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Central and peripheral activity of cholinesterase inhibitors as revealed by yawning and fasciculation in rats

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Abstract

This study was designed to investigate the central and peripheral activity profile of cholinesterase inhibitors in rats. Intravenous injection of cholinesterase inhibitors caused fasciculation, a fine involuntary muscular movement. This peripheral cholinergic sign was tightly correlated with in vitro anti-acetylcholinesterase activity by cholinesterase inhibitors, suggesting that fasciculation is a valid index of peripheral cholinergic activation. Yawning, used as a marker of central cholinergic activation, was also monitored. E2030 (3-(2-(1-(1,3-dioxolan-2-ylmethyl)-4-piperidyl)ethyl)-2 *H*-3,4-dihydro-1,3-benzoxazin-2,4-dione hydrochloride) elicited yawning at more than 4 mg/kg, while fasciculation was significantly intensified only at a dose of 16 mg/kg. Donepezil and tacrine induced both yawning and fasciculation at doses greater than 4 mg/kg, whereas physostigmine induced both behaviors at a dose of 8 mg/kg and above. Finally, ipidacrine elicited yawning at a dose of 16 mg/kg and fasciculation at doses greater than 8 mg/kg. Thus, all putative centrally acting cholinesterase inhibitors elicited yawning. TAK-147 (3-[1-(phenylmethyl)-4-piperidinyl]-1-(2,3,4,5-tetrahydro-1 *H*-benzazepin-8-yl)-1-propanone fumarate) did not significantly elicit yawning at doses under 16 mg/kg, but elicited fasciculation at a dose of more than 4 mg/kg. Distigmine, a peripherally acting cholinesterase inhibitor, evoked fasciculations, but not yawning. When mild to moderate fasciculation was evoked, donepezil and E2030 elicited more than nine yawns over 30 min, while the other cholinesterase inhibitors elicited approximately five yawns at most during this period. These results indicated that E2030 and donepezil exhibited the most marked preferential central cholinergic activity, relative to peripheral activity, among cholinesterase inhibitors tested. Scopolamine, a centrally acting antimuscarinic drug, completely inhibited E2030-induced yawning, while peripherally acting methylscopolamine did not. Haloperidol, a dopamine receptor antagonist, partially blocked E2030-induced yawning, but did not block donepezil-induced yawning. These results suggest that central cholinergic and, in part, dopaminergic mechanisms are involved in E2030-induced yawning.

Keywords: Cholinesterase inhibitor; Donepezil; E2030; Yawning; Fasciculation; (Rat)

1. Introduction

Cholinesterase inhibitors are, so far, the only successful strategy for the symptomatic treatment of Alzheimer's disease (Nordberg and Svensson, 1998; Whitehouse, 1998; Doody, 1999). Cholinesterase is the enzyme that is responsible for the hydrolysis of acetylcholine. Cholinesterase inhibitors both block this metabolism of acetylcholine, thus increasing the level of acetylcholine in the synaptic cleft, and activate cholinergic transmission. An important prerequisite for the use of cholinesterase inhibitors in Alzheimer's disease therapy is a preferential action in the central, versus peripheral, nervous system.

Activation of central cholinergic systems elicits yawning in experimental animals. Urba-Holmgren et al. (1977) described that pilocarpine, a muscarinic receptor agonist, and physostigmine, a cholinesterase inhibitor, induced yawning in rats, while neostigmine, which passes the blood–brain barrier with difficulty, did not induce yawning. Scopolamine, a centrally acting muscarinic receptor antagonist, inhibited yawning induced by cholinergic activation, but the peripheral muscarinic antagonist, methylscopolamine, did not (Urba-Holmgren et al., 1977; Yamada and Furukawa, 1980). These findings suggest that the yawning is induced by central cholinergic activation.

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Thus, the elicitation of yawning by cholinesterase inhibitors can be used as a useful index for their action within the central nervous system.

Cholinesterase inhibitors cause a variety of peripheral cholinergic effects. Fasciculation is characterized by a fine involuntary movement of muscles and is a consistent measurement among peripheral cholinergic signs (Taylor, 1996; Kosasa et al., 1999a). Facilitation of cholinergic transmission at the neuromuscular junction by cholinesterase inhibitors leads to fasciculation (Sprouse and Baker, 1985).

We now demonstrated the tight relationship between induction of fasciculation by intravenous administration of cholinesterase inhibitors and in vitro inhibitory activity on acetylcholinesterase. It was suggested that fasciculation is a reliable index of peripheral cholinergic activation by cholinesterase inhibitors. In addition, we compared the potencies of several cholinesterase inhibitors to activate central and peripheral cholinergic system by concurrent monitoring of yawning and fasciculation. This experimental design allows for a clear assessment of the central and peripheral profiles of cholinesterase inhibitors. Furthermore, the involvement of cholinergic and dopaminergic mechanisms in yawning induced by E2030, (3-(2-(1-(1,3-dioxolan-2-ylmethyl)-4-piperidyl)ethyl)-2 *H*-3,4-dihydro-1,3-benzoxazin-2,4-dione hydrochloride), a novel centrally acting cholinesterase inhibitor, was investigated.

2. Materials and methods

2.1. Subjects

Male Wistar rats (6-weeks old, Charles River , Japan), weighing 160 to 220 g, served as experimental animals. They were housed in groups of five or six per cage maintained at 24 ± 2°C on a 12-h light/12-h dark cycle. Food and water were provided ad libitum.

2.2. Compounds

E2030, donepezil hydrochloride, TAK-147 (3-[1-(phenylmethyl)-4-piperidinyl]-1-(2,3,4,5-tetrahydro-1 *H*-benzazepin-8-yl)-1-propanone fumarate), tacrine hydrochloride, ipidacrine (9-amino-2,3,5,6,7,8-hexahydro-1 *H*-cyclopenta[b] quinoline hydrochloride hydrate) (all compounds synthesized by Eisai), physostigmine (eserine, Sigma, MO, USA) and distigmine (Ubretid, Torii Pharm, Tokyo, Japan) were all dissolved in sterile distilled water. Scopolamine hydrobromide (Wako, Osaka, Japan) and methylscopolamine (scopolamine *N*-methylbromide, Sigma) were dissolved in 0.9% saline. Doses are expressed in terms of the salts. Haloperidol (injectable Serenace, Dai-Nippon, Tokyo, Japan) was diluted in distilled water. Animals treated with physiological saline in the same way as with test compounds served as controls.

2.3. Behavioral test procedures

In the study of relationship between fasciculation and cholinesterase inhibition, test compounds were administered intravenously and the occurrence of fasciculation was observed for 15 min immediately after administration. The experimenter observed closely any fine muscular movement of the digits of the four limbs while the trunk of the animal was being held. A common ratio of doses tested was the square root of two. Five to six rats were used per group.

In the comparative study of cholinesterase inhibitors, the animals were put individually in small plastic observation cages ($20 \times 20 \times 15$ cm). At least 2 h later, cholinesterase inhibitors were administered orally and, 30 min after drug administration, the number of yawns was counted during a 30-min period. Yawning is characterized by a wide and momentary opening of the mouth (Urba-Holmgren et al., 1977; Ushijima et al., 1984). Immediately after the 30-min session, fasciculation was monitored. The

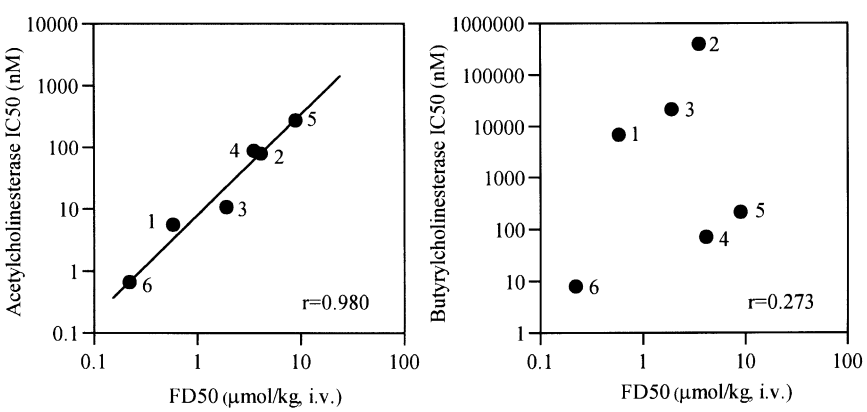


Fig. 1. Relationship between fasciculation and inhibition of acetylcholinesterase. IC_{50} values for acetylcholinesterase and butyrylcholinesterase inhibition used here, which were quoted from our previous study (Ogura et al., 2000b), were as follows: (1) donepezil (5.7 nM for acetylcholinesterase, 7100 nM for butyrylcholinesterase), (2) E2030 (90 nM, 410 000 nM), (3) TAK-147 (11 nM, 22 000 nM), (4) tacrine (81 nM, 73 nM), (5) ipidacrine (280 nM, 220 nM), and (6) physostigmine (0.68 nM, 8.1 nM).

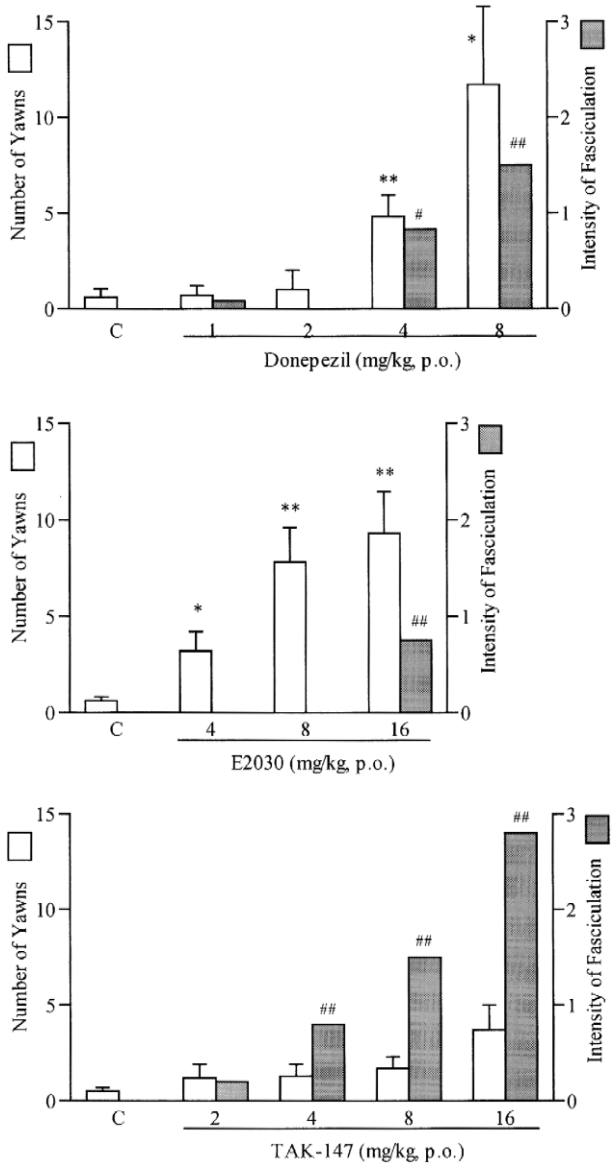


Fig. 2. Induction of yawning and fasciculation after treatment with piperidine-based cholinesterase inhibitors, donepezil, E2030, and TAK-147 in rats. *, **: $p < 0.05$, 0.01 (*t*-test), #, ##: $p < 0.05$, 0.05 (Mann–Whitney *U*-test).

intensity of fasciculation was classified into four grades as follows: 0: none. 1: mild and intermittent fasciculation on some of the digits. 2: moderate fasciculation on limbs and digits. 3: severe fasciculation observed all over the body. When it was difficult to attribute a specific grade, an intermediate rating was given.

Scopolamine or methylscopolamine was administered intraperitoneally 15 min after the administration of E2030, and yawning was observed 15 min later for 30 min as described above. Haloperidol was administered 30 min prior to the administration of donepezil or E2030, and yawning was observed 30 min later for 30 min. Fasciculation was scored at the end of each yawning observation period as above.

All behavioral experiments were carried out under blind conditions. All animal studies described herein were reviewed and approved by the ethical committee in our laboratories and were performed following institutional guidelines concerning the care and handling of experimental animals.

2.4. Data analysis

FD_{50} value (dose estimated to cause fasciculation in 50% of animals) was calculated by the probit method. The number of yawns was analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. Fasciculation scores were analyzed with a two-tailed Mann–Whitney *U*-test.

3. Results

3.1. Fasciculation, a peripheral cholinergic sign

The doses of drugs required to cause fasciculation after i.v. administration in 50% of rats were as follows:

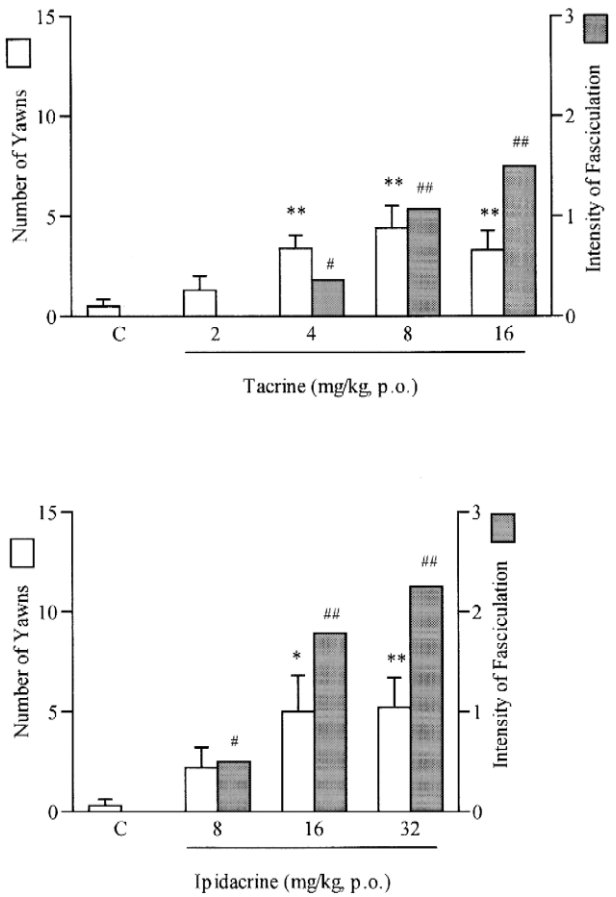
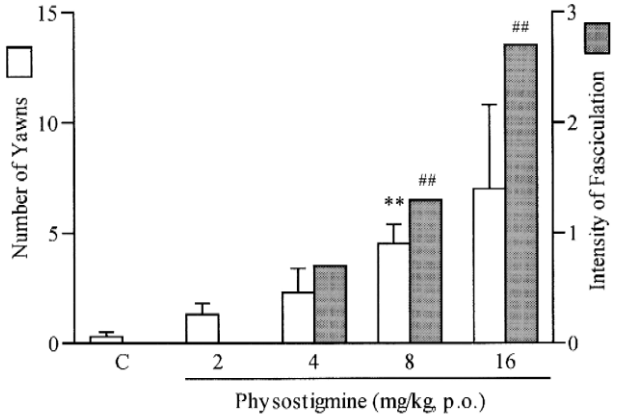


Fig. 3. Induction of yawning and fasciculation after treatment with tricyclic cholinesterase inhibitors, tacrine, and ipidacrine in rats. *, **: $p < 0.05$, 0.01 (*t*-test), #, ##: $p < 0.05$, 0.05 (Mann–Whitney *U*-test).



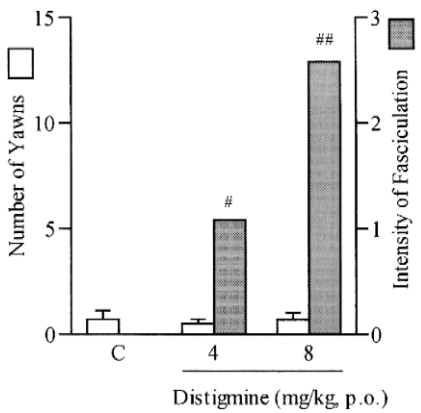


Fig. 4. Induction of yawning and fasciculation after treatment with carbamate cholinesterase inhibitors, physostigmine, and distigmine in rats. *, **: $p < 0.05$, 0.01 (*t*-test), #, ##: $p < 0.05$, 0.05 (Mann–Whitney *U*-test).

donepezil: 0.58 μmol/kg, E2030: 3.5 μmol/kg, TAK-147: 1.9 μmol/kg, tacrine: 4.1 μmol/kg, ipidacrine: 8.9 μmol/kg, and physostigmine: 0.22 μmol/kg. There was a 3–10-s delay in the appearance of fasciculation after physostigmine administration; all other compounds caused fasciculation immediately after administration. The IC_{50} values for acetylcholinesterase and butyrylcholinesterase inhibition for each compound were taken from our previous study (Ogura et al., 2000b).

Fig. 1 shows the relationship between potency of cholinesterase inhibition and FD_{50}. The correlation coefficient between acetylcholinesterase inhibition and FD_{50} was investigated and a tight relationship between indices was revealed ($r = 0.980$, $P < 0.02$). In contrast, there was no correlation between FD_{50} values and butyrylcholinesterase inhibition.

3.2. Cholinesterase inhibitors induced yawning

Donepezil elicited yawning ($F(4,26) = 6.17$, $P = 0.0012$, 4 mg/kg: $t = 3.72$, $P = 0.0034$, 8 mg/kg: $t = 2.93$, $P = 0.014$) and fasciculation significantly at doses of more than 4 mg/kg (Fig. 2). E2030 increased the number of yawns significantly at doses greater than 4 mg/kg ($F(3,21) = 8.29$, $P = 0.00079$, 4 mg/kg: $t = 2.71$, $P = 0.020$, 8 mg/kg: $t = 4.36$, $P = 0.0011$, 16 mg/kg: $t = 4.40$, $P = 0.0011$) and mild fasciculation was elicited only after treatment with the highest dose, 16 mg/kg (Fig. 2). TAK-147 significantly induced fasciculation at doses of more than 4 mg/kg (Fig. 2). Yawning was not significantly elicited by TAK-147 ($F(4,25) = 2.49$, $P = 0.069$), although the highest dose, 16 mg/kg, caused 3.7 ± 1.3 counts of yawns with severe fasciculation. Tacrine elicited yawning at doses greater than 4 mg/kg ($F(4,25) = 3.49$,

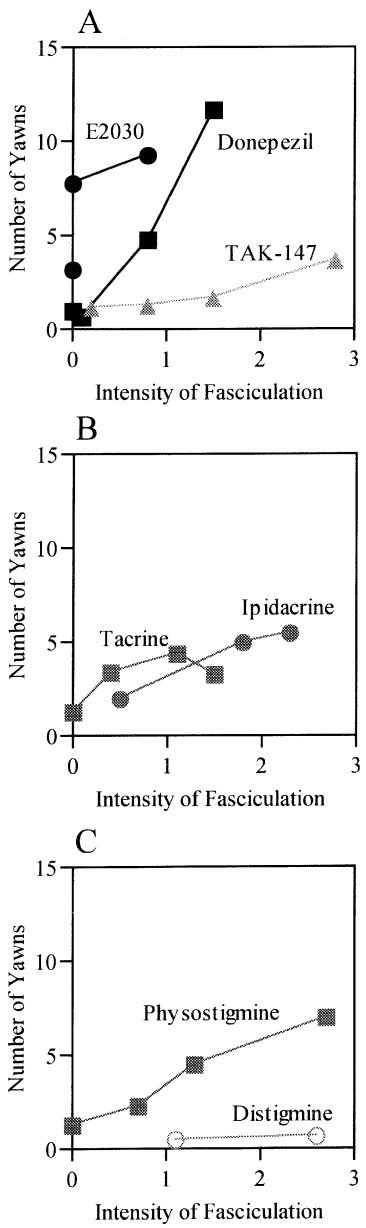


Fig. 5. Relation between elicitation in yawning and fasciculation in rats after administration of cholinesterase inhibitors. (A) Donepezil, E2030, and TAK-147, (B) tacrine and ipidacrine and (C) physostigmine and distigmine.

$P = 0.021$, 4 mg/kg: $t = 3.79$, $P = 0.0030$, 8 mg/kg: $t = 3.15$, $P = 0.0092$, 16 mg/kg: $t = 3.13$, $P = 0.0097$) (Fig. 3). The dose–response plot for tacrine-induced yawning showed a plateau effect, while tacrine induced fasciculation dose dependently at doses of more than 4 mg/kg. Ipidacrine elicited yawning at doses greater than 16 mg/kg ($F(3,22) = 3.24$, $P = 0.042$, 16 mg/kg: $t = 2.2$, $P = 0.047$, 32 mg/kg: $t = 4.0$, $P = 0.0018$), and also fasciculation at doses higher than 8 mg/kg (Fig. 3). Physostigmine elicited yawning at the 8 mg/kg dose ($F(3,20) = 5.81$, $P = 0.0050$, 8 mg/kg: $t = 4.58$, $P = 0.0010$) but the effect of the highest dose, 16 mg/kg, was not significant because of a wide variation in responses (Fig. 4). Fasciculation was elicited by physostigmine at doses greater than 8 mg/kg. Distigmine did not cause yawning at 4 and 8 mg/kg ($F(2,15) = 0.082$, $P = 0.92$), although strong fasciculation was evoked at these doses (Fig. 4).

Elicitation of yawning and fasciculation is plotted in Fig. 5. In this analysis, the closer a plotted curve of a compound is to the vertical line, the greater its ability to preferentially activate central cholinergic transmission. The response curves for donepezil and E2030 were close to the vertical axis, suggesting a preferentially central site of action. The curves for tacrine, ipidacrine, and physostigmine, found at the center of the figure, also suggest a predominantly central cholinergic activity. In contrast, TAK-147 and distigmine produced response curves close to the horizontal line, meaning that they mainly act on peripheral, rather than central, cholinergic systems.

3.3. Effects of scopolamine and haloperidol on E2030-induced yawning

Fig. 6 shows the effects of muscarinic receptor antagonists on E2030-induced yawning. E2030 elicited yawning dose dependently at doses of 8 and 16 mg/kg, while it did not induce fasciculation significantly. Scopolamine, a muscarinic receptor antagonist, completely abolished E2030-induced yawning (8 mg/kg: $t = 3.75$, $P = 0.0038$, 16 mg/kg: $t = 3.24$, $P = 0.0089$). Methylscopolamine, a peripherally acting muscarinic receptor antagonist, failed to block yawning induced after administration of 16 mg/kg of E2030. The effects of haloperidol, a dopamine receptor antagonist, on donepezil- and E2030-induced yawning were

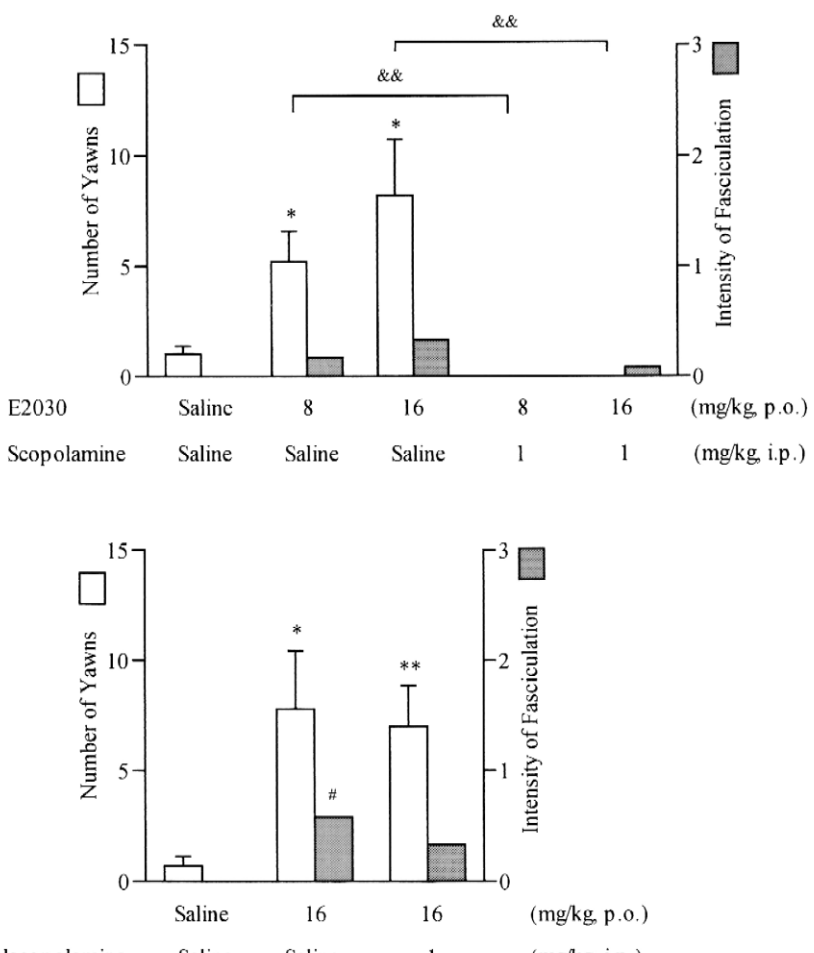


Fig. 6. Effects of scopolamine and methylscopolamine on E2030-induced yawning in rats. *, **, &&: $p < 0.05$, 0.01, 0.01 (*t*-test), #, ##: $p < 0.05$, 0.01 (Mann–Whitney *U*-test).

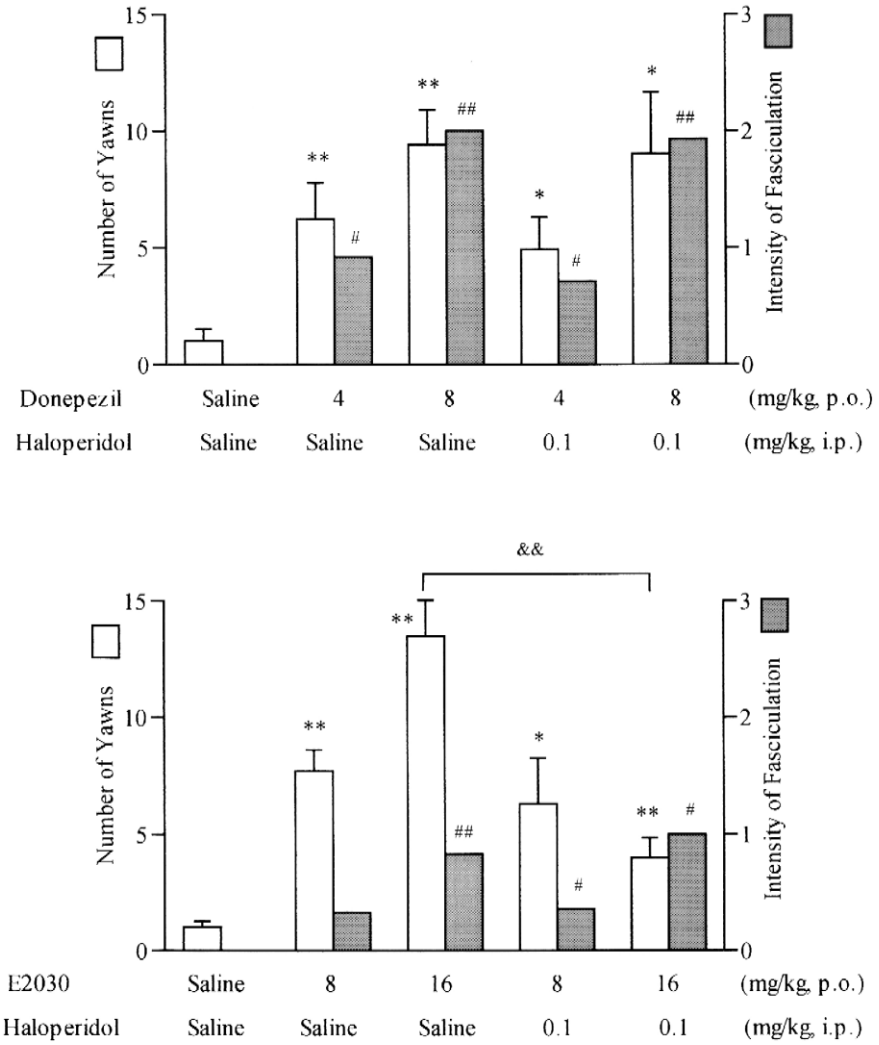


Fig. 7. Effects of haloperidol on donepezil- and E2030-induced yawning in rats. *, **, &&: $p < 0.05$, 0.01, 0.01 (t-test), #, ##: $p < 0.05$, 0.01 (Mann–Whitney U-test).

also investigated (Fig. 7). The ability of donepezil to elicit yawning dose dependently was confirmed. Haloperidol did not inhibit donepezil-induced yawning. E2030 induced yawning significantly. Haloperidol inhibited yawning induced by 16 mg/kg of E2030 ($t = 5.62$, $P = 0.00016$), but not yawning induced by 8 mg/kg. Fasciculation produced by either donepezil or E2030 was not affected by haloperidol treatment.

4. Discussion

In this study, the proportion of central versus peripheral cholinergic activity of a number of cholinesterase inhibitors was clarified. E2030 and donepezil were found to potently and preferentially activate the central cholinergic system, as indicated by a marked induction of yawning in rats, while having relatively little effect on the intensity of fasciculation, a peripheral cholinergic marker.

Fasciculation is observed when cholinergic transmission at the neuromuscular junction is prompted (Sprouse and Baker, 1985; Taylor, 1996). The subtype of acetylcholine receptors in muscles is nicotinic, and it is thus not surprising that scopolamine did not inhibit the fasciculation induced by donepezil or E2030, as shown here, or by physostigmine and distigmine (data not shown). In this study, fasciculation produced by intravenous cholinesterase inhibitors was tightly correlated with the in vitro activity of acetylcholinesterase inhibition, but not of butyrylcholinesterase inhibition. This suggests that the occurrence of fasciculation depends on the in vitro activity of cholinesterase inhibitors, as well as their plasma concentrations, and quite independently of the brain permeability of each compound. This evidence supports the use of fasciculation as a good marker of peripheral cholinergic activation. Furthermore, it suggests that acetylcholinesterase, but not butyrylcholinesterase, is involved in cholinergic transmission at the neuromuscular junction.

Results of this study further support the idea that yawning elicited by cholinesterase inhibitors is a result of central cholinergic activation. For example, among several cholinesterase inhibitors tested, distigmine, which is a peripherally acting cholinesterase inhibitor (Cameron, 1966; Kikuchi et al., 1971), did not elicit yawning. This observation was consistent with results of an early study (Urba-Holmgren et al., 1977) showing that the peripherally acting cholinesterase inhibitor, neostigmine, did not cause yawning. Moreover, the centrally acting muscarinic receptor antagonist, scopolamine, completely blocked E2030-induced yawning, while the peripherally acting muscarinic receptor antagonist, methylscopolamine, had no effect on yawning. Similar results were obtained in the case of other cholinesterase inhibitors as well as muscarinic agonists (Urba-Holmgren et al., 1977; Yamada and Furukawa, 1980; Kimura et al., 1992). It is not clear which brain sites are involved in yawning induced by cholinergic activation. Oxytocin was found to induce yawning when injected into the hippocampus (Melis et al., 1992). Wood et al. (1978, 1979) showed that yawning could be induced by neuropeptides like α-MSH (α-melanocyte stimulating hormone) or ACTH (adrenocorticotropic hormone), and this was associated with an increase in hippocampal acetylcholine turnover rate. Some cholinesterase inhibitors used in the present study have also been shown to enhance the basal concentration of extracellular acetylcholine in the hippocampus of rats (Kawashima et al., 1994; Kosasa et al., 1999b). Therefore, it is possible that the hippocampus is an important site involved in yawning induced by cholinomimetics.

Putative centrally acting cholinesterase inhibitors were found to elicit yawning, suggesting that the study design we used here meets the criterion of face validity to assess central cholinergic activity. When comparing the effective doses to elicit yawning and fasciculation, E2030 was the only compound that elicited yawning at a dose (4 mg/kg), which was less than the dose (16 mg/kg) necessary to induce fasciculation. Donepezil, tacrine, and physostigmine elicited both yawning and fasciculation at the same doses. Ipidacrine elicited fasciculation more effectively than yawning (i.e., half the dose). We found, however, that TAK-147, which is currently in clinical trials for Alzheimer's disease in Japan (Miyamoto et al., 1996), was not very effective to cause yawning. We previously reported on the effects of donepezil, tacrine and TAK-147 on extracellular acetylcholine concentrations in the cerebral cortex of rats (Kosasa et al., 1999a). The rank order comparing the values of the ratio of the minimum effective dose for acetylcholine-increasing action to that for the fasciculation-producing action was: donepezil > tacrine > TAK-147. These results correspond well with the results of the present study.

Analysis of central and peripheral balance classified cholinesterase inhibitors approximately into three categories. Donepezil and E2030 are in the first group, and show a strong preference toward central cholinergic system activation. Compounds like tacrine, ipidacrine, and physostigmine are in the second category and have an equipotent degree of central and peripheral action. Distigmine can be classified in a third category of peripherally acting cholinesterase inhibitors. In this context, TAK-147 may lie in the middle of the second and third categories. The central and peripheral balance for each compound depends mainly on brain permeability and partly on selectivity for acetylcholinesterase. We previously showed that donepezil was more potent than tacrine to improve learning impairments in some hypocholinergic models using rats (Ogura et al., 2000a). This finding, in general, is consistent with the present results.

It is well known that dopaminergic systems also are involved in the induction of yawning (Ushijima et al., 1988; Zarrindast and Poursoltan, 1989; Furukawa, 1996; Argiolas and Melis, 1998). Scopolamine blocks yawning induced by both cholinomimetics and dopamine receptor agonists such as apomorphine, while haloperidol, a dopamine receptor antagonist, inhibits yawning induced by dopamine receptor agonists, but not cholinomimetics such as physostigmine, tacrine, ipidacrine, and pilocarpine (Ushijima et al., 1984; Ushijima et al., 1985). In this study, we confirmed that E2030-induced yawning was blocked by scopolamine. Interestingly, haloperidol, which did not affect donepezil-induced yawning, partially inhibited E2030-induced yawning. It is not apparent why haloperidol inhibited yawning only after the highest dose of E2030, but only high doses of E2030 may produce additional dopamine-mediated effects on yawning, which can be blocked by haloperidol. These results suggest that the dopaminergic system might be partially involved in E2030-induced yawning, and that the underlying mechanism is different from that of donepezil.

This study sheds light on the central and peripheral balance of action of cholinesterase inhibitors. Among cholinesterase inhibitors tested, donepezil and E2030 have a superior ability to enhance central cholinergic transmission preferentially. E2030 may have a suitable balance of central cholinergic action for symptomatic therapy in the treatment of Alzheimer's disease as well as donepezil, which is prescribed worldwide.

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