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Nicotine binding to brain tissue from drug-naive and nicotine-treated rats

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Two nicotine binding sites with dissociation constants for nicotine of approximately 3 nM and 12 μM respectively have been found in homogenates of rat hippocampus, hypothalamus, parietal cortex and mesencephalon, the greatest density of high affinity binding sites being in parietal cortex (30·0 ± 3·0 fmol (mg protein)$^{-1}$), the lowest in hypothalamus (16·1 ± 1·0 fmol (mg protein)$^{-1}$). The density of the low affinity sites (approx. 20 pmol (mg protein)$^{-1}$) did not show any regional variation. Neither site was present in homogenates of medulla oblongata. The accumulation of radioactivity following the subcutaneous administration of [^{3}H]nicotine (0·4 mg kg^{-1}) was rapid, the highest concentrations being found in the brain regions with the highest density of high affinity binding sites. Medulla oblongata did not accumulate radioactivity above the concentration found in plasma. The chronic administration of nicotine (0·4 mg kg^{-1} s.c. daily for 39 days) had no significant effects on [^{3}H]nicotine binding to brain tissue or its accumulation into brain following subcutaneous administration. It is concluded that nicotine readily passes from plasma into brain tissue and is accumulated in the areas containing high affinity binding sites for the compound. It is also concluded that the biochemical and behavioural effects reported previously in response to the chronic daily administration of nicotine do not depend upon changes in its uptake or binding by brain tissue.

Previous studies have shown that chronic nicotine administration causes regionally-specific changes in the concentration and biosynthesis of 5-hydroxytryptamine (5-HT) in rat hippocampus (Benwell & Balfour 1979, 1982a, b). This region of the brain is also reported to accumulate large amounts of nicotine following systemic administration (Schmitterlow et al 1967) and to be especially rich in α-bungarotoxin binding sites (Hunt & Schmidt 1978; Morley et al 1979; Salvaterra & Foders 1979). However, recent studies suggest that radioactive α-bungarotoxin may not be an appropriate ligand for the nicotine binding sites reported to be present in the brain (Romano & Goldstein 1980; Marks & Collins 1982; Costa & Murphy 1983). In the present study, therefore, [^{3}H]nicotine has been used to examine the possibility that the regionally-specific changes, previously observed in the hippocampus of nicotine-treated rats, might be the result of regional differences in the number, properties or subcellular distribution of nicotine acceptor sites in the brain or in the accumulation of the alkaloid following systemic administration.

MATERIALS AND METHODS

Materials

Radiolabelled D,L[N-methyl ^{3}H]nicotine (specific activity: 61·2 Ci mmol^{-1}) was purchased from the New England Nuclear Corporation and was stored frozen in the presence of a 3-fold molar excess of D-tartaric acid. The purity of the labelled compound was routinely checked by thin-layer chromatography using 2 different solvent systems (system 1: $CHCl_3$–ethanol–NH_4OH (85 : 25 : 0·25); system 2: methanol–NH_4OH (99 : 1)). Lobeline sulphate, methacholine hydrochloride, mecamylamine hydrochloride, decamethonium bromide, hexamethonium bromide, corticosterone, α-bungarotoxin and poly-L-lysine (type V) were obtained commercially from Sigma Chemical Company (UK); carbamyl choline chloride, atropine sulphate and physostigmine sulphate from British Drug Houses Ltd; dimethylphenylpiperazinium (DMPP) from Parke Davis and Co.; (+)-tubocurarine from Aldrich Chemical Company Ltd. D-(+)-nicotine hydrogen tartrate was a generous gift from Dr Ian Stolerman, Institute of Psychiatry, London; chlorisondamine was a generous gift from Ciba Geigy Ltd.

Methods

Male Wistar rats (Charles River UK Ltd) were used. For those involving pretreatment, the animals received nicotine (0·4 mg kg^{-1} day^{-1}) subcutaneously in the form of its hydrogen tartrate as described previously (Benwell & Balfour 1979). The animals, which weighed approximately 150 g at the beginning of the experiment, were given daily

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injections of nicotine or saline (controls) for 39 days. On day 40, 24 h after their last injection, the rats were killed by cervical dislocation and the brains rapidly removed for dissection and radioligand binding assay.

Studies on nicotine binding sites

Brains from either chronically-treated or untreated rats were dissected, over ice, into the hippocampal formation, hypothalamus, parietal cortex, mesencephalon (midbrain and pons), striatum and medulla according to the method of Glowinski & Iversen (1966). Tissue samples were prepared as either a crude homogenate (50 mg ml^{-1} 0·32 M sucrose) or were subfractionated into the nuclear, myelin, synaptosomal, mitochondrial and microsomal fractions using the procedure described by Whittaker (1965).

Binding assays were performed using samples of tissue suspension (200 µl containing 300–800 µg of protein) which were preincubated with 0·7 ml Krebs-Ringer solution buffered to pH 7·4 with phosphate buffer (20 mM), for 5 min to allow the samples to reach the incubation temperature (25 °C). Binding was initiated by the addition of [^{3}H]nicotine (final concentration approximately 4 nM), the final incubation volume being 1 ml. The incubation was halted after 30 min by the addition of 4 ml of ice-cold Krebs-Ringer solution and the bound ligand was rapidly separated from free by filtration under vacuum through Whatman GF/C filters presoaked in 0·05% solution of poly-L-lysine in phosphate buffer to reduce the non-specific filter binding (Romano & Goldstein 1980). The filters were washed with 3 × 4 ml aliquots of ice cold buffer solution and counted by scintillation spectrometry using NE 260 scintillation fluid (New England Nuclear). Non-displaceable nicotine binding was determined in the presence of 100 µM unlabelled L-(−)-nicotine and specific nicotine binding defined as the difference between the total and non-displaceable binding to the tissue. Other unlabelled drugs were added to some of the incubations to characterize the binding sites.

The protein concentrations of the samples were estimated using the method of Lowry et al (1951) with bovine serum albumin as standard.

Data analysis

All equilibrium dissociation constants (K_D), estimates of maximum specific binding (B_{max}) and equilibrium inhibition constants (K_I) were estimated using computer-based curve fitting programmes which evaluated the best fit to the Scatchard data. Differences between K_D or B_{max} values with different treatments were assessed using the Student's *t*-test as modified by Cochran to take account of differences in the variation (Snedecor & Cochran 1967).

Studies on nicotine uptake into brain

Groups of rats (n = 6 for each) were pretreated with nicotine (0·4 mg kg^{-1}) or saline for 39 days as described above. On day 40, all the rats received subcutaneous injections of [^{3}H]nicotine (0·4 mg kg^{-1}: specific activity 0·49 µCi $mmol^{-1}$) and were then killed by cervical dislocation 15 or 90 min after the injection. Blood samples were taken from the neck and the brains were rapidly removed, chilled on ice and dissected into hippocampus, hypothalamus, parietal cortex, mesencephalon, striatum, medulla and cerebellum as described in the previous section. The brain tissues were weighed and dissolved in NCS tissue solubilizer (New England Nuclear) and the radioactivity counted by scintillation spectrometry. Samples of whole blood and plasma were also treated with solubilizer and, where necessary, decolourized with hydrogen peroxide prior to counting.

RESULTS

Studies on nicotine binding sites

Under the conditions used, specific [^{3}H]nicotine binding reached equilibrium after 15 min incubation and increased linearly (r = 0·93) with protein over the range of concentrations tested (0·2–1·0 mg/incubation). Curvilinear Scatchard plots were obtained for specific [^{3}H]nicotine binding to hippocampal homogenates (Fig. 1a) suggesting the presence of multiple binding sites for nicotine in this brain region. Computer analysis of the data suggested they were best fitted to a two site model with K_D values of 2·9 ± 0·9 nM and 11·9 ± 5·0 µM and B_{max} values of 7·3 ± 3·0 fmol mg^{-1} protein and 16·2 ± 5·0 pmol mg^{-1} protein respectively. A Hill plot of the data (Fig. 1b) produced a coefficient of 0·76. High and low affinity binding was also observed in the parietal cortex, hypothalamus, mesencephalon and striatum but not in the medulla (Table 1).

Displaceable nicotine binding was found in all subfractions of the brain regions studied (Fig. 2). High concentrations were found in the synaptosome fraction of all three brain regions with microsomal and mitochondrial fractions of the hypothalamus and the microsomal fraction of the parietal cortex having comparable levels of binding.

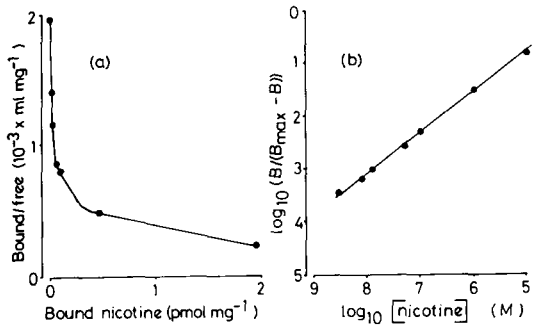

FIG. 1. Scatchard and Hill plots of nicotine binding to hippocampal homogenates. The plots were prepared from the results of 3 experiments carried out in triplicate, in which non-radioactive L-(−)-nicotine (3 nM–10 μM) was used to displace [^{3}H]nicotine (3 nM). The Hill coefficient (0·76) was determined by the method of least squares.

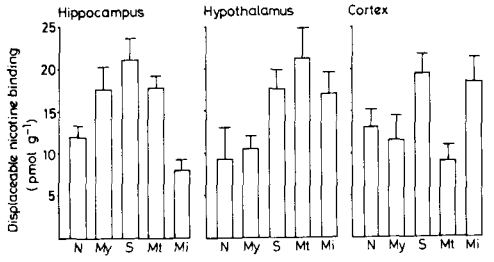

FIG. 2. Subcellular distribution of nicotine binding to discrete brain regions. Specific [^{3}H]nicotine binding was measured in nuclear (N), myelin (My), synaptosomal (S), mitochondrial (Mt) and microsomal (Mi) tissue using the procedure described in the methods section. Results are means ± s.e.m. of 3 experiments carried out in duplicate.

Table 1. Computer estimated K_I values for inhibitors of nicotine binding to high and low affinity sites in discrete brain regions.

Drug	K_I for high affinity binding site (nM) Hippocampus	Hypothalamus	Parietal cortex
(−)-Nicotine	2·2 ± 1·0	1·6 ± 0·1	2·6 ± 1·2
(+)-Nicotine	53·8 ± 6·7	188·1 ± 48·8	87·2 ± 8·7
DMPP	11·2 ± 4·6	20·1 ± 11·2	42·4 ± 48·9
Lobeline	62·8 ± 10·5	63·7 ± 19·2	5 2 ± 1·8
Carbachol	193·1 ± 46·5	89·6 ± 28·4	128·5 ± 33·6
Decamethonium	127·0 ± 78·0	208·0 ± 38·0	254·0 ± 87·0
Mecamylamine	503·0 ± 457·0	350·0 ± 483·0	391·0 ± 46·0
Hexamethonium	>1000	>1000	>1000
(+)-Tubocurarine	111·0 ± 5·0	81·0 ± 22·0	534·0 ± 199
α-Bungarotoxin	NM	NM	NM
Atropine	231·0 ± 168·0	>1000	173·0 29
	K_I for low affinity binding site (μM)		
(−)-Nicotine	1·6 ± 1·6	0·32 ± 0·02	0·5 ± 0·3
(+)-Nicotine	19·2 ± 8·8	31·3 ± 40·2	37·3 ± 18·1
DMPP	37·0 ± 23·0	2·5 ± 3·1	9·5 ± 12·3
Lobeline	5·8 ± 2·5	14·1 ± 7·9	3·5 ± 3·6
Carbachol	71·8 ± 26·2	55·5 ± 21·4	10·4 ± 5·7
Decamethonium	37·0 ± 25·0	49·0 ± 10·0	19·0 ± 6·0
Mecamylamine	25·0 ± 26·0	291·0 ± 93·0	65·0 ± 4·0
Hexamethonium	>1000	>1000	>1000
(+)-Tubocurarine	42·0 ± 49·0	39·0 ± 19·0	539·0 ± 960·0
α-Bungarotoxin	NM	NM	NM
Atropine	117·0 ± 20·0	860·0 ± 15·0	165·0 ± 11·0

Results are means ± s.e.m. from two separate experiments each performed in triplicate. NM: no displaceable binding could be measured.

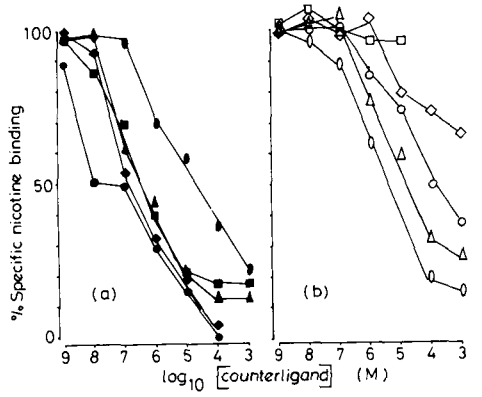

FIG. 3. Competition for [^{3}H]nicotine binding to hippocampal homogenates by cholinergic drugs. (a) Hippocampal homogenates were incubated with [^{3}H]nicotine (5 nM) in the presence of increasing concentrations of the cholinergic agonists L-(−)-nicotine (—●—), D-(+)-nicotine (—◆—), DMPP (—■—), lobeline (—▲—) and carbachol (—⬮—) Panel A. (b) Or the cholinergic blocking drugs, (+)-tubocurarine (—○—), α-bungarotoxin (—□—), hexamethonium (—◇—), mecamylamine (—△—) and decamethonium (—⬭—) Panel B. Specific binding is expressed as a percentage of the control binding (i.e. with no competing drug present). Curves are derived from at least 2 separate experiments, assayed in triplicate.

A preliminary examination of a wide range of pharmacological agents showed that drugs which interact with cholinoreceptors, especially nicotinic receptors agonists, were the most effective inhibitors of nicotine binding to brain tissue. More detailed studies showed that, of the nicotinic agonists tested, L-(−)-nicotine had the highest and carbachol the lowest affinity for the nicotine acceptor sites and that agonists were more effective inhibitors of binding than antagonists (Fig. 3a, b). Table 1 summarizes the computer estimates of the K_I values for the counter-ligands at the two binding sites measured in three regions of the brain. These results show that, while the properties of the acceptor sites in the three brain regions were generally similar, some of the ligands did appear to exhibit interregional differences in their affinities for the sites.

Both binding sites appeared to have a lower affinity for D-(+)-nicotine than the L-(−)-isomer although, because of the variation in the estimates of the K_I values for the low affinity sites, the differences between the two enantiomers only reached statistical significance ($P < 0·01$) for the high affinity sites. The

Table 2. Effects of chronic pretreatment with nicotine on the properties of nicotine binding sites in discrete brain regions.

Brain region	Chronic treatment	High affinity site K_D (nM)	High affinity site B_{max} (fmol (mg protein)$^{-1}$)	Low affinity site K_D (μM)	Low affinity site B_{max} (pmol (mg protein)$^{-1}$)
Hippocampus	Saline	8·6 ± 1·0	20·0 ± 2·0	40·0 ± 10·0	19·0 ± 4·0
	Nicotine	7·6 ± 1·0	19·0 ± 2·0	35·0 ± 10·0	20·0 ± 4·0
Hypothalamus	Saline	4·4 ± 0·5	16·1 ± 1·0	22·0 ± 3·0	18·0 ± 2·0
	Nicotine	6·1 ± 1·1	20·0 ± 2·0	26·0 ± 9·0	22·0 ± 6·0
Parietal cortex	Saline	9·7 ± 2·1	30·0 ± 3·0	33·0 ± 17·0	20·0 ± 8·0
	Nicotine	10·1 ± 2·1	29·0 ± 5·0	36·0 ± 6·0	22·0 ± 3·0
Striatum	Saline	8·1 ± 2·6	24·0 ± 4·0	21·0 ± 4·0	16·0 ± 2·0
	Nicotine	8·7 ± 2·1	19·0 ± 4·0	36·0 ± 18·0	23·0 ± 9·0
Mesencephalon	Saline	11·2 ± 2·6	24·0 ± 4·0	27·0 ± 8·0	20·0 ± 5·0
	Nicotine	9·0 ± 2·7	21·0 ± 5·0	25·0 ± 7·0	16·0 ± 3·0

Rats were treated with either saline or nicotine (0·4 mg kg^{-1} day^{-1}) for 39 days. On day 40, the animals were killed and nicotine binding measured as described in the methods section. Results are means ± s.e.m. of 6 experiments in each group.

Table 3. Effects of chronic nicotine treatment on nicotine accumulation by rat brain.

	[^{3}H]Nicotine Concn (nmol ml^{-1} or g^{-1}) Control 15 min	Control 90 min	Nicotine-treated 15 min	Nicotine-treated 90 min
Whole blood	2·9 ± 0·6	1·9 ± 0·2	2·7 ± 0·7	2·4 ± 0·6
Plasma	3·3 ± 0·7	2·2 ± 0·3	3·4 ± 0·7	2·0 ± 0·3
Hippocampus	6·0 ± 1·2	3·1 ± 0·4	5·6 ± 1·3	3·0 ± 0·4
P. cortex	6·4 ± 1·3	3·2 ± 0·5	6·4 ± 1·4	3·5 ± 0·5
Hypothalamus	5·0 ± 0·9	2·8 ± 0·3	5·0 ± 1·1	2·4 ± 0·5
Mesencephalon	4·8 ± 1·0	3·1 ± 0·7	4·9 ± 1·2	2·6 ± 0·4
Striatum	5·4 ± 1·0	3·2 ± 0·8	4·9 ± 1·4	3·0 ± 0·5
Medulla	3·4 ± 0·7	2·1 ± 0·3	3·8 ± 0·9	2·0 ± 0·4
Cerebellum	4·0 ± 0·6	2·7 ± 0·5	4·2 ± 0·9	2·3 ± 0·4

The tissue concentrations were measured in control and nicotine-treated (0·4 mg kg^{-1} s.c. for 39 days) rats 15 and 90 min after the injection of [^{3}H]nicotine (0·4 mg kg^{-1} s.c.) and are expressed as moles of nicotine which were equivalent to radioactivity measured in the samples. The results are means ± s.e.m. of 6 observations.

stereospecificity was most apparent in the hypothalamus which had a significantly lower affinity ($P < 0·05$) for D-(+)-nicotine than hippocampus.

The chronic administration of nicotine had no significant effects on the numbers of affinities of either of the acceptor sites in any of the brain regions studied (Table 2).

Studies on nicotine accumulation by brain tissue

Of the six brain regions investigated, parietal cortex accumulated the most radioactivity following the subcutaneous administration of [^{3}H]nicotine (Table 3). With the exception of the medulla, all the regions accumulated radioactivity above the concentration found in the plasma. The amount of radioactivity present in the brain samples 15 and 90 min after the nicotine injections was not significantly affected by chronic pretreatment with nicotine for 39 days (Table 3).

DISCUSSION

The characteristics of the nicotine binding sites, described in the present report, are similar to those reported by others (Yoshida & Imura 1979; Abood et al 1980; Romano & Goldstein 1980; Marks & Collins 1982) to the extent the data have shown that nicotine can bind to two sites in the brain, one with a high affinity for the alkaloid and the other having a much lower affinity. The results also agreed with those of Romano & Goldstein (1980) and Marks & Collins (1982) in that they showed that the high affinity site bound nicotine stereospecifically and had a high affinity for drugs which stimulate ganglionic nicotinic receptors whereas, in comparison, nicotinic antagonists had a low affinity for the site. However, the K_D and B_{max} values for the high affinity site reported here both appear to be somewhat lower than those reported in these previous papers. The Hill coefficient of less than unity, derived from the data, suggests that the two K_D values do not represent two allosteric conformations of the same site. Both sites were absent from the medulla but present in the remaining brain regions examined and, therefore, it is possible they form part of the same macromolecular complex. This, however, seems unlikely since the low affinity site did not show the regional differences in density observed for the high affinity site.

High and low affinity nicotine binding sites were found in all but one of the brain regions examined and there was little evidence to suggest that the sites located in the hippocampus were different to those

found elsewhere in the brain. In addition the study has shown that, while the hippocampus does appear to be a major site of nicotine accumulation in the brain other areas, notably parietal cortex and striatum, also accumulated at least as much radioactivity following the subcutaneous administration of [^{3}H]nicotine. The regions which accumulated most radioactivity were those which had the highest concentration of high affinity nicotine binding sites, whereas the medulla, which was devoid of these sites, failed to accumulate radioactivity above the concentration found in the plasma. The data suggest, therefore, that nicotine can enter the brain readily but that its accumulation in brain tissue may depend upon binding to acceptor sites present in the tissue. Therefore, the results would suggest that the regionally-specific changes in the concentration and biosynthesis of 5-HT in the hippocampus, reported previously (Benwell & Balfour 1979, 1982a), cannot be attributed to a regionally-specific nicotine acceptor site or to a specific accumulation of the alkaloid in that region of the brain.

Marks et al (1983) have reported that chronic infusions of nicotine cause an increase in the number of high affinity nicotine binding sites in rat brain and these authors have suggested that tolerance to some of the pharmacological responses to the drug may be associated with this change in acceptor site number. Similarly Schwartz & Kellar (1983) have shown that chronic subcutaneous injections of nicotine can increase the number of nicotinic acetylcholine binding sites in rat brain. In contrast the present investigation, in which nicotine was administered less frequently and at a considerably lower dose than those used in the reports cited above, has failed to show any effect of chronic nicotine administration on the number of nicotine binding sites or their affinities for nicotine. Previous studies, using an identical dosing schedule to that used in the present investigation have shown that rats become tolerant to the depressant effects of the alkaloid on locomotor activity (Morrison & Stephenson 1972; Stolerman et al 1973) and to its stimulatory effects on corticosterone secretion (Benwell & Balfour 1979). In addition the schedule has been used in experiments which provided behavioural and biochemical evidence that nicotine influences the mechanism by which rats adapt to repeated exposure to a psychological stress (Morrison 1974; Balfour & Morrison 1975; Benwell & Balfour 1982), effects that have been tentatively associated with the development of nicotine dependence (Morrison 1974; Balfour 1982; Benwell & Balfour 1982). Clearly the results reported here would suggest that these biochemical and behavioural responses to chronic nicotine administration are not related to changes in the number or binding characteristics of nicotine acceptor sites within the brain.

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