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Effect of acute and subchronic nicotine treatment on cortical acetylcholine release and on nicotinic receptors in rats and guinea-pigs

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1 The effect of acute and chronic (16 days) administration of nicotine on cortical acetylcholine (ACh) release, gross behaviour and brain nicotinic binding sites was investigated in rats and guinea-pigs.

2 The drug, injected either subcutaneously (0.45–0.90 mg kg^{-1}) or intracerebroventricularly (1, 3 and 5 μg) increased the cortical ACh release, in a dose-dependent manner, through mecamylamine-sensitive receptors for 1–2 h in both species.

3 Chronic treatment significantly increased basal ACh release in the rat and slightly lowered it in the guinea-pig, but the response to a challenging dose of nicotine was proportionally maintained in both species.

4 The number of nicotinic receptors was four times higher in the rat than in the guinea-pig and was not dependent on the radioligand used ([^{3}H]-nicotine or [^{3}H]-ACh, in the presence of atropine) to determine this. The nicotinic binding sites showed an apparent increase in chronically treated rats but no change in guinea-pigs.

5 Tolerance to the inhibitory effect of the drug, assessed with the T maze test, was found in the rat. No apparent change in gross behaviour was detected in the guinea-pig.

6 It is concluded that chronic nicotine treatment causes evident tolerance to its inhibitory effect on behaviour in the rat, but no adaptation to its excitatory properties on the cholinergic brain structures in rats and guinea-pigs.

Introduction

Nicotine is known to cause behavioural stimulation and EEG desynchronization in many animal species by interacting with specific CNS receptors, identified and distinguished in different subtypes with proper ligands (Domino, 1973; Morley *et al.*, 1979; Morley, 1981; Aceto & Martin, 1982; Hall, 1982; Larsson & Nordberg, 1985; Adem *et al.*, 1987; Wonnacott, 1987; Nordberg *et al.*, 1988a,b). The functional significance of these receptors still remains to be fully elucidated. Most of them are heteroreceptors present, for example, on noradrenergic, dopaminergic and 5-hydroxytryptaminergic cell bodies and axons (Schwartz *et al.*, 1984; Clarke & Pert, 1985; Härfstrand *et al.*, 1988). When stimulated, they increase the neuronal firing rate and transmitter release (Hall & Turner, 1972; Goodman, 1974; Giorguieff *et al.*, 1979; Yoshida *et al.*, 1980; Engberg & Swensson, 1980; Balfour, 1982; Westfall *et al.*, 1983; Clarke *et al.*, 1985; Egan & North, 1986; Rowell *et al.*, 1987; Mereu *et al.*, 1987). Mecamylamine and (sometimes) (+)-tubocurarine are effective blocking agents, suggesting the coexistence of ganglionic and neuromuscular junction subtypes in the same brain area (Morley, 1981; De La Garza *et al.*, 1987). Autoreceptors too seem to exist. This inference is based on binding studies carried out in human brains with spontaneous lesions of the basal cholinergic nuclei (Whitehouse *et al.*, 1986; Nordberg & Winblad, 1986) and on neurochemical investigations performed in animal brain synaptosomes (Rowell & Winkler, 1984; Moss & Wonnacott, 1985) and cortical slices (Beani *et al.*, 1985; Araujo *et al.*, 1987). The actual relevance of nicotine-induced facilitation of acetylcholine (ACh) release remains, however, to be firmly established in physiological, integrated models. In fact, *in vitro* studies might show the presence of nicotinic auto- and hetero-receptors which are devoid of any role in normal conditions. Also, no

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detailed data are available about the effects of nicotine on ACh release from the brain of the whole animal. Only preliminary observations have been carried out by Armitage *et al.* (1969) and Erickson *et al.* (1973). In addition, there are few results on the tolerance of the cholinergic systems to chronic nicotine.

The aim of the present study was to determine the effect of acute and chronic administration of nicotine on the cortical ACh release in two animal species, guinea-pigs and rats. Concomitantly, studies were performed to check for possible changes in behavioural responses and in the number of nicotinic binding sites following chronic treatment.

A preliminary account of this investigation has been recently published (Nordberg *et al.*, 1988c).

Methods

Wistar rats of either sex weighing 300–400 g and guinea-pigs of either sex weighing about 400 g were used. The animals were kept under a 12 h light/dark cycle and had free access to food and water.

Epidural cups

The epidural cup implantation on the left or right parietal cortex was performed according to the method described for the rabbit and guinea-pig as well as for the rat (Beani *et al.*, 1968; Beani & Bianchi, 1970; Casamenti *et al.*, 1980). In some animals an intraventricular cannula was also implanted into the ventricle on the opposite side (Beani & Bianchi, 1970).

Acute experiments

Two days after surgery rats and guinea-pigs underwent the experiment. Ringer solution containing 0.3 mM physostigmine (0.3 ml) was placed in the cup and renewed every 30 min. After 3–4 basal samples had been collected, (−)-nicotine was injected either subcutaneously (0.45–0.90 mg kg^{-1}, as base) or intracerebroventricularly (1, 3 or 5 μg base) and its effect was followed for at least 2 h. Changes in gross behaviour were evaluated by direct inspection of the animals. The amount of ACh present in the samples was determined either by bioassay on tetrodotoxin pretreated guinea-pig ileum (Beani *et al.*, 1978) or by an automatic luminometric assay method (Lundin *et al.*, 1984; Blomqvist *et al.*, 1987).

Chronic treatment

Rats and guinea-pigs were injected subcutaneously with nicotine 0.45 mg kg^{-1} twice daily for 16 days. Saline-treated animals were run in parallel. On the 15th day of treatment an epidural cup was implanted and the experiment on ACh release was carried out two days later. The same protocol as for the acute nicotine treatment experiments was followed.

Tolerance test

A T-maze consisting of a central space (24 × 24 × 28 cm) and two arms (27 × 8 × 8 cm) was used for testing of tolerance to the effect of nicotine in naïve rats and chronic nicotine-treated rats. On the morning of the 17th day each rat received a subcutaneous injection of nicotine (0.45 mg kg^{-1}) or saline. Five minutes later the rat was placed in the central space of the maze and the number of entrances (at least half of the body) into one of the arms was recorded for 5 min, according to the method described by Stollerman *et al.* (1973). The number of rears was also recorded during the same time period.

Nicotine receptor binding studies

Twelve hours after the last injection of nicotine the chronically-treated rats and guinea-pigs were killed by decapitation, their brains were taken out and placed on an ice-cold glass plate. The cerebral cortex was dissected and frozen at −70°C until binding experiments were performed. [^{3}H]-(−)-nicotine (7 nM; specific activity 80 Ci $mmol^{-1}$, Amersham, UK) was incubated with the prepared P2 fraction at 4°C for 40 min according to a method described previously (Larsson & Nordberg, 1985; Zhang *et al.*, 1987). [^{3}H]-ACh (10 nM, specific activity 81 Ci $mmol^{-1}$, Amersham, UK) binding was carried out with the P1 fractions at 4°C for 40 min in the presence of atropine 10^{-6} M (Adem *et al.*, 1987). The samples were filtered through Whatman GF/C glass filters presoaked in a 0.05% polyethylenimine solution. The filters were washed twice, dried and placed in scintillation tubes. Then 5 ml HP-B scintillation fluid was added and the radioactivity counted in a Nuclear Chicago scintillation counter. Specific binding for both ligands was calculated by subtracting the values for non-specific binding in the presence of unlabelled nicotine 10^{-3} M. All assays were performed in triplicate.

Measurement of nicotine and cotinine in plasma

Plasma nicotine and cotinine were analysed according to the method described by Curvall *et al.* (1983).

Drugs

Freshly prepared solutions of the following drugs were used: (−)-nicotine bitartrate, mecamylamine

Table 1 Effect of (−)-nicotine (0.45 mg kg^{-1}) on cortical release of acetylcholine (ACh) in naïve and chronically nicotine-treated guinea-pigs and rats

Experimental conditions	*No. of expts*	*ACh release* (ng cm^{-2} 30 min^{-1}) *Basal*	*30 min after (−)-nicotine*	*% increase*
Guinea-pigs				
Naïve	10	23.9 ± 3.1	36.0 ± 4.4*	150 ± 5.9
Nicotine-treated	10	19.0 ± 1.9	25.2 ± 2.5*	132 ± 5.7**
Rats				
Naïve	8	26.0 ± 6	40.0 ± 6*	153 ± 11
Nicotine-treated	8	44.0 ± 6**	62.0 ± 10*	140 ± 16

*Significantly different from pretreatment values, $P < 0.01$.
**Significantly different from naïve animals, $P < 0.05$.

HCl, physostigmine sulphate (Sigma), (+)-tubocurarine chloride (Wellcome).

Statistical methods

Statistical differences were checked by use of Student's *t* test for paired and unpaired data.

Results

The basal release of endogenous ACh from the cerebral cortex of naïve freely moving rats and guinea-pigs was similar (Table 1). No consistent differences in the values were detected when the assays were run in parallel with automatic luminometric assay and with bioassay on the guinea-pig ileum. The cortical ACh release remained at about the same level in control animals for some hours (see Beani & Bianchi, 1970). Therefore, the initial 2–3 samples before injecting nicotine were taken as internal standards in each experiment.

Acute nicotine treatment

Nicotine at doses as low as 0.10–0.20 mg kg^{-1} s.c. gave uncertain responses both in rats and guinea-pigs. However, when injected into rats at doses of 0.45 and 0.90 mg kg^{-1}, nicotine reduced spontaneous motor activity (Table 1) and increased cortical ACh outflow (Figure 1b). The maximal neurochemical effect was obtained in the first collection period (30 min) after injection and vanished within 120 min. Similar results on ACh release were obtained in the guinea-pig (Figure 1A) which, however, did not show any reduction in locomotor activity. In order to exclude peripheral effects, experiments were carried out in which nicotine was injected directly into the right or left ventricular space. As shown in Figure 2c and d the drug was able to increase, in a dose-dependent manner, the cortical ACh release in both animal species. Clearly the guinea-pig displayed a

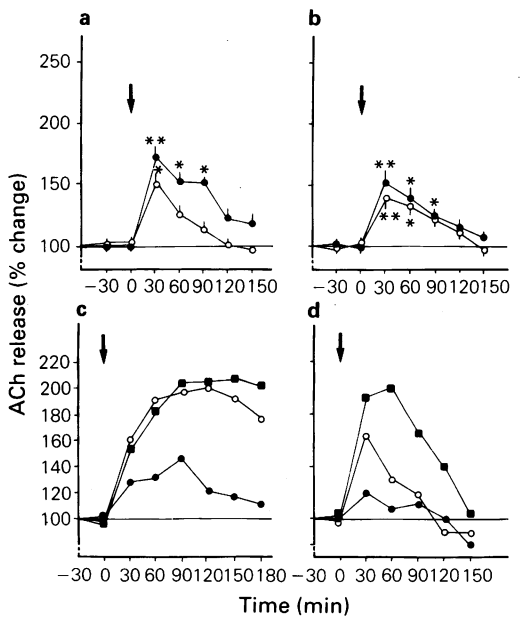

Figure 1 Effect of nicotine on the release of acetylcholine from the parietal cortex of naïve freely moving guinea-pigs (a and c) and rats (b and d). The drug was injected (arrow) at 0.45 (○) and 0.90 (●) mg kg^{-1} s.c. in (a) and (b) and at 1 (●), 3 (○) and 5 (■) μg in the lateral ventricles in (c) and (d). The values are expressed as percentages of the basal release (see Table 1) and are the mean (vertical lines show s.e.mean) of 5–10 experiments. Significantly different from the pre-injection values, $*P < 0.05$; $**P < 0.01$, Student's *t* test for paired data.

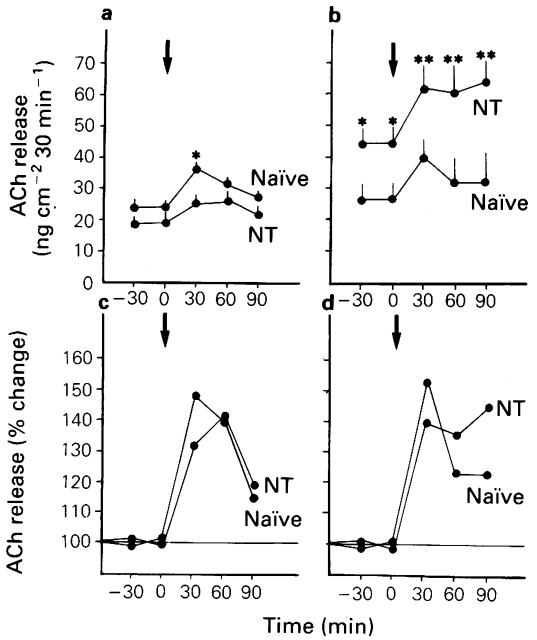

Figure 2 Effect of nicotine 0.45 mg kg^{-1} s.c. on the release of acetylcholine from the parietal cortex of sub-chronically treated (NT) guinea-pigs (a) and rats (b). The values are the mean (vertical lines show s.e.mean) of 5–10 experiments. For comparison, the response of naïve animals to the same dose is shown (see also Table 1). The data normalized with respect to the pre-injection release are presented in (c) (guinea-pigs) and (d) (rats). *$P < 0.05$; **$P < 0.01$.

greater and longer-lasting responsiveness compared with the rat. Again, the rat showed reduced locomotor activity and, after 5 μg nicotine, reduced muscle tone (mainly to the hind limbs). Mecamylamine (2 mg kg^{-1}, s.c.) injected one hour before nicotine (0.90 mg kg^{-1} s.c.) completely prevented the increase in ACh release both in guinea-pigs ($n = 4$) and in rats ($n = 4$).

Chronic nicotine treatment

The gross behaviour of chronically nicotine-treated animals and guinea-pigs (locomotor activity, responsiveness to tactile stimulation, interest for food and environment) did not differ from that of naïve animals. Also, no difference in body weight was found compared to saline-treated controls. When the rats were tested for behavioural effects of nicotine in a T-maze, partial tolerance in chronically treated rats was found (Table 2). In fact the number of entries and rears was drastically reduced by nicotine in naïve rats but was less evident in chronically treated rats.

The basal release of endogenous ACh was significantly higher in chronically treated rats in comparison with saline-treated controls (Figure 2b; Table 1), whereas a lower ACh release was found in chronically treated guinea-pigs (Figure 2b; Table 1). Nicotine (0.45 mg kg^{-1} s.c.) caused a similar, absolute increase of endogenous ACh release in naïve and chronically treated rats. In contrast, the drug evoked a lower response in chronically treated guinea-pigs. However, when the values were normalized with respect to the initial basal release a moderate, but not significant, reduction of the neurochemical response was evident (Figure 2c and d; Table 1).

Effect on nicotinic receptor binding

Unexpectedly, a consistent difference in the number of cortical high affinity nicotinic binding sites was found to exist between the two animal species, when the cerebral cortex was incubated with a certain concentration of the radioligand [^{3}H]-(−)-nicotine or [^{3}H]-ACh plus atropine. The number of binding sites for both ligands was four times higher in rat than in guinea-pig brain tissue. The reason for the higher values obtained with [^{3}H]-nicotine as compared to [^{3}H]-ACh have been reviewed and discussed previously (Wonnacott 1987; Nordberg *et al.*, 1988a). The difference between rat and guinea-pig might be due to differences in receptor affinity. After

Table 2 Effect of nicotine on the T-maze test performed on naïve and chronically treated rats

Groups	*No. of rats*	*No. of entries*	*No. of rears*
Controls	5	9.6 ± 1.2	17.2 ± 1.5
Naïve	5	2.1 ± 0.4**	0.6 ± 0.3*
Chronic treated	9	6.1 ± 0.8*	9.7 ± 2.1*

The chronically treated rats were injected with nicotine 0.45 mg kg^{-1} s.c. twice daily for 16 days. The drug was injected 5 min before the test (see Methods).
Significantly different from controls, *$P < 0.05$; **$P < 0.01$.

Table 3 Nicotinic receptors in the cerebral cortex following chronic treatment with nicotine (0.45 mg kg^{-1}, twice daily for 16 days)

Animal species	*Experimental conditions*	*Receptors* [^3H]-*ACh*	[^3H]-*nicotine*
Guinea-pig			
	Controls (6)	4.0 ± 0.45	7.1 ± 0.54
	Nicotine (7)	4.7 ± 0.52	8.3 ± 0.65
Rat			
	Controls (5)	16.9 ± 0.76	31.2 ± 1.4
	Nicotine (9)	20.5 ± 0.82*	40.0 ± 0.94*

The number of animals used is shown in parentheses.
Significantly different from control, *$P < 0.05$.

chronic nicotine treatment a significant increase in the number of nicotinic binding sites was detected only in the rats (Table 3).

Discussion

The main findings of this study are: (1) Nicotine, injected either s.c. or i.c.v. increases the cortical ACh release in a dose-dependent manner in both rats and guinea-pigs. (2) Chronic drug treatment significantly increased the basal ACh release in rats and slightly reduced it in guinea-pigs, but the response to nicotine was proportionally maintained in both species. (3) The number of nicotinic binding sites was found to be four times higher in rat brain than in guinea-pig brain (at a certain radioligand concentration). Also, only the receptors of the rat underwent apparent upregulation following chronic treatment. (4) Partial tolerance to nicotine developed, as judged from the cholinergic response and specifically from the T-maze test performed in the rat.

These four points require some comments. The increase of cortical ACh release found by Armitage *et al.* (1969) and by Erickson *et al.* (1973) was confirmed in the present study, which, in addition, demonstrated the central site of drug action. Obviously a peripheral afferent component (Hajos & Engeberg, 1988) cannot be excluded. The receptors involved in the cholinergic response are of the ganglionic type. This last finding is at variance with a previous study which showed that (+)-tubocurarine antagonised nicotine facilitation of ACh release in guinea-pig cortical slices (Beani *et al.*, 1985). Nicotine probably increases cortical ACh release in the whole animal by changing the firing rate of the subcortical cholinergic neurones (which have receptors of the ganglionic type), whereas at high concentrations, it is able to enhance transmitter release in cortical slices by acting upon receptors of the neuromuscular junction type. It has been suggested that this latter type of receptors might be present on the intracortical cholinergic nerve endings (Rowell & Winkler, 1984; Araujo *et al.*, 1987). However, the antagonism by tetrodotoxin of the effect of nicotine on unstimulated guinea-pig cortical slices (Beani *et al.*, 1985), together with some negative results on binding and biochemical studies on synaptosomes after lesioning of the subcortical cholinergic nuclei of the rat (Schwartz *et al.*, 1984; Meyer *et al.*, 1987), cast some doubts about such definite or exclusive localization. Further studies are needed to clarify if these supposed autoreceptors are also upstream (i.e. along the cholinergic axons), or in other nervous structures which in turn activate ACh release. Rats and guinea-pigs react differently to chronic nicotine treatment. When a complete washout of the plasma drug levels was achieved, the rat showed a higher basal ACh release whereas the guinea-pig displayed a lower transmitter outflow (Table 1). The reason for these opposite changes is not clear. Studies carried out in cortical slices demonstrate that the electrically-evoked ACh release is unaffected by chronic treatment (Nordberg, Romanelli, Sundwall, Bianchi & Beani, unpublished observations). Consequently, the repeated administration of nicotine seems to affect differently the average firing rate of the subcortical cholinergic neurones, but to leave the release process unchanged as such. In addition, the responsiveness of these neurones to nicotine is maintained.

After 16 days of nicotine administration the number of receptors, as measured with the two nicotinic radioligands, was apparently increased in the rat but not in the guinea-pig. This finding is in keeping with the different adaptative responses of these two animal species in terms of gross behaviour and ACh release. A shift of low affinity to high affinity binding sites due to desensitization (Changeux *et al.*, 1984) or the synthesis of new receptors might explain the increase found in the rat. Receptor competition experiments support the first hypothesis

(Romanelli *et al.*, 1988). The plasticity of the rat brain receptors is similar to that of the human brain. Recent studies carried out with positron emission tomography demonstrated an increased uptake of [^{11}C]-(−)-nicotine in the brain of smokers compared to non-smokers (Nybäck *et al.*, 1989). Similarly, an increased number of binding sites has been detected, with the standard techniques, in specimens of cerebral cortex of smokers compared to non-smokers (Benwell *et al.*, 1988). However, the reason why the guinea-pigs do not show nicotinic receptor adaptation remains to be explained. Interestingly, this species is provided with a smaller number of receptors (lower receptor affinity?) than the rat, but shows a greater responsiveness to nicotine. These points raise the question as to the actual significance of the nicotinic binding sites (and related changes) as assessed by standard techniques. Probably their total number includes physiological synaptic receptors, pharmacologically responsive extrasynaptic receptors, and, finally, extraneuronal (glial?) binding sites (Morley, 1981). Therefore, in order to bypass the limits of the binding technique, nicotine tolerance must be evaluated by measuring functional and/or biochemical responses to the drug in normal and chronically treated animals.

The experiments performed with the T-maze test have shown tolerance to the inhibitory behavioural effect of the drug in the rat. In contrast, the increase of ACh release (considered as an excitatory response) was not consistently modified by the chronic treatment, if the results were normalized with respect to the basal values. This means that moderate or no tolerance to the stimulating properties of this drug was evident (Ksir *et al.*, 1985). This fact may explain the persistent reward detected in animals and man submitted to self administration tests (Goldberg *et al.*, 1981; Henningfield *et al.*, 1983) and can justify the profile of nicotine as a drug of abuse.

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