pyramidal neurons (Chen and Johnston, 2005) and Kir2.2 channels, which are expressed in granule cells (Karschin et al., 1996), but these channels might be expressed in only a limited numbers. GTPγS-activated K^+ currents showed inward rectification when incremental voltage steps were applied, and were blocked by 500 μM Ba^{2+} (Fig. 5A and B). These results suggest that the GTPγS-activated K^+ currents correspond to GIRK currents. Ethosuximide at 10 mM reduced the GTPγS-activated K^+ currents in a voltage-dependent manner (Fig. 5B). Fig. 5C shows that ethosuximide, at 100 μM–20 mM, significantly suppressed GTPγS-activated GIRK currents in a concentration-dependent manner ($IC_{50} = 3.15$ mM; $n_H = 0.72$; $n = 9$). In contrast, whole-cell currents in GTP-treated granule cells were not significantly affected by 10 mM ethosuximide (Fig. 5A). These results suggest that ethosuximide inhibits neuronal GIRK channels at similar concentrations observed in the recombinant channels.

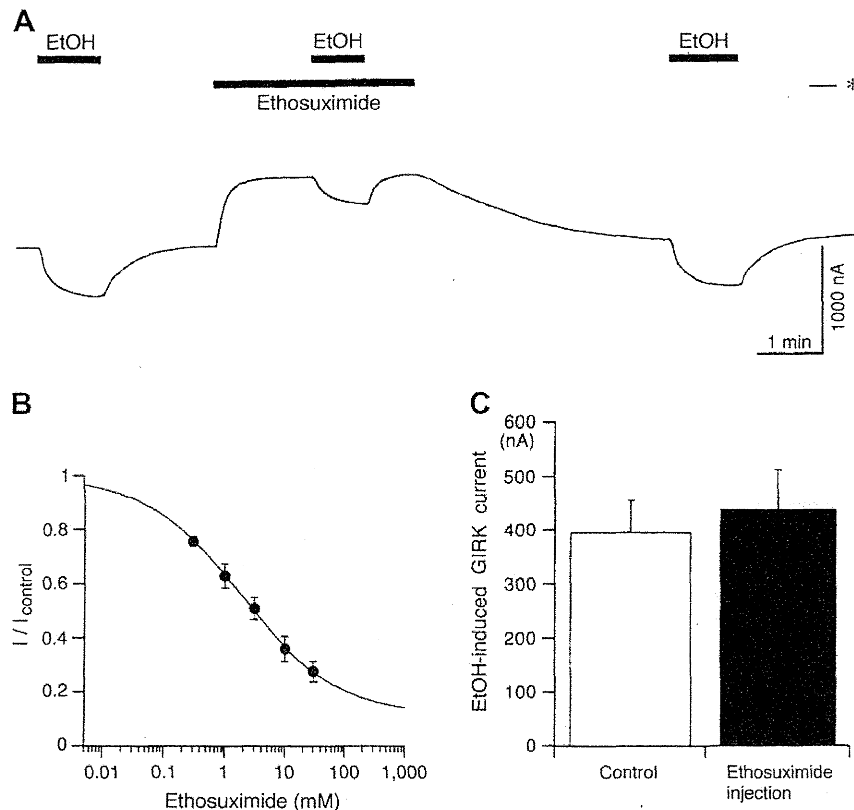

Fig. 4. Effect of ethosuximide on ethanol-induced GIRK currents. (A) In an oocyte injected with GIRK1 and GIRK2 mRNAs, current responses to 100 mM ethanol (EtOH), 100 mM EtOH in the presence of 3 mM ethosuximide, and 100 mM EtOH are shown. Asterisk indicates the zero current level. Bars show the duration of application. (B) Concentration-dependent inhibition of EtOH-induced GIRK currents by ethosuximide. $I_{control}$ is the amplitude of GIRK currents induced by 100 mM EtOH (326.9 ± 25.3 nA, $n = 10$), and I is the current amplitude in the presence of ethosuximide. (C) Lack of effect of intracellular ethosuximide on EtOH-induced GIRK currents. There was no significant difference in 100 mM EtOH-induced GIRK currents between the groups before and after intracellular ethosuximide injection ($P > 0.05$, $n = 6$, paired t-test). Current responses were measured at a membrane potential of −70 mV in an hK solution containing 96 mM K^+. All values represent the mean and S.E.M.

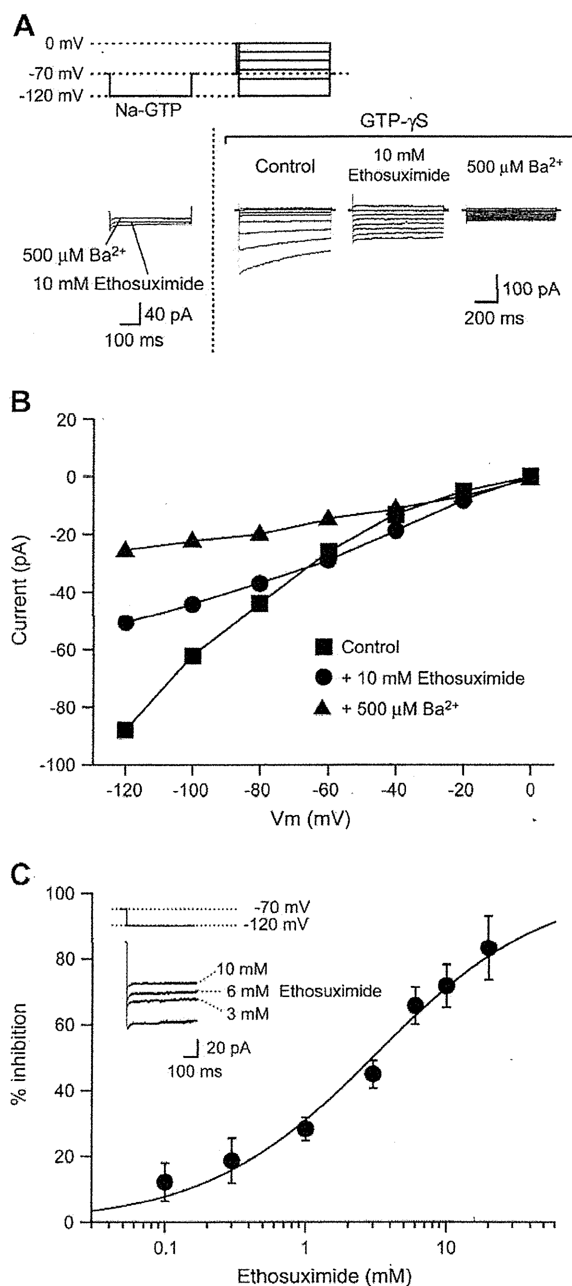

Fig. 5. Inhibitory effect of ethosuximide on GTPγS-activated GIRK currents in cerebellar granule cells. (A) Effects of external ethosuximide and Ba^{2+} in granule cells treated intracellularly with GTP (left) or with GTPγS (right). Whole-cell currents were elicited by voltage step(s) from −120 to 0 mV from a holding potential of −70 mV. (B) Current–voltage relationships of GTPγS-activated currents in the presence or absence of 10 mM ethosuximide or 500 μM Ba^{2+}. (C) Concentration-dependent inhibition of GTPγS-activated GIRK currents by ethosuximide. Changes in inward currents at the end of a voltage step to −120 mV in the presence of different concentrations of ethosuximide were measured. The magnitudes of inhibition of GIRK currents by ethosuximide were compared to the 500 μM Ba^{2+}-sensitive current components.

3.4. Inhibition of platelet aggregation by ethosuximide

ADP and epinephrine cause platelet aggregation by coactivation of $P2Y_1$ and $P2Y_{12}$ receptors (Jin and Kunapuli, 1998) and activation of α_2 adrenergic receptors (Yang et al., 2002), respectively. GIRK1, GIRK2 and GIRK4 subunits are expressed in human platelets (Shankar et al., 2004). $P2Y_{12}$ and α_2 adrenergic receptors activate GIRK channels (Jeong and Ikeda, 1998; Shankar et al., 2004). Recent studies have shown that two structurally distinct GIRK inhibitors: SCH23390 and U50488H, inhibit ADP- and thrombin-induced platelet aggregation (Shankar et al., 2004), suggesting that GIRK channels may be important for platelet functional responses. In the present study, we examined the effects of ethosuximide on ADP- and epinephrine-induced aggregation responses of human platelets in a Ca^{2+}-free extracellular solution. Ethosuximide concentration-dependently inhibited 2 μM ADP-induced platelet aggregation (Fig. 6). In addition, 10 mM ethosuximide by itself had no significant platelet aggregation response (−1.7 ± 0.3% light transmission). Activation of $P2Y_1$ and α_2 adrenergic receptors elevates intracellular Ca^{2+} levels (Kimura and Okuda, 1994; Shankar et al., 2004), possibly leading to activation of Ca^{2+}-activated K^+ channels in human platelets (Fine et al., 1989). Ethosuximide also inhibits Ca^{2+}-activated K^+ channels in neurons (Crunelli and Leresche, 2002). Therefore, the effects of charybdotoxin, a platelet Ca^{2+}-activated K^+ channel blocker (Fine et al., 1989), on ADP-induced platelet aggregation were examined. ADP-induced platelet aggregation was not significantly affected by the presence of 50 nM charybdotoxin (95.5 ± 1.3% of control response), and 50 nM charybdotoxin by itself had no significant effect on platelet aggregation (−4.5 ± 1.3% light transmission). Moreover, 1 μM epinephrine-induced platelet aggregation responses were delayed by 0.3–3 mM ethosuximide, and 10 mM ethosuximide inhibited the final aggregation responses (74.2 ± 5.9% inhibition; Fig. 6A), whereas the responses were not significantly affected by 50 nM charybdotoxin (96.2 ± 4.0% of control response). Together these results suggest that ethosuximide has an antiplatelet property.

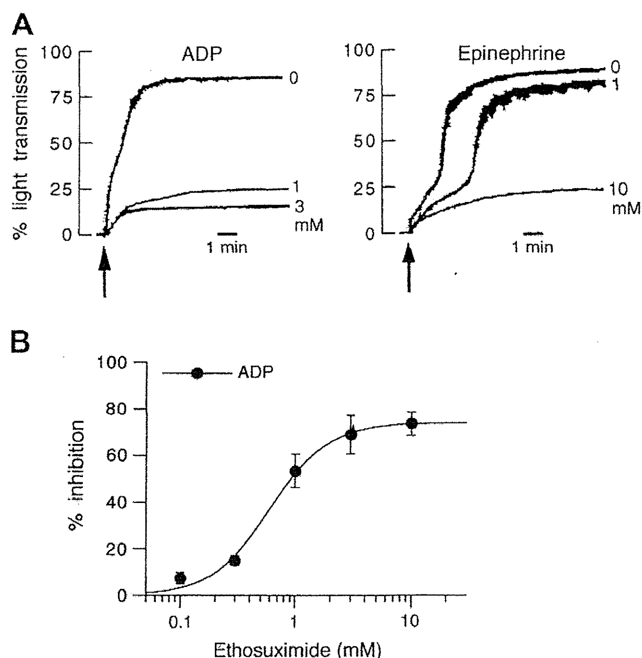

Fig. 6. Inhibitory effect of ethosuximide on agonist-induced platelet aggregation. (A) Representative traces of platelet aggregation induced by ADP or epinephrine in the presence or absence of different ethosuximide concentrations. Platelet aggregation was determined by measuring percentage light transmission at 37 °C in an aggregometer. Concentrations of ADP and epinephrine were 2 μM and 1 μM, respectively. Ethosuximide was added 5 min prior to the addition of an agonist. Arrow indicates the addition of an agonist. Ethosuximide concentrations are shown in mM on the right of each trace. (B) Concentration-dependent inhibition of ADP-induced platelet aggregation by ethosuximide. Control aggregation responses of platelets induced by 2 μM ADP and 1 μM epinephrine were 80.2 ± 1.8% and 85.6 ± 4.1% light transmission, respectively. Data were obtained from 3 different donors.

4. Discussion

In the present study, we found that among various chemical classes of antiepileptic drugs, ethosuximide significantly inhibited brain-type GIRK1/2 and GIRK2 channels and cardiac-type GIRK1/4 channels expressed in *Xenopus* oocytes. Kir1.1 and Kir2.1 channels in other Kir channel subfamilies were insensitive to all the drugs tested. The inhibition by ethosuximide was concentration-dependent but not complete even at high concentrations, voltage-independent, and time-independent during each voltage pulse. These observations suggest that ethosuximide may cause an allosteric conformation change in GIRK channels even before the voltage pulses, rather than simply occlusion of the open channel as is the case with extracellular Ba^{2+} and Cs^{+} (Lesage et al., 1995), resulting in the inhibition of GIRK channels. In addition, the incomplete blockade by ethosuximide might be due to such an action mechanism. In cerebellar granule cells, ethosuximide significantly inhibited GTPγS-activated GIRK currents at similar concentrations. However, the inhibition of neuronal GIRK channels was voltage-dependent. Although neuronal GIRK channels are considered to consist predominantly of GIRK1/2 channels (Liao et al., 1996; Lüscher et al., 1997), cerebellar granule cells express GIRK1, GIRK2 and GIRK3 subunits (Aguado et al., 2008). Part of the GIRK1 protein is glycosylated not only in brain and heart tissues (Krapivinsky et al., 1995; Aguado et al., 2008) but also in *Xenopus* oocytes expressed with cloned GIRK1 subunits (Pabon et al., 2000). Furthermore, GIRK channels are regulated by not only G proteins but also phosphatidylinositol 4,5-bisphosphate in the cell membrane, polyamines and protein kinases (Kobayashi and Ikeda, 2006). The difference in the voltage-dependency might be influenced by differences in composition of the channel subunits, levels of glycosylation of GIRK1 subunits, and interaction with membrane and intracellular factors between the *Xennopus* oocyte expression system and the cerebellar granule neurons.

The therapeutic plasma concentrations of ethosuximide are reported to be approximately 250–750 μM in most patients, and occasionally require up to approximately 1.1 mM in some treatment-resistant cases without toxicity (Sherwin et al., 1973; Crunelli and Leresche, 2002; Neels et al., 2004). The concentrations of ethosuximide in the brain were similar to those in blood (El Sayed et al., 1978). The present study demonstrated that at clinically relevant concentrations, ethosuximide significantly inhibited GIRK channels. In contrast, the effective concentrations of the other drugs in inhibiting GIRK channels were substantially higher than blood levels observed in toxic cases (Neels et al., 2004). Since GIRK channels play an important role in regulating neuronal excitability, even partial inhibition of GIRK channels by ethosuximide might affect some brain functions.

T-type Ca^{2+} channels are considered to play a critical role in the generation of $GABA_B$ receptor-mediated spike-and-wave discharges in the thalamus, the hallmark of absence seizures (Kim et al., 2001). Activation of $GABA_B$ receptors produces long-lasting K^{+}-dependent inhibitory postsynaptic potentials that are mediated by GIRK currents (Crunelli and Leresche, 1991; Lüscher et al., 1997). GIRK channel-induced hyperpolarization plays an important role in the generation of low-threshold Ca^{2+} potentials by removing inactivation of T-type Ca^{2+} channels, leading to burst firing (Crunelli and Leresche, 1991). Recent studies using animal models of absence epilepsy have shown that neuronal excitation in a specific cortical focus spreads to the cortex, followed by immediate activation of the corticothalamic network, the progression of spike-and-wave discharges (Manning et al., 2003). T-type Ca^{2+} channels are widely distributed in the brain (Talley et al., 1999), and the distribution of GIRK channels (Kobayashi et al., 1995; Karschin et al., 1996) overlaps in many brain regions, including the cerebral cortex and thalamus. The present study showed that ethosuximide at clinically relevant concentrations inhibited GIRK currents in neurons. Taken together, ethosuximide might aid in the treatment of absence seizures by reducing T-type Ca^{2+} current induction via GIRK channel inhibition in addition to directly inhibiting T-type Ca^{2+} channels.

It has been suggested that ethosuximide, when used alone in mixed types of absence epilepsy with tonic–clonic seizures, may exacerbate tonic–clonic seizures in some of the patients (Perucca et al., 1998). GIRK2 knockout mice show spontaneous seizures and are more susceptible to seizures induced by pentylenetetrazol than wild type mice (Signorini et al., 1997). Ethosuximide also inhibits Ca^{2+}-activated K^{+} channels (Crunelli and Leresche, 2002). In addition, ethosuximide fails to control tonic–clonic seizures, although ethosuximide is highly effective in the treatment of absence seizures (McNamara, 2001). Therefore, inhibition of neuronal GIRK channels and Ca^{2+}-activated K^{+} channels by ethosuximide may contribute to the exacerbation of tonic–clonic seizures in some patients with epilepsy. Since GIRK channels are expressed in various brain regions such as the cerebral cortex, amygdala, cerebellum and vestibular nuclei (Kobayashi et al., 1995; Karschin et al., 1996), GIRK channel inhibition by ethosuximide might be partly related to the occurrence of other neurological side effects, such as ataxia, dizziness, euphoria and insomnia (Neels et al., 2004).

Ethosuximide was shown to suppress alcohol withdrawal signs in mice (Kaneto et al., 1986). GIRK2 knockout mice show reduced ethanol-induced conditioned taste aversion and conditioned place preference and are less sensitive than wild types to some of acute ethanol-induced effects, including anxiolysis, habituated locomotor stimulation and handling-induced convulsions (Hill et al., 2003). In the present study, ethosuximide inhibited ethanol-induced GIRK currents, suggesting that it may suppress some GIRK-related ethanol effects. Interestingly, GIRK knockout mice also show reduced self-administration of cocaine (Morgan et al., 2003). GIRK channels are activated by opioid and CB_1 cannabinoid receptors (Kobayashi and Ikeda, 2006), and ethosuximide inhibited methamphetamine-induced stereotyped behavior (Jadhav et al., 1981). Ethosuximide, therefore, might play a role in the treatment of drug addiction.

In the heart, GIRK channels cause slowing of the heart rate in response to activation of M_2 muscarinic receptors through acetylcholine release from the stimulated vagus nerve (Kubo et al., 1993b; Krapivinsky et al., 1995). GIRK4 knockout mice are resistant to atrial fibrillation caused by vagal stimulation without showing any changes in atrioventricular nodal function and ventricular arrhythmias (Kovoor et al., 2001). Ethosuximide and quinidine reduce maximum driving frequency of rabbit atria, indicating their ability to increase the effective refractory period of atria (Shembekar and Deshpande, 1974). Quinidine, which is used for treating atrial fibrillation, inhibits GIRK channels (Kobayashi and Ikeda, 2006). Tertiapine, a selective GIRK blocker in the heart, terminates atrial fibrillation with atrial effective refractory period prolongation (Hashimoto et al., 2006). In the present study, ethosuximide inhibited cardiac-type GIRK1/4 channels at clinically relevant plasma levels, although the corresponding heart tissue concentrations have not been determined. Ethosuximide, therefore, might have therapeutic value in preventing and terminating atrial fibrillation without causing major cardiac side effects.

Recent studies have shown that GIRK channels are expressed on platelets and that two structurally distinct GIRK inhibitors: SCH23390 and U50488H, inhibit ADP- and thrombin-induced platelet aggregation (Shankar et al., 2004). In the present study, ethosuximide inhibited GIRK channels and platelet aggregation responses induced by ADP or epinephrine. In addition, ADP- and epinephrine-induced platelet aggregation was not significantly affected by charybdotoxin, a platelet Ca^{2+}-activated K^{+} channel

blocker, suggesting that the inhibition of platelet aggregation was not due to inhibition of Ca^{2+}-activated K^+ channels by ethosuximide. These results support the involvement of GIRK channels in platelet functions. Interestingly, the inhibitory effects of ethosuximide on platelet aggregation were observed at clinically relevant blood levels. Plasma protein binding of ethosuximide is negligible with such blood levels (El Sayed et al., 1978). The antiplatelet effect of ethosuximide might affect the maintenance of hemostasis and thrombotic events.

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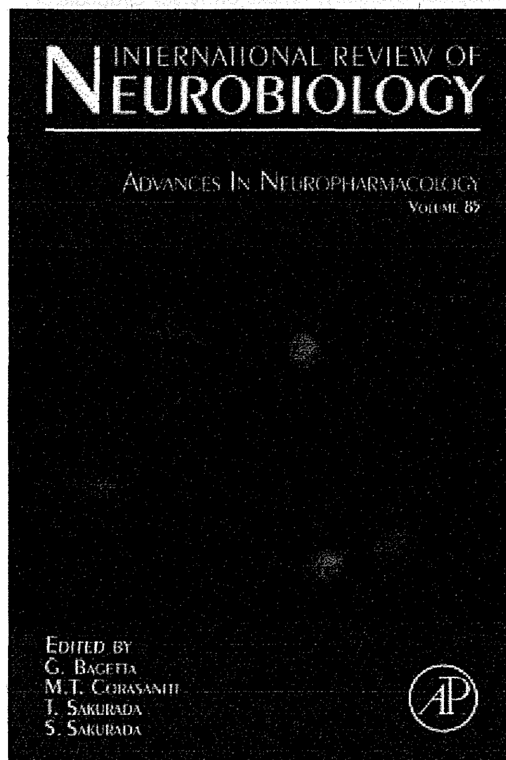

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MONOAMINE TRANSPORTER AS A TARGET MOLECULE FOR PSYCHOSTIMULANTS

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I. Introduction
II. MAP-Induced Behavioral Sensitization
III. MAP-Induced Hyperthermia and Neuronal Toxicity
References

Methamphetamine (MAP), a drug of abuse known worldwide for its addictive effects and neurotoxicity, causes somatic and psychiatric disorders. MAP enters terminals/neurons via monoamine transporters, displaces both vesicular and intracellular monoamines, and facilitates the release of monoamines into the extraneuronal space through synaptic transport via the monoamine transporters. Chronic psychostimulant abusers exhibit psychotic features, including delusions and auditory hallucinations. The dopamine transporter (DAT) and the vesicular monoamine transporter 2 (VMAT2) play pivotal roles in the action of MAP, including locomotor effects. The deletion of DAT attenuates the locomotor effects of MAP and may play larger role in behavioral responses to MAP compared to the deletion of VMAT2. MAP produces hyperthermia and/or neuronal toxicity in most species. The effects of MAP in DAT or serotonin transporter (SERT) single knockout (KO) mice and DAT/SERT double KO mice suggested that DAT and SERT are key molecules for hyperthermia and neuronal toxicity of MAP.

I. Introduction

Methamphetamine (MAP) is a psychostimulant that induces enhanced arousal and euphoria acutely, and psychosis and addiction chronically. MAP enters the terminals/neuron via the monoamine transporters (dopamine transporter: DAT, serotonin transporter: SERT, or norepinephrine transporter: NET), displaces

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both vesicular and intracellular monoamines, and facilitates release of monoamines into the extraneuronal space by synaptic transport in the monoamine transporters (Seiden *et al.*, 1993). The large release of monoamine produced by psychostimulant is thought to contribute to the drug's effects in the brain.

II. MAP-Induced Behavioral Sensitization

The acute and chronic pharmacological consequences of MAP in human users have been observed in behavioral experiments in animals, including both hyperactivity and sensitization of locomotor responses (Segal and Schuckit, 1983). Behavioral sensitization is a phenomenon whereby repeated intermittent exposure to MAP-like psychostimulant elicits a progressive enhancement of those responses, which persists for extended time periods following withdrawal from the drug and are easily reinstated by exposure to the drug or psychosocial stress (Robinson and Becker, 1986). This process closely resembles the course of the relapse in MAP-induced psychosis or schizophrenia, thus sensitization in animals has been suggested to model these psychoses (Sato *et al.*, 1983). Behavioral sensitization is thought to be an early and enduring manifestation of neuronal plasticity associated with changes in mesolimbic dopamine neurotransmission (Kalivas *et al.*, 1993). MAP induces dopamine release through exchange diffusion of plasma membrane DAT (Seiden *et al.*, 1993), and release of vesicular dopamine into the cytosol by acting on the vesicular monoamine transporter 2 (VMAT2) (Sulzer *et al.*, 2005). The dopamine releasing effect of MAP has been postulated to mediate its locomotor stimulant and rewarding effects (White and Kalivas, 1998). Therefore, DAT and VMAT2 should play pivotal roles in the mechanisms underlying the actions of MAP.

DAT knockout (KO) mice and VMAT2 KO mice have been used to investigate the roles of DAT and VMAT2 in dopamine neurotransmission and pharmacological mechanisms underlying the actions of psychostimulants. Homozygous deletion of the DAT gene has been reported to produce a 10-fold increase (Shen *et al.*, 2004) or fivefold elevation (Jones *et al.*, 1998) of extracellular dopamine concentrations in the striatum measured by *in vivo* microdialysis, while heterozygous deletion of DAT was not found to significantly increase extracellular dopamine (Shen *et al.*, 2004) or to produce a smaller twofold elevation (Jones *et al.*, 1998) of dopamine in the striatum. Homozygous DAT KO mice show growth retardation and hyperactivity, whereas heterozygous DAT KO mice did not show gross abnormalities in either development or baseline behavioral parameters (Sora *et al.*, 1998). Habituated homozygous DAT KO mice do not show any significant cocaine-induced increase in locomotion (Sora *et al.*, 1998, 2001; Uhl *et al.*, 2002).