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Effect of dried bonito (katsuobushi) and some of its components on $GABA_A$ receptors

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Abstract

Katsuobushi, a popular Japanese food additive and traditional flavour enhancer, is produced from a fish, bonito, by a variety of processes, including boiling, sun drying, smoking and mould culturing. Aqueous katsuobushi (AK), which is produced from katsuobushi powder by extraction with water, and some of its aroma components, such as 2-ethyl-3-methylpyrazine and phenol derivatives, potentiated dose-dependently the response of the $GABA_A$ receptors expressed in *Xenopus* oocytes. When AK, 2-ethyl-3-methylpyrazine or 3-methoxyphenol were injected into mice prior to an intraperitoneal administration of pentobarbital, the pentobarbital-induced sleeping time increased. In an elevated plus maze test, intraperitoneal administration of 2-ethyl-3-methylpyrazine to mice increased significantly both the number of entries into the open arms and the duration of stay in the open arms, indicating anti-anxiety activity. Katsuobushi and its aroma components may modulate human mood or consciousness through acting on $GABA_A$ receptors in the brain.

Keywords: Dried bonito; Elevated plus maze; Flavour; $GABA_A$ receptor; Pentobarbital-induced sleep

1. Introduction

Katsuobushi (dried bonito) contains umami, i.e., glutamic acid and inosinic acid, and is a traditional Japanese food additive (Ohta, 1992). It is also used widely as a flavour enhancer. It is produced by a variety of processes including boiling, sun drying, smoking, and mould culturing. The volatile flavours in katsuobushi contain components such as acids, phenols, pyridines, pyrazines, and thiazoles (Sakakibara, 1991; Yajima, Nakamura, Sakakibara, Ide, Yanai, & Hayashi, 1983; Yajima, Nakamura, Sakakibara, Yanai, & Hayashi, 1981). Moreover, proteolytic digestion of dried bonito muscle with thermolysin produces a hydrolysate with strong angiotensin-converting enzyme (ACE) inhibitory activity and is the basis of a dietary supplement with antihypertensive activity (Curtis, Dennis, Waddell, MacGillivray, & Ewart, 2002; Kouno, Hirano, Kuboki, Kasai, & Hatae, 2005; Yokoyama, Chiba, & Yoshikawa, 1992). A major portion of the ACE activity was shown to arise from the peptide Leu-Lys-Pro-Asn-Met (LKPNM).

We have reported that many fragrances in teas (Hossain, Aoshima, Koda, & Kiso, 2004; Hossain, Hamamoto, Aoshima, & Hara, 2002), coffee (Hossain, Aoshima, Koda, & Kiso, 2003), whiskey (Hossain, Aoshima, Koda, & Kiso, 2002; Koda, Hossain, Kiso, & Aoshima, 2003), and beer (Aoshima, Takeda, Okita, Hossain, & Koda, 2006) potentiate the responses of the $GABA_A$ receptors expressed in *Xenopus* oocytes, and we have proposed that the fragrances act on the receptors after absorption into the brain, because they are hydrophobic and easily pass the blood-brain barrier. Since $GABA_A$ receptors are main inhibitory neurotransmitter receptors (Martin & Olsen, 2000; Nicholls, 1994) and the targets of drugs such as tranquillisers, sleeping drugs, and anaesthetics, fragrances may modulate human mood or consciousness (Chebib & Johnston, 2000).

In the present study, we examined the effects of the volatile flavour components of katsuobushi on the response of

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the $GABA_A$ receptors expressed in *Xenopus* oocytes. We also examined their effects both on sleeping time induced by pentobarbital in mice (Aoshima et al., 2006; Hossain et al., 2004; Koda et al., 2003; Matsumoto, Satoh, Bing, Ohta, & Watanabe, 1991; Yamada, Watanabe, Aoyagi, & Ohta, 2001) and on mouse behaviour in an elevated plus maze.

2. Materials and methods

2.1. Samples and chemicals

Katsuobushi was produced from bonito by a variety of processes, including boiling, sun drying, smoking, and mould culturing, by Yamaki Co., Ltd. (Iyo, Ehime, Japan). A powder of solid katsuobushi was produced with a grinder. Aqueous katsuobushi (AK) was produced commercially at Yamaki Co., Ltd., by extracting powdered katsuobushi with water. The AK contained 92.0% (w/w) water, 5.6% proteins, 1.4% carbohydrate, 1.0% ash and fragrant compounds less than 1%, of which hexanal, 1-penten-3-ol, phenol, 2-methylphenol, 3-methylphenol, 4-methylphenol, 2-methoxyphenol, 2-methoxy-4-methylphenol, 2,5-dimethylpyrazine, and 2,6-dimethylpyrazine were detected by GC–MS. It has been reported that 339 fragrant compounds were detected in katsuobushi by GC–MS (Sakakibara, 1991; Yajima et al., 1981, 1983).

Since katsuobushi contained a small amount of GABA, we prepared a diethyl ether extract of katsuobushi. Katsuobushi powder (50 g) was extracted with 100 ml of deionised water for 1 h at room temperature. After filtration through a glass filter with suction, an equal amount of diethyl ether was added to the filtrate. This was followed by 1 min of vigorous shaking. The upper diethyl ether phase was separated from the aqueous phase by a separating funnel. The diethyl ether was removed by evaporation and the solid was dissolved in 400 μl of ethanol. This sample was named EK and did not induce the $GABA_A$ receptor response. The sample solution was stored in a freezer until its effect on the potentiation of the response of $GABA_A$ receptors was examined. All chemicals were of guaranteed reagent quality, purchased from either Nacalai Tesque, (Kyoto), Wako Chemical Industry, (Osaka), Katayamakagaku Co. (Osaka), or Sigma–Aldrich, Co. (Tokyo).

2.2. Preparation of cRNA and Xenopus oocytes

The cRNAs of the α_1 and β_1 subunits of the bovine $GABA_A$ receptor were synthesised from cloned cDNAs of bovine brain receptors with RNA polymerase (Promega, Madison, WI), according to standard procedures (Gurevoch, Pokrovskaya, Obukhova, & Zozulya, 1991). The cloned cDNAs were provided by Prof. Eric A. Barnard at the Medical Research Council Centre, London, UK.

Adult female frogs (*Xenopus laevis*) were purchased from Hamamatsu Seibutsu Kyozai Co. (Hamamatsu, Japan). The oocytes were dissected from adult frog ovaries that had been kept in ice for 1 h. They were manually detached from the inner ovarian epithelium and follicular envelope after incubation in collagenase (Type I, 1 mg/ml; Sigma) solution for 1 h, according to the procedure of Kusano, Miledi, and Stinnarkre (1982). The oocytes were microinjected with cRNAs in sterilised water and then incubated in modified Barth's 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 electrophysiological measurements (Aoshima & Hamamoto, 1999).

2.3. Electrophysiological measurements of the response

The membrane current of the receptors evoked by GABA was measured by the voltage clamping method, with a voltage clamp amplifier (TEV-200A, Dagan Co., Minneapolis, USA), according to the procedure described in a previous paper (Hossain et al., 2003). To examine the effect of AK on the $GABA_A$ receptors, the AK samples diluted various fold with normal frog Ringer solution (115 mM NaCl, 1 mM KCl and 1.8 mM $CaCl_2$ in 5 mM Tris at pH 7.2) were applied to oocytes expressing the $GABA_A$ receptors. The electrical responses induced by AK were compared with the response caused by 20 μM GABA and the concentration of GABA in the AK was estimated. To examine the effect of AK, its flavour components, and the diethyl ether extract of katsuobushi (EK) on the GABA-elicited response, they were added to 10 or 20 μM GABA solutions. One or the other of the solutions was selected by switching a valve in the flow system. The control response was obtained by perfusing a 10 or 20 μM GABA solution without the extract or compounds, and was taken as 100%. The effect of the AK, EK or flavour compounds on the response of the receptors was measured by using a mixture of GABA with AK, EK or the flavour compounds. When their dose dependences were measured, different amounts of the samples were added to the GABA solution. The measurement was repeated several times with the same oocyte, and control values were measured after every two or three measurements. Values of data were expressed as the means of four experiments. A Student's *t*-test was used to evaluate the significance of differences between the mean values of the sample and those of the control.

2.4. Animals

Male ICR mice (Clea Japan, Tokyo) aged 7–10 weeks and weighing 32–40 g were housed in plexiglas cages (10 mice/cage) with a stainless-steel mesh top and excelsior bedding (Clea Japan). Commercial solid (Clea Japan) and tap water were available *ad libitum*. The cages were placed in a room artificially illuminated by fluorescence lamps on a 12L:12D schedule (light period: 07:00–19:00), at a temperature of 25 ± 1 °C (Umezu & Morita, 2003).

All experiments proceeded in accordance with the guidelines of the Ethics Committee for Experimental Animals of the National Institute for Environmental Studies, Japan.

2.5. Measurement of pentobarbital-induced sleep in mice

Pentobarbital-induced sleep was measured, as reported by Matsumoto et al. (1991). Compounds were dissolved in a physiological solution of sodium chloride. AK was administered to mice intraperitoneally as it was. The compounds dissolved in the solution (100 mg/kg mouse weight) were administered intraperitoneally 30 min before the intraperitoneal injection of pentobarbital (30 mg/kg mouse weight). As a control, the physiological solution was administered intraperitoneally. The injection volumes of the compounds and pentobarbital were usually 1 ml/100 g mouse weight, but 2 ml/100 g of AK was also administered because the AK had a weaker effect than the other drugs. Sleeping time was measured as the time between the disappearance and recovery of the righting reflex. Five measurements were done for each sample. A Student's *t*-test was used to evaluate the significance of differences between the mean values of the sample and those of the control.

2.6. Measurement of anti-anxiety activity with an elevated plus maze

The plus maze (O'Hara and Co., Tokyo) was made of plexiglass and consisted of two open arms 25 × 8.5 cm, and two enclosed arms 25 × 8.5 × 12 cm (Tokumo, Tamura, Hirai, & Nishio, 2006). The arms extended from a central platform, 5 × 5 cm. The apparatus was mounted on an iron stand, raising it 65 cm above the floor. The surface of the arms was white and the movement of the mouse was monitored by a CCD camera for 15 min and analysed by a computer. The time spent in the open arms and the number of entries to the open arms were calculated. 3-Methoxyphenol and 2-ethyl-3-methylpyrazine were dissolved in a physiological solution of sodium chloride and administered by intraperitoneal injection at 100 mg/kg mouse weight. AK was administered intraperitoneally as it was. As a control, a physiological solution of sodium chloride was administered to the mice. One millilitre compound solution/100g mouse weight was injected intraperitoneally into ICR mice, but 2 ml/100 g of AK was injected into the mice because of the low level of active compounds in it. These samples were administered similarly every day twice and the measurements were done on the third day after the first injection, to reduce the effect of the injection on the measurements. Ten measurements were done for each sample. A Student's *t*-test was used to evaluate the significance of differences between the mean values of the sample and those of the control.

3. Results

$GABA_A$ receptors were expressed in *Xenopus* oocytes by injecting cRNAs of the α_1 and β_1 subunits of bovine $GABA_A$ receptors. Fig. 1 shows some examples of the potentiation of the responses of the $GABA_A$ receptors by GABA and the mixture of GABA and a component of

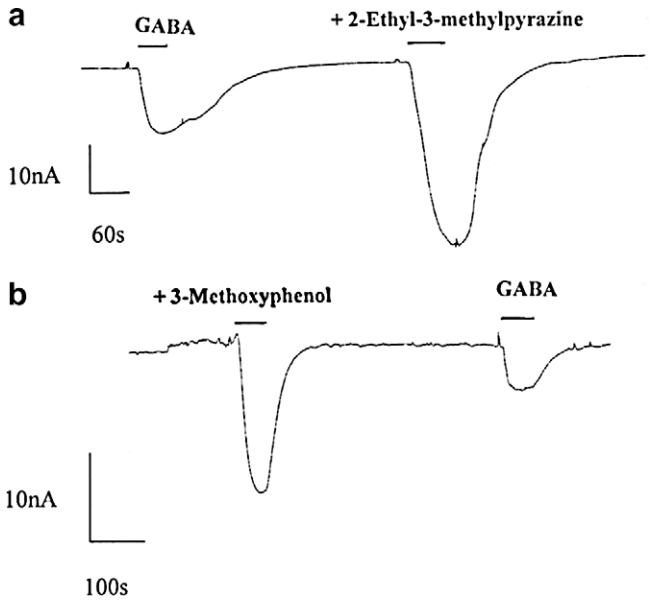

Fig. 1. Examples of the potentiation of GABA-elicited current by components in dried bonito (katsuobushi). The response of the $GABA_A$ receptors caused by 20 μM GABA was compared with that caused by 20 μM GABA with (a) 0.8 mM 2-ethyl-3-methylpyrazine or (b) 0.93 mM 3-methoxyphenol. The upper bars show when GABA or a mixture of GABA and the compound was applied on the oocytes.

katsuobushi. Aqueous katsuobushi (AK) itself induced very small electrical responses of the $GABA_A$ receptors, possibly because of the presence of a very small amount of GABA, whose concentration in AK was estimated to be 369 μM, from the comparison with the control GABA-elicited response. The dose–potentiation relationship of AK was examined in Fig. 2, where the same concentration of GABA as that measured in AK was added to the control, a 20μM GABA solution. The dissociation constant (K_p) and the maximum potentiation of the receptors (V_m), when all the potentiation sites of the receptors were occupied by the compounds, were estimated to be 1.4%

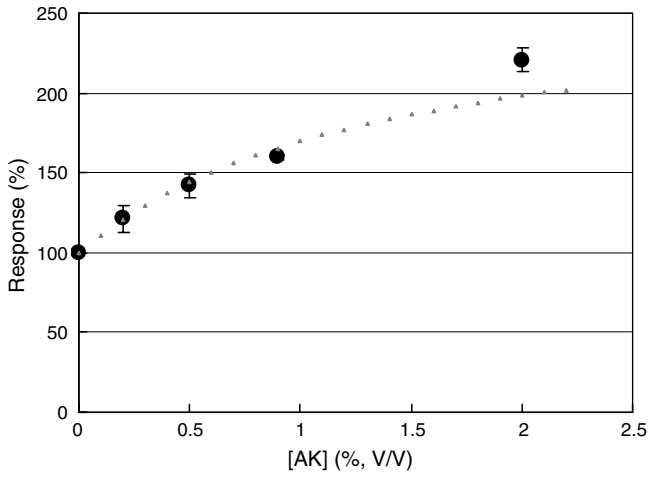

Fig. 2. Dose–potentiation relationship of aqueous extract of katsuobushi (AK).

(v/v) and 267%, respectively, from the results, with the assumption of a simple equilibrium between the compounds and the receptors (Aoshima, Hossain, Hamamoto, Yokoyama, & Yamada, 2001). Further, to prove directly that katsuobushi includes compounds which potentiate the response of $GABA_A$ receptors, a diethyl ether extract of katsuobushi (EK) was prepared. The EK induced no response, but potentiated the response induced by 10 μM GABA as follows: 0.2% (v/v) EK, 138 ± 9%, and 0.5% EK, 214 ± 21%.

More than 300 volatile flavour components have already been identified in katsuobushi (Sakakibara, 1991; Yajima et al., 1981, 1983). The effects of 13 of these components, which can be purchased commercially, on the responses of the $GABA_A$ receptors were examined by adding them to the 20 μM GABA solution (Fig. 3). It was found that phenol derivatives, especially 3-methoxyphenol and 2-ethyl-3-methylpyrazine, strongly potentiated the response. The dose dependency of the potentiation by 2-ethyl-3-methylpyrazine, 3-methoxyphenol, and 3-ethylphenol were examined; the maximum potentiation (V_m) and the dissociation constant (K_p) were estimated to be 555% and 1.14 mM for 2-ethyl-3-methylpyrazine, 535% and 1.04 mM for 3-methoxyphenol and 224% and 0.14 mM for 3-ethylphenol, respectively (Fig. 4).

Since AK and some of its components potentiated the response, we examined whether they act on the $GABA_A$ receptors *in vivo*. It is known that pentobarbital induces sleep in mice by potentiating the response of $GABA_A$ receptors (Martin & Olsen, 2000; Nicholls, 1994). Fig. 5 shows the effect of AK and its components on the sleeping time induced by the intraperitoneal injection of pentobarbital (30 mg/kg). Their intraperitoneal injection (100 mg/kg)

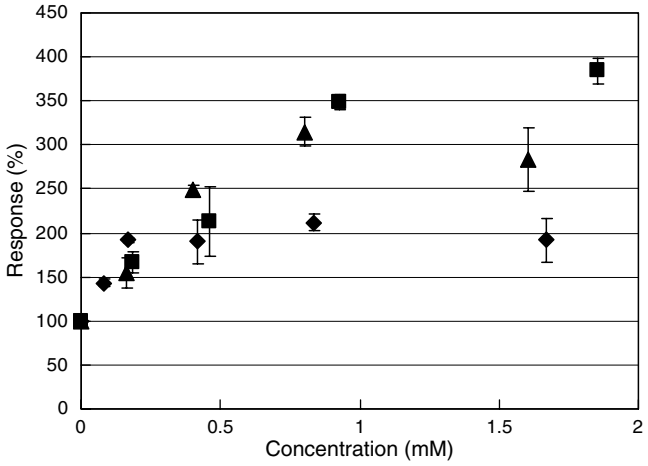

Fig. 4. Dose–potentiation of some components in katsuobushi: 2-ethyl-3-methylpyrazine (▲), 3-methoxyphenol (■) and 3-ethylphenol.

prolonged sleeping time in mice, as compared to the control (pentobarbital only). Since 2-ethyl-3-methylpyrazine markedly prolonged sleeping time of mice, the dose dependency of its effect was examined (data not shown). 2-Ethyl-3-methylpyrazine at 25 mg/kg doubled the sleeping time, compared to the control. Injection of AK at 1 ml/100 g did not induced significant extension of sleeping time of mice. However, injection of AK into mice at 2 ml/100 g induced significant extension of sleeping time of mice.

It is known that muscimol, an agonist of $GABA_A$ receptors, and diazepam, a benzodiazepine agonist, reduce anxiety in an elevated plus maze. The effect of katsuobushi and its components, which potentiated the response of $GABA_A$ receptors and prolonged the sleeping time induced by pen-

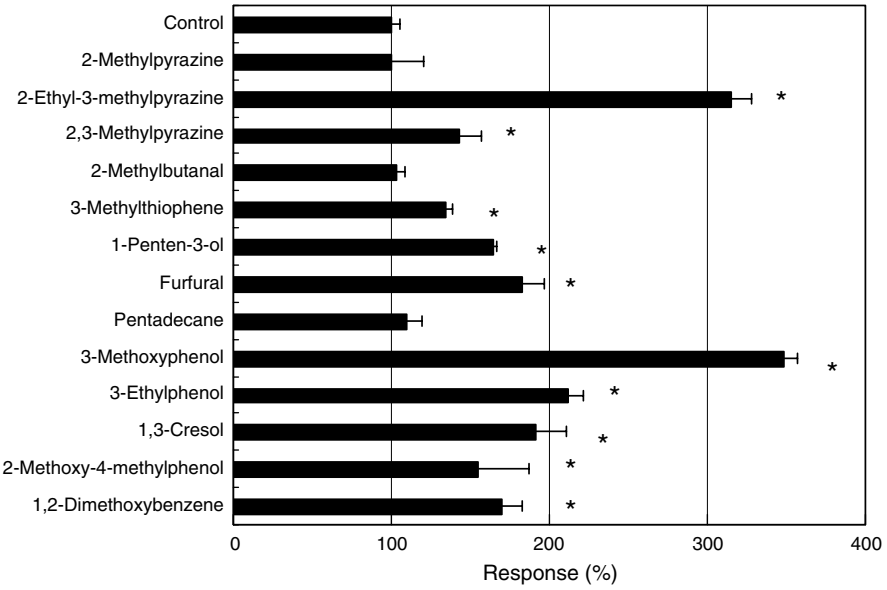

Fig. 3. Effect of volatile components of katsuobushi on the response of $GABA_A$ receptors elicited by 20 μM GABA. The response elicited by 20 μM GABA was taken as a control (100%). The concentration of the volatile components of katsuobushi was 0.01% (v/v). $^*p < 0.05$ for the difference between the control and the sample by Student's *t*-test.

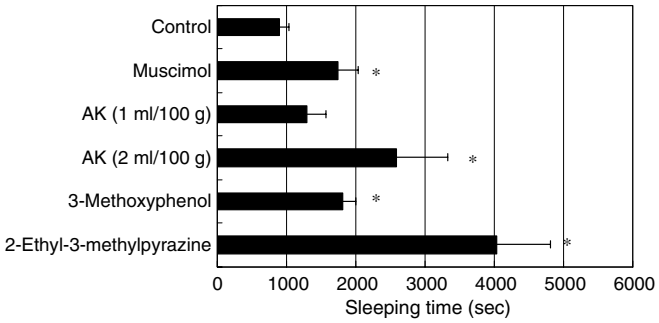

Fig. 5. Effect of katsuobushi and its components on sleeping time induced by pentobarbital in mice. The sample concentrations were muscimol, 0.5 mg/kg; 3-methoxyphenol and 2-ethyl-3-methylpyrazine, 100 mg/kg. Data are means ± SD from five experiments. $^{*}p < 0.05$ for the difference between the control and the samples by Student's *t*-test.

tobarbital, on mouse behaviour was examined with an elevated plus maze. The intraperitoneal injection of 100 mg/kg of 2-ethyl-3-methylpyrazine significantly increased both the number of entries into the open arms and the duration of stay in the open arms, while the injection of 100 mg/kg 3-methoxyphenol decreased both the number of entries and the duration, as shown in Fig. 6. The injection of AK had no significant effect on mouse behaviour.

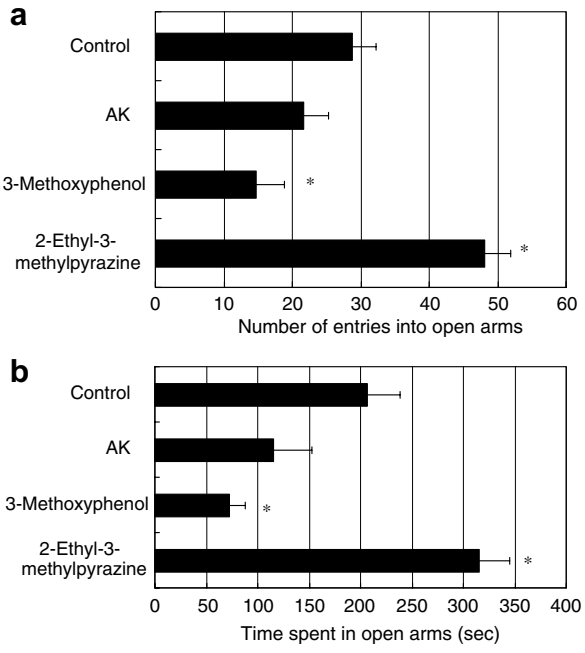

Fig. 6. Effect of katsuobushi and its components on behaviour in an elevated plus maze showing the number of entries (a) into the open arms and the time spent (b) in the open arms, recorded for 15 min. 3-Methoxyphenol and 2-ethyl-3-methylpyrazine were injected at 100 mg/kg into mice. Data are mean ± SD values from 10 experiments. $^{*}p < 0.05$ for the difference between the control and the samples by Student's *t*-test.

4. Discussion

Katsuobushi, which is produced from the sea fish bonito, is an important seasoning, together with sea tangles and dried mushrooms, in Japanese cuisine (Ohta, 1992). These seasonings contain glutamic acid, inosinic acid and guanylic acid, and activate the umami receptor in the tongue. Katsuobushi also includes many flavour compounds (Sakakibara, 1991; Yajima et al., 1981, 1983). We have already found that many flavour compounds in beverages potentiate the responses of $GABA_A$ receptors, the main inhibitory neurotransmitter receptors in the brain; the binding of fragrances to the receptors increased the affinity of the GABA-binding sites, shifting the dose-response curve of GABA to a lower concentration (Aoshima et al., 2006; Hossain et al., 2002, 2003, 2004; Hossain, Hamamoto, et al., 2002; Koda et al., 2003). So we have examined the effect of AK and its flavour components on the response of $GABA_A$ receptors and found the response to be potentiated. It is well established that the potentiation of the response of $GABA_A$ receptors by drugs, such as benzodiazepine, barbiturates, anaesthetics, and ethanol induces tranquillising, sleeping, and anaesthetic effects, depending on the dose (Chebib & Johnston, 2000; Martin & Olsen, 2000; Nicholls, 1994). So when one drinks katsuobushi soup, the fragrant compounds in it may be absorbed into the blood, cross the blood-brain barrier, enter the brain, and potentiate the response, which may modulate mood or consciousness, as ethanol does (Martin & Olsen, 2000).

Though katsuobushi is used as a food additive only in certain areas of Asia, mainly in Japan, the volatile components in katsuobushi are present in various popular foods and beverages. Many phenol derivatives are present in hams, sausages, bacons and smoked cheeses or fishes, since these compounds are added during the smoking process. Some pyrazine derivatives are present in coffee, tea, cocoa, and soy sauce (Maarse, 1991).

The amount of fragrant compounds in AK is much lower than that of ethanol in alcohols, but the potency and efficacy with which these fragrances affect $GABA_A$ receptors is much greater than those of ethanol (Aoshima et al., 2001; Hossain et al., 2002). In our experiments, $\alpha_1\beta_1$ $GABA_A$ receptors were expressed in *Xenopus* oocytes and used for examining the effects of fragrant components of katsuobushi on the receptors, since the effects of many fragrant compounds on these receptors were similar to those on the $GABA_A$ receptors expressed in the oocytes by injecting poly(A)$^+$RNA prepared from rat whole brain, as reported before (Aoshima et al., 2001). However, it is reported recently that ethanol at low concentration (3 mM) enhances the response of special combinations of subunits of the $GABA_A$ receptor, i.e., $\alpha_4\beta_3\delta$ in extrasynaptic regions (Wallner, Hanchar, & Olsen, 2003). So it is likely that the fragrant compounds also act on $\alpha_4\beta_3\delta$GABA$_A$ receptors at lower concentrations in the brain than ethanol does and modulate human mood or consciousness (Wallner, Hanchar, & Olsen, 2006).

Since sleep-inducing drugs potentiate the response of $GABA_A$ receptors and induce sleep in mice, we examined the effect of the intraperitonial coadministration of AK and its components, 2-ethyl-3-methylpyrazine and 3-methoxyphenol, on pentobarbital-induced sleeping time (Aoshima et al., 2006; Hossain et al., 2004; Koda et al., 2003; Matsumoto et al., 1991; Yamada et al., 2001). As expected, they increased the sleeping time, possibly because they also potentiate the response of $GABA_A$ receptors. However, the toxicity of 3-methoxyphenol may modulate the pentobarbital-induced sleeping time of mice. The intracerebroventricular administration of alkylpyrazine derivatives, such as 2,5-dimethylpyrazine, 2-chloro-3,6-dimethylpyrazine and 2-fluoro-3,6-dimethylpyrazine, lengthened the pentobarbital-induced sleeping time of mice and also extended the interval before the appearance of picrotoxin-induced convulsions (Yamada et al., 2001). The accumulation of essential oil components in the mouse brain was found when they were given by means of pancutaneous or vapour exposure absorption (Inoue, Oshihara, Uchida, & Yamaguchi, 2000; Inoue & Yamaguchi, 2000). These results suggest that the lipophilic components in katsuobushi enter the brain and potentiate the response of $GABA_A$ receptors.

To investigate the effect of katsuobushi and its components on mouse behaviour, an elevated plus maze was used to examine anti-anxiety properties (Tokumo et al., 2006). It is known that the injection of muscimol and diazepam into mice reduces anxiety, increasing the number of entries into open arms and the time spent in these arms in an elevated plus maze. The intraperitonial injection of 2-ethyl-3-methylpyrazine increased significantly both the number of entries into and the time spent in open arms. Unexpectedly, the intraperitonial injection of 3-methoxyphenol decreased significantly both these parameters. At present, we cannot explain exactly why 2-ethyl-3-methylpyrazine and 3-methoxyphenol had opposite effects on behaviour in the elevated plus maze, though both compounds potentiated the response of $GABA_A$ receptors and also increased the pentobarbital-induced sleeping time of mice. One plausible explanation is that 3-methoxyphenol is toxic at high concentrations and altered mouse behaviour. The intraperitonial injection of AK did not have a significant effect on mouse behaviour in the elevated plus maze, possibly because the concentrations of fragrant compounds are very low in AK.

A direct effect of fragrant compounds on $GABA_A$ receptors was suggested by a study showing that inhaling chamomile, lavender, and lemon oil vapour decreased stress-induced increases in the plasma adrenocorticotropic hormone levels of ovariectomized rats, as did diazepam, a benzodiazepine derivative (Yamada, 2004; Yamada, Miura, Mimaki, & Sashida 1996). It has also been reported that rose oil and its components had anticonflict effects in a behaviour test, when injected intraperitonially into mice (Umezu, 2000).

AK has brown pigments, suggesting the presence of antioxidative activity. As expected, it scavenged 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals with an EC_{50} of 15% (v/v) (data not shown). Since it is usually diluted 15-fold during cooking, AK possibly contributes to radical scavenging in foods.

5. Conclusions

Katsuobushi as a food additive acts to: (1) increase nutrition by adding proteins, amino acids, vitamin B and minerals; (2) stimulate umami receptors through glutamic acid and inosinic acid, which enhance taste; (3) improve flavour and potentiate the response of the $GABA_A$ receptors, leading to a more tranquil mood; (4) provide anti-oxidative activity *via* pigment; and (5) achieve an antihypertensive effect after proteolytic digestion by thermolysin.

Acknowledgments

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