Effect of acute and subchronic nicotine treatment on cortical efflux of [^3H]-D-aspartate and endogenous GABA in freely moving guinea-pigs

¹Lorenzo Beani, Sergio Tanganelli, Tiziana Antonelli, Luca Ferraro, Michele Morari, Piero Spalluto, *Agneta Nordberg & Clementina Bianchi

Department of Pharmacology, University of Ferrara, Via Fossato di Mortara 23, 44100 Ferrara, Italy and *Department of Pharmacology, University of Uppsala, Sweden

1 The [^3H]-D-aspartate preloading of the parietal cortex of freely moving guinea-pigs equipped with epidural cups makes it possible to investigate drug effects on the efflux of this radiolabel, assumed as a marker of the glutamatergic structures underlying the cup. In the same model, the efflux of [^3H]- γ -aminobutyric acid ([^3H]-GABA) and endogenous GABA can be measured.

2 Nicotine, 0.9–3.6 mg kg⁻¹, s.c., or 3–5 μg , i.c.v., increased the efflux of [^3H]-D-aspartate but reduced that of GABA.

3 These effects were mediated through mecamylamine-sensitive receptors but the ganglionic blocking agent was devoid of any primary activity.

4 The inhibition of GABA efflux induced by nicotine 3.6 mg kg⁻¹, s.c., was abolished by methysergide 2 mg kg⁻¹, i.p. and was reduced by naloxone 3 mg kg⁻¹, i.p. pretreatment, suggesting the involvement of tryptaminergic and opioid systems. In contrast, muscarinic and catecholamine antagonists were ineffective.

5 Chronic treatment with nicotine (3.6 mg kg⁻¹, twice daily for 16 days) reduced the facilitatory effect of [^3H]-D-aspartate and abolished the inhibition of endogenous GABA efflux.

6 A slight increase in the number of nicotinic binding sites (by use of [^3H]-nicotine as ligand) was found in the neocortex of chronically treated guinea-pigs.

7 The higher degree of tolerance to chronic nicotine treatment shown by GABA as compared with [^3H]-D-aspartate efflux suggests that adaptative changes of the inhibitory neuronal pools prevail. This may contribute to the reinforcing and addictive properties of nicotine.

Keywords: Nicotine; guinea-pig cortex; mecamylamine; methysergide; naloxone; GABA efflux; D-aspartate efflux

Introduction

Nicotine is known to increase the firing rate of many neuronal pools and to enhance the release of a number of putative neurotransmitters, by interacting with receptors located on the cell bodies and/or nerve terminals (see Nordberg *et al.*, 1989). However, only a few investigations have been devoted to analyzing effects of nicotine on the amino acid transmitters. To date, the increased firing rate of the cerebellar glutamatergic cells (de la Garza *et al.*, 1987a), as well as enhanced glutamate release from n. accumbens slices (Gongwer *et al.*, 1987) by nicotine, have been reported. Conversely, the available data regarding γ -aminobutyric acid (GABA) are conflicting. Behavioural (Austin *et al.*, 1988) and electrophysiological studies *in vivo* (de la Garza *et al.*, 1989b) and *in vitro* (Freund *et al.*, 1988) suggest that nicotine inhibits the GABAergic structures, possibly by releasing dopamine (Austin *et al.*, 1988) or endogenous opioids (Brennan *et al.*, 1980; Dewar *et al.*, 1987). On the other hand, Limberger *et al.* (1986) and Wonnacott *et al.* (1987, 1989) reported that nicotine increased GABA efflux from caudatal slices and hippocampal synaptosomes, whereas Gongwer *et al.* (1987) did not find any effect on n. accumbens slices. These discrepancies may be due to the different brain areas studied and to the more or less integrated models employed. Since direct measurements of nicotine-induced changes of glutamate and GABA release *in vivo* are lacking, it was considered of interest to study nicotine effects on [^3H]-D-aspartate (assumed as a marker of endogenous glutamate) and on endogenous GABA overflow from the cerebral cortex of freely moving guinea-pigs, equipped with epidural cups.

The data here summarized demonstrate that nicotine, at doses considerably higher than those affecting acetylcholine (ACh) release (Nordberg *et al.*, 1989), increases the efflux of [^3H]-D-aspartate but inhibits that of GABA, through mecamylamine-sensitive receptors. These effects are reduced by chronic drug treatment, particularly as regards nicotine inhibition of GABA overflow. A preliminary account of this investigation has been published (Beani *et al.*, 1989).

Method

Guinea-pigs of either sex weighing 400–500 g were used. The animals were kept under a 12 h light/dark cycle and had free access to food and water. Implantation of the epidural cup on the left or right parietal cortex was performed under nembutal (30 mg kg⁻¹, i.p.) plus morphine (2 mg kg⁻¹, i.p.) anaesthesia, as previously described (Beani *et al.*, 1968; Beani & Bianchi, 1970). In some animals an intracerebroventricular guide cannula was also placed on the side contralateral to the cup to perform i.c.v. injections. Two days after surgery the animals were used for the release experiments.

[^3H]-D-aspartate efflux

Preliminary trials were carried out by placing a saline solution of [^3H]-D-aspartate (final concentration 0.6 $\mu\text{mol l}^{-1}$, specific activity 13.5 Ci mmol⁻¹) into the epidural cup for 5 h, in order to load the underlying cortex and to study drug effects over the washout profile curve (Beani *et al.*, 1989). Subsequently, with the aim of increasing the radiolabel uptake and efflux, the following procedure was applied: at the beginning, 0.4 ml of KCl 50 mmol l⁻¹ was placed into the cup for 30 min; then, 0.4 ml of Krebs solution containing [^3H]-D-aspartate

¹ Author for correspondence.

$0.3 \mu\text{mol l}^{-1}$ was kept in contact with the cortex for 4 h. After repeated and careful washing, 0.4 ml of normal Krebs solution was placed into the cup and renewed every 15 min. Drug treatment was performed after 4 collection periods of 15 min each, i.e., when the washout curve was levelling off to almost steady values.

The [^3H]-D-aspartate content of the samples (added to the scintillation liquid) was counted with a beta spectrometer (Beckman LS 1800). Some samples were run through a Dowex column CH_3COO^- form (Gaitonde, 1973), and eluted with CH_3COOH , 2 mol l^{-1} , so as to establish that their radioactivity was associated with [^3H]-D-aspartate standards. In addition, the amounts of [^3H]-D-aspartate present at the different depths of the normal and KCl-pretreated cortex were measured. After the animals were killed, the disk (3 mm diameter) of the whole cortex underlying the cup was excised immediately before starting the washout curve. Slices $400 \mu\text{m}$ thick were cut along a plane parallel to the cortical surface, dissolved in protosol and counted for their radioactivity.

GABA efflux

To study the efflux of endogenous GABA, 0.4 ml of Krebs solution containing ethanolamine-O-sulphate 2 mmol l^{-1} (to inhibit GABA metabolism, Beani *et al.*, 1988) was placed in the cup and renewed every 30 min for 3–4 h. The GABA content of the samples was measured with the GC-MS technique as detailed previously (Beani *et al.*, 1988).

Since the analysis of nicotine effects on glutamatergic neurones was carried out for convenience (see Discussion) by prelabelling the cortex with [^3H]-D-aspartate, some experiments were performed by measuring [^3H]-GABA outflow, so as to compare nicotine-induced changes in endogenous and ^3H -labelled amino acid. To this purpose, ethanolamine-O-sulphate pretreatment was performed as above for 3–4 h. KCl, 50 mM was then applied for 30 min, followed by [^3H]-GABA $0.3 \mu\text{mol l}^{-1}$ (50 Ci mmol^{-1}) preloading for 4 h. After careful washing, 0.4 ml of Krebs solution (containing ethanolamine-O-sulphate 2 mmol l^{-1}) was placed in the cup and renewed every 15 min. Nicotine was injected s.c. after 4 collection periods when the tritium efflux had become relatively steady. Since GABA metabolism was inhibited by ethanolamine-O-sulphate pretreatment, no attempt was made to ascertain to what extent the effluent tritium remained associated with GABA. In fact, under similar experimental conditions about 80%–90% of tritium proved to be represented by [^3H]-GABA (Young *et al.*, 1990). The samples were then measured for their tritium content as for [^3H]-D-aspartate (see above).

Drug treatment

In naive animals, (–)-nicotine bitartrate was injected s.c. (0.5 ml of saline solution adjusted to pH 7) 60–90 min after starting sample collection so as to determine the basal values of [^3H]-D-aspartate and GABA overflow as well as the drug effect on them. The time schedule of other drug treatments has been specified in Results.

Other animals were submitted to chronic nicotine treatment. The drug was injected s.c., twice daily for 16 days, at 3.6 mg kg^{-1} per day, i.e. at a dosage level 8 times higher than that previously tested for the experiments on acetylcholine release (Nordberg *et al.*, 1989). Saline-treated animals were run in parallel. On the 15th day, an epidural cup was implanted and the [^3H]-D-aspartate and GABA release experiments were carried out two days later. On the day of the trial, the same protocol of drug treatment as for acute nicotine experiments (injection at 9 h 00 min) was followed.

Nicotine receptor binding studies

Twelve hours after the last injection of nicotine, the animals were killed, their cerebral cortex dissected and processed for binding measurements with [^3H](–)-nicotine (7 nmol l^{-1} ;

specific activity 80 Ci mmol^{-1} , Amersham, U.K.) and [^3H]-acetylcholine (10 nmol l^{-1} ; specific activity 81 Ci mmol^{-1} , Amersham, U.K.) in the presence of atropine $1 \mu\text{mol l}^{-1}$ (Nordberg *et al.*, 1989). The rationale for using two ligands was to label high affinity ([^3H]-acetylcholine) as well as additional low affinity ([^3H]-nicotine) binding sites (Nordberg *et al.*, 1988; Romanelli *et al.*, 1988) which have been found to be significantly increased in rats following chronic nicotine treatment at the low dose of 0.45 mg kg^{-1} but not in guinea-pigs (Nordberg *et al.*, 1989).

Drugs

Freshly prepared solutions of the following drugs were used: (–)-nicotine bitartrate, mecamylamine HCl, ethanolamine-O-sulphate, atropine sulphate, haloperidol and methysergide maleate (Sigma Chemical Co., St. Louis, MO, U.S.A.), naloxone (Salars, Italy). The doses are expressed as the bases. The radiochemicals and protosol were purchased from NEN.

Statistical analysis

Statistical differences were checked by ANOVA analysis followed by Dunnett's test and Newman-Keuls multiple range test, as specified in the table and figure legends.

Results

Assessment of the [^3H]-D-aspartate overflow method

The hypotonic KCl solution applied for 30 min gave rise to mild signs of local irritation, as shown by the appearance of occasional clones in the foreleg contralateral to the cup and by the development of spiking in the unipolar EEG, recorded from inside the cup (for technical details see Beani *et al.*, 1968). These signs rapidly disappeared soon after removal of the KCl solution. The total amount and the intracortical distribution of [^3H]-D-aspartate was nearly the same in KCl-treated and in control animals. However, the [^3H]-D-aspartate released was larger in KCl-treated than in control guinea-pigs (Table 1). In both instances, the tritium of the cup samples was exclusively represented by [^3H]-D-aspartate, as checked with the Dowex column separation (data not given), but its concentrations were higher and drug induced changes were more uniform in KCl-treated animals (Table 1). Interestingly, the tritium ratio between the loading solution of [^3H]-D-aspartate in the epidural cup (0.3 nmol ml^{-1}) and the superficial layers of the cortex 30 min after washing out the radiolabel (about 18 pmol g^{-1} see Table 1) was about 17:1, not far from the ratio between the loading solution in the microdialysis fibre and the tissue surrounding the probe as reported by Nielsen *et al.* (1989).

Table 1 Effect of KCl pretreatment on [^3H]-D-aspartate content per g of fresh tissue and the total efflux (150 min) from the cerebral cortex of freely moving guinea-pigs

Experimental conditions	N° of expts.	[^3H]-D-aspartate (pmol)		Efflux as % of content
		content	efflux	
KCl pretreatment	5	27.8 ± 3.7^1	1.49 ± 0.27	66 ± 2.8
No pretreatment	5	27.7 ± 4.6^1	1.08 ± 0.32	$46 \pm 7.9^*$

* Statistically significant difference from the efflux of the non pretreated cortex, $P < 0.05$. ¹ The [^3H]-D-aspartate content in the first (superficial), second, third and fourth (deep) slice (thickness $400 \mu\text{m}$), calculated as percentage of the total content in the cortical disk (see methods) was 65 ± 10 (i.e., 18 pmol g^{-1}), 21 ± 3 , 10 ± 3 and 3 ± 1 , respectively both in normal and KCl-treated animals.

Effect of (–)-nicotine on [^3H]-D-aspartate

Nicotine, at doses below 0.9 mg kg^{-1} , did not change the [^3H]-D-aspartate overflow. Starting from 0.9 mg kg^{-1} , the drug induced a short-lasting (15–20 min) dose-related increase in the amino acid outflow, which reached the value of +70% above the basal levels at 3.6 mg kg^{-1} (Figure 1a). An evident increase in [^3H]-D-aspartate was also detected by injecting nicotine i.c.v. (Figure 2). The response to nicotine was prevented by local pretreatment of the cerebral cortex with tetrodotoxin $2\text{ }\mu\text{mol l}^{-1}$, which also reduced the basal overflow by about 40% (Figure 1b). This suggests that the nerve conducted activity contributed both to the basal and to drug-

Figure 1 (a) Correlation between nicotine doses and [^3H]-D-aspartate efflux from the parietal cortex of normal guinea-pigs (●; $b = 82.4$, $r = 0.62$, $P < 0.01$) and mecamlamine-pretreated (8 mg kg^{-1} , i.p. 60 min before) animals (■; $b = 94.2$, $r = 0.72$, $P < 0.01$). The effect of nicotine on subchronically pretreated animals (see methods) is also shown (□). (b) Effect of topical application of tetrodotoxin (●, TTX) on the [^3H]-D-aspartate efflux induced by nicotine 3.6 mg kg^{-1} (at arrow). The effect of nicotine alone is shown for comparison (□). The spontaneous efflux in normal animals is also shown (○). Each point is the mean of 5–10 animals; vertical lines show s.e.mean. The values are normalized with respect to pre-injection efflux (the average efflux of [^3H]-D-aspartate before treatment was $0.694 \pm 0.1\text{ pmol}$ in 15 min). The difference from control is statistically significant (* $P < 0.05$), according to ANOVA and Dunnett's test. The difference from nicotine alone is statistically significant († $P < 0.05$) according to ANOVA and Newman-Keuls multiple range test.

Figure 2 Effect of nicotine ($5\text{ }\mu\text{g}$ at arrow) injected i.c.v. on the [^3H]-D-aspartate efflux from the parietal cortex of freely moving guinea-pigs. Each point is the mean of 3 animals; vertical lines show s.e.mean. The values are normalized with respect to the pre-injection release (average efflux of [^3H]-D-aspartate before treatment was $0.71 \pm 0.06\text{ pmol}$ in 15 min). The difference from basal efflux is statistically significant (* $P < 0.05$, ** $P < 0.01$) according to ANOVA and Dunnett's test.

induced release. The local application of the toxin did not have any discernible behavioural effect. Mecamlamine, 2 mg kg^{-1} i.p. injected 30 min before nicotine, did not itself change the profile of the washout curve but reduced the effectiveness of nicotine in a competitive fashion, shifting the dose-response curve to the right by a factor of about 2.5 (Figure 1a).

Effect of nicotine on GABA overflow

At the same doses that increased the [^3H]-D-aspartate efflux, nicotine reduced [^3H]-GABA (Figure 3a) and endogenous GABA overflow to similar extents (Figure 3b). The effect of the drug on the endogenous amino acid release was dose-related at 90 min ($r = 0.65$, $P < 0.05$). At variance with the time course of [^3H]-D-aspartate efflux, the inhibition of GABA overflow was long-lasting (about 2 h). Similar results were found when nicotine was injected i.c.v. (Figure 3c). Mecamlamine 2 mg kg^{-1} completely prevented the inhibition (Figure 3b). The local treatment with tetrodotoxin ($0.5\text{ }\mu\text{mol l}^{-1}$ in the epidural cup for 30 min) consistently reduced the basal efflux as previously observed (Antonelli *et al.*, 1986) and prevented any further GABA reduction by nicotine 3.6 mg kg^{-1} (Figure 3d). Since nicotine is known to excite directly most of the neurones to which it is applied (Egan, 1989), the inhibition of GABA outflow suggested a drug-induced release of other transmitters which, in turn, inhibited GABA cells. Therefore, the possible involvement of some neuronal pools was tested by pretreating the animals with the proper antagonists. First of all atropine, haloperidol and prazosin were checked. None of these drugs modified the nicotine-induced GABA overflow reduction (Figure 4a). Conversely naloxone 3 mg kg^{-1} partially prevented it, while methysergide 2 mg kg^{-1} abolished any nicotine effect (Figure 4a and b).

Figure 3 γ -Aminobutyric acid (GABA) efflux from the parietal cortex of freely moving guinea-pigs. (a) Effect of nicotine 3.6 mg kg^{-1} (arrow, $\bullet$) injected s.c. on $[^3\text{H}]$ -GABA efflux. The spontaneous efflux in controls is also shown ($\circ$). (b) Effect of nicotine (arrow) injected s.c. (Δ , 0.9 ; $\square$, 1.8 ; $\circ$, 3.6 mg kg^{-1} and $\bullet$, 3.6 mg kg^{-1} 30 min after mecamylamine 8 mg kg^{-1}). (c) Effect of nicotine ($3 \mu\text{g}$ $\bullet$, and $5 \mu\text{g}$ Δ , arrow) injected i.c.v. (d) The topical application of tetrodotoxin (TTX) prevents the inhibition of GABA efflux by nicotine 3.6 mg kg^{-1} (arrow). Each point is the mean of 5–6 animals; s.e.mean shown by vertical lines. The values are normalized with respect to the pre-injection release (average efflux before treatment was $0.036 \pm 0.0039 \text{ pmol}$ in 15 min for $[^3\text{H}]$ -GABA and $26 \pm 3.9 \text{ pmol cm}^{-2} \text{ min}^{-1}$ for endogenous GABA). The difference from the basal efflux is statistically significant ($*P < 0.05$; $**P < 0.01$) according to ANOVA and Dunnett's test. The difference from spontaneous efflux in controls is statistically significant ($\dagger P < 0.05$; $\dagger\dagger P < 0.01$) according to ANOVA and Newman-Keuls multiple range test.

Effect of chronic (–)-nicotine treatment

In chronically treated animals a challenging dose of nicotine 3.6 mg kg^{-1} , administered 12 h after the last injection (according to the 16 day treatment schedule) caused an increase in $[^3\text{H}]$ -D-aspartate efflux of only +25% (instead of

Figure 4 (a) Effect of nicotine 3.6 mg kg^{-1} ($\circ$) (arrow) on γ -aminobutyric acid (GABA) efflux from the cerebral cortex of freely moving guinea-pigs pretreated: with prazosin $15 \mu\text{g kg}^{-1}$ (Δ), methysergide 2 mg kg^{-1} ($\bullet$) (injected 30 min before); atropine 10 mg kg^{-1} ($\blacksquare$) and haloperidol 2 mg kg^{-1} ($\square$) (injected 60 min before). (b) Effect of nicotine 1.8 mg kg^{-1} ($\square \cdots \square$) and 3.6 mg kg^{-1} ($\circ \cdots \circ$) (arrow) in guinea-pigs pretreated; with naloxone 3 mg kg^{-1} i.p. ($\blacksquare$) 15 min before nicotine 1.8 mg kg^{-1} and ($\bullet$) before nicotine 3.6 mg kg^{-1} . Each point is the mean of 5–6 animals; s.e.mean shown by vertical lines. The values are normalized with respect to the pre-injection release. The average release before treatment was $27.5 \pm 2.6 \text{ pmol cm}^{-2} \text{ min}^{-1}$. The difference from nicotine alone is statistically significant ($*P < 0.05$) according to ANOVA and Newman-Keuls multiple range test.

+75% detected in naive animals). The recovery of the initial response was obtained with a dose of 7.2 mg kg^{-1} , indicating the development of moderate tolerance (Figure 1a). Conversely in chronically-treated guinea-pigs, nicotine 3.6 mg kg^{-1} no longer inhibited GABA outflow.

Binding studies

An apparent up-regulation of nicotinic binding sites was previously found in rats but not in the guinea-pigs chronically treated with nicotine 0.45 mg kg^{-1} (Nordberg *et al.*, 1989). Such up-regulation was also evident in this latter species treated with nicotine 3.6 mg kg^{-1} (Table 2), but the extent of this change barely passed the limits of statistical significance and was restricted to the experiments with $[^3\text{H}]$ -nicotine as a ligand.

Discussion

In a previous study (Nordberg *et al.*, 1989) it was shown that the time course and extent of the nicotine (0.45 – 0.9 mg kg^{-1}) effect on acetylcholine release from the cortex of rats and guinea-pigs was much the same, suggesting a similarity in the responsiveness of the two species to this drug. Since guinea-pigs are more suitable for the cortical cup technique, the decision was taken to make further investigations into the effect of nicotine on amino acid release in this last species alone.

The unmetabolizable analogue of glutamate, $[^3\text{H}]$ -D-aspartate, has been widely used to study the activity of the glutama-

Table 2 Nicotinic receptors in the guinea-pig cerebral cortex following chronic treatment with nicotine (3.6 mg kg^{-1}), twice daily for 16 days

Experimental conditions	Receptors	
	$[^3\text{H}]$ -ACh	$[^3\text{H}]$ -nicotine
Controls	1.8 ± 0.2 (5)	7.4 ± 0.50 (12)
Nicotine	2.4 ± 0.30 (5)	9.7 ± 1.08 (5)*

The values are $\text{pmol g}^{-1} \text{ protein} \pm \text{s.e.mean}$. The number of animals is shown in parentheses. *Significantly different from controls, $P < 0.05$.

tergic neurones *in vitro* and *in vivo* (Davies & Johnston, 1976; Huxtable *et al.*, 1985; Neilsen *et al.*, 1989). Recently, identical drug effects on the release of endogenous glutamate and [^3H]-D-aspartate from hippocampal slices have been reported (Marchi *et al.*, 1990). The choice of [^3H]-D-aspartate seems particularly convenient for studying glutamatergic neurone function *in vivo* since the glutamate coming from the blood can make a significant contribution to the overall amount of the amino acid present in the cup samples. This interference may overshadow drug effects on the neuronal release of this putative transmitter. The simple analysis of the [^3H]-D-aspartate washout curve was considered the best way to obtain information about the direction of nicotine-induced changes in amino acid release. In fact, the local application of selective inhibitors of glutamate uptake and/or metabolism (in order to amplify the responses) increases its extracellular concentration and, consequently, influences the activity of the entire neuronal network under the cup, thus complicating interpretation of the results. Topical pretreatment with a hypotonic KCl solution appears to be a procedure which increases the validity of the method, as shown by the higher amounts of [^3H]-D-aspartate released and by the more uniform responses in comparison with animals not pretreated.

The present findings clearly show that nicotine dose-dependently affected, in the opposite direction, [^3H]-D-aspartate and endogenous (or ^3H -labelled) GABA overflow from the cerebral cortex of naive, freely moving guinea-pigs. Its effect appears to be central in origin (Figures 2 and 3c) since nicotine injected i.c.v. gave rise to a response similar to that after s.c. administration.

Clearly, a peripheral component, as suggested by Hajos & Engberg (1988), cannot be ruled out. The doses required to change GABA and [^3H]-D-aspartate efflux were higher than those able to increase ACh release, indicating that the cholinergic structures impinging upon the neocortex are more responsive to nicotine than are the amino acid neurones. These neurones clearly respond to nicotine 3.6 mg kg^{-1} s.c., a dose which is 7–9 times lower than the LD_{50} (s.c.) in rats and guinea-pigs (Negherbon, 1959). As previously shown for the increase in cortical ACh *in vivo* (Nordberg *et al.*, 1989), these effects, are also mediated through nicotinic receptors of the neuronal type, as they are prevented by mecamylamine. On the other hand, the lack of any influence of mecamylamine on the basal [^3H]-D-aspartate efflux (as well as on basal GABA efflux) seems to rule out any physiological cholinergic nicotinic control over glutamate and GABA neurones in normal animals.

The excitatory properties generally attributed to nicotine, may be directly responsible for the increased [^3H]-D-aspartate overflow, the prevailing neuronal origin of which has been demonstrated (Figure 1). Thus, the present data are in accordance with those of de la Garza *et al.* (1987a) *in vivo* and with the report of Gongwer *et al.* (1987) *in vitro*. Conversely, the inhibition of GABA release in the conscious animal appears to be an indirect effect, due to the stimulation of nicotinic receptors located on other neuronal systems which, in turn, inhibit GABA cells. Clearly, this mechanism may no longer be operative in brain slices and is surely absent in synaptosomes.

Therefore, these simplified preparations may display excitatory responses to nicotine (Limberger *et al.*, 1986; Wonnacott *et al.*, 1989) provided some nicotinic receptors are present

in the structures releasing the transmitter under study. Since atropine, haloperidol and prazosin did not prevent the reduction in GABA release, it is conceivable that the well-documented changes in acetylcholine, dopamine and noradrenaline release induced by nicotine (Balfour, 1982; Grenhoff & Svensson, 1989) are not the reason for the reduction in GABA overflow from the guinea-pig parietal cortex. Instead, the present results agree with the conclusion of Brennan *et al.* (1980) and Dewar *et al.* (1987) that the endogenous opioids released by nicotine are involved in the GABA response (Figure 4a and b). In addition, the results shown in Figure 4a demonstrate for the first time that 5-hydroxytryptamine (5-HT) plays a crucial role in the nicotine-induced inhibition of GABA release. In fact, methysergide prevented nicotine effects. In view of the existence of many 5-HT receptor subtypes, and taking into account the complexity of the *in vivo* model, further studies are needed to define the subtype and the location of these receptors.

The different duration of nicotine-induced changes in [^3H]-D-aspartate and GABA overflow is another point requiring some comment. In fact, the facilitation of [^3H]-D-aspartate is short-lived while the inhibition of GABA release is long lasting. Considering the different time courses of nicotine effects on the release of other putative transmitters (Imperato *et al.*, 1986; Nordberg *et al.*, 1989) a complex neurochemical (and behavioural) picture arises. This may explain the biphasic nicotine influence on locomotor activity of the rat (inhibition followed by facilitation, see Freeman *et al.*, 1987; Clarke & Kumar, 1983) as well as the increased alertness and decreased anxiety, the two overlapping stages of behavioural and subjective signs caused by nicotine in smokers (Pomerleau, 1986).

The results of the studies carried out in chronically treated guinea-pigs fit well with the expectations. In fact, as previously reported for the nicotine-induced increase in cortical ACh release (Nordberg *et al.*, 1989), even the increase in [^3H]-D-aspartate was less evident after chronic treatment. However, the inhibition of GABA efflux was no longer evident. This different degree of tolerance displayed by the glutamatergic and GABAergic cells confirms previous observations on the prevailing tolerance to the inhibitory nicotine effects on behaviour (Morrison & Stephenson, 1972). The persistent glutamatergic response may contribute to the maintenance (or enhancement) of the locomotor activation in chronically treated animals and may be another factor determining the reward in man (Pomerleau & Pomerleau, 1984; Russell, 1989), together with the well documented maintenance of the dopamine response (Clarke, 1990).

The moderate increase in the number of the nicotinic binding sites, which is limited to [^3H]-nicotine as ligand and can be detected only after 3.6 mg kg^{-1} per day for 16 days (but not after 0.45 mg kg^{-1} per day, see Nordberg *et al.*, 1989), confirms that, in the guinea-pig, the adaptation to chronic nicotine is not associated with consistent changes in the number or affinity of the receptors, as in other animal species, including man (Nordberg *et al.*, 1989; Wonnacott, 1990). However, in every instance, reduced responsiveness to the chronic drug administration develops.

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