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Effect of Chronic Nicotine Treatment Against Repeated Immobilization Stress

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YAMANAKA, K., I. MURAMATSU AND S. KIGOSHI. *Effect of chronic nicotine treatment against repeated immobilization stress.* PHARMACOL BIOCHEM BEHAV 26(2) 259–263, 1987.—Alpha$_2$ and beta adrenoceptors, and muscarinic cholinoceptors in 2 brain regions (cerebral cortex and hippocampus) were measured in rats which received either tap water or nicotine added to the drinking water (5–8 mg/kg/day) for 4 weeks, and immobilization stress (daily 2 hr) for the last 5 days. The repeated stress induced a reduction in the maximum number of binding sites (Bmax) for (^{3}H)dihydroalprenolol (DHA) in the cerebral cortex of rats with tap water, without affecting (^{3}H)clonidine binding. Nicotine-treatment also caused a decrease in the Bmax of cortical (^{3}H)DHA binding comparable to the case of stress, and increased the (^{3}H)clonidine binding. However, the combination of nicotine- and stress-treatments failed to induce further no changes in the 2 radioligands binding. The binding of (^{3}H)quinuclidinyl benzilate in the cerebral cortex and of the 3 radioligands in the hippocampus was unaltered by nicotine- and/or stress-treatments. These results indicate that long-term administration of nicotine induces down-regulation of cortical beta adrenoceptors and seemingly attenuates the receptor alteration by repeated stress.

Nicotine Chronic treatment Repeated stress Alpha$_2$ and beta adrenoceptors Cerebral cortex

ACUTE exposure of animals to different types of stress causes increased activities of catecholamine-neurons in brain. This is shown by the evidence of a reduced level of catecholamines in several brain regions [15, 16, 19, 22]. Acute administration of nicotine also induces a decreased level and an increased turnover of catecholamines in the hypothalamus [1,2,10] and nigrostriatal system [4, 12, 13]. Therefore, nicotine may modify the local changes in catecholamine turnover induced by stress in brain. In support of this, nicotine was found to attenuate partially the reduction of norepinephrine in the rat hypothalamus induced by acute immobilization stress [9].

Recently, we found that nicotine on chronic administration alters adrenoceptors and muscarinic cholinoceptors in rat brain [24], suggesting changes in neurotransmission through the receptors. Receptor alteration is well known to occur during exposure of rats to chronic stress, as evidenced by a reduced density of brain beta adrenoceptors which may be the result of an excessive release of norepinephrine [17, 20, 21].

Thus, the present study was attempted to determine whether or not the chronic nicotine-treatment may modify the receptor alteration in rat brain induced by repeated stress. For this purpose, alpha$_2$ and beta adrenoceptors and muscarinic cholinoceptors were measured in the cerebral cortex and hippocampus which are sensitive to chronic nicotine-treatment and stress [4, 5, 8, 17, 20, 21, 24].

METHOD

Male Wistar rats (6 weeks of age: initial body weight of 160–180 g) were housed in groups of 3 animals with 12 hr day/night cycle and free access to the usual chow diet and tap water. One week later, four different groups of rats were designed; that is, non-treated, stressed, nicotine-treated and stressed nicotine-treated rats. The former two groups of the animals were given tap water for drinking, ad lib, but the latter two groups were provided with tap water containing nicotine in a dose of 100 mg/liter (as nicotine base) for 4 weeks. The nicotine solution provided for drinking was freshly prepared daily by dissolving bitartrate in the drinking water, with an adjustment of pH to 5.5–6.0. The average daily consumption of nicotine by each animal was calculated to be between 5–8 mg/kg. The groups of rats with stress-treatment received immobilization stress for the last 5 days (2 hr daily).

The immobilization stress was induced by restraining the rats in plastic tubes (length, 185 mm; inside diameter, 59 mm) which were the equipments of a Heiner. Borgwaldt Hamburg inhalation apparatus (model R14.01, Germany). In this method, the rat was restrained in a prone position by pressing the hinder part of the animal from one end of the tube, using a small circular plate manipulated through a rubbery tube stopper. The head motion was partially limited by wide metal loops fixed over the neck area at the other end of the tube. The immobilization stress was forced between the 2 hours of 0900–1100.

The rats were killed by decapitation within 5 min after completing the last (5th) stress session. Two brain regions (cerebral cortex and hippocampus) were rapidly dissected on ice, and their membrane fractions were prepared for the binding assays, as described previously [24].

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TABLE 1

SPECIFIC BINDING OF (^{3}H)CLONIDINE AND (^{3}H)DHA TO THE CEREBRAL CORTEX AND HIPPOCAMPUS FROM NON-TREATED, STRESSED (2 HR × 5 DAYS), NICOTINE-TREATED (4 WEEKS) AND STRESSED NICOTINE-TREATED RATS

^{3}H-Ligand	Treatments	Cerebral Cortex	Hippocampus
(^{3}H)Clonidine[a]	None	84.1 ± 0.4	54.6 ± 2.9
	Stress	88.3 ± 4.9	64.0 ± 3.3
	Nicotine	96.4 ± 3.2*	71.8 ± 3.9
	Nicotine + stress	102.5 ± 1.9*	72.6 ± 5.1
(^{3}H)DHA[b]	None	134.2 ± 6.9 (237.6 ± 15.1/0.67 ± 0.07)	70.6 ± 1.7 (122.9 ± 5.9/0.77 ± 0.15)
	Stress	109.5 ± 6.4* (195.3 ± 10.8*/0.60 ± 0.02)	71.2 ± 3.5 (128.2 ± 5.1/0.99 ± 0.11)
	Nicotine	110.3 ± 2.0* (168.3 ± 7.9*/0.61 ± 0.03)	71.5 ± 3.4 (125.3 ± 6.3/1.02 ± 0.09)
	Nicotine + stress	107.9 ± 3.5* (176.6 ± 6.6*/0.64 ± 0.05)	62.7 ± 3.1 (113.6 ± 5.7/0.93 ± 0.05)

[a]The binding experiments were performed with 2.44 ± 0.03 nM of (^{3}H)clonidine, and the values (fmol/mg protein) are the mean ± S.E.M. from 5 animals. The results of a 3-way ANOVA indicated only a significant overall effect of nicotine-treatment, $F(1,4)=8.68$, $p<0.05$, in the cerebral cortex. Asterisks (*) show the significant difference from the value of non-treated rats obtained by a further test of WSD. There were no other significant effects of the factors and their interactions analyzed in the cerebral cortex (F values=0.13–3.92, $p>0.05$) and hippocampus (F values=0.49–6.05, $p>0.05$).

[b]The binding experiments were performed with 1.29 ± 0.01 nM of (^{3}H)DHA, and the values (fmol/mg protein) are the mean ± S.E.M. from 6 animals. The results of a 3-way ANOVA indicated significant overall effects of nicotine, $F(1,5)=7.66$, $p<0.05$,- and stress, $F(1,5)=25.45$, $p<0.05$,-treatments in the cerebral cortex. Asterisks (*) show the significant difference from the value of non-treated rats obtained by a further test of WSD. There were no other significant effects of the factors and their interactions analyzed in the cerebral cortex (F values=0.27–4.69, $p>0.05$) and hippocampus (F values=0.12–1.91, $p>0.05$). The values in parenthesis show the B_{max}/K_D (nM) values determined from 3 saturation experiments with 0.09–1.79 nM of the radioligand. Asterisks (*) represent the significant difference from the value of non-treated rats obtained by a WSD test.

Alpha$_2$ and beta adrenoceptors, and muscarinic cholinoceptors were measured from the binding assays with (^{3}H)clonidine, (^{3}H)dihydroalprenolol (DHA) and (^{3}H)quinuclidinyl benzilate (*l*-isomer, QNB), respectively. In most of the binding assays with (^{3}H)clonidine and (^{3}H)DHA, the tissue homogenates (5 or 7.5 mg of tissue wet wt.) were incubated with a single concentration of each radioligand ((^{3}H)clonidine, 2.44±0.03 nM; (^{3}H)DHA, 1.29±0.01nM) for 30 min at 25°C, in a final 2-ml volume of 50 mM Tris/HCl buffer (pH 7.7). For detailed assays of (^{3}H)DHA binding, saturation experiments were performed with various concentrations of the radioligand (0.09–1.79 nM). In the (^{3}H)QNB binding assays, the homogenates (0.5 mg of tissue wet wt.) were incubated with 326.9±2.4 pM of the radioligand for 60 min at 37°C, in a total 2-ml volume of 50 mM Na/K phosphate buffer (pH 7.4). All assays were done in duplicate or quadriplicate. The binding was terminated by rapid filtration over Whatman GF/B filters, under reduced pressure, followed by 4±4 ml rinses with ice-cold incubation buffer. After drying the filters, the radioligand retained on the filters was extracted by scintillation fluid and the radioactivity was counted in a scintillation spectrometer. Nonspecific binding of each radioligand was defined as the binding in the presence of L-norepinephrine (10 μM), propranolol (0.1 μM) and atropine (1 μM) for the binding assays of alpha$_2$ and beta adrenoceptors and muscarinic cholinoceptors, respectively. The specific binding as percentage of total ranged 85–96%, 68–70% and 98–99% in the case of (^{3}H)clonidine, (^{3}H)DHA (except 47–53% for hippocampus) and (^{3}H)QNB binding, respectively. Protein measurement was performed by the method of Lowry *et al.* [14], using bovine serum albumin as a standard.

Saturation isotherms constructed from saturation experiments were analyzed by estimation of the maximum number of binding sites (Bmax) and the apparent dissociation constant (K_D), according to the Scatchard method described by Bennet [6]. Hill coefficients were also estimated from the data by Hill analysis. Analyses of Scatchard and Hill plots were carried out using a method of linear least-squares regression analysis. Statistical evaluation of the data between different groups was examined by a 3-way of analysis of variance (ANOVA), using three factors of nicotine- and stress-treatments, and variation within the individual group. In those analyses, there were not significant overall effects of the variation within the individual group and its interactions with other two factors analyzed. Therefore, when significant overall effects of nicotine- and stress-treatments were found at a level of $p<0.05$, the significance of the differences between the individual groups was further analyzed using a test of wholly significant difference (WSD).

Materials

Radioligands ((^{3}H)clonidine, 20.5 Ci/mmol; (^{3}H)DHA, 34.1 Ci/mmol; (^{3}H)QNB, 33.2 Ci/mmol) were purchased from New England Nuclear (Boston, MA) and other agents

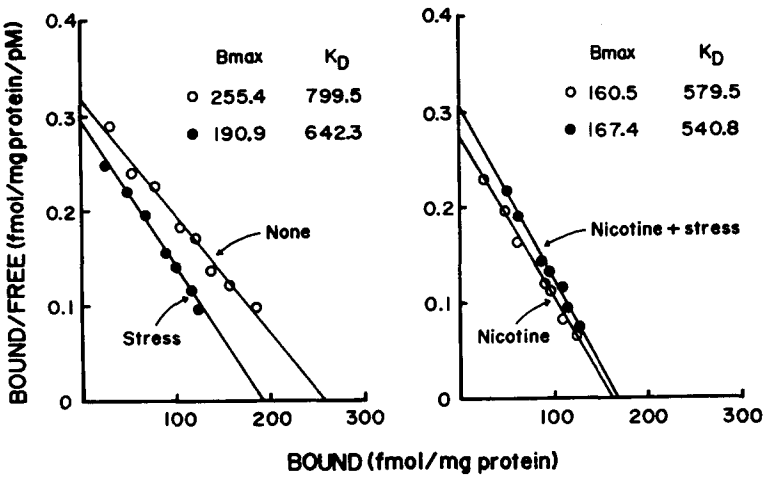

FIG. 1. Representative Scatchard plots of specific (^{3}H)DHA binding to the cerebral cortex from non-treated, stressed (2 hr × 5 days), nicotine-treated (4 weeks) and stressed nicotine-treated rats. The saturation experiments were performed with 0.09–1.79 nM of (^{3}H)DHA. The saturation isotherms were transformed into Scatchard plots, and the maximum number of binding sites (Bmax, fmol/mg protein) and equilibrium dissociation constant (K_D, pM) were estimated from the linear regression line indicated. Further Hill analysis gave coefficients of 0.90 to 1.01.

used in this study were obtained from commercial sources; atropine sulfate and D,L-propranolol hydrochloride (Nakarai Chemicals, Ltd., Kyoto, Japan), and L-norepinephrine bitartrate (Sigma Chemicals, St. Louis, MO).

RESULTS

Water Intake, Nicotine Consumption and Body Weight Changes

The daily intake of water by each animal during the first week ranged from 35 to 40 ml, but it decreased to be between 9–18 ml on day 1 after switching tap water to nicotine solution. This level of water intake recovered to be in a range of 18–25 ml by day 5 and thereafter was maintained throughout the period of nicotine-treatment. The daily consumption of nicotine by each animal calculated from the water intake, was between 5–6 mg/kg on day 1 of nicotine-treatment and increased to a range of 7–8 mg/kg within a week. This level, thereafter, was constant throughout the period of nicotine-treatment.

In parallel to the changes in water intake, the gain in body weight of rats was suppressed especially during the first week of nicotine-treatment and thereafter increased at a similar rate to the case of rats provided with tap water. Thus, the body weight of rats at the beginning of 1st stress-treatment was 346.8±12.4 g and 319.0±9.2 g for rats provided with tap water and with nicotine solution, respectively. The two groups of rats respectively gained in body weight for an additional 5 days, by 13.6±2.3 g (n=5) and 16.0±4.6 g (n=5). On the other hand, the application of repeated immobilization stress (daily 2 hr) for the 5 days induced a decrease in the body weight of rats. The weight loss in stressed nicotine-treated rats (13.8±1.1 g, n=5) was slightly less than that in stressed rats provided with tap water (20.4±2.1 g, n=5), but there was no significant difference between the two groups. No significant change of nicotine consumption was observed during the 5 days of stress-treatment.

Alpha$_2$ Adrenoceptors

The specific (^{3}H)clonidine (2.44±0.03 nM) binding to both the cerebral cortex and hippocampus exhibited no significant difference, $F(1,4)=1.84$ and 4.83 for stress-treatment, respectively; $p>0.05$, between non-treated and stressed rats, and between nicotine-treated and stressed nicotine-treated rats (Table 1). However, the latter nicotine groups of rats gave significantly, $F(1,4)=8.67$ for nicotine-treatment, $p<0.05$, high level of (^{3}H)clonidine binding in the cerebral cortex (by 14.7% and 21.8%) as compared with non-treated rats (Table 1). Similar increase in the (^{3}H)clonidine binding was observed in the hippocampus from nicotine groups of rats, but this change was not statistically significant, $F(1,4)=6.05$ for nicotine-treatment, $p>0.05$. There were no significant overall effects of the interaction between nicotine- and stress-treatments in both the cerebral cortex, $F(1,4)=0.13$, $p>0.05$, and hippocampus, $F(1,4)=1.68$, $p>0.05$.

Beta Adrenoceptors

The specific (^{3}H)DHA (1.29±0.01 nM) binding to the cerebral cortex was significantly, $F(1,5)=25.45$ for stress-treatment, $p<0.05$, lower (by 18.4%) in stressed rats than in non-treated rats (Table 1). However, there was no difference in the cortical (^{3}H)DHA binding between nicotine-treated and stressed nicotine-treated rats; instead the nicotine groups of rats gave significantly, $F(1,5)=7.66$ for nicotine-treatment, $p<0.05$, lower (^{3}H)DHA binding (by 17.8% and 19.6%) as compared with non-treated rats. The extent of the reduction of (^{3}H)DHA binding was comparable to the case of stressed rats. There was no significant overall effect of the interaction between nicotine- and stress-treatments, $F(1,5)=4.69$, $p>0.05$.

Further saturation experiments indicated a single class of binding sites for (^{3}H)DHA in the four distinct groups, as evidenced by Hill coefficients of close to unity (0.95±0.03–

1.11±0.04). However, there was a significant reduction in the maximum number of (^{3}H)DHA sites (Bmax) in the cerebral cortex of stressed, nicotine-treated and stressed nicotine-treated rats, as compared with the Bmax value for non-treated rats (Fig. 1, Table 1). The equilibrium dissociation constant (K_D) for (^{3}H)DHA binding was similar between the four distinct groups. The specific (^{3}H)DHA binding to the hippocampus was unaltered after nicotine- and/or stress-treatments of rats, as shown by the similar Bmax and K_D values between the four distinct groups (Table 1).

Muscarinic Cholinoceptors

The specific (^{3}H)QNB (326.9±2.4 pM) binding in the two brain regions tested, were mostly the same between non-treated and stressed rats; cerebral cortex (fmol/mg protein, n=5), 907.3±52.7 and 871.1±52.8, and hippocampus (fmol/mg protein, n=5), 694.8±35.2 and 651.0±45.0, for non-treated and stressed rats, respectively. These values were unaltered in rats treated with nicotine and repeated stress (data not shown).

DISCUSSION

Repeated immobilization stress caused a decrease in beta adrenoceptors in the cerebral cortex, but not hippocampus, of rats provided with tap water, as seen by a reduced number (Bmax) of (^{3}H)DHA sites. Chronic nicotine-treatment of rats also reduced the level (Bmax) of (^{3}H)DHA binding in the cerebral cortex, as comparable to the case of stressed rats. However, the combination of nicotine- and stress-treatments produced further no reduction of (^{3}H)DHA binding, and seemingly the stress failed to alter the cortical beta adrenoceptors in rats receiving nicotine. Thus, the effects of the two treatments were neither additive nor synergistic with each other.

The cortical beta adrenoceptors are regulated by the activity of noradrenergic neurons which originate primarily from the locus coeruleus (L.C.) [7,23]. Repeated stress is well known to activate brain noradrenergic neurons, and the reduced receptor density is presumed as an adaptation to an excessive and sustained release of norepinephrine [20,21]. Accordingly, it is likely that the repeated stress in this study produced a sustained increase in the noradrenergic activity from the L.C. At present, it is unclear how nicotine-treatment altered the cortical beta adrenoceptors. Nevertheless, it may be associated with the modification by nicotine of either noradrenergic activity from the L.C or the transmission through these neurons at the level of the cerebral cortex, because nicotine causes an increased turnover of catecholamines in several brain regions [1, 2, 4, 10, 12, 13]. Alternatively, the reduction of cortical beta adrenoceptors might be a result from the actions of nicotine on other neuronal systems which regulate the cortical beta adrenoceptors [11,18]. Further studies, however, are necessary to clarify the possibilities.

In contrast to the beta adrenoceptors, long-term administration of nicotine caused an increase in the (^{3}H)clonidine binding in the cerebral cortex, as described previously [24]. This effect of nicotine was not shared by repeated stress and unaltered by the combination with stress. Therefore, an alteration of alpha$_2$ adrenoceptors seems to be a feature of long-term effect of nicotine, although the physiological significance of this change in unknown.

There are several reports indicating that repeated stress increases the number of muscarinic cholinoceptors in the hippocampus [8], and that nicotine causes an increase in acetylcholine release at the cerebral cortex [3,4]. However, (^{3}H)QNB binding in the two brain regions was unaltered in rats treated with nicotine and stress. The reason for the discrepancy is unclear.

Conclusively, the present study indicates that long-term administration of nicotine induces down-regulation of cortical beta adrenoceptors and seemingly attenuates the receptor alteration by repeated stress.

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