

## Tolerance to the Convulsions Induced by Daily Nicotine Treatment in Rats

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**ABSTRACT**—Development of tolerance to the nicotine-induced convulsions in rats was examined. Acute intraperitoneal (i.p.) administration of nicotine (2.5, 3.75 and 5 mg/kg) produced convulsions in a dose-dependent manner. Mecamylamine (1 mg/kg, i.p.) antagonized the convulsions, but hexamethonium (5 mg/kg, i.p.) did not modify them. Daily nicotine administration (2.5, 3.75 and 5 mg/kg, i.p.) once a day for 6 days developed tolerance to the convulsions induced by nicotine. After the daily administrations of nicotine for 6 days, the effects of a challenge administration of nicotine (2 mg/kg) on the nicotine-induced convulsions were tested on the 7th-day. Further tolerances were also developed by the 7th-day challenge administration. After the 7th-day test, nicotine levels of the brain and blood 15 min after the challenge injection were measured. With nicotine (5 mg/kg once a day)-treatment, nicotine levels of all the brain regions were increased. In contrast, a similar challenge injection had no effect on blood nicotine level. These results indicate that the development of tolerance to the nicotine-induced convulsions is produced relatively earlier and day by day by daily administrations to rats, which is closely related with the increase in brain nicotine level.

**Keywords:** Nicotine, Tremor, Convulsion, Tolerance, Brain nicotine level

A variety of studies have already demonstrated the nicotine-induced behaviors in rodents (1–3). Acute systemic injection of nicotine at high concentrations depresses locomotor activity (3, 4) and produces tremors (1), prostration (5) and convulsions (1, 2) in rats and mice. In pharmacological studies, it has been shown that the nicotine-induced convulsions are blocked by small doses of ganglion blocking agents such as mecamylamine and chlorisondamine (6, 7). With intracerebroventricular (i.c.v.) injections of nicotine, Caulfield and Higgins (8) reported that the nicotine-induced convulsions in mice possessed a similar pharmacological profile to those by an activation of ganglionic ( $C_6$ ) receptors, rather than neuromuscular ( $C_{10}$ ) receptors. Beleslin and Krestic (9) also demonstrated that the i.c.v.-injection of mecamylamine and  $C_6$  antagonized the convulsions evoked by nicotine in cats. These results suggest that the nicotine-induced convulsions may be mediated by nicotinic acetylcholine receptors (nAChRs) in the brain.

There is a relationship between the nicotine-induced

convulsive movements and nicotine levels of the blood and brain. Mice exhibiting a higher nicotine concentration in the brain suffered severe convulsions with a shorter latency than mice exhibiting a lower nicotine level after an acute i.p.-injection of nicotine, although the nicotine levels of the blood were unaffected (10).

On the other hand, the tolerance to the nicotine-induced behavior, especially the depressant effect on the locomotor activity, in rats and mice has also been studied (3, 11, 12). In a study of tolerance to nicotine-induced convulsions, Miner and Collins (13) reported that mice pretreated with a single dose of nicotine showed an increase in the  $ED_{50}$  for nicotine-induced seizures. However, there is little information on the time- and dose-dependent development of tolerance to the convulsions induced by daily administrations of nicotine. Therefore, we would like to determine if and how the tolerance to nicotine-induced convulsions is developed. Also, we tried to characterize the relationships between nicotine levels of the brain and blood and development of tolerance to the nicotine-induced convulsions.

## MATERIALS AND METHODS

### *Animals*

Male Wistar rats (Kiwa Experimental Laboratories, Wakayama), weighing 180–200 g, were used. Rats were acclimated to an environmentally controlled vivarium at  $25 \pm 2^\circ\text{C}$ , and they were provided food (MF: Oriental Yeast Co., Tokyo) and water ad libitum for one week. Cycles of light and dark were controlled by a fluorescent lamp, and the light time was from 6:00 A.M. to 6:00 P.M.

### *Drugs*

Nicotine (Maruwaka Kagaku, Osaka), Mecamylamine (Sigma, Chemical Co., St. Louis, MO, U.S.A.) and hexamethonium (Sigma) were dissolved in physiological saline (Otsuka, Naruto). All rats received intraperitoneal (i.p.) injections of the drugs in a volume of 1 ml/kg. The drugs solutions were freshly prepared each time.

### *Behavioral procedure*

Rats were injected with nicotine (2.5, 3.75 and 5 mg/kg, i.p.) or saline once a day for 6 successive days. The drugs were constantly administered between 9:30 A.M. and 1:00 P.M. After placing each chronically drug administered rat in a test-cage ( $37 \times 21 \times 15$  cm) for 30 min, they were observed for 30 min after an injection of nicotine. The onset time and duration of tremor and clonic convulsions were recorded, and the incidence of tonic convulsions were also observed. In addition, the recovery time from the paralysis of rat hind legs after the injection of nicotine was measured. All experiments were done between 10:00 A.M. and 2:00 P.M. After rats were daily injected with nicotine (2.5, 3.75 and 5 mg/kg), their nicotine-induced convulsive movements for 6 successive days were recorded. On the 7th-day, both nicotine- and saline-treated rats were challenged with nicotine (2 mg/kg) and immediately monitored for their nicotine-induced convulsions for 15 min.

### *Antagonists test*

After placing each naive rat in a test-cage for 30 min, mecamlamine (1 mg/kg, i.p.) and hexamethonium (5 mg/kg, i.p.) were given to naive rats 15 min before a single injection of nicotine (3.75 mg/kg). Their convulsive movements were observed for 30 min after a single injection of nicotine. Control rats were injected with saline 15 min before the injection of nicotine.

### *Measurement of blood and brain levels of nicotine*

On the 7th-day, a single challenge dose of nicotine (2 mg/kg, i.p.) was injected to both nicotine- and single-

treated rats. Rats were sacrificed by decapitation 15 min after an injection of a single challenge dose of nicotine. The blood was immediately collected and centrifuged at  $1,000 \times g$  for 20 min to obtain the serum. The brain was removed rapidly, dissected on ice into the cortex, midbrain, cerebellum, medulla, hypothalamus, hippocampus and striatum, according to the method of Glowinski and Iversen (14), and then each section was weighed. Serum and brain tissues were stored at  $-100^\circ\text{C}$  until the assay.

### *Tissues and serum preparation*

The brain tissues were homogenized in 10 volumes of 0.05 N trichloroacetic acid for 2 min at  $4^\circ\text{C}$ , and the solution was centrifuged at  $10,000 \times g$  at  $2^\circ\text{C}$  for 30 min to obtain the resultant supernatant. Nicotine was extracted with diethyl ether from the supernatants and the serum, and then was measured by a gas chromatographic method, a modification of the procedures described by Jacob et al. (15). A 1-ml aliquot of the serum or supernatant was added to centrifuge tubes containing 0.5 ml of 2 N sodium hydroxide and  $1 \mu\text{g}$  of quinoline as an internal standard. Two milliliters of diethyl ether was then added to each tube, which was mechanically shaken for 20 min and centrifuged at  $3,000 \times g$  for 10 min. The extraction with ether was performed three times. After the combined ether layers were transferred to tubes containing 1 ml of 1 N HCl, the tubes were mechanically shaken and centrifuged, and then the ether layers were removed and discarded. One milliliter of 2 N sodium hydroxide and 1 ml of ether were added to the aqueous layer; the tubes were again shaken. The ether layers were separated and concentrated by a centrifugal concentrator (Taiyo Chemical Industrial Co., Ltd., model VC-360) to a final volume of  $50 \mu\text{l}$ . A  $1\text{-}\mu\text{l}$  aliquot of the ether layer was injected onto the chromatographic column. The ratio of the peak height of nicotine to that of quinoline were determined.

A Hitachi model 163 gas chromatograph equipped with a flame thermionic detector was used. The column was a  $2\text{ m} \times 3\text{ mm}$  glass tubing packed with 10% Apiezon L and 10% KOH on WAW 80–100 mesh. The operating conditions were as follows: injection block temperature,  $300^\circ\text{C}$ ; column temperature,  $190^\circ\text{C}$ – $240^\circ\text{C}$  ( $5^\circ\text{C}/\text{min}$ ); detector temperature,  $300^\circ\text{C}$ ; carrier gas (helium) flow rate, 50 ml/min; air flow rate, 85 ml/min; and hydrogen flow rate, 1.8 ml/min.

The calibration curves, which were constructed by adding nicotine and quinoline as internal standards to blank solutions of sample type, was linear over the working range. The recovery of nicotine was 93%. The retention time for quinoline and nicotine were 2.5 and 3.0 min, respectively.

### Statistical analysis

Values are the means  $\pm$  S.E.M. Tolerance to the nicotine-induced convulsive movements was assessed by  $\chi^2$  analysis or two-way analysis of variance (ANOVA). Effects of daily nicotine treatment on nicotine concentrations in the brain or serum at different concentrations were analyzed by one-way ANOVA. All other comparisons were made by Student's *t*-test.

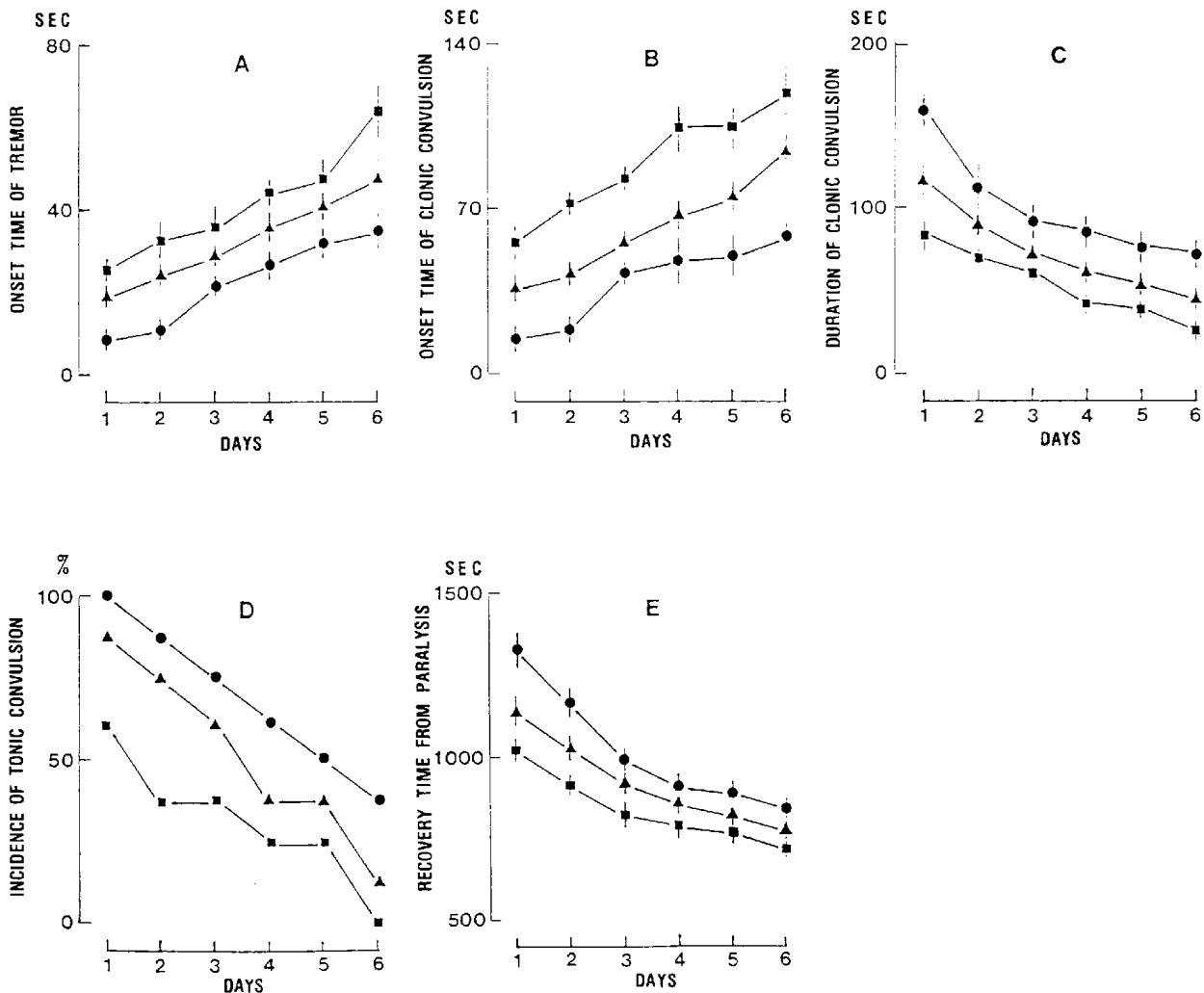
## RESULTS

### Development of tolerance to nicotine-induced convulsions

The development of tolerance to the nicotine-induced convulsions in rats was examined (Fig. 1). On the first

day, after i.p.-injections of nicotine (2.5, 3.75 and 5 mg/kg), typical convulsions were dose-dependently produced in all the rats. Prostration, tremor and clonic convulsion followed by tonic convulsion were observed in the rats. Effects of antagonists on the nicotine (3.75 mg/kg)-induced convulsions were studied (Table 1). Pretreatment of mecamylamine (1 mg/kg, i.p.) antagonized all the nicotine-induced convulsive effects (tremor, prostration, and clonic and tonic convulsions), but hexamethonium (5 mg/kg, i.p.) did not affect them. These data are summarized in Table 1.

In ANOVA analysis for daily nicotine administration, the onset time of tremor was significantly slowed time-dependently [ $F(5,126) = 13.262$ ,  $P < 0.01$ ] and dose-dependently [ $F(2,126) = 20.663$ ,  $P < 0.01$ ] (Fig. 1A).



**Fig. 1.** Development of tolerance to the convulsive effects induced by nicotine in rats. Nicotine (2.5, 3.75 and 5 mg/kg) was injected (i.p. once a day) for 6 successive days. A: Onset time of tremor, B: Onset time of clonic convulsion, C: Duration of clonic convulsion, D: Incidence of tonic convulsion, E: Recovery time from the paralysis of rat hind legs. Symbols indicate: at 2.5 mg/kg (—■—), at 3.75 mg/kg (—▲—) and at 5 mg/kg (—●—) of nicotine. Each point shows the mean  $\pm$  S.E.M. (n = 8).

The onset time of clonic convulsion was also slowed time-dependently [ $F(5,126) = 22.182$ ,  $P < 0.01$ ] and dose-dependently [ $F(2,126) = 46.082$ ,  $P < 0.01$ ] (Fig. 1B). In the duration of clonic convulsion, time- and dose-dependences were observed [ $F(5,126) = 81.395$ ,  $P < 0.01$ , and  $F(2,126) = 116.443$ ,  $P < 0.01$ , respectively] (Fig. 1C). The recovery time from the paralysis of the hind legs was shortened time- and dose-dependently [ $F(5,126) = 86.993$ ,  $P < 0.01$ , and  $F(2,126) = 74.197$ ,  $P < 0.01$ ] (Fig. 1E). In addition, the incidence of tonic convulsion was decreased significantly (Fig. 1D).

Effects of the challenge injection on the 7th-day on the development of tolerance were examined. The incidence of clonic and tonic convulsions significantly decreased at only the concentration of 5 mg/kg, as shown in Table 2. Both the durations of clonic convulsion and the recovery time from the paralysis of the hind legs were decreased in a dose-dependent manner.

#### *Effects of chronic nicotine treatment on nicotine levels of blood and brain*

The nicotine concentrations of the blood and brain 15 min after the challenge injection of nicotine (2 mg/kg) were measured in the nicotine- and the saline-treated rats (Fig. 2). The nicotine level of the blood in the nicotine-treated rats was not modified significantly, but the brain nicotine level was elevated with an increase in nicotine concentration induced by the daily treatment. These data are analyzed by ANOVA. The nicotine levels were dose-dependent in the cortex [ $F(3,28) = 6.71$ ,  $P < 0.01$ ]; midbrain [ $F(3,28) = 2.98$ ,  $P < 0.05$ ]; cerebellum [ $F(3,28) = 3.93$ ,  $P < 0.05$ ]; medulla [ $F(3,28) = 3.63$ ,  $P < 0.05$ ]; hypothalamus [ $F(3,28) = 3.93$ ,  $P < 0.05$ ]; hippocampus [ $F(3,28) = 14.48$ ,  $P < 0.01$ ]; and striatum [ $F(3,28) = 6.91$ ,  $P < 0.01$ ]. No significant change in serum nicotine level occurred [ $F(3,28) = 0.62$ ,  $P > 0.05$ ]. The values in the saline-treated rats were taken as a control value (indicated as 0). With the daily treatment of nicotine (3.75 mg/kg), nicotine levels were significantly increased in the cortex, striatum and hippocampus.

**Table 1.** Effects of antagonists on nicotine-induced convulsant effects in rats

	Tremor (/n)	Prostration (/n)	Convulsion	
			Clonic (/n)	Tonic (/n)
Saline	8/8	8/8	8/8	7/8
Mecamylamine (1 mg/kg)	0/8**	0/8**	0/8**	0/8**
Hexamethonium (5 mg/kg)	8/8	8/8	8/8	3/8

Antagonists or saline were intraperitoneally (i.p.) administered 15 min before an injection of nicotine (3.75 mg/kg, i.p.) in naive rats. \*\* $P < 0.01$ : Significantly different from the saline groups. Data were analyzed by the  $\chi^2$ -test. n: number of rats.

**Table 2.** Effects of challenge injections on convulsant effects induced by chronic nicotine treatments in rats

	Duration of clonic convulsion (sec)	% of convulsion		Recovery time (sec)
		Clonic (/n)	Tonic (/n)	
Saline	93.1 $\pm$ 9.4	100 (8/8)	62.5 (5/8)	845.4 $\pm$ 13.1
Nicotine (2.5 mg/kg)	46.3 $\pm$ 4.5**	100 (8/8)	25 (2/8)	727.8 $\pm$ 25.8**
Nicotine (3.75 mg/kg)	19.5 $\pm$ 4.4**	87.5 (7/8)	12.5 (1/8)	642.8 $\pm$ 19.3**
Nicotine (5 mg/kg)	5.5 $\pm$ 3.5**	50 (4/8)*	0 (0/8)*	482.1 $\pm$ 22.2**

Rats, intraperitoneally (i.p.) treated with 2.5, 3.75 or 5 mg/kg of nicotine or saline daily for 6 successive days, were given a challenge injection of nicotine (2 mg/kg, i.p.) on the 7th day. Data represent the mean  $\pm$  S.E.M. (n = 8). \* $P < 0.05$ , \*\* $P < 0.01$ , with respect to the control. Data were analyzed by Student's *t*-test or the  $\chi^2$ -test. n: number of rats.

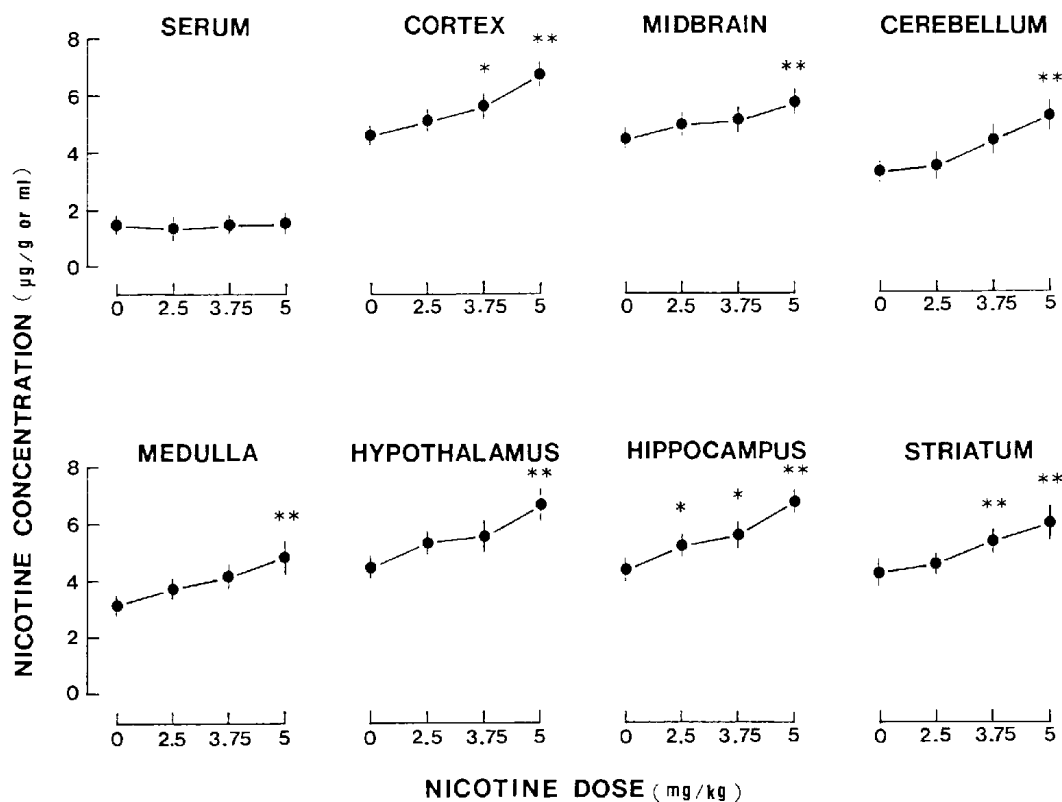


Fig. 2. Changes in nicotine levels of several brain regions and serum by chronic injections of nicotine in rats. Rats were daily injected with nicotine (2.5, 3.75 and 5.0 mg/kg, i.p.) or saline; and then they were sacrificed 15 min after the challenge injection of nicotine (2.0 mg/kg, i.p.) on the 7th-day. Measurement of nicotine concentration was described in Materials and Methods. Each point shows the mean  $\pm$  S.E.M. ( $n = 8$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , with respect to the control (the saline-treated rats).

## DISCUSSION

It has been shown that an acute injection of nicotine elicits depressed locomotor activity in rats (3, 4, 16). In the present experiments, on the first day, the injections with nicotine (2.5, 3.75 and 5 mg/kg, i.p.) produced tremor with prostration rapidly followed by clonic and tonic convulsions. The convulsive effects were consistent with the results of several reports (1, 5–7, 17). Mecamylamine antagonized the nicotine-induced convulsive effects, but hexamethonium did not antagonize it. These results indicate that the nicotine-induced convulsive effects are due to the stimulation of nAChRs in the brain, but not due to neuromuscular activation.

In general, chronic drug treatment often results in alterations in the intensity of response to the drug. The development of tolerance to drugs might play a critical role in facilitating the continued use of the drugs. In this study, repeated injections of nicotine also produced the defined development of tolerance to the nicotine-induced convulsive effects. The tolerance was developed relatively rapidly during the first 2–3 days of nicotine

treatment. This rapid tolerance was similar to development of tolerances to locomotor activity and hypothermia (16). Marks et al. (18) have reported that with chronic nicotine treatment, an increase in the receptor number is induced time- and dose-dependently, and the increase in receptors in DBA/2 mice roughly parallels the development of physiological and behavioral tolerances to nicotine. It was also reported that the increase in nAChR binding correlated with the development of tolerance to body temperature and locomotor activity (16). In addition, Collins et al. (19) suggested that chronic administrations of nicotine might cause prolonged desensitization and inactivation of the nAChRs, resulting from either an increase in the rate of receptor synthesis or a decrease in the rate of receptor catabolism. In the present experiments, therefore, it seems that the repeated nicotine treatment may produce an increase in the number of nAChRs in the brain. The great elevation in the brain nicotine concentration suggests that the desensitization of nAChRs may probably be elicited. Furthermore, up-regulation of nAChRs by chronic nicotine treatment has been widely supported

by several investigators (18, 20, 21).

After a challenge injection, repeated injections with nicotine produced opposite effects on brain nicotine levels and those of the blood. Daily injections of nicotine increased the nicotine levels of all brain regions. However, no change in serum nicotine level occurred. It appears that the increase in brain nicotine levels in the nicotine-treated rats may be due to the increase in the absolute number of nAChRs, which were desensitized. The elevation of the nicotine level in the brain, but not in the blood, is evidence that the nicotine-induced convulsions were caused by the activation of nAChRs in the brain, especially in the cortex, hippocampus and striatum. Several investigators reported that nicotine-induced convulsive movements were concerned with upper brain regions (cortex, hippocampus and diencephalon) (22–24). Furthermore, it has been shown that nicotine may increase the release of adrenal steroids (19). Certain steroids may alter GABA receptor binding and functions. Thus, the tolerance to chronic nicotine treatment might be influenced by the effects of adrenal steroids on response to nicotine and nicotine receptor binding. In addition, Yamamoto et al. (25) reported that the dopamine level in whole rat brain was significantly increased when nicotine induced convulsions in rats. Nicotine-induced convulsions also might be associated with the dopaminergic system in rat brain. Further experiments are required to elucidate the complex mechanisms.

In conclusion, these results have demonstrated that the repeated injections with nicotine cause a dose-dependent development of tolerance to nicotine-induced convulsive effects. The tolerance appeared relatively earlier after the nicotine injections. The development of tolerance to the nicotine-induced convulsions are closely related to the increase in brain level of nicotine, presumably resulting from the increase in the number of nAChRs and from the desensitization of nAChRs in the brain.

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