Potentiation of GABA<sub>Α</sub> Receptors Expressed in Xenopus Oocytes by Perfume and Phytocnic

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To study the effects of perfume and phytocnic on GABA<sub>Α</sub> receptors, ionotropic GABA<sub>Α</sub> receptors were expressed in Xenopus oocytes by injecting mRNAs that had been prepared from rat whole brain. Essential oil, perfume and such phytocnic as leaf alcohol, hinokitiol, pinene, eugenol, citronellol and citronellal potentiated the response in the presence of GABA at low concentrations (10 and 30 μm), possibly because they bound to the potentiation-site in GABA<sub>Α</sub> receptors and increased the affinity of GABA to the receptors. Since it is known that the potentiation of GABA<sub>Α</sub> receptors by benzodiazipine, barbiturate, steroids and anesthetics induces the anxiolytic, anticonvulsant and sedative activity or anesthetic effect, these results suggest the possibility that the intake of perfume or phytocnic through the lungs, the skin or the intestines modulates the neural transmission in the brain through ionotropic GABA<sub>Α</sub> receptors and changes the frame of the human mind, as alcohol or tobacco does.

Key words: aromatherapy; essential oil; perfume; phytocnic; GABA<sub>Α</sub> receptor

The frame of the human mind is affected by the intake of alcohol or tobacco. The effects of ethanol on the neurotransmitter receptors have been extensively studied because of the importance of behavior disorders related to alcohol abuse.1,2) Although many kinds of compound are added to processed foods to protect against oxygen and microorganisms, or to give the foods a desirable fragrance and a good taste, little is known about the effects of such food additives on the receptors. We have examined in previous studies the effects of food additives on the nicotinic acetylcholine<sup>3</sup> and glutamate<sup>4</sup> receptors expressed in Xenopus oocytes and found that some food additives inhibited these receptors competitively or noncompetitively. We then examined the effects of linoleic acid and its hydroperoxide<sup>5</sup> and of food additives on the ionotropic γ-aminobutyric acid (GABA<sub>Α</sub>) receptors.<sup>6</sup> Caffeine and vanillin inhibited the GABA<sub>Α</sub> receptors. However, 13,1-t-hydroperoxy- and hydroxy-linoleic acid, and alcohol, aldehyde and ester which are often used for flavoring potentiated the response of the GABA<sub>Α</sub> receptors in the presence of low concentrations (5 and 10 μm) of GABA.<sup>6</sup> The potentiation of the response depended strongly on the functional group of the compounds.

Essential oils, which include various kinds of alcohol, aldehyde and ester, are used as a perfume and are also used for aromatherapy to induce mental tranquility and aid sleep.<sup>7</sup> Trees and plants in the forest produce and emanate many volatile compounds such as (3Z)-hexen-1-ol (leaf alcohol), (−)-α-pinene and hinokitiol (β-thujaplicin) which are generally called phytocnics.<sup>8</sup> It is known that walking in a forest with the scent of a phytocnic induces anxiolytic, anticonvulsant and sedative activity. So it would be interesting to learn whether essential oils, perfumes and phytocnics could also potentiate the GABA<sub>Α</sub> receptors.

In this paper, GABA<sub>Α</sub> receptors were expressed in Xenopus oocyte by injecting mRNAs prepared from rat brain. The responses elicited by GABA were examined in the presence of various compounds composing perfumes and phytocnics. It was assumed that these compounds would be taken into the brain through the lungs, skin or intestines, and potentiate the GABA<sub>Α</sub> receptors directly, resulting in modulation of the frame of the human mind.

Materials and Methods

Materials. Essential oils and perfumes: Lavender Drome, Lavandin Abrial Drome, English Lavender

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Ingredient</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lavender Drome</td>
<td>Essential oil of Lavandula officinalis; linalyl acetate (33-35%), linalool (15-20%), 3-octanone (1.3-3%), limonene, borneol, camphor</td>
</tr>
<tr>
<td>2</td>
<td>Lavandin Abrial Drome</td>
<td>Essential oil of Lavandula hybrida; linalool (44%), linalyl acetate (20%), camphor (6-10%), cineole (3-5%), borneol (5%), terpene (5%)</td>
</tr>
<tr>
<td>3</td>
<td>English Lavender (Yardley Co. Ltd.)</td>
<td>Perfume of lavender; mixture of essential oil of lavender and ethanol (unknown ingredients)</td>
</tr>
<tr>
<td>4</td>
<td>Northfolk Lavender (Northfolk Lavender Ltd.)</td>
<td>Perfume of Lavender; ethanol, fragrance, 2-methylpropane-2-ol, coloring matter</td>
</tr>
<tr>
<td>5</td>
<td>Lavender Theraphy (Coty Co. Ltd.)</td>
<td>Cologne of lavender (unknown ingredients)</td>
</tr>
</tbody>
</table>

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Abbreviation: GABA, γ-aminobutyric acid
(Yardley Co., London), Northfolk Lavender (Northfolk Lavender Co.), and Lavender Therapy (Coty Co., Paris) are characterized in Table 1, having been kindly supplied by Takasago International Co. (Tokyo). GABA, (−)-α-pinene, (−)-β-pinene, eugenol, citronellol, citronellal and hinokitiol (β-thujaplicin) were purchased from Nacalai Tesque (Kyoto). (3Z)-Hexen-1-ol (leaf alcohol) was purchased from Sigma Chemical Co. (St. Louis). All chemicals were of guaranteed reagent quality.

Preparation of poly(A)+RNA and Xenopus oocytes. Whole brains were obtained from male adult Wistar rats (weighing about 100 g) after they had been anesthetized with diethyl ether. Poly(A)+RNA was prepared from the brains by the procedure described by Maniatis et al.9

Adult female frogs (Xenopus laevis) were purchased from Hamamatsu Seibutsu Kyoza (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 by the procedure of Kusano et al.10 The oocytes were microinjected with about 50 ng of poly(A)+RNA in sterilized water and then incubated in a modified Barth solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.33 mM Ca(NO3)2, and 0.41 mM CaCl2 in 5 mM Tris at pH 7.6) containing 25 mg/1 of penicillin and 50 mg/1 of 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 with a voltage clamp amplifier (CEZ-1100; Nihon Kohden Kogyo, Tokyo, Japan). An oocyte was placed on the net of a small chamber (about 0.3 ml) and impaled with two microelectrodes filled with 3 M KCl, one for monitoring the membrane potential and the other for passing current for clamping the membrane potential, usually at −60 mV. The electrical response of the current was filtered at 1–5 Hz by a P-84 filter (NF Electronic Instruments, Kanagawa, Japan). The oocyte placed on the net was continuously perfused from the bottom with a normal frog Ringer solution (115 mM NaCl, 1 mM KCl, and 1.8 mM CaCl2 in 5 mM Tris at pH 7.2) by a gravity feed system, usually at a flow rate of about 2 ml/min.11

Measurement of the receptor response. GABA was dissolved in a normal frog Ringer solution. To examine the effect of such compounds as essential oils and phytocids on the GABA-elicited response, each was added to the Ringer solution and GABA solution which were shaken vigorously for a minute. One or other of the solutions was selected by switching a cock in the flow system. The control response was obtained by perfusing the GABA solution without any compound and is taken as 100%. The effect of the compound on the response of the receptors was measured by using a mixture of GABA and the compound; in some cases, the compound was added for 1 min before co-application with GABA when desensitization of the receptors was significantly induced before attaining equilibrium of the compound binding.12 The measurement was repeated several times with the same oocyte, and control values were obtained after every two or three measurements. To eliminate desensitization of the receptors, the oocyte was washed for more than 10 min in a normal frog Ringer solution before the next measurement, since desensitization of the ionotropic GABAA receptors is a reversible process and the receptors usually recover after about 10 min of washing.13

Results

Figure 1 shows the electrical responses of the GABA receptors expressed in Xenopus oocytes by injecting rat whole brain mRNA. These responses are thought to have been induced by ionotropic GABA receptors (GABAA receptors), since the response of metabotropic receptors (GABAB receptors) can not be detected by electrophysiological measurements on a Xenopus oocyte.14

The addition of 1 µl of essential oil of lavender (No. 1) or leaf alcohol to 10 ml of a solution of 100 µM GABA showed little effect on the response of the receptor in the oocyte when each compound was applied for 1 min before co-application with 100 µM GABA. This protocol was used because 100 µM GABA induced desensitization of the GABAA receptors before attaining equilibrium of the essential oil binding.12,13 On the other hand, the addition of 1 µl of an essential oil or leaf alcohol to 10 ml of a solution of 10 µM GABA potentiated the response of the GABAA receptors. Since the rate of desensitization of the GABAA receptors induced by 10 µM GABA was slow, preliminary treatment of the oocyte with the essential oil had no significant effect on potentiation by the oil (data not shown). Table 2 shows the effects of GABA concentration on the potentiation of the GABAA receptors caused by the various kinds of essential oil and per-

![Fig. 1](image-url)
Table 2. Effect of Fragrant Compounds on GABA Response

The response without the fragrant compound was taken to be 100%. However, values in parentheses are the relative response when the response caused by 1 mM GABA without any compound was taken to be 100%, this being estimated from the results of the previous paper. Data are mean ± SD values from four experiments. Nos. 1–5 are characterized in Table 1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>10 μM GABA</th>
<th>30 μM GABA</th>
<th>100 μM GABA</th>
<th>1 mM GABA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(14.9)</td>
<td>(49.5)</td>
<td>(93.6)</td>
<td>(100)</td>
<td></td>
</tr>
<tr>
<td>0.1 μl/ml of No. 1</td>
<td>208±3</td>
<td>148±14</td>
<td>75±4</td>
<td>95±4</td>
</tr>
<tr>
<td>0.1 μl/ml of No. 2</td>
<td>223±21</td>
<td>160±3</td>
<td>86±6</td>
<td>78±8</td>
</tr>
<tr>
<td>0.1 μl/ml of No. 3</td>
<td>196±38</td>
<td>152±15</td>
<td>105±3</td>
<td>89±2</td>
</tr>
<tr>
<td>0.1 μl/ml of No. 4</td>
<td>172±21</td>
<td>143±5</td>
<td>116±5</td>
<td>90±4</td>
</tr>
<tr>
<td>0.1 μl/ml of No. 5</td>
<td>188±12</td>
<td>147±11</td>
<td>113±6</td>
<td>91±4</td>
</tr>
<tr>
<td>0.63 mM α-Pinene</td>
<td>149±16</td>
<td>157±9</td>
<td>98±2</td>
<td>117±12</td>
</tr>
<tr>
<td>0.65 mM Eugenol</td>
<td>356±58</td>
<td>234±21</td>
<td>140±3</td>
<td>102±10</td>
</tr>
<tr>
<td>0.55 mM Citronellol</td>
<td>301±32</td>
<td>146±12</td>
<td>101±5</td>
<td>92±2</td>
</tr>
<tr>
<td>0.56 mM Citronellal</td>
<td>224±11</td>
<td>123±6</td>
<td>108±7</td>
<td>90±1</td>
</tr>
<tr>
<td>0.34 mM Leaf alcohol</td>
<td>218±11</td>
<td>142±1</td>
<td>135±5</td>
<td>94±2</td>
</tr>
<tr>
<td>0.61 mM Hinokitiol</td>
<td>297±16</td>
<td>132±6</td>
<td>91±0</td>
<td>102±2</td>
</tr>
</tbody>
</table>

Fig. 2. Dose-Response Curve in the Presence and Absence of 0.1 μl/ml of Perfume No. 3 (solid line) or 0.55 mM Citronellol (dashed line).

The response caused by 1 mM GABA without any compound was taken to be 100%. The solid curve is the theoretical one estimated in the previous paper. The values in the presence of the fragrant compounds are calculated from the data in Table 2. Data are mean ± SD (bar) values from four experiments.

The presence of 10 μM GABA, although its concentrations may not be accurate since they have little solubility in an aqueous solution. Even a very small amount (20 ppm, i.e. about 100 μM) of the fragrance induced a significant potentiation of the response of the GABA<sub>A</sub> receptors. Thus, their effects on the GABA<sub>A</sub> receptors depended on the concentrations of both the compounds and GABA.

Since stereoselectivity has been reported for anesthetic and barbiturate activity, we examined the potentiation of the GABA<sub>A</sub> receptors by both α- and β-pinene in the presence of 10 μM GABA. However, no significant difference was apparent in their potentiation (0.13 mM pinene: α, 117±4%; β, 122±18%; 0.63 mM pinene: α, 149±16%; β, 145±28%).

Since 13-1-hydroperoxylinoleic acid had increased the rate of desensitization by GABA, the rate of desensitization caused by 50 μM GABA was measured in the presence and absence of the essential oil of Lavender Drome, α-pinene, eugenol and leaf alcohol. However, these compounds showed no effect on the desensitization of the GABA<sub>A</sub> receptors (data not shown).

Discussion

The GABA<sub>A</sub> receptors are ionotropic neurotransmitter receptors and conduct Cl<sup>−</sup> when the ligands are bound. They are inhibitory receptors and antagonize the tendency of excitatory receptors to sufficiently depolarize the membrane to reach the threshold at which action potentials fire, since GABA<sub>A</sub> channel activation moves the membrane potential closer to the Cl<sup>−</sup> equilibrium potential, which is usually quite negative (−60 to −80 mV).

The GABA<sub>A</sub> receptors have multiple binding sites and a complex pharmacology. GABA agonists bind to two binding sites which are possibly present between the α and β subunits. Antagonists also bind to these sites and competitively inhibit the response. Hydrophobic compounds such as vanillin and divalent cations noncompetitively inhibit the response. Benzodiazepine, barbiturate, steroids, ethanol and anesthetics bind to multiple al-
pharmacological profile observed in general anesthetics.

In aromatherapy, an essential oil is externally applied through the skin or by respiration for medical and health treatment. This method is thought to be effective for relaxing mental and physical strain or stress by a mainly psychological action through stimulation of the olfactory system. Some fragrant herbs and spices are sometimes used for cooking and are also eaten. Trees and herbs emanate many fragrant compounds such as leaf alcohol, pinene and hinokitiol, which are generally named phytoncids and have sterilizing power. Walking in the forest and breathing phytoncids is also known to be helpful for mental and physical health mainly by a psychological action.

Our experiments have shown that the essential oil of lavender, perfume and phytoecids potentiated the GABA_\text{A} receptor-response caused by a low concentration of GABA as well as benzodiazepine, barbiturate, steroids, ethanol and anesthetics. Since the fragrant compounds shifted the GABA-dose response curve to a lower concentration (Fig. 2), they possibly increased the affinity of GABA to the receptors. These results suggest the possibility that fragrances and phytoncids induce the anxiolytic, anticonvulsant and sedative activity in the brain by potentiating the GABA_\text{A} receptors if they have been absorbed into blood and carried to the brain.

In aromatherapy, an essential oil can penetrate into the blood through the skin if it has been applied. Linalool and linalyl acetate were reportedly detected in the blood 10–20 min after application to the skin. Caution against too much use of an essential oil is usually given as an aromatherapy protocol. If it is added to bath water or put on underwear, it is evaporated and absorbed into the blood by respiration through the lungs. A phytoncid is also absorbed into the blood through the lungs when walking in a forest where many trees and herbs emanate the various volatile compounds called phytoncids. An adult person usually takes about 41/min of air, i.e., 240 l/h of air by respiration. This implies an intake of about 120 μl of leaf alcohol during a walk for an hour in the forest, if air contains 0.01% of leaf alcohol emanating from plants. Fragrant herbs are sometimes cooked and eaten with food, while fragrance is also added to foods as a flavoring. These compounds are absorbed into the blood through the stomach and the intestines. Since fragrant compounds are usually lipophilic, they can easily pass the blood-brain barrier. In fact, ethanol and general anesthetics are absorbed into the blood through the stomach and lungs, taken into the brain and cause their remarkable effects on the brain, although their concentration is possibly much higher than that of a perfume or phytoncid, and their effect on the brain is much stronger than that of a fragrant compound. These hypotheses are summarized in Fig. 4.

Since amount of the fragrance taken into the brain is possibly very small, compared with drugs, ethanol and anesthetics, potentiation of the response of the GABA_\text{A} receptors by a fragrance must be very little. However, the degree of ethanol or anesthetic potentiation of the GABA_\text{A} receptors depended on their subunit composi-
The fragrant compounds stimulate the olfactory system and affect the olfactory system and affect the frame of the human mind psychologically. However, the olfactory system adapt to this stimulation by fragrance in a short time. A fragrant compound will be absorbed into the blood through the lungs by respiration, through the skin when applied, and through the stomach and intestines when added to foods. Then they will be carried to the brain through the blood-brain barrier and potentiate the GABA<sub>A</sub> receptors, which will induce anxiolytic, anticonvulsant and sedative activity in the frame of the human mind, as benzodiazepine, barbiturate, steroids, ethanol and anesthetics do.

21) The distribution of subunits throughout the central nervous system varied greatly. So it is likely that the potentiation by a fragrance would also be greater in some brain regions than that observed in our experiments, by which total mRNAs from whole brain were injected into the oocyte. Moreover, a neuron forms thousands of both excitative and inhibitory synapses in the brain and its excitation depends on the balance between the excitatory and inhibitory inputs through the synapses. So even a small degree of potentiation of the GABA<sub>A</sub> receptors may prevent the excitation of some neurons in a great many ones, exhibit anxiolytic, anticonvulsant and sedative activity, and change the frame of the human mind. To prove these hypotheses, it would be necessary to measure the amount of the fragrance in the blood and brain. Behavioral tests to measure anxiety in rats or mice treated with an essential oil or phytoncid by using a plus-maze or mirrored chamber will be also necessary in the future.

Acknowledgments
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References
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