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Long-term nicotine treatment reduces cerebral cortical vasodilation mediated by α4β2-like nicotinic acetylcholine receptors in rats

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ABSTRACT

Regional cortical cerebral blood flow is increased via activation of brain nicotinic acetylcholine receptors. Acute intravenous injection of nicotine increases cortical blood flow, without changing systemic blood pressure in anesthetized rats. Here, we examined whether the nicotine-induced cerebral cortical vasodilation is affected by chronic nicotine treatment. Rats received chronic subcutaneous nicotine (at a low or a high-dose) short-term (1 h) or long-term (14 days). Under urethane anesthesia, blood flow in the frontal cortex, before and after bolus injection of nicotine (0.3–30 μg/kg, i.v.) was measured by laser Doppler flowmetry. The threshold dose of nicotine (3 μg/kg, i.v.) producing vasodilation was not affected by chronic nicotine treatment. However, the vasodilation induced by nicotine at 30 μg/kg was reduced after long-term nicotine treatment (but not after short-term exposure). The degree of reduction was marked and was statistically significant with high-dose (100 μg/kg/h) nicotine; low-dose (33 μg/kg/h) nicotine had a small effect that was not statistically significant. In contrast, the vasodilation in the cortical vessels obtained by hypercapnia (inhalation of 10% CO_2) was not changed by chronic nicotine treatment. The nicotine-induced cortical vasodilation was not influenced by methyllycaconitine, an α7-selective nicotinic antagonist, while it was completely abolished by dihydro-β-erythroidine, an α4β2-preferring nicotinic antagonist. We conclude that long-term nicotine treatment reduces the functional activity of α4β2-like nicotinic receptors that mediate cortical vasodilation.

1. Introduction

Nicotinic acetylcholine receptors in the brain play a crucial role in the vasodilation in the cerebral cortex that is induced by basal forebrain cholinergic activation or nicotine injection (Biesold et al., 1989; Sato and Sato, 1992; Linville et al., 1993; Uchida et al., 1997). For example, intravenous injection of a small dose of nicotine in rats produces an increase in cortical blood flow, without significant changes in systemic blood pressure (Uchida et al., 1997). This effect of nicotine on blood flow is probably due to an activation of nicotinic receptors in the brain tissues; in fact, the response is not influenced by a nicotinic receptor antagonist (hexamethonium) that is not permeable to the blood-brain barrier, while it is abolished by a nicotinic receptor antagonist (mecamylamine) that crosses the blood-brain barrier (Uchida et al., 1997). Furthermore, this cerebral cortical vasodilation mediated by nicotinic receptors is less marked in aged rats (Uchida et al., 1997). The decrease in cortical blood flow response in aged animals is probably due to a down-regulation (decrease in number) of nicotinic receptors in the cortex, as has been observed in humans (Araujo et al., 1990; Nordberg et al., 1992).

In contrast, an up-regulation (increase in number) of nicotinic receptors of the brain, especially in the cerebral cortex, is observed in rodents after chronic exposure to nicotine (Wonnacott, 1990; Sanderson et al., 1993). Similarly, an up-regulation of nicotinic receptors is found in the brain of human smokers by comparison with non-smokers (Benwell et al., 1988; Perry et al., 1999). However, the data concerning functional activity of the increased nicotinic receptors after chronic nicotine treatment are at variance with each other, reporting an increased response, or no change or a decreased response (Hulihan-Giblin, 1990; Marks et al., 1993; Arnold et al., 2003; Nguyen et al., 2004; Grilli et al., 2005; Vann et al., 2006). For example, Grilli et al. (2005) working on synaptosomes from the brain of rats chronically exposed to nicotine, found that nicotine stimulation produces a greater release of noradrenaline but reduces a release of acetylcholine, from hippocampal synaptosomes, while it has no effect on dopamine release from striatal synaptosomes, by comparison with the synaptosomes of untreated rats. It is still unknown what effect a chronic nicotine treatment has on the vasodilative response in the cerebral cortex mediated by nicotinic receptors. Therefore, the present study aimed, first, to clarify whether the nicotine-induced cortical vasodilation is affected by chronic nicotine at a dose known to

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increase the number of nicotinic receptors in the cerebral cortex (Dávila-García et al., 2003; Hernandez and Terry, 2005).

Of the several subtypes of nicotinic receptors, the α4β2 and the α7 subtypes are the most abundant and widespread in the mammalian brain (Picciotto et al., 2001). Recent studies suggest that the changes in functional activity of brain nicotinic receptors after chronic nicotine are different in the different receptor subtypes (Grilli et al., 2005; Besson et al., 2007). The subunit composition of the nicotinic receptors mediating vasodilatation in the cerebral cortex has not yet been established. Therefore, the present study aimed, secondarily, to determine whether either or both the α4β2-like or the α7-subtype is involved in the vasodilative response.

2. Materials and methods

The experiments were performed on 31 male adult Wistar rats (body weight, 285–425 g, 4–7 months old). All animal experiments were conducted according to the Guidelines for Animal Experimentation prepared by the Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology.

The effect of chronic nicotine treatment on the cortical vasodilation mediated by nicotinic receptors was tested in 4 groups of rats; a short-term (1 h) high-dose (125 μg/kg/h) nicotine-treated group ($n=4$), two long-term (14 days) nicotine-treated groups either low-dose (33 μg/kg/h, $n=6$) or high-dose (100 μg/kg/h, $n=6$), and a long-term (14 days) saline-treated control group ($n=6$). The cortical vasodilation mediated by nicotinic receptors was assessed via the cortical blood flow response to bolus i.v. nicotine (0.3–30 μg/kg). The cortical vasodilation that is not mediated by nicotinic receptors was also tested in a part of rats ($n=4$ rats for each group) via the cortical blood flow response to hypercapnia (10% CO_2 inhalation).

2.1. Long-term nicotine treatment

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and a minipump (Alzet, model #2002, 0.5 μl/h) containing either (−) nicotine (Tokyo Kasei Kogyo, Japan) (low-dose: 4.6–5.9 mg/200 μl or high-dose: 12–18 mg/200 μl, calculated as the free base) or saline was inserted into a subcutaneous pocket via a small incision made over the shoulders. The wound was sutured with cotton thread and the rats were returned to their cage. After awakening, the animals were housed at an ambient temperature of 22 ± 2 °C and fed laboratory food with water ad libitum. 14 days after minipump implantation, the cerebral cortical vasodilative responses were tested under urethane anesthesia.

2.2. Measurement of vasodilative responses in the cerebral cortex

Cortical blood flow was measured under general anesthesia, as described elsewhere (Uchida et al., 1997). Briefly, rats were anesthetized with urethane (1.1 g/kg, i.p.). Respiration was maintained by means of an artificial respirator (SN-480-7, Shinano, Tokyo) through a tracheal cannula. End-tidal CO_2 concentration was maintained at 3.0–4.0% by monitoring it with a respiratory gas monitor (Microcap, Oridion Medical, Jerusalem, Israel). Arterial blood pressure was measured through a catheter inserted into a femoral artery with a pressure transducer (TP-400T, Nihon Kohden, Tokyo). Body temperature was measured rectally and continuously with a thermistor and kept at around 37.5 °C by means of an infrared lamp and a heater system (ATB-1100, Nihon Kohden). The depth of anesthesia was adjusted by additional urethane doses (100 mg/kg, i.v. via a catheter inserted into a femoral vein) when necessary and by monitoring body movement, stability of blood pressure and respiratory movement. The head of the rat was fixed on a stereotaxic instrument (SR-5, Narishige, Tokyo) in the prone position. After craniotomy, a probe (diameter 0.8 mm) of a laser Doppler flowmeter (ALF2100, Advance, Tokyo) was placed on the surfaces of the frontal lobe. The flowmeter probe was fixed with a balancing holder (ALF-B, Advance). The output of the laser Doppler flowmeter was expressed in mV and recorded on a polygraph. Cortical blood flow response to a bolus nicotine (0.3–30 μg/kg, i.v.), or to hypercapnia (10% CO_2 inhalation for 2 min) was tested. Injection of nicotine at doses of 0.3, 3 and 30 μg/kg was tested usually 1 trial (sometimes 2–3 trials) in each rat at randomized order. We waited for about 10–30 min between each trial, or waited until all effects of the drug had disappeared.

2.3. Short-term nicotine treatment

Under urethane anesthesia (1.1 g/kg, i.p.), nicotine at a dose of 125 μg/kg/h was continuously infused via the catheter implanted into a subcutaneous space over the shoulder using an infusion pump (1 μl/min). One hour after the start of nicotine infusion, the cerebral cortical vasodilative responses were tested. Subcutaneous nicotine infusion was continued during the experiment.

2.4. Determination of α4β2-like and α7 subtypes of nicotinic receptors

In other 9 rats, the contribution of α4β2-like nicotinic receptors (5 rats) or α7 nicotinic receptors (4 rats) on the bolus nicotine-induced increase in cortical blood flow was tested. The α4β2-preferring nicotinic receptor antagonist dihydro-β-erythroidine hydrobromide (Sigma, St. Louis, USA, 1.5–4 mg/kg) or the α7 selective nicotinic receptor antagonist methyllycaconitine citrate (Tocris, Ellisville, USA, 2 mg/kg) was calculated as compounds with the base, dissolved in saline and administered intravenously. After waiting 20 min, we tested the cortical blood flow response to a bolus nicotine (30 μg/kg, i.v.). The dosages of 1.5–4 mg/kg dihydro-β-erythroidine and 2 mg/kg methyllycaconitine were in the same range as the doses previously reported to be effective in blocking each specific subtype of nicotinic receptors in the brain (Solinas et al., 2007; Rao et al., 2008).

2.5. Statistical analysis

All values are given as means ± S.E.M. Statistical comparisons were carried out by means of a repeated-measures ANOVA followed by a Dunnett's multiple comparison test, two-way repeated-measures ANOVA followed by a Bonferroni correction, paired *t*-test, or unpaired *t*-test. A *P*-value of <0.05 was considered to be statistically significant.

3. Results

3.1. Effect of short-term and long-term nicotine treatment on the nicotine-induced increase in cortical blood flow

The basal cortical blood flow and the mean arterial pressure, before testing the cortical vasodilative responses in the saline-treated group were 398 ± 49 mV and 88 ± 6 mmHg, respectively. These parameters were not significantly changed by chronic nicotine treatment, either short-term or long-term (low or high-dose).

In a saline-treated rat, bolus i.v. injection of nicotine (30 μg/kg) produced a marked increase in cortical blood flow (Fig. 1A) without significant changes in systemic blood pressure. Fig. 1C summarizes the time courses of the responses of cerebral blood flow measured every 2–5 min in 6 rats. The significant increase of cortical blood flow appeared 2 min after the bolus nicotine injection and reached its maximum of 58 ± 10% at about 5–10 min, and lasted until 25 min after injection before returning to the basal level (Fig. 1C closed circles). In rats receiving long-term treatment with high-dose nicotine (Fig. 1B, C open circles), bolus i.v. injection of nicotine (30 μg/kg) produced an increase in cortical blood flow which was smaller and more short-lasting than that in the saline-treated group. The significant increase of cortical blood flow appeared 2 min after the bolus nicotine injection and reached its maximum of 33 ± 3% at 5 min, and lasted until 10 min

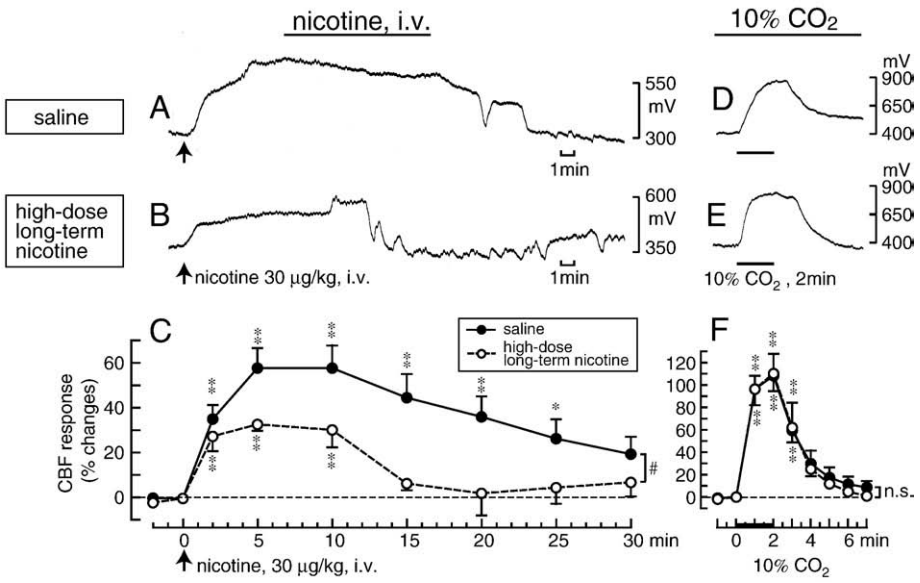

Fig. 1. Responses of cortical blood flow (CBF) in the frontal cortex to bolus i.v. nicotine (A–C) or hypercapnia (D–F) in rats chronically injected with either saline (A, D, closed circles in C and F) or high-dose nicotine (B, E, open circles in C and F). A, B, D, E: Sample recordings of CBF. C, F: Summary of the responses (C; $n=6$, F; $n=4$). Changes in cortical CBF were calculated every 1–5 min. Each point and vertical bar represents a mean ± S.E.M. In each rat, averaged data obtained from 1–2 trials were summarized. $^{*}P<0.05$, $^{**}P<0.01$; significantly different from basal CBF values just prior to the nicotine injection, or to the hypercapnia, using one-way repeated ANOVA followed by Dunnett's multiple comparison test. $\#P<0.01$; significant difference between the saline-treated group and high-dose nicotine-treated group, tested by two-way repeated ANOVA.

after injection before returning to the basal level. (Fig. 1C open circles). There was a significant difference in the cerebral cortical vasodilatory response to bolus nicotine injection (30 μg/kg, i.v.) between high-dose long-term nicotine-treated rats and saline-treated rats ($P<0.01$, two-way repeated ANOVA). In contrast, the magnitude and time course of the increased responses of cortical blood flow to hypercapnia stimulation (inhalation of 10% CO_2) were similar in both saline-treated and high-dose long-term nicotine-treated rats (Fig. 1D–F).

The peak (maximum) responses of cerebral blood flow measured during the 2–10 min after the beginning of different doses of bolus nicotine injection or the beginning of hypercapnia were summarized in Fig. 2. Cortical blood flow was increased by nicotine injection (3–30 μg/kg) in a dose-dependent manner in all 4 groups (Fig. 2A–C). The threshold dose of nicotine (3 μg/kg, i.v.) producing vasodilation was not influenced by short-term and long-term continuous nicotine treatments (Fig. 2B). Long-term (at either low- or high-dose) but not short-term continuous nicotine treatment attenuated the increase in cortical blood flow to 30 μg/kg of i.v. nicotine (Fig. 2C). The increase in cortical blood flow after bolus nicotine i.v. injection (30 μg/kg) was

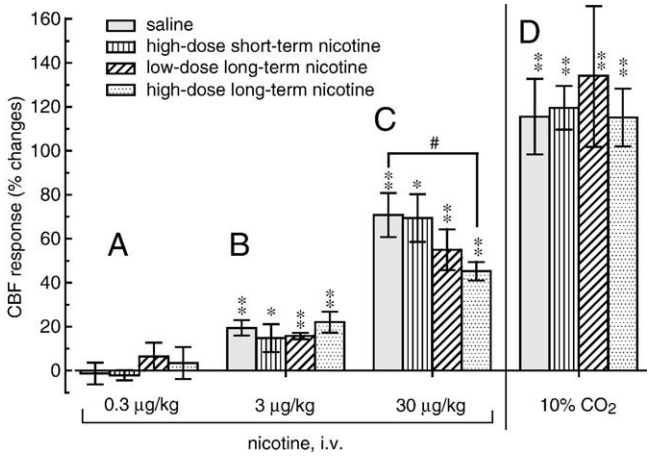

Fig. 2. Effect of continuous nicotine treatment on the cortical CBF responses induced by different doses of bolus i.v. nicotine (A–C: 0.3–30 μg/kg) or hypercapnia (D). A–C; $n=6$ for long-term nicotine or saline-treated groups, $n=4$ for short-term nicotine-treated group. D; $n=4$ for all groups. Peak responses of CBF were measured during the 2–10 min after the beginning of stimulation (bolus i.v. nicotine injection or hypercapnia). Each column and vertical bar represents the mean ± S.E.M. In each rat, averaged data obtained from 1–3 trials were summarized. $^{*}P<0.05$; $^{**}P<0.01$; significantly different from basal CBF values just prior the stimulation, using paired t-test. $\#P<0.05$; significantly different from the response in saline-treated group, using unpaired t-test.

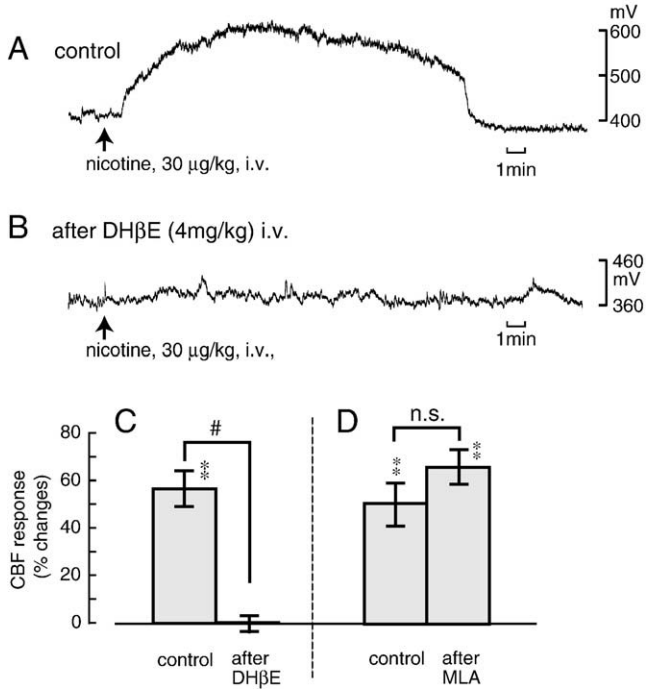

Fig. 3. Effects if i.v. administration of nicotinic receptor antagonists (DHβE: dihydro-β-erythroidine and MLA: methyllycaconitine) on bolus i.v. nicotine-induced increase in CBF in the frontal cortex. A, B: Sample recordings of CBF to bolus i.v. injection of nicotine before (A) and 20 min after i.v. administration of DHβE (4 mg/kg, B). C, D: Summary of CBF responses (C; $n=5$, D; $n=4$). Responses of CBF were calculated 10 min after the beginning of bolus i.v. nicotine. The data after administration of DHβE or MLA were taken 20–90 min after injection of these drugs. Other details are as in Fig. 2.

highest in saline-treated group (71 ± 10%) and in the short-term nicotine-treated group (69 ± 11%). In the low-dose long-term nicotine-treated group (55 ± 9%), the response was slightly smaller than in the controls but the difference was not statistically significant. In the high-dose long-term nicotine-treated group, there was a statistically significant reduction of the response (45 ± 4%), when compared with the response of saline-treated group. In contrast, the increases in cortical blood flow induced by hypercapnia (10% CO_2) were equivalent across all groups (Fig. 2D).

3.2. Effect of α4β2-preferring and α7 selective nicotinic receptor antagonists on the bolus nicotine-induced increase in cortical blood flow

Dihydro-β-erythroidine, an α4β2-preferring nicotinic receptor antagonist, was tested in a total of 5 rats. Three rats were tested with 4 mg/kg and other rats were tested with 1.5 mg/kg or 3.3 mg/kg. No differences in any of the measures were observed across dosages. I.v. injection of dihydro-β-erythroidine (1.5–4 mg/kg) abolished nicotine-induced increase in cortical blood flow, when tested 20 min and 60–90 min after injection of dihydro-β-erythroidine (Fig. 3A–C). In contrast, nicotine-induced increase in the cortical blood flow was not significantly influenced by i.v. injection of methyllycaconitine (2 mg/kg), an α7 selective nicotinic receptor antagonist (Fig. 3D). The basal cortical blood flow and blood pressure were not significantly changed following administration of dihydro-β-erythroidine or methyllycaconitine (data not shown).

4. Discussion

The present study demonstrates for the first time that the cerebral cortical vasodilatation mediated by nicotinic receptors is markedly reduced by long-term treatment with nicotine. The effect occurs at doses that are known to increase the number of nicotinic receptors in the cerebral cortex, and here we discuss below the possible correlation between the two events.

In the present study, we show that the increase in cortical blood flow by bolus i.v. nicotine was not influenced by methyllycaconitine, an α7-selective nicotinic receptor antagonist, but was completely abolished by dihydro-β-erythroidine, an α4β2-preferring nicotinic receptor antagonist. Clearly, the cortical vasodilatation is mediated via α4β2-like nicotinic receptors while the α7 nicotinic receptors seem not to be involved. Although dihydro-β-erythroidine is often used as α4β2-preferring nicotinic receptor antagonist (Nott and Levin, 2006; Wang et al., 2006), we cannot exclude the possible contribution of other heteromeric nicotinic receptors, since dihydro-β-erythroidine is known to bind to heteromeric neuronal nicotinic receptors containing not only α4 and β2 subunits but also α3 subunit (Dwoskin and Crooks, 2001).

The present study shows that long-term nicotine treatment does not change the increase in cortical blood flow induced by hypercapnia, but reduces the increase in cortical blood flow induced by bolus i.v. nicotine. Therefore, we can assume that the reduction of the acute effect of nicotine is due to the decreased signal transmission through the α4β2-like nicotinic receptors. Our observation that the long-term nicotine treatment decreases the functional activity of β2-subunit-containing nicotinic receptors, agrees with the previous studies by Grilli et al. (2005) and Besson et al. (2007). Grilli et al. (2005) reported that long-term nicotine treatment decreased nicotine-evoked release of acetylcholine from rat hippocampal synaptosomes, and that the processes involve α4β2 subtype nicotinic receptors. Besson et al. (2007) demonstrated that long-term nicotine treatment decreases the functional activity of β2-subunit-containing nicotinic receptors but increases the functional activity of α7-containing nicotinic receptors, in the dopaminergic system of ventral tegmental area in mice. However, there has been no studies of the nicotinic receptor subtypes involved in the nicotine-induced cerebral cortical vasodilation, and no study has examined the effects of long-term nicotine on the function of these nicotinic receptors. The present results show not only that α4β2-like nicotinic receptors but not α7 nicotinic receptors are involved in nicotine-induced cerebral cortical vasodilative response, but also that this functional activity of α4β2-like nicotinic receptors is reduced by long-term nicotine treatment.

The reduction of nicotinic receptor-mediating vasodilation that we observed after chronic nicotine treatment depends on both the dose of chronic nicotine and the duration of treatment. We showed that reduction of nicotinic receptor-mediating cerebral cortical vasodilative response is induced after long-term (14 days) but not short-term (1 h) nicotine treatment. The results obtained from the short-term (1 h) experiment demonstrate that the reduction of vasodilative response to bolus nicotine in the long-term (14 days) experiment is not due to tachyphylaxis. Furthermore, the degree of reduction is marked and is statistically significant with high-dose (100 μg/kg/h) nicotine; low-dose (33 μg/kg/h) nicotine has a small effect that is not statistically significant. The reduction of nicotinic receptor-mediated cerebral vasodilation after long-term nicotine treatment may have a role for feedback inhibition of signal transduction. Up-regulation (increase in number) of nicotinic receptors by chronic nicotine treatment depends on both the dose of nicotine and the duration of treatment. Nguyen et al. (2004) observed that the number of nicotinic receptors (determined by [^{3}H]epibatidine binding) in the cerebral cortex is increased following 14 days but not 16 h chronic treatment of nicotine at doses of 6 mg/kg/day. Dávila-García et al. (2003) reported that chronic nicotine treatment (14 days) at doses of 1.2 mg/kg/day and 3.5 mg/kg/day increases the number of nicotinic receptors (determined by [^{3}H]epibatidine binding) in the cerebral cortex by 40% and 70%, respectively. We can assume that, in our experimental conditions, the number of nicotinic receptors in the cerebral cortex is increased following 14 days but not 1 h of nicotine treatment, and the degree of increase is higher in the high-dose (100 μg/kg/h; nearly 2.4 mg/kg/day) nicotine-treated group than in the low-dose (33 μg/kg/h; nearly 0.8 mg/kg/day) nicotine-treated group. Although the mechanism of the reduction of nicotinic receptor-mediating cortical vasodilation by long-term nicotine treatment is not clear, nicotinic receptor desensitization and subsequent up-regulation may be involved in this process, as suggested previously (Dani and Heinemann, 1996). However, since we did not measure receptor number in the present study, a direct determination of this relationship is not possible.

We have already shown that the effect of nicotine on cortical blood flow is less marked in aged rats (Uchida et al., 1997). The decrease in the cortical blood flow response in the aged animals is probably due to a decline in the number of nicotinic receptors in the cortex, as has been observed in humans (Araujo et al., 1990; Nordberg et al., 1992). Because the nicotinic receptors in the cerebral cortex decline with age, there may be a beneficial effect for old people in receiving a treatment with a nicotinic receptor agonist; this intervention would support or enhance to cholinergic vasodilative mechanisms. Furthermore, increase of cortical blood flow induced by nicotinic receptor agonist should provide sufficient oxygen and glucose to the cerebral cortex, and these sufficient nourishments appear to be beneficial for protection of the cortical neurons (Hotta et al., 2002) and would lead to enhancement of cognitive function (Rezvani and Levin, 2001). However, while it is known that chronic nicotine treatment up-regulates cortical nicotinic receptors (Wonnacott, 1990), this study has shown that the nicotine treatment also reduces the functional activity of the nicotinic receptors that are involved in cortical vasodilation. Our results show that functional changes in nicotinic receptors are not always in the same direction as the changes in number; the observations highlight the importance of investigating not only the changes in receptor numbers but also those in their functional activity.

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