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On the Action of Nicotine and Cotinine on Central 5-Hydroxytryptamine Neurons

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FUXE K., B. J. EVERITT AND T. HÖKFELT. *On the action of nicotine and cotinine on central 5-hydroxytryptamine neurons.* PHARMAC. BIOCHEM. BEHAV. **10**(5) 671–677, 1979.— The actions of nicotine, and its main metabolite cotinine, on 5-hydroxytryptamine (5-HT) neurons in the brain of the rat have been assessed biochemically (on turnover, uptake, release, overflow and binding of 5-HT in brain) and functionally (on extensor reflex activity, which is 5-HT dependent). Nicotine and cotinine in repeated doses of 2 mg/kg caused a reduction of brain 5-HT turnover, which was not blocked by pretreatment with mecamylamine, and nicotine significantly inhibited the effects of norfenfluramine and 5-methoxydimethyltryptamine on extensor reflex activity, effects counteracted by mecamylamine. In low concentrations cotinine weakly inhibits the uptake and retention of 5-HT and also increases its spontaneous release in vitro. The biochemical findings suggest that the reduction of 5-HT turnover caused by high doses of nicotine are mediated, at least in part, by its main metabolite cotinine. The experiments on extensor reflexes indicate that nicotine can block the functional expression of 5-HT receptor activity in the spinal cord by an action beyond the 5-HT receptor at nicotine-like cholinergic receptors whose location is also discussed.

Nicotine 5-HT turnover, release, re-uptake Cotinine Extensor reflex Mecamylamine

THE INVOLVEMENT of cholinergic mechanisms in neuroendocrine systems has been implied by the results of many experiments (see [4] for review). It is also well known that monoamine transmitters are intimately related to the control of anterior pituitary function and sexual behaviour [14, 18, 26]. However, the extent to which cholinergic and monoaminergic mechanisms interact, if at all, is unclear. Evidence has, however, recently been obtained which suggests that nicotine can reduce LH and prolactin secretion, at least in part by selective activation of an inhibitory dopaminergic mechanism known to be located in the median eminence [11, 12, 18–20]. It has also been shown that the tryptophan hydroxylase inhibitor p-chlorphenylalanine completely inhibits the rise in plasma prolactin which is induced by suckling [24]. Particularly interesting, then, was the finding that nicotine also blocks the suckling-induced rise of circulating prolactin in lactating rats [5]. This raises the possibility, therefore, that the effects of nicotine on prolactin secretion may also involve a 5-HT-mediated mechanism. That nicotine in low doses can enhance sexual behaviour in the female rat [21] is at first sight consistent with this view, since the inhibitory influences of 5-HT pathways on sexual receptivity is well accepted [13–15, 26]. It is important, therefore, to determine whether cholinergic (nicotine) receptors are indeed linked to 5-HT pathways in these contexts.

Evidence has, in fact, already been published which shows that nicotinic receptor blockers can prevent the carbachol (10^{-5} M) stimulated release of newly synthesized ^{3}H-5-HT in hypothalamic slices [23]. In this paper, however, we present experiments which were undertaken to investigate the actions of nicotine, and its main metabolite cotinine, on 5-HT turnover and receptor activity.

METHOD

Male, specific pathogen-free Sprague-Dawley rats (Anticimex, Sweden; body weight 150–200 g) have been used. The rats were kept under a regular day and night cycle (lights off at 8 p.m. and on at 6 a.m.). The experiments were performed between 8 and 12 a.m. Nicotine was given as the tartrate or the salicylate salt.

Biochemical Experiments

In vivo.

5-HT turnover. Changes in 5-HT turnover after nicotine and cotinine were evaluated by studying their influence on the depletion of brain 5-HT after treatment with α-propyldopacetamide (H 22/54, 500 mg/kg, IP, 3 hr before killing, see ref. [3]). The 5-HT stores were measured by means of spectrophotofluorimetry after cation exchange chromatography [1,6]. Nicotine or cotinine were given IP either alone or together with H 22/54. For details on the various treatment schedules, see Tables 1 and 2. In one experiment the ganglion blocking agent mecamylamine (1 mg/kg) was given prior to nicotine to establish if the nicotine induced changes in

TABLE 1

THE EFFECT OF NICOTINE ON THE DEPLETION OF BRAIN 5-HT FOLLOWING TREATMENT WITH α-PROPYLDOPACETAMIDE (H 22/54)

Treatment	Dose of nicotine mg/kg (No. of injections in parenthesis)	Brain 5-HT in per cent	Statistical significance
No drug treatment		100 ± 5.2 (8)	
Nicotine	2 (×3)	103 ± 3.7 (8)	ns
Nicotine	1 (×1)	95 ± 4.4 (3)	ns
H 22/54		64 ± 3.4 (7)	
Nicotine + H 22/54	2 (×3)	83 ± 3.6 (4)	$p<0.01$
Nicotine (all injections before H 22/54) + H 22/54	2 (×3)	108 ± 4.6 (4)	$p<0.001$
No drug treatment		100 ± 8.4 (4)	
H 22/54		51 ± 1.9 (4)	
Nicotine + H 22/54	1 (×1)	46 ± 5.0 (4)	ns
Nicotine + H 22/54	0.5 (×1)	55 ± 3.5 (4)	ns
Nicotine + H 22/54	0.1 (×1)	57 ± 3.8 (4)	ns

Nicotine was injected IP 5 min before H 22/54 (500 mg/kg, IP 3 hr) or saline and 1 and 2 hr after H 22/54 or saline. In one experiment nicotine (2 mg/kg, IP) was given 2 hr, 1 hr and 5 min before H 22/54 injection. When single doses were used they were given 5 min before H 22/54 or saline. In the H 22/54 alone group saline instead of nicotine was given 5 min before and 1 and 2 hr after H 22/54. Means ± SEM are given in per cent of untreated control group means (421 ± 35 ng/g). N=number of animals within parenthesis. Statistical analysis was made according to Student's *t*-test after establishing significant differences using a one-way analysis of variance. All comparisons have been made with the respective untreated and H 22/54 alone groups.

TABLE 2

THE EFFECT OF COTININE ON THE H 22/54 INDUCED 5-HT DEPLETION IN WHOLE BRAIN

Treatment	Dose of cotinine mg/kg (No. of injections in parenthesis)	Brain 5-HT in per cent	Statistical significance
No drug treatment		100 ± 5	
Cotinine	2 (×3)	111 ± 4	
H 22/54		36 ± 3	
Cotinine + H 22/54 (at the same time)	2 (×3)	47 ± 1	$p<0.01$
Cotinine + H 22/54 (5 min apart)	2 (×3)	48 ± 3	$p<0.05$

Cotinine was given IP in 3 doses of 2 mg/kg with 1 hr intervals, the last dose given 1 hr before killing. The first dose was given at the same time as the H 22/54 injection or 5 min earlier. H 22/54 was given in a dose of 500 mg/kg 3 hr before killing. In the H 22/54 alone group saline was given instead of cotinine. Means ± SEM are shown. Number of animals are given in parenthesis. The brain 5-HT levels in the untreated group was 502 ± 26 ng/g. Statistical analysis was made according to Student's *t*-test after one-way analysis of variance. All comparisons have been made with the untreated or the H 22/54 alone group.

TABLE 3

THE EFFECT OF NICOTINE AND COTININE ON THE UPTAKE AND RETENTION OF ^{3}H-5-HT IN SLICES OF DORSAL NEOCORTEX

Treatment	Amount of radioactivity per slice in per cent
Buffer alone	100 ± 11.6 (8)
Nicotine 10^{-7} M	106 ± 14.9 (4)
Nicotine 10^{-6} M	86 ± 9.4 (4)
Nicotine 10^{-5} M	91 ± 4.0 (4)
Buffer alone	100 ± 2.8 (14)
Cotinine 10^{-7} M	92 ± 1† (4)
Cotinine 10^{-6} M	83 ± 2.8§ (8)
Cotinine 10^{-5} M	99 ± 7.5 (8)

The slices were preincubated in a Krebs-Ringer bicarbonate buffer for 15 min with nicotine at +37°C, after which ^{3}H-5-HT (2×10^{-8} M) was added. Incubation was continued for 15 min, and, after rapid rinsing, the slices were taken for liquid scintillation counting [20]. The values are corrected for uptake at 0°C. Number of experiments in parenthesis. Means ± SEM are given. Student's t-test: *$p<0.05$; †$p<0.01$; §$p<0.001$. All comparisons are made with the respective buffer alone uptake value.

TABLE 4

THE EFFECT OF COTININE ON SPONTANEOUS RELEASE OF ^{3}H-5-HT FROM SLICES OF THE DORSAL PART OF THE NEOCORTEX

Treatment	Amount of radioactivity per slice in per cent
Buffer alone	100 ± 5.9 (8)
Cotinine 10^{-7} M	73 ± 6.4* (4)
Cotinine 10^{-6} M	88 ± 4.7 (3)
Cotinine 10^{-5} M	100 ± 10 (4)

The slices were incubated with ^{3}H-5-HT (10^{-7} M) for 15 min at +37°C, then after rapid rinsing the slices were incubated for 30 min with a Krebs-Ringer bicarbonate buffer containing various concentrations of cotinine. The slices were then taken for liquid scintillation counting. Number of experiments in parenthesis. Means ± SEM are given in per cent of untreated group means. Student's t-test: *$p<0.05$. All comparisons are made with the respective buffer alone value.

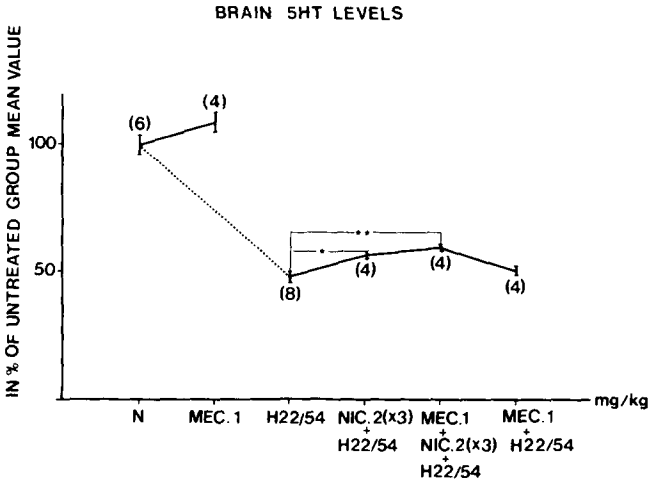

FIG. 1 The effect of mecamylamine on the reduction of the H 22/54 induced 5-HT depletion in whole brain produced by nicotine treatment. Nicotine (2 mg/kg, IP) was administered 5 min before and 1 hr and 2 hr after α-propyldopacetamide (H 22/54; 500 mg/kg, IP, 3 hr before killing). Mecamylamine (1 mg/kg, IP) was administered 15 min before H 22/54. When administered alone it was given 3 hr and 15 min before killing. Means ± SEM. Number of animals within parenthesis. The untreated group mean value was 496 ±20 ng/g. Students's t-test after a one-way analysis of variance. All statistical comparisons have been made with the untreated or the H 22/54 alone group.

5-HT turnover involved the activation of nicotine-like cholinergic receptors. For details, see text to Fig. 1.

In vitro.

^{3}H-5-HT uptake. ^{3}H-5-HT (10 Ci/mmol, Amersham, England) uptake was studied in slices of the dorsal part of the neocortex. It is well-known that the radioactivity present in the slices after incubation is mainly due to the presence of unaltered ^{3}H-5-HT [22]. A Krebs-Ringer bicarbonate buffer was used. For further details, see Table 3.

Spontaneous release of ^{3}H-5-HT. Slices from the dorsal part of the neocortex were made and the amount of radioactivity remaining after incubation with cotinine or a Krebs-Ringer bicarbonate buffer alone was determined. For further details, see Table 4.

Spontaneous overflow of ^{3}H-5-HT. Slices from the most ventral hypothalamus were made excluding the DA rich median eminence area. The slices were superfused with Krebs-Ringer bicarbonate buffer alone or for 100 min with the buffer containing nicotine (10^{-5} M). Fractions were collected every 5 min and taken for liquid scintillation counting (see Fig. 2).

(5-^{3}H)HT and (D-^{3}H)LSD binding. The radioligands (5-^{3}H) HT and (D-^{3}H)LSD are known to label mainly postsynaptic 5-HT receptors, 5-HT binding to the agonist state while d-LSD binds to both the agonist and the antagonist state [5]. The homogenization and binding procedures were performed according to Bennett and Snyder [5] using the dorsal neocortex. Specific binding was defined as the difference between the total counts in the absence of unlabelled ligand and the counts obtained in the presence of 1μM unlabelled d-LSD or 10 μM unlabelled 5-HT. Saturation of (5-^{3}H)HT and (D-^{3}H)LSD binding revealed that the dissociation constants and the maximal number of binding sites for (5-^{3}H)HT (K_D=16 nM; B_{max}=18 pmole/g tissue) and (D-^{3}H)LSD (K_D=6 nM; B_{max}=39 pmole/g tissue) were similar to those previously reported [5]. Five to seven concentrations of nicotine and cotinine were used to compete with the binding of both radioactive ligands. Each experiment was repeated once and at each concentration five replicates were made. The concentration used of (5^{3}H)-HT (16-17 Ci/mM) was 5 nM and that of (D-^{3}H)LSD (11.4 Ci/mol) was 2–3 nM. (5-^{3}H)HT was purchased from Amersham-Searle, England and (D-^{3}H)LSD from New England Nuclear, U.S.A.

Functional Experiments

Hindlimb extensor reflex activity.

In the acutely spinalized rat this reflex has been shown to be highly dependent on 5-HT receptor activity [2,25]. The

TABLE 5

THE EFFECTS OF NICOTINE ON THE NORFENFLURAMINE AND 5-METHOXY-DIMETHYLTRYPTAMINE-INDUCED INCREASE IN THE EXTENSOR HINDLIMB REFLEX ACTIVITY OF ACUTELY SPINALIZED UNTREATED RATS

Treatment	Dose of nicotine mg/kg	Magnitude of Increase in Extensor Reflex Time after 5-MeO-DMT 5 min	10 min	15 min
5-MeO-DMT		1.5(3) 2(1) 2.5(1)	2(3) 2.5(2)	2(2) 2.5(3)
Nicotine + 5-MeO-DMT	1	0(1) 0.5(4)*	0.5(2) 1(3)*	0.5(1) 1(4)*
5-MeO-DMT		1(1) 1.5(3) 2.5(1)	1.5(2) 2(1) 2.5(1) 3(1)	1.5(2) 2(1) 2.5(1) 3(1)
Nicotine + 5-MeO-DMT	0.5	1(1) 1.5(4)	1.5(1) 2(1) 2.5(3)	1.5(1) 2(1) 2.5(3)
Nicotine + 5-MeO-DMT	0.1	1.5(2) 2(2) 2.5(1)	2.5(3) 3(1)	2(1) 2.5(3) 3(1)
		Time after NF 15 min	30 min	60 min
NF		0.5(1) 1(2) 1.5(2)	1.5(1) 2(1) 2.5(3)	1.5(1) 2(1) 2.5(3)
Nicotine + NF	1	0(2) 0.5(3)	0(1) 0.5(1) 1(3)*	0(0) 0.5(2) 1(2)*
NF		1.5(1) 2(4)	1.5(1) 2(3) 2.5(1)	1.5(1) 2(2) 2.5(1) 3(1)
Nicotine + NF	0.5	0.5(3) 1(2)*	1(2) 1.5(2) 2(1)	1(2) 1.5(1) 2(1) 2.5(1)
Nicotine + NF	0.1	2(4) 2.5(1)	2.5(3) 3(2)	2.5(2) 3(3)

The rats were acutely spinalized at the midthoracic level 2 hr before drug treatment. Nicotine was given IP in a dose of 1 mg/kg 5 min before the injection of norfenfluramine (NF; 5 mg/kg, IP) and 5-methoxy-dimethyltryptamine (5-MeO-DMT; 1 mg/kg, IP). In the case of the NF treated animals nicotine treatment was continued with a total of 3 injections given at 30 min time intervals. The strength of the extensor hindlimb reflex, evoked by pressing the base of the tail, was evaluated semiquantitatively on coded animals. 3=strong; 2=moderate; 1=weak; 1/2=very weak. Number of animals within parenthesis. Statistical significance according to Tukey's quick test. All statistical comparisons are made with the corresponding control group treated with NF or 5-MeO-DMT alone. In the table the increase produced by the drug treatment is shown. Before NF or 5-MeO-DMT treatment the reflex activity was zero or very weak. This basal activity has been subtracted from the values obtained after NF or 5-MeO-DMT treatment.

rats were transected at midthoracic level 2-3 hr before injection of nicotine or cotinine. In the pharmacological analysis norfenfluramine (NF), a granular 5-HT releasing agent (17), was used and also 5-methoxy-dimethyltryptamine (5-MeO-DMT), a 5-HT receptor stimulating agent (16). A ganglion blocking agent, mecamylamine, was used to block the effects of nicotine. For further details, see Tables 5 and 6.

RESULTS

Biochemical Experiments

In vivo.

5-HT turnover. As seen in Table 1, nicotine in repeated doses of 2 mg/kg caused a reduction of the H 22/54-induced 5-HT depletion, particularly when all the injections had been made before the H 22/54. In lower doses, down to 0.05 mg/kg, when nicotine was also given after the H 22/54 injection, no significant changes in the 5-HT depletion were obtained. The nicotine-induced reduction of 5-HT turnover was not counteracted by pretreatment with mecamylamine (1 mg/kg; Fig. 1) which, when given alone (1 mg/kg), similarly had no effects on 5-HT levels or H 22/54-induced 5-HT depletion. Cotinine in the same doses and treatment schedule as nicotine (3×2 mg/kg) also significantly reduced 5-HT turnover (Table 2).

In vitro.

^{3}H-5-HT uptake. Nicotine (10^{-7}–10^{-5} M) did not block the uptake and retention of ^{3}H-5-HT by cortex slices (Table 3). Cotinine caused a slight but significant inhibition of ^{3}H-5-HT uptake and retention in the lower concentrations studied

TABLE 5 (Con't)

Treatment	Dose of nicotine mg/kg	Magnitude of Increase in Extensor Reflex Time after 5-MeO-DMT 20 min	25 min
5-MeO-DMT		1.5(2) 2(2) 2.5(1)	0.5(4) 1(1)
Nicotine + 5-MeO-DMT	1	0.5(1) 1(4)*	0(5)*
5-MeO-DMT		0.5(2) 1(1) 2(2)	0(2) 0.5(2) 1(1)
Nicotine + 5-MeO-DMT	0.5	0.5(1) 1.5(2) 2(2)	0(4) 0.5(1)
Nicotine + 5-MeO-DMT	0.1	1(1) 1.5(1) 2(2) 2.5(1)	0(1) 0.5(3) 1(1)
		Time after NF 90 min	120 min
NF		1.5(2) 2(1) 2.5(2)	1(2) 1.5(3)
Nicotine + NF	1	0(2) 0.5(2) 1(2)*	0(3)* 0.5(2)
NF		2(3) 2.5(2)	2(4) 2.5(1)
Nicotine + NF	0.5	1(2) 1.5(1) 2(1) 2.5(1)	0.5(1) 1(1) 1.5(2) 2.5(1)
Nicotine + NF	0.1	2.5(2) 3(3)	2.5(4) 3(1)

TABLE 6

EFFECTS OF COTININE, NICOTINE AND MECAMYLAMINE PLUS NICOTINE ON THE 5-METHOXY-DIMETHYLTRYPTAMINE INDUCED INCREASE IN THE EXTENSOR HINDLIMB REFLEX ACTIVITY OF ACUTELY SPINALIZED RATS

Treatment	Dose mg/kg	Peak increase in extensor reflex after 5-MeO-DMT
5-MeO-DMT	1	2 (5)
Nicotine + 5-MeO-DMT	1 1	0 (4)
Mecamylamine + nicotine + 5-MeO-DMT	5 1 1	1.5 (2) 2 (3)
Cotinine + 5-MeO-DMT	2 1	1.5 (1) 2 (4)

Nicotine (1 mg/kg, IP) and cotinine (2 mg/kg, IP) were administered 5 min before 5-MeO-DMT. Mecamylamine (5 mg/kg, IP) was administered 30 min before 5-MeO-DMT. For further explanations and details see text to Table 5. Mecamylamine significantly counteracted the effects of nicotine (§: $p<0.001$; Tukey's quick test). Before 5-MeO-DMT treatment the extensor reflex activity was zero or very weak.

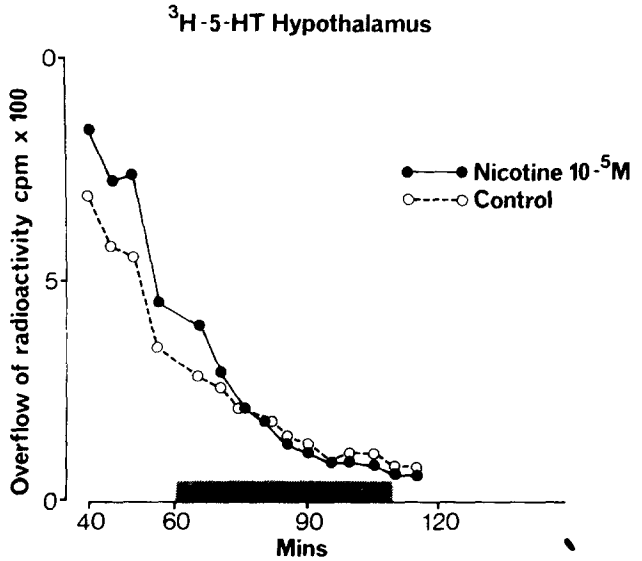

FIG. 2. The effect of nicotine (10^{-5} M) on the tritium overflow from slices of hypothalamus, preincubated for 30 min with ^{3}H-5-HT (10^{-7} M), is shown. The overflow is measured every fifth minute. Values shown are means of 3 experiments. The variability (SEM) was in the order of 10–15% of the mean value. The shaded area represents the time period of nicotine infusion.

(10^{-6}–10^{-7} M). However, in the highest concentration (10^{-5} M) cotinine had no effect.

Spontaneous release of ^{3}H-5-HT. In the lowest concentration (10^{-7} M) used, cotinine caused a significant increase in the release of ^{3}H-5-HT. This did not, however, occur in the higher concentrations (Table 4).

Spontaneous overflow of ^{3}H-5-HT. Nicotine in a concentration of 10^{-5} M was not found to cause any clearcut increase in spontaneous tritium overflow from hypothalamic slices (Fig. 2).

(5-^{3}H)HT and (D-^{3}H)LSD binding. Nicotine and cotinine in concentrations up to 10,000 nM did not displace specific 5-HT and d-LSD binding in the cortex cerebri.

Functional Experiments

Extensor hindlimb reflex activity.

Nicotine and cotinine had no effects on extensor reflex activity. However, nicotine but not cotinine (1 mg/kg) significantly reduced both the norfenfluramine-and 5-MeO-DMT-induced increases in this reflex (Tables 5 and 6). In a lower dose (0.1 mg/kg) nicotine had no significant effects (see Table 5). As seen in Table 6, mecamylamine (5 mg/kg) significantly blocked the inhibitory effects on nicotine (1 mg/kg), although, by itself, mecamylamine did not increase extensor reflex activity.

DISCUSSION

Both nicotine and cotinine in repeated doses of 2 mg/kg, but not in lower doses, were found to reduce brain 5-HT turnover. This is consistent with the data of Rosecrans [28] which showed that repeated doses of nicotine reduced the rate of accumulation of 5-HT after monoamine oxidase inhibition. However, the nicotine-induced reduction of 5-HT turnover was not blocked by the ganglion blocking agent mecamylamine in a dose that completely blocked the effects of nicotine on both sexual behaviour [21] and arousal [10]. Thus, it appears that the effects of nicotine on 5-HT turnover are probably mediated by its main metabolite cotinine and not directly by activating a nicotine-like cholinergic receptor, at least not in this high dose range. The mechanism by which cotinine reduces 5-HT turnover is unclear at present, but the weak inhibition of 5-HT uptake and retention and the increase in spontaneous 5-HT release produced by cotinine in low concentrations in vitro could be contributary factors. None-the-less, it is difficult to explain why cotinine in a high concentration (10^{-5} M) was without effect on these two mechanisms at the 5-HT synapse. Clearly, any direct effects of cotinine and nicotine on 5-HT receptors are unlikely, since both compounds had no effects on 5-HT and D-LSD binding in the cortex.

Thus, at least after treatment with high doses of nicotine, the behavioural pharmacology of this drug is complicated by the fact that its main metabolite cotinine has effects of its own on central 5-HT neurons. However, these effects on the 5-HT systems are probably not important in mediating the nicotine-induced increases in sexual behaviour [21] or decreases in prolactin secretion [7], since cotinine given alone changes neither paramenter. It is much more likely that these effects of nicotine are a result of direct stimulation of central cholinergic receptors with nicotinic properties, since they are reduced after pretreatment with mecamylamine ([21], unpublished data). Such a mechanism existing distal to the spinal cord 5-HT receptors could also explain why nicotine (1 mg/kg) was able to counteract the actions not only of a 5-HT releasing agent but also of a 5-HT receptor stimulating agent in the extensor reflex experiments. A direct receptor blocking action of nicotine at central 5-HT receptors is unlikely to explain these findings for a number of reasons: first a reduction and not an increase of 5-HT turnover was observed, secondly nicotine has no affinity for d-LSD binding sites, and thirdly the action of nicotine in the spinal cord was antagonized by mecamylamine. The nicotinic cholinergic receptor involved is possibly located on the Renshaw nerve cells which inhibit activity in the α-motoneurons and are innervated by recurrent cholinergic collaterals from the α-motoneurons. Thus, any 5-HT receptor mediated activation of the α-motoneurons will be counteracted by nicotine activity directly on the Renshaw cell.

In conclusion, the present study suggests that nicotine in high doses reduces 5-HT turnover and that these effects are mediated, at least partly by its main metabolite cotinine, which can directly although weakly influence 5-HT uptake and release in low concentrations. In the spinal cord nicotine reduces the increase in α-motoneuron activity induced by both direct or indirect stimulation of 5-HT receptors probably by an action at nicotine-like cholinergic spinal cord receptors. The possible location of these receptors is also discussed.

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