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Aging of Whiskey Increases the Potentiation of $GABA_A$ Receptor Response

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It is known that the target of most mood-defining compounds such as ethanol is an ionotropic γ-aminobutyric acid receptor ($GABA_A$ receptor). The potentiation of the response of these inhibitory neurotransmitter receptors induces anxiolytic, sedative, and anesthetic activities in the human brain. Because both extracts of whiskey by pentane and fragrant components in whiskey potentiate the $GABA_A$ receptor-mediated response, $GABA_A$ receptors were expressed in *Xenopus* oocyte by injecting cRNAs prepared from the cloned cDNA for the α_1 and β_1 subunits of the bovine receptors in order to study the effects of whiskey itself on the $GABA_A$ receptor-mediated response. Whiskey itself also potentiated the electrical responses of $GABA_A$ receptors generally more than ethanol at the same concentration as that of the whiskey. The potentiation of the $GABA_A$ receptor-mediated response increased with the aging period of the whiskey. Inhalation of whiskey to mice increased the sleeping time induced by pentobarbital more than that of the same concentration of ethanol as the whiskey. These results suggest that not only ethanol but also minor components in whiskey play an important role in the potentiation of $GABA_A$ receptor-mediated response and possibly the sedative effect of whiskey. Although the minor components are present in extremely small quantities compared with ethanol in alcoholic beverages, they may modulate the mood or consciousness of humans through the potentiation of the $GABA_A$ receptor response after absorption into the brain, because these hydrophobic compounds are easily absorbed into the brain across the blood−brain barrier and are several thousands times as potent as ethanol in the potentiation of the $GABA_A$ receptor-mediated response.

KEYWORDS: Aging of whiskey; $GABA_A$ receptor; potentiation; whiskey fragrance; *Xenopus* oocyte

INTRODUCTION

It has been reported that some components of foods or beverages such as caffeine, capsaicin, and menthol can act on receptors, channels, and enzymes in the brain to modulate human consciousness (*1*). Various types of liquor are produced and consumed around the world. The main component in liquor, ethanol, potentiates the response of ionotropic γ-aminobutyric acid receptors ($GABA_A$ recptors) (*2*), whereas it inhibits that of *N*-methyl-D-aspartate (NMDA) receptors (*3*). G-Protein-coupled inwardly rectifying K^+ channels are also opened by ethanol (*4*, *5*). It has been reported that the fragrance in whiskey (*6*) and wine (*7*) affects human brain function, altering moods and relaxing consciousness possibly through the stimulation of the olfactory system. In a previous paper (*8*), we expressed $GABA_A$ receptors in *Xenopus* oocytes by injecting them with rat whole-brain mRNA or cRNA prepared from cDNA for the α_1 and β_1 subunits of bovine $GABA_A$ receptors and examined the effects of various fragrant compounds in whiskey on the electrical response of these expressed receptors. Most fragrant compounds in whiskey potentiated the response of the $GABA_A$ receptors, although with differences in efficiency. Thus it is important to know whether whiskey itself potentiates the response of the $GABA_A$ receptors and affects neural transmission via a modulation of receptor function in the brain to change moods or consciousness.

It is known that a sharp or raw odor typical of fresh whiskey distillates is modified to a rounded, soft, and mellow one and that colorless distillates turn to amber ones during aging in oak barrels (*9*), suggesting the changes in the flavor composition during the aging of whiskey (*10*, *11*). Aged whiskey and wine are usually highly valued, so it is important to know whether the potency in potentiating the $GABA_A$ receptor response by whiskey also changes during the aging. It is also important to clarify how much the minor components in whiskey contribute to the potentiation of $GABA_A$ receptor responses by whiskey.

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GABA is a major inhibitory neurotransmitter in the brain and is essential for the overall balance between neuronal excitation and inhibition. It is known that many mood-defining drugs target $GABA_A$ receptors in the brain. The $GABA_A$ receptors have a complex pharmacology (*12*, *13*), with binding sites for direct GABA agonists and antagonists together with multiple allosteric sites for benzodiazepine tranquilizers, barbiturate central nervous system depressants, both synthetic and endogenous steroids (*14*), general anaesthetics (*15*), and ethanol (*2*). In our previous papers, we reported $GABA_A$ receptors that were expressed in *Xenopus* oocytes by injecting rat whole-brain mRNA or cRNA prepared from cDNA of bovine $GABA_A$ receptor subunits and showed that the responses of these receptors were inhibited by caffeine and catechin derivatives (*16*) or potentiated by various compounds such as alcohols (*17*) and phenol derivatives (*18*) which are present in food additives or essential oils (*19*, *20*). Because *Xenopus* oocytes, which are round and have a diameter of >1 mm, are larger, more stable, and simpler in shape than neurons, electrophysiological measurements of the responses of the receptors expressed in oocytes can be taken easily and repeatedly over a long period.

In the present study, we measured the effect of both whiskey and ethanol at the same concentration as that of the whiskey on the potentiation of $GABA_A$ receptor-mediated response in order to examine the contribution of minor components in whiskey to the potentiation. We also measured the effect of whiskies after various aging periods on the potentiation in order to find the relationship between the aging period and the potentiation. We examined the effect of whiskey inhalation in mice on the sleeping time induced by pentobarbital to learn whether whiskey components modulate $GABA_A$ receptor response in the brain. These experiments suggest that minor components in whiskey play an important role in the potentiation of $GABA_A$ receptor-mediated response and intoxication.

MATERIALS AND METHODS

Materials. Japanese whiskies after various aging periods were supplied by Suntory Ltd., Osaka, Japan. The single-malt whiskey was produced from only barley malt. The blended whiskey was produced by blending the barley malt whiskey with grain whiskey produced from the mixture of barley malt and grain such as corn or wheat. They were aged in a puncheon barrel (usual size = 96 cm maximum diameter × 107 cm length, 480 L volume) made of white oak imported from North America. Various types of whiskies, grain, Bourbon, Tennessee, Canadian, Irish, Scotch (S; single malt), and Scotch (B; blended), were purchased from a local market in Osaka, Japan. The whiskies contained 40−43% (v/v) ethanol.

Preparation of cRNA and *Xenopus* Oocytes. The cDNAs for the α_1 and β_1 subunits of $GABA_A$ receptors cloned from bovine brain were gifts from Prof. Eric A. Barnard (MCR Center, U.K.). The cRNAs for the α_1 and β_1 subunits of $GABA_A$ receptors were synthesized from these cloned cDNAs by RNA polymerase according to standard procedures.

Adult female frogs (*Xenopus laevis*) were purchased from Hamamatsu Seibutsu Kyozai (Hamamatsu, Japan). The oocytes were dissected from the ovaries of adult female frogs that had been kept in ice for 1 h. They were manually detached from the inner ovarian epithelium and follicular envelope after incubation in a collagenase (type I, 1 mg/mL; Sigma) solution for 1 h using the procedure of Kusano et al. (*21*). The oocytes were microinjected with ~50 ng of cRNAs in sterilized water and then incubated in a modified Barth solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, and 0.41 mM $CaCl_2$ in 5 mM Tris at pH 7.6] containing 25 mg/L penicillin and 50 mg/L streptomycin at 15−18 °C for 2−7 days before the electrophysiological measurements.

Electrophysiological Measurements. The membrane current of the receptors evoked by GABA was measured by the voltage clamping method using a voltage clamp amplifier (CEZ-1100; Nihon Kohden Kogyo, Tokyo, Japan). An oocyte was placed on a net in a small chamber (~0.3 mL) and impaled with two microelectrodes filled with 3 M KCl, one for monitoring the membrane potential and the other for passing a current for clamping the membrane potential, usually at −40 mV. The oocyte placed on the net was continuously perfused from the bottom with normal frog Ringer's solutions (115 mM NaCl, 1 mM KCl, and 1.8 mM $CaCl_2$ in 5 mM Tris at pH 7.2) by a gravity feed system at a flow rate of ~2 mL/min (*22*).

Measurement of the Receptor Response. GABA was dissolved in normal frog Ringer's solution. To examine the effect of whiskey or ethanol on the GABA-elicited response, either whiskey or ethanol was added to the Ringer's solution and GABA solution. The solutions were then shaken vigorously for 1 min. The solutions were selected by switching a cock in the flow system. The control response was obtained by perfusing the GABA solution without any added solution, and this was taken as 100%. The effect of whiskey or ethanol on the response of the receptors was measured by using a mixture of GABA and whiskey or ethanol. Because whiskies contain 40−43% (v/v) ethanol, we compared the effect of whiskies on the response with the effect of ethanol at the same concentration as that of the whiskey. We added 99.5% ethanol, whose volume was 43% of the whiskey, instead of whiskies to the GABA solution for comparison between whiskey and ethanol. Because some whiskey caused a small nonspecific current even in noninjected oocyte at high concentrations, we also measured the response caused by whiskey dissolved in normal frog Ringer's solution. If the nonspecific current was observed, it was subtracted from the responses caused by a mixture of GABA and whiskey when the potentiation currents were calculated. When a significant desensitization of the receptors was induced before the binding equilibrium of the whiskey was attained in the presence of high concentrations (>20 μM) of GABA, the whiskey dissolved in normal frog Ringer's solution was applied 1 min before the coapplication with GABA. Because there was no difference between the presence and absence of preapplication of whiskey or ethanol for 1 min when the mixture of the whiskey or ethanol and GABA at <10 μM was applied, we usually measured the effect of the whiskey or ethanol on the response elicited by GABA <10 μM without the preapplication (*23*). The measurement was repeated several times with the same oocyte, and control values were obtained after every two or three measurements. To reverse the desensitization of the receptors, the oocyte was washed for >10 min in a normal frog Ringer's solution before the next measurement, because desensitization of the $GABA_A$ receptors is a reversible process and the receptors usually recover completely after ~10 min of washing (*24*).

The values given were usually as the means of four experiments. Student's *t* test was used to evaluate the significance of the mean values, in comparison with the control.

Measurement of Pentobarbital-Induced Sleep in Mice. Male ddY mice at the age of 28 days (Japan SLC Co., Shizuoka, Japan) were obtained. They were housed five per cage under a standardized light−dark cycle condition (lights on at 7:00 a.m., off at 7:00 p.m.) at 24 ± 1 °C and 60 ± 10% humidity with food and water ad libitum. All animals received humane care in accordance with Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacology Society.

Pentobarbital-induced sleep was measured as previously reported by Matsumoto et al. (*25*). The inhalation apparatus was a lidded cage ($L = 31.5 \times W = 21.0 \times H = 21.0$ cm).

The aroma of Japanese whiskey (ethanol concentration = 43%) aged for 30 years or 43% ethanol was generated by bubbling it using an air-pump. The air containing the fragrance of the whiskey or ethanol was introduced into the cage. Male ddY strain male mice (7 weeks old) were exposed to the aroma 30 min prior to the administration of pentobarbital, because it possibly takes some time before the aroma and ethanol are absorbed into the blood, pass the blood−brain barrier, and reach the brain. Sodium pentobarbital (50 mg/kg) was administered to mice intraperitoneally (ip). Sleeping time was measured under inhalation of the aroma. Sleeping time was calculated as the time between disappearance of the righting reflex and recovery of the righting reflex.

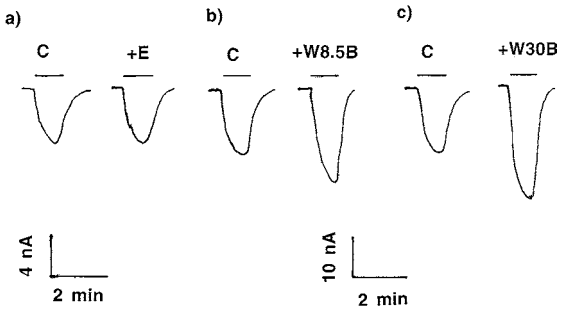

Figure 1. Effects of ethanol (E; a), blended whiskey aged for 8.5 years (W8.5B; b), and blended whiskey aged for 30 years (W30B; c) on the 0.25 μM GABA-mediated current of $GABA_A$ receptors expressed in *Xenopus* oocytes by injecting cRNAs prepared from cDNA for the α_1 and β_1 subunits of bovine $GABA_A$ receptors. All traces were obtained with a voltage clamp at −40 mV. An inward current is shown as a downward curve. The upper bars show 0.25 μM GABA (C) or a mixture of GABA and the sample applications. The concentrations of whiskey and ethanol were 1% (v/v) and 0.43% (v/v), respectively, because whiskies contain 43% ethanol. Both responses in a given panel were obtained from the same injected oocyte, but the responses in panels a–c were from different oocytes. No detectable nonspecific currents by the whiskies were observed in these measurements.

RESULTS

Potentiation of the $GABA_A$ Receptor Response by Japanese Whiskey. The $GABA_A$ receptors expressed in *Xenopus* oocytes by injection of poly(A)$^+$RNA prepared from rat whole brains express an enormous number of pentamers combinations for $GABA_A$ receptors, which show a different and complex pharmacology (*12*, *13*). The potentiation induced in these receptors by the whiskey components was similar to those expressed by injecting the cRNA of only the α_1 and β_1 subunits of bovine receptors (*8*). Therefore, we used simple $GABA_A$ receptors composed of only the α_1 and β_1 subunits to examine the effects of whiskey on the $GABA_A$ receptor responses. **Figure 1** shows some examples of the electrical responses of the $GABA_A$ receptors expressed in *Xenopus* oocytes by the injection of cRNAs for the α_1 and β_1 subunits of the bovine $GABA_A$ receptors. Because the oocytes were injected by synthesized cRNAs and many $GABA_A$ receptors were usually expressed there, we usually examined the effect of whiskey on the electrical responses elicited by a very low concentration of GABA at 0.25 μM, which corresponds to approximate GABA $EC_{0.4\%}$. Addition of 1% (v/v) Japanese whiskey, especially that aged for 30 years, potentiated the response elicited by 0.25 μM GABA more than the addition of ethanol with the same concentration as that of the whiskey. **Figure 2** shows the dose dependency of the potentiation in the $GABA_A$ receptor-elicited response by the ethanol and whiskies aged for 0, 8.5, or 30 years. High concentrations [>1% (v/v)] of whiskies dissolved in frog normal Ringer's solution sometimes caused small electrical responses, which were not inhibited by the addition of biccuculline, a $GABA_A$ receptor antagonist. Similar responses were also observed even in noninjected oocytes. These nonspecific electrical responses were sometimes observed before, when saponin, tannic acid, catechin, or lactone derivatives at high concentrations were applied to the oocytes (*8*, *26*). They showed large variations in size, depending on the individual frog and oocyte. These values were subtracted from the responses caused by a mixture of GABA and whiskey when the potentiation currents were calculated. The potentiation of the response by whiskies aged for 8.5 and 30 years was usually

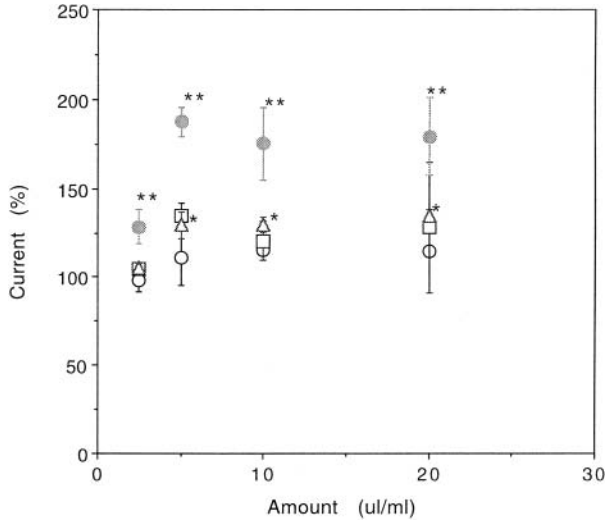

Figure 2. Dose–potentiation relationship of ethanol and whiskey in the presence of 0.25 μM GABA. $GABA_A$ receptors were expressed in *Xenopus* oocytes by injecting cRNAs prepared from cDNA for the α_1 and β_1 subunits of bovine $GABA_A$ receptors. The samples at various concentrations were applied simultaneously with 0.25 μM GABA. The control response was obtained by perfusing the GABA solution without any added sample and was taken as 100%. Data are mean ± SD (bars) values from four experiments: (○) 43% Ethanol; (□) whiskey aged for 0 years; (△) whiskey aged for 8.5 years; (shaded circle) whiskey aged for 30 years. $p^{**} < 0.002$ between the ethanol and whiskey aged for 30 years by Student's *t* test. $p^{**} < 0.03$ between whiskey aged for 8.5 years and one aged for 30 years by Student's *t* test. $p^{**} < 0.05$ between nonaged whiskey and one aged for 30 years by Student's *t* test. $p^{*} < 0.08$ between the ethanol and whiskey aged for 8.5 years by Student's *t* test.

greater than that by the ethanol at the same concentration present in the whiskey. To examine the effect of whiskies on the GABA dose−response curve, various amounts of the $GABA_A$ receptors were expressed in the oocyte by injecting various amounts of cRNAs. The addition of Japanese whiskies aged for 30 years shifted the GABA dose−response curve to a lower concentration, as did most whiskey fragrant components (**Figure 3**). The shift of the curve by whiskey was less than that by whiskey components such as quercus lactone b or 1,1-diethoxy-2-methylpropane, because the potentiation by whiskey was less than that by these compounds (*8*).

Aging-Dependent Potentiation of the $GABA_A$ Receptor Response by Japanese Whiskey. It has been reported that aging of whiskies in oak barrels changes their color and odor, that is, pigments and fragrant components (*9*−*11*), so we examined the effect of the aging period on the potentiation of the $GABA_A$ receptor responses caused by 0.25 μM GABA. The potentiation of the response by nonaged whiskey was usually greater than that by the ethanol, but not significant ($P = 0.37$ by Student's *t* test). However, the potentiation of the response by Japanese whiskies aged for >8.5 years was significantly greater than that by the ethanol ($P < 0.02$). Moreover, the potentiation of the response increased with the whiskey aging period (**Figure 4**). The correlation factors between the potentiation currents and years of aging for single-malt whiskies (△) and blended malt whiskies (○) were estimated to be 0.960 and 0.931, respectively. In these samples, the potentiation of the $GABA_A$ receptor responses by single-malt whiskies increased with their aging period more than that by blended malt whiskies.

Effect of Japanese Whiskey Aroma on Sleeping Time Induced by Pentobarbital in Mice. It is known that pento-

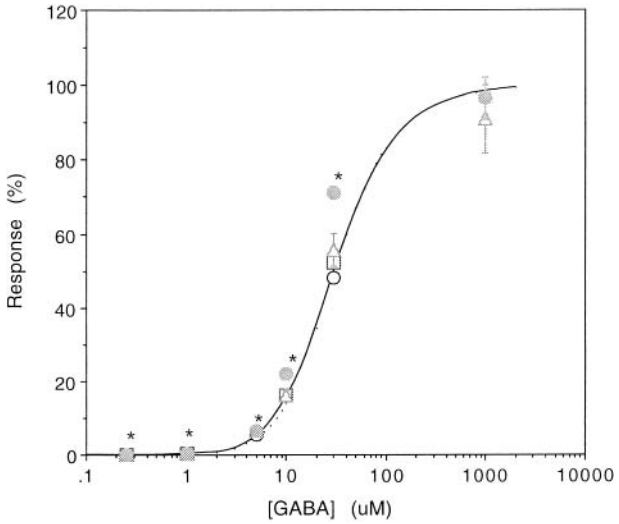

Figure 3. Effects of ethanol (○), whiskey aged for 0 year (□), whiskey aged for 8.5 years (△), and whiskey aged for 30 years (shaded circle) on the GABA dose–response curve. The concentrations of whiskies and ethanol were 1% (v/v) and 0.43% (v/v), respectively. The theoretical GABA dose–response curve without any compound was drawn using a dissociation constant (K_1) for the GABA receptor complex of 59 μM on the basis of the minimal model reported previously (*24*). The maximum response elicited by a high concentration of GABA without any compound was taken as 100. p^* < 0.02 between the ethanol and whiskey aged for 30 years by Student's *t* test.

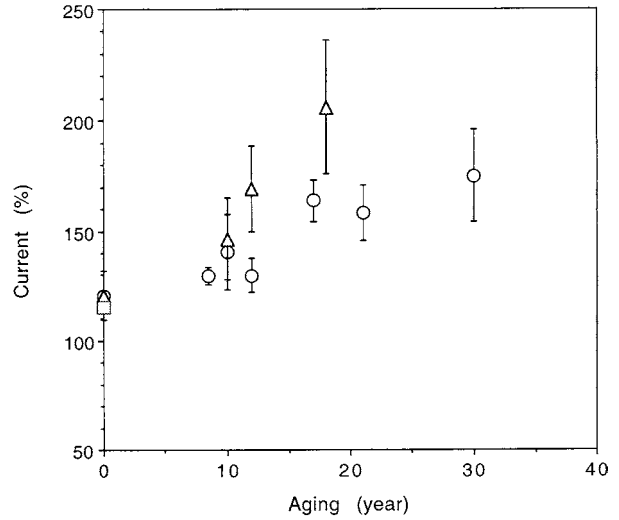

Figure 4. Effect of aging period of whiskey on the potentiation of the $GABA_A$ receptor responses caused by 0.25 μM GABA. $GABA_A$ receptors were expressed in *Xenopus* oocytes by injecting cRNAs prepared from cDNA for the α_1 and β_1 subunits of bovine $GABA_A$ receptors. The concentrations of whiskies were 1% (v/v), respectively. The control response was obtained by perfusing the GABA solution without any whiskey and was taken as 100%. The correlation factors between the potentiation currents and aged years for single-malt whiskies (△) and blended malt whiskies (○) were estimated to be 0.960 and 0.931, respectively. The square (□) shows the potentiation by 0.43% ethanol. Data are mean ± SD (bars) values from eight experiments: p < 0.05 between nonaged whiskey and aged whiskies by Student's *t* test.

barbital induces sleep by potentiating the $GABA_A$ receptor responses (*25*). **Figure 5** shows the effect of whiskey aroma and the aroma of ethanol, at the same concentration as in Japanese whiskey, on the sleeping time of mice induced by injection of pentobarbital. Inhalation of Japanese blended whiskey aged for 30 years significantly prolonged the sleeping time induced by pentobarbital in mice compared to the control or ethanol.

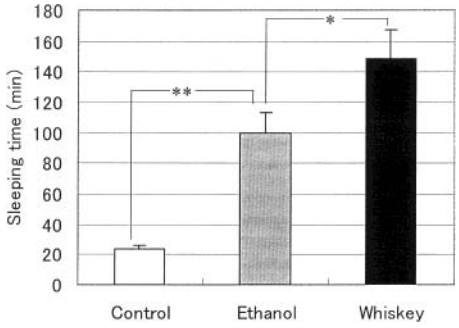

Figure 5. Effect of whiskey aroma on sleeping time induced by pentobarbital in mice. Sodium pentobarbital (50 mg/kg) was injected ip 30 min after inhalation of the aroma of Japanese blended whiskey aged for 30 years (ethanol concentration = 43%) and 43% ethanol. Data are mean ± SD (bars) values from four experiments. p^* < 0.05 and p^{**} < 0.001 by Student's *t* test.

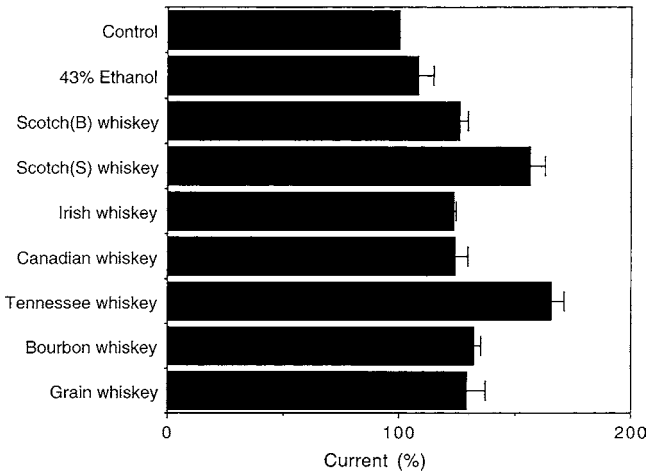

Figure 6. Effect of various whiskies on the GABA-elicited response of the $GABA_A$ receptors expressed in the oocytes. The concentrations of GABA and whiskies were 0.25 μM and 1% (v/v), respectively. The concentration of ethanol was 0.43% (v/v), because whiskies contain 43% ethanol. The control was obtained by perfusing the GABA solution without ethanol and whiskies and was taken as 100%. p < 0.01 between the ethanol and each whiskey by Student's *t* test.

Potentiation of the $GABA_A$ Receptor Response by Various Types of Whiskies. We measured the effects of various types of 1% (v/v) whiskies on the $GABA_A$ receptor responses to examine whether the $GABA_A$ receptor response is potentiated only by Japanese whiskies or not. **Figure 6** shows that all of the whiskies examined potentiated the response elicited by 0.25 μM GABA significantly (P < 0.01 by Student's *t* test) more than ethanol at the same concentration as in the whiskey. However, the degree of the potentiation by these whiskies showed some variations from 124% (Irish and Canadian whiskies) to 165% (Tennessee whiskey).

DISCUSSION

In a previous study (*8*), we found that a pentane extract of Japanese whiskey and most fragrant components in whiskey such as ethyl phenylpropanoate, lactone, and ethoxy derivatives potentiated the response of $GABA_A$ receptors expressed in *Xenopus* oocytes. Because very volatile substances with low boiling points may be lost during the pentane extraction and

the evaporation of pentane with suction, we measured the effect of whiskey itself on the $GABA_A$ receptor responses in this study. The potentiation of the $GABA_A$ receptor-elicited response by Japanese whiskies increased with their concentration and reached the saturation level at ~0.5% (v/v) (**Figure 2**). However, the dose−potentiation curve of whiskey may become complex, because whiskey includes various components with different dissociation constants and different maximum potentiations for the $GABA_A$ receptors (*8*, *17*). We found that even a very low concentration (0.25%) of the whiskey aged for 30 years induced significant potentiation of the responses ($P < 0.002$ against the ethanol by Student's *t* test). We hypothesized that minor components, possibly fragrant compounds, in whiskey played an important role in the potentiation of the $GABA_A$ receptor responses because the potentiation by whiskey was much greater than that by ethanol at a concentration the same as that in whiskey. The potentiation of the response by Japanese whiskies increased with their aging period with high correlation between the potentiation and years of aging (**Figure 4**). These minor components in whiskey possibly come from the wooden barrel in which the whiskey is aged, although some minor components in whiskey may be produced or changed by slow chemical reactions during storage for a long period (*9*−*11*). The change in the flavor of the maturing spirits is due to changes in the composition and concentration of compounds influencing the taste and aroma. The production of whiskey lactone [5-butyl-4-methyl-2(3*H*)-furanone] from oak has been studied in detail and reported (*27*). The concentrations of the following compounds in Japanese whiskies increased with the aging period when they were measured by gas chromatography (unpublished data): 1,1-diethoxyethane, 1,1-diethoxyhexane, 1,1-diethoxyheptane, 1,1-diethoxy-2-propane, 1,1,3-triethoxypropane, quercus lactone b, eugenol, and ethyl phenylpropanoate, which potentiated the $GABA_A$ receptor responses in a previous study (*8*). It seems that single-malt whiskies potentiate the $GABA_A$ receptor responses more potently than the blended whiskies (**Figures 4** and **6**), possibly because grain whiskey has a calm and light taste (silent spirit) with less fragrance, the reason for which we cannot explain at present. Additional support for the importance of minor components in the potentiation of the $GABA_A$ receptor responses was given by the measurement of mouse sleeping time induced by pentobarbital (**Figure 5**). Addition of the whiskey aged for 30 years significantly extended the sleeping time of mice caused by pentobarbital compared to the control or ethanol. However, because the sleeping time of mice is possibly settled by various steps and conditions, direct quantitative comparison between the sleeping time and the potentiation of the $GABA_A$ receptor responses must be meaningless. Assuming that all of the components are absorbed into the blood and that the weight of a drinker is 52 kg, ingestion of only 10 mL of whiskey aged for 30 years reaches a concentration [0.25% (v/v)] in the blood, which affects the $GABA_A$ receptor responses significantly, because the total amount of blood in humans is usually $^1/_{13}$ of their weight.

As the experiments in **Figures 1**−**5** were done by using Japanese whiskies, it is necessary to show that the $GABA_A$ receptor responses are generally potentiated by whiskies more than by ethanol at the same concentration as that of the whiskies. Different types of whiskies also potentiated the response to different degrees and more than ethanol at the same concentration as in the whiskies (**Figure 6**). The variations of the potentiation possibly come from those of both the composition and concentration of minor components in whiskies, because ethanol concentrations in whiskies were almost the same, 40−43% (v/v). The results in **Figure 6** do not show the general order of the whiskey potency in the potentiation of $GABA_A$ receptor responses; the measured whiskies must have different aging periods, which we could not know exactly.

$GABA_A$ receptors are ligand-gated ion channels, the subunits of which have amino acid sequences similar to those of ionotropic nicotinic acetylcholine, serotonin (type 3), and glycine receptors (*12*). A number of structurally diverse compounds enhance the action of GABA on $GABA_A$ receptors; these compounds include benzodiazepine, barbiturates, pregnane steroids, general anesthetics, and ethanol (*2*, *12*−*15*). In animal models, these compounds exhibit anxiolytic, anticonvulsant, and sedative activities (*28*, *29*), because $GABA_A$ receptors are the main inhibitory neurotransmitter receptors in the brain. In our previous papers (*8*, *16*, *17*, *20*), we found that many fragrant compounds potentiated the response of $GABA_A$ receptors expressed in *Xenopus* oocytes. Although the γ subunit is essential for the potentiation of the $GABA_A$ receptor-mediated response by benzodiazepine (*30*, *31*), the potentiation site for fragrant compounds in whiskey must be present in $GABA_A$ receptors that are composed of only α_1 and β_1 subunits, because these compounds potentiated the responses of both types of receptors expressed in *Xenopus* oocytes by injecting poly(A)$^+$RNA prepared from rat whole brains or cRNA for the α_1 and β_1 subunits of the bovine receptors (*8*, *32*). The potentiation site for whiskey fragrances is possibly a region of 45 amino acid residues within the TM2 and TM3 domains of the subunit (*33*, *34*).

Although ethanol is possibly the main liquor component that modulates the moods and consciousness of humans through acting on $GABA_A$ receptors (*2*), NMDA receptors (*3*), and K^+ channels (*4*, *5*), our results showed that minor components in whiskey also played an important role in the potentiation of the $GABA_A$ receptor responses. Most minor components are hydrophobic and easily absorbed into the brain, so they may also potentiate the $GABA_A$ receptor responses and in part modulate the mood and consciousness when humans drink whiskey. The accumulation of essential oil components in the mouse brain was found after percutaneous or vapor exposure absorption (*35*, *36*). The direct effect of fragrant compounds on $GABA_A$ receptors was suggested by a study showing that inhaling chamomile and lemon oil vapor decreased restriction-stress-induced increases in the plasma adrenocorticotropic hormone level of ovariectomized rats as did diazepam, a benzodiazepine derivative (*37*). Our measurements of convulsions induced by pentetrazole (*8*) and the sleeping time induced by pentobarbital in the presence and absence of whiskey components or whiskey also suggested an effect on $GABA_A$ receptors. It was also reported that rose oil and its components showed anticonflict effects in a mouse behavior test (*38*, *39*).

Different liquors have different fragrant components. Both ethanol and fragrant compounds potentiate the $GABA_A$ receptor-elicited responses, but only ethanol may open G-protein-coupled inwardly rectifying K^+ channels (*4*, *5*). Thus, a small amount of minor fragrant components in liquors may contribute to the taste of each liquor through not only the stimulation of the olfactory system but also the potentiation of $GABA_A$ receptor-elicited responses. The potentiation of the $GABA_A$ receptor responses by whiskey increased with aging in a wooden barrel possibly because of the increase of fragrant components, which may be the main reason aged whiskey and wine are highly valued. Aged whiskey or wine may induce a sedative effect with less toxicity than ethanal (acetaldehyde) produced from ethanol by alcohol dehydrogenase. In the future, it will be

necessary to clarify the main target of liquors when they are ingested, although $GABA_A$ receptors are thought to be the first candidate for the main target at present (*40*). Recently, it was reported that the response of $GABA_A$ receptors composed of α_4, β_2, and δ subunits is potentiated by low concentrations (1−3 mM) of ethanol (*41*). Examining the effects of whiskey on the response of $GABA_A$ receptors composed of various combinations of subunits will provide interesting results.

ABBREVIATIONS USED

GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate.

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