ELSEVIER

European Journal of Pharmacology 408 (2000) 19–23

ejp

www.elsevier.nl/locate/ejphar

Involvement of 5-hydroxytryptamine$_{1A}$ receptors in nicotine-induced tail tremor in rats

Katsuya Suemaru, Hiroaki Araki *, Yutaka Gomita

Department of Hospital Pharmacy, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

Received 30 March 2000; received in revised form 30 August 2000; accepted 31 August 2000

Abstract

Involvement of the serotonergic system in tail tremor induced by repeated administration of nicotine was investigated in rats. Tail tremor induced by nicotine (0.5 mg/kg, s.c.) was suppressed by a 5-HT$_{1A}$ receptor antagonist, *N*-{2-[4-(2-methoxyphenyl)-1-piperazinyl-]ethyl}-*N*-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride (WAY-100635; 0.3–3 mg/kg, i.p.), but not by a 5-HT$_2$ receptor antagonist, ketanserin (0.1–0.3 mg/kg, i.p). The 5-HT$_{1A}$ receptor agonists, buspirone (1–20 mg/kg, i.p.), gepirone (1–10 mg/kg, i.p.), tandospirone (1–10 mg/kg, i.p.) and (±)-8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT; 0.01–0.1 mg/kg, s.c.), enhanced the tail tremor. The enhancement of tail tremor by buspirone (10 mg/kg, i.p.) was blocked by WAY-100635 (0.3–3 mg/kg, i.p.). These findings suggest that nicotine-induced tail tremor is mediated by 5-HT$_{1A}$ receptors and that 5-HT$_{1A}$ receptor antagonists are effective in the treatment of tremor.

Keywords: Nicotine; Tail tremor; 5-HT$_{1A}$ receptor; (Rat)

1. Introduction

The central serotonergic system is implicated in various types of human movement disorders such as myoclonus, hyperreflexia, shivering and tremor (Sternbach, 1991). Clinically, β-adrenoceptor antagonists such as propranolol, which has an antagonistic action to both β-adrenoceptors and 5-HT$_{1A}$ receptors (Weinstock et al., 1977), are effective in the treatment of essential tremor (Hallet, 1991; Ogawa et al., 1987), lithium-induced tremor (Zubenko et al., 1984) and alcohol withdrawal-induced tremor (Zilm et al., 1975). In behavioral animal studies, β-adrenoceptor antagonists have been shown to reduce the tremors evoked by stimulation of the central cholinergic (Alkondon et al., 1985; Iwata et al., 1993) and serotonergic systems (Hallberg, 1986) in rodents. However, it is not clear whether 5-HT$_{1A}$ receptors are involved in the mechanisms of the anti-tremorogenic effect of β-adrenoceptor antagonists.

We previously reported that repeated exposure to cigarette smoke or nicotine causes a tremor only in the tail (tail tremor) of rats (Gomita et al., 1988, 1991). This tremor is accompanied by locomotor hyperactivity and not by rigidity or immobility of the whole body (Suemaru et al., 1994), suggesting that the nicotine-induced tail tremor model might be useful for study of tremors associated with movement. The nicotine-induced tail tremor is suppressed by the β-adrenoceptor antagonists, propranolol and pindolol (Suemaru et al., 1993, 1997). However, the involvement of the serotonergic system in this phenomenon remains unknown. Thus, in the present study, we investigated the effects of 5-HT$_{1A}$ receptor agonists or antagonists on nicotine-induced tail tremor in rats.

2. Materials and methods

2.1. Animals

Male Wistar rats (Charles River Lab., Atsugi, Japan) weighing 180–230 g were housed in groups of three or four per plastic cage (26 × 36 × 25 cm) in a room kept at

* Corresponding author. Tel.: +81-86-235-7641; fax: +81-86-235-7796.

E-mail address: haraki@hospital.okayama-u.ac.jp (H. Araki).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00685-3

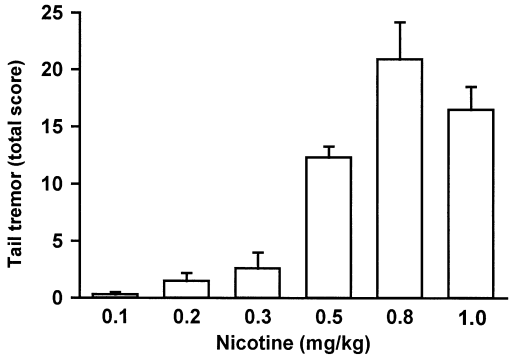

Fig. 1. Dose–response for nicotine on tail tremor in rats. Various doses of nicotine were subcutaneously administered to rats pretreated with nicotine (0.5 mg/kg/day, s.c.) for 10 days. Each column represents the mean total score of tail tremor for 15 min with S.E.M. for five rats.

22 ± 2°C with an alternating 12-h light/dark cycle (lights on at 6:00 AM). Food and water were given ad libitum.

2.2. Observation of tail tremor

Tail tremor begins 3 min after nicotine administration and reaches a peak at approximately 7–9 min after the administration. The tail tremor disappears about 15 min after nicotine administration (Gomita et al., 1988). Nicotine-induced tail tremor was observed in individual cages (20 × 15 × 15 cm) for 15 min immediately after the subcutaneous administration of nicotine, as described previously (Gomita et al., 1988). The degree of tail tremor was scored once every minute as follows: no tremor = 0; occasional slight tremor = 1; moderate intermittent tremor = 2; gross tremor but with occasional quiescent periods = 3 and gross intense continuous tremor = 4. All observations were made by one observer who was unaware of the treatment schedule. Tremor intensity was expressed as the total sum of the score per min for 15 min.

2.3. Experimental procedure

The intensity of the nicotine-induced tail tremor is increased by daily administration of nicotine (Suemaru et al., 1997). In the present study, the rats were given nicotine (0.5 mg/kg, s.c.) once daily for 10 days, and 5-HT receptor agonists and antagonists were administered subcutaneously or intraperitoneally only once, 30 min before the 10th nicotine administration.

2.4. Drugs

(−)-Nicotine tartrate (Sigma, St. Louis, MO, USA), buspirone hydrochloride (Bristol–Myers Research Institute, Tokyo, Japan), 1-(2-pyrimidinyl)-piperazine dihydrochloride (1-PP, Bristol–Myers), gepirone hydrochloride (Bristol–Myers), tandospirone (Sumitomo Pharmaceutical, Osaka, Japan), (±)-8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT, Sigma), clomipramine hydrochloride (CIBA-GEIGY Pharmaceutical, Basel, Switzerland) and *N*-{2-[4-(2-methoxyphenyl)-1-piperazinyl-]ethyl}-*N*-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride (WAY-100635, Taishou Pharmaceutical, Tokyo, Japan) were dissolved in 0.9% saline. 1-(2-methoyphenyl)-4-[4-(2-phthalimido) butyl]piperazine hydrobromide (NAN-190, Sigma) was suspended in 0.5% sodium carboxymethylcellulose. All drugs were injected in a volume of 0.1 ml/100 g body weight.

2.5. Statistical analysis

Statistical significance was evaluated by Kruskal–Wallis analysis of variance followed by the Mann–Whitney *U*-test. Probability values less than 0.05 were considered significant.

3. Results

The subcutaneous administration of nicotine at doses of 0.1–0.8 mg/kg to rats pretreated with nicotine (0.5 mg/kg/day, s.c.) for 10 days caused a dose-dependent increase in tail tremor with the maximal effect occurring at

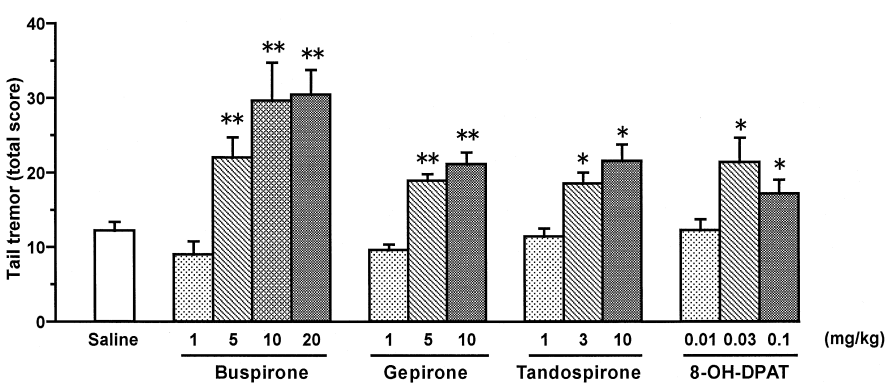

Fig. 2. Effects of 5-HT_{1A} receptor agonists on nicotine-induced tail tremor in rats. Buspirone, gepirone, tandospirone, 8-OH-DPAT and saline were administered 30 min before the 10th nicotine (0.5 mg/kg, s.c.) treatment. Each column represents the mean total score of tail tremor for 15 min with S.E.M. ($n = 6$ for each). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with saline.

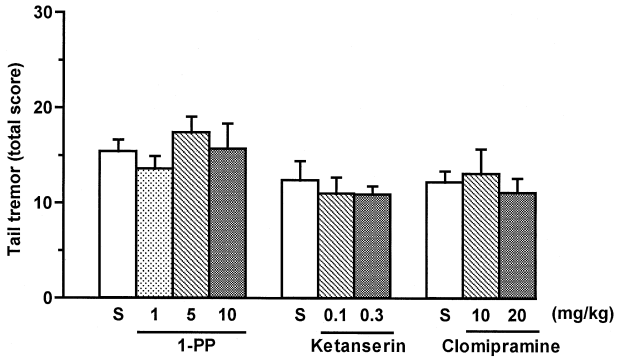

Fig. 3. Effects of 1-PP, ketanserin and clomipramine on nicotine-induced tail tremor in rats. 1-PP, ketanserin, clomipramine and saline were administered intraperitoneally 30 min before the 10th nicotine (0.5 mg/kg, s.c.) treatment. Each column represents the mean total score of tail tremor for 15 min with S.E.M. ($n = 6$ for each).

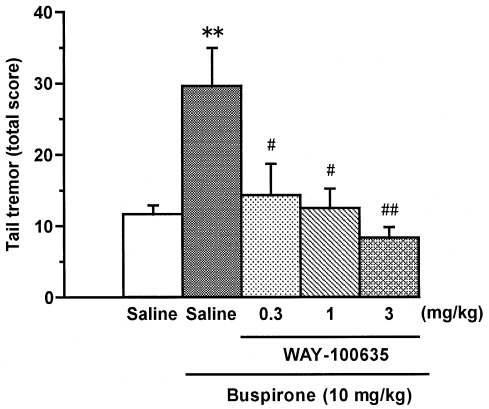

Fig. 5. Effect of WAY-100635 on nicotine-induced tail tremor in buspirone-treated rats. WAY-100635 and saline were administered intraperitoneally 40 min before the 10th nicotine (0.5 mg/kg, s.c.) treatment. Buspirone (10 mg/kg) was intraperitoneally injected 10 min after administration of WAY-100635 or saline. Each column represents the mean total score of tail tremor for 15 min with S.E.M. ($n = 5–6$ for each). $^{**}P < 0.05$ compared with saline. $\#P < 0.05$, $\#\#P < 0.01$ compared with saline plus buspirone.

a dose of 0.8 mg/kg (Fig. 1). The tail tremor induced by nicotine (0.5 mg/kg, s.c.) was dose-dependently enhanced by 5-HT_{1A} receptor agonists, buspirone (1–20 mg/kg, i.p.), gepirone (1–10 mg/kg, i.p.), tandospirone (1–10 mg/kg, i.p.) and 8-OH-DPAT (0.01–0.1 mg/kg, s.c.) (Fig. 2). Neither these 5-HT_{1A} receptor agonists nor saline caused tail tremor. Furthermore, the tail tremor was unaffected by the buspirone metabolite, 1-PP (1–10 mg/kg, i.p.), the 5-HT_2 receptor antagonist, ketanserin (0.1 and 0.3 mg/kg, i.p.), and 5-HT uptake inhibitor, clomipramine (10 and 20 mg/kg, i.p.) (Fig. 3).

Figs. 4 and 5 show the effects of 5-HT_{1A} receptor antagonists, WAY-100635 and NAN-190. WAY-100635 (0.3–3 mg/kg, i.p.) dose-dependently suppressed nicotine-induced tail tremor, however, NAN-190 (1 and 4 mg/kg, i.p.) did not have any effect on the tail tremor. The enhancement of tail tremor by nicotine plus buspirone (10 mg/kg, i.p.) was suppressed by pretreatment with WAY-100635 (0.3–3 mg/kg, i.p.), and there was a significant difference ($P < 0.05$) at a dose of 0.3 mg/kg, which had no effect on nicotine-induced tail tremor.

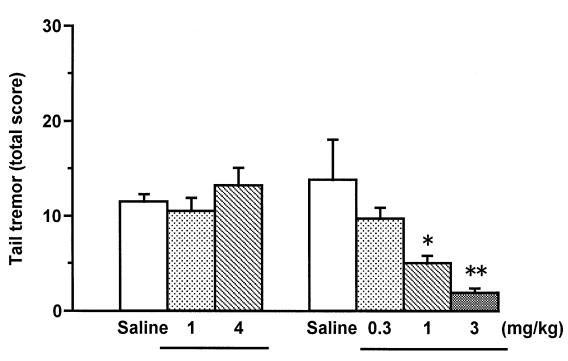

Fig. 4. Effects of 5-HT_{1A} receptor antagonists on nicotine-induced tail tremor in rats. WAY-100635, NAN-190 and saline were administered intraperitoneally 30 min before the 10th nicotine (0.5 mg/kg, s.c.) treatment. Each column represents the mean total score of tail tremor for 15 min with S.E.M. ($n = 6$ for each). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with saline.

4. Discussion

Systemic administration of nicotine to rodents produced whole body tremors. With higher doses, these tremors were followed by convulsions (Silvette et al., 1962). Repeated administration of nicotine at small doses does not produce whole body tremors or convulsions, but causes a tail tremor (Suemaru et al., 1994). Central nicotinic receptors and β-adrenoceptors (Suemaru et al., 1993, 1997) are involved in the mechanisms responsible for the tail tremor. Previous studies have shown that depletion of 5-HT by *para*-chlorophenylalanine reduces the tremor induced by nicotine in pilocarpine-treated rats (Dohi and Tsujimoto, 1978), suggesting the involvement of serotonergic mechanisms in the tremorogenic actions of nicotine. In addition, it has been reported that nicotine accelerates 5-HT release in the rat brain (Li et al., 1998; Yu and Wecker, 1994), and that 5-HT_{1A} receptor agonists, 8-OH-DPAT and buspirone, reduce the antinociceptive effects of nicotine in mice (Damaj et al., 1994). In the present study, the 5-HT_{1A} receptor agonists, buspirone, gepirone, tandospirone and 8-OH-DPAT, enhanced the nicotine-induced tail tremor, and a 5-HT_{1A} receptor antagonist, WAY-100635, suppressed the tremor. However, 5-HT_2 receptor antagonist, ketanserin, and 5-HT reuptake inhibitor, clomipramine, had no effect. These results indicate that nicotine-induced tail tremor is mediated by the 5-HT_{1A} receptor. However, a direct interaction with nicotine receptors may not be involved during the enhancing effect by 5-HT_{1A} receptor

agonists, since the scores for tail tremor induced by nicotine (0.5 mg/kg) plus buspirone (10 and 20 mg/kg) were markedly higher compared with the maximal effect of nicotine (0.8 mg/kg). In addition, binding studies have shown that buspirone and 8-OH-DPAT have no affinity at central nicotinic receptors (Damaj et al., 1994). Previous findings and those of the present study indicate that nicotine-induced tail tremor is facilitated via 5-HT_{1A} receptors. Therefore, 5-HT_{1A} receptors may be involved in the mechanisms of the anti-tremorogenic effect of β-adrenoceptors such as propranolol and pindolol.

1-PP is the major common metabolite of buspirone, gepirone and tandospirone and appears rapidly in the plasma and brain after administration of these drugs (Caccia et al., 1982). The metabolite, which has negligible affinity to the 5-HT_{1A} receptor (Hoyer, 1988), has been reported to have a potent α_2-adrenoceptor antagonistic effect and to contribute to the pharmacological effects of buspirone (Bianchi et al., 1988; Bianchi and Garattini, 1988). However, 1-PP did not affect the tail tremor induced by nicotine in the present study.

Previous binding studies indicated that buspirone, gepirone and tandospirone bound to 5-HT_{1A} receptors with high affinities and also to dopamine D_2 receptors (Piercey et al., 1994). The action of dopamine receptors has been reported to be related to some behavioral actions of buspirone (McMillen and McDonald, 1983). However, it appears that a dopaminergic antagonistic action is not involved in the enhancing mechanisms of these drugs on the tail tremor induced by nicotine, since a dopamine receptor antagonist, haloperidol, suppresses nicotine-induced tail tremor (Suemaru et al., 1994). This concept is further supported by the finding that a selective 5-HT_{1A} receptor agonist, 8-OH-DPAT, enhanced the nicotine-induced tail tremor.

The 5-HT_{1A} receptors are situated both post-synaptically to serotonergic neurons and pre-synaptically as inhibitory autoreceptors on dendrites of raphe-localized serotonergic perikarya (Saxena, 1995). Although NAN-190 displays antagonist properties only in post-synaptic 5-HT_{1A} receptors, it produces agonist-like responses at pre-synaptic somatodendritic 5-HT_{1A} receptors, thereby inhibiting raphe neuronal firing (Gobert et al., 1995) and attenuating 5-HT release at axon terminals (Hjorth and Sharp, 1990). In contrast, WAY-100635, which is a potent and selective 5-HT_{1A} receptor antagonist at both the pre- and post-synaptic level, increases the firing rate of raphe nuclei cells (Fletcher et al., 1996; Forster et al., 1995). In the present study, nicotine-induced tail tremor was suppressed by WAY-100635 but not by NAN-190. Furthermore, the enhancement of tail tremor by buspirone was blocked by WAY-100635. Together, previous findings and those of the present study suggest that at least pre-synaptic 5-HT_{1A} receptors were involved in the mechanisms underlying the tail tremor induced by nicotine. However, further study is required to provide clear evidence, since post-synaptic 5-TH receptors have been reported to mediate the 5-HT syndrome induced by 5-HT_{1A} receptor agonists in rats (Hjorth and Sharp, 1990) and control 5-HT release in rat brain (Casanovas et al., 1999).

Systemic administration of 5-HT_{1A} receptor agonists causes a 5-HT syndrome consisting of reciprocal forepaw treading, flattened body posture, hindlimb abduction, Straub tail and tremor (Smith and Peroutka, 1986). 8-OH-DPAT elicits this full 5-HT syndrome, whereas buspirone and other azapirones only induce appreciable low body posture and hindlimb abduction (Smith and Peroutka, 1986). None of the 5-HT_{1A} compounds, to our knowledge, has been reported to induce tail tremor.

Previous studies have shown the involvement of the spinal cord in the antinociceptive effect of nicotine in rats (Aceto et al., 1986). Furthermore, results of studies of brain transections (Jacobs and Klemfuss, 1975) and depletion of spinal 5-HT by the intrathecal or intracerebroventricular administration of 5,7-dihydroxytryptamine (Sawynok and Reid, 1990) have suggested that the presumed pathophysiological mechanisms of hindlimb abduction, Straub tail and tremor involve brainstem and/or spinal cord activation of the 5-HT_{1A} receptor. Therefore, it may be that the spinal 5-HT_{1A} receptors are associated with the enhancement of nicotine-induced tail tremor by 5-HT_{1A} receptor agonists.

In conclusion, the findings of the present study demonstrate an involvement of 5-HT_{1A} receptors in the tail tremor induced by nicotine. Moreover, they suggest the possibility that 5-HT_{1A} receptor antagonists could be effective in the treatment of tremor. Further behavioral and neurochemical experiments should be undertaken to examine the role of the 5-HT_{1A} system and its manipulation by drugs in another model.

Acknowledgements

This study was supported, in part, by a grant-in-aid for Drug Abuse Research from the Ministry of Health and Welfare, Japan. The authors thank Taishou Pharmaceutical Company for donating WAY-100635 used in this study.

References

Aceto, M.D., Bagley, R.S., Dewey, W.L., Fu, T.C., Martin, B.R., 1986. The spinal cord as a major site for the antinociceptive action of nicotine in the rat. Neuropharmacology 25, 1031–1036.

Alkondon, M., Ray, A., Sen, P., 1985. Cholinergic involvement in the modulation of oxotremorine-tremor in mice by propranolol. Arch. Int. Pharmacodyn. 277, 161–167.

Bianchi, G., Garattini, S., 1988. Blockade of α_2-adrenoceptors by 1-(2-pyrimidinyl)-piperazine (PmP) in vivo and its relation to the activity of buspirone. Eur. J. Pharmacol. 147, 343–350.

Bianchi, G., Caccia, S., Vedova, F.D., Garattini, S., 1988. The α2-adrenoceptors antagonist activity of ipsapirone and gepirone is mediated by their common metabolite 1-(2-pyrimidinyl)-piperazine (PmP). Eur. J. Pharmacol. 151, 365–371.

Caccia, S., Garattini, S., Mancinell, A., Muglia, M., 1982. Identification and guantiation of 1-(2-pyrimidinyl)-piperazine, an active metabolite of the anxiolytic agent buspirone in rat plasma and brain. J. Chromatogr. 252, 310–314.

Casanovas, J.M., Hervas, I., Artigas, F., 1999. Postsynaptic 5-HT_{1A} receptors control 5-HT release in the rat medial prefrontal cortex. NeuroReport 14, 1441–1445.

Damaj, M.I., Glennon, R.A., Martin, B.R., 1994. Involvement of the serotonergic system in the hypoactive and antinociceptive effects of nicotine in mice. Brain Res. Bull. 33, 199–203.

Dohi, T., Tsujimoto, A., 1978. Possible mechanisms of sustained tremor induced by nicotine in pilocarpine treated animals. Arch. Int. Pharmacodyn. 235, 62–72.

Fletcher, A., Forster, E.A., Bill, D.J., Brown, G., Cliffe, I.A., Hartlet, J.E., Jones, D.E., McLenachan, A., Stanhope, K., Critchley, D.J.P., Childs, K.J., Middlefel, V.C., Lanfumey, L., Corradetti, R., Laporte, A.M., Gozlan, H., Hamon, M., Dourish, C.T., 1996. Electrophysiological, biochemical, neurohormonal and behavioral studies with WAY 100635, a potent, selective and silent 5-HT_{1A} receptor antagonist. Behav. Brain Res. 73, 337–353.

Forster, E.A., Cliffe, I.A., Bill, D.J., Dover, G.M., Jones, D., Reilly, Y., Fletcher, A., 1995. A pharmacological profile of the selective silent 5-HT_{1A} receptor antagonist, WAY-100635. Eur. J. Pharmacol. 281, 81–88.

Gobert, A., Lejeune, F., Rivet, J.M., Audinot, V., Newman-Tancredi, A., Millan, M.J., 1995. Modulation of the activity of central serotonergic neurons by novel serotonin$_{1A}$ receptor agonists and antagonists: a comparison to adrenergic and dopaminergic neurons in rats. J. Pharmacol. Exp. Ther. 273, 1032–1046.

Gomita, Y., Suemaru, K., Furuno, K., Araki, Y., 1988. Nicotine-induced tail tremor and drug effects. Pharmacol., Biochem. Behav. 34, 817–821.

Gomita, Y., Suemaru, K., Furuno, K., Araki, Y., 1991. Tail-tremor induced by exposure to cigarette smoke in rats. Pharmacol., Biochem. Behav. 40, 453–455.

Hallberg, H., 1986. Central β-adrenoceptors can modulate 5-hydroxytryptamine-induced tremor in rats. Br. J. Pharmacol. 87, 403–408.

Hallett, M., 1991. Classification and treatment of tremor. JAMA 226, 1115–1117.

Hjorth, S., Sharp, T., 1990. Mixed agonist/antagonist properties of NAN-190 at 5-HT_{1A} receptors: behavioral and vivo brain microdialysis studies. Life Sci. 46, 955–963.

Hoyer, D., 1988. Functional correlates of 5-HT_1 recognition sites. J. Recept. Res. 8, 59–81.

Iwata, S., Nomoto, M., Fukuda, T., 1993. Effects of β-adrenergic blockers on dug-induced tremors. Pharmacol., Biochem. Behav. 44, 611–613.

Jacobs, B.L., Klemfuss, H., 1975. Brain stem and spinal cord mediation of a serotonergic behavioral syndrome. Brain Res. 100, 450–457.

Li, X., Rainnie, D.G., McCarley, R.W., Greene, R.W., 1998. Presynaptic nicotinic receptors facilitate monoaminergic transmission. J. Neurosci. 18, 1904–1912.

McMillen, B.A., McDonald, C.C., 1983. Selective effects of buspirone and molindone on dopamine metabolism and function in the striatum and frontal cortex of the rat. Neuropharmacology 22, 273–278.

Ogawa, N., Takayama, H., Yamamoto, M., 1987. Comparative studies on the effects of β-adrenergic blokers in essential tremor. J. Neurol. 235, 31–33.

Piercey, M.F., Smith, M.W., Lum-Ragan, J.T., 1994. Excitation of noradrenergic cell firing by 5-hydroxytryptamine$_{1A}$ agonists correlates with dopamine antagonists properties. J. Pharmacol. Exp. Ther. 268, 1297–1303.

Sawynok, J., Reid, A., 1990. Supersensitivity to intrathecal 5-hydroxytryptamine, but not noradrenaline, following depletion of spinal 5-hydroxytryptamine by 5,7-dihydroxytryptamine administered into various sites. Naunyn-Schmiedeberg's Arch. Pharmacol. 342, 1–8.

Saxena, P.R., 1995. Serotonin receptors: subtypes, functional responses and therapeutic relevance. Pharmacol. Ther. 66, 339–368.

Silvette, H., Hoff, E.C., Larson, P., Haag, H.B., 1962. The action of nicotine on central nervous system functions. Pharmacol. Rev. 14, 137–173.

Smith, L.H., Peroutka, S.J., 1986. Differential effects of 5-hydroxytryptamine$_{1A}$ selective drugs on the 5-HT behavioral syndrome. Pharmacol., Biochem. Behav. 24, 1513–1519.

Sternbach, H., 1991. The serotonin syndrome. Am. J. Psychiatry 148, 705–713.

Suemaru, K., Gomita, Y., Furuno, K., Araki, Y., 1993. Effects of β-adrenergic receptor antagonists on nicotine-induced tail tremor in rats. Pharmacol., Biochem. Behav. 46, 131–133.

Suemaru, K., Oishi, Y., Gomita, Y., 1994. Characteristics of tail tremor induced by nicotine in rats. Naunyn-Schmiedeberg's Arch. Pharmacol. 350, 153–157.

Suemaru, K., Kawasaki, H., Oishi, Y., Gomita, Y., Tanizaki, Y., 1997. Role of central nicotinic and β-adrenergic receptors in the onset and further development of tail tremor induced by repeated nicotine administration to rats. Naunyn-Schmiedeberg's Arch. Pharmacol. 355, 571–575.

Weinstock, M., Weiss, C., Gitter, S., 1977. Blockade of 5-hydroxytryptamine receptors in the central nervous system by β-adrenoceptor antagonists. Neuropharmacology 16, 273–276.

Yu, Z.J., Wecker, L., 1994. Chronic nicotine administration differentially affects neurotransmitter release from rat striatal slices. J. Neurochem. 63, 186–194.

Zilm, D.H., Sellers, E.M., MacLeod, S.M., Degani, N., 1975. Propranolol effect on tremor in alcoholic withdrawal. Ann. Intern. Med. 83, 234–236.

Zubenko, G.S., Cohen, B.M., Lipinski, J.F., 1984. Comparison of metoprolol and propranolol in the treatment of lithium tremor. Psychiatry Res. 11, 163–164.