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Research Report

Nicotine-induced changes of glutamate and arginine in naive and chronically alcoholized rats: An in vivo microdialysis study

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ABSTRACT

The effects of nicotine, when administered either acutely or chronically, at doses of 0.15, 0.3 or 0.6 mg/kg, on the release of glutamate and arginine in the rat nucleus accumbens have been studied in microdialysis experiments. Glutamate release significantly increased after acute nicotine injection, 0.3 mg/kg, which was accentuated if there was a priming regime of saline for the previous 27 days. This is possibly related to the rewarding effects of nicotine. Five hours after cessation of chronic oral nicotine administration, there were significant increases in glutamate content, which was possibly reflective of a withdrawal process. Significant decreases in nucleus accumbens arginine release were evident, between 1 and 2 h, after chronic nicotine administration. When nicotine was co-administered to rats during chronic ethanol intoxication, at either 0.15 mg/kg or 0.3 mg/kg doses, glutamate release did not increase during the first 12 h of withdrawal. However, a decrease in arginine microdialysate content was still observed with all nicotine doses. The nicotine-induced changes in glutamate and arginine release in nucleus accumbens highlights the complex neuropharmacological interactions evoked by this compound and also identified its possible modulating effect on glutamate release during the initial stages of chronic ethanol withdrawal.

1. Introduction

The neuropharmacology of addiction and withdrawal involves complex interactions between a variety of neurotransmitters and their receptors in a number of specific brain regions (Weiss, 2005). Drugs, such as cocaine, amphetamines and nicotine are psychomotor stimulants (Yamada and Nabeshima, 2004). Opiates and alcohol are more generally considered to be sedative compounds (Fantegrossi et al., 2005; Maas et al., 2005; Viviand and Garnier, 2004), although they can increase locomotor activity within a narrow dose range. Such drugs, however, may have a common mode of action by their interaction with related substrates (Kovacic, 2005). Their reinforcing effects are mediated by enhanced activity of the cortico-mesolimbic dopamine system (Clark and Little, 2004). Other neurotransmitter systems, e.g., the cholinergic system, as well as GABA, glutamate, serotonin, endogenous opioids and nitric oxide, are also involved in the addictive properties of these drugs (Kalivas, 2004; Markou and Kenny, 2002; Salin-Pascual et al., 2003). Co-administration of such drugs may

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result in neurochemical and behavioral interactions which leads to aggregation of certain effects, while withdrawal will induce a variety of biochemical effects. These range from deficits in accumbal dopamine release, interaction between cholinergic and opioid receptor systems, and changes in serotonin, glutamate and nitric oxide release in specific brain regions (reviewed in Kenny and Markou, 2001).

It is well known that there is a high rate of co-occurrence between smoking and alcohol (de Fiebre and de Fiebre, 2003; Little, 2001); in fact between 70 and 90% of alcoholic subjects also smoke (Batel et al., 1995; Sher et al., 1996) which may indicate that common genes may influence both alcohol and tobacco abuse (reviewed in Madden and Heath, 2002). Such cross-tolerance may be caused by increasing the rewarding effects or decreasing the aversive action of the other; for example, in animal studies Al Rejaie and Dar (2006) showed that there were significant attenuations of ethanol-induced motor impairment after 5 daily intracerebellar nicotine injections while Clark and Little (2004) identified a complex interaction in the ability of these two drugs, alone or in combination, at certain concentrations, to alter the firing rate of ventral tegmental dopamine neurones. Therefore, complex alcohol–tobacco interactions may exist.

Both of these drugs activate nicotinic acetylcholine receptors (nAChRs), which play a significant role in mediating both their rewarding and withdrawal effects. Nicotinic cholinergic receptors, nAChRs, are pentameric ligand-gated ion channels composed of α (α2–10) and β (β2–4) subunits. Studies where nicotinic antagonists have been used, and more recently genetic studies, have implicated β2 and α7 nAChR subunits in nicotine reward (reviewed in Corringer et al., 2006). Indeed, mice lacking the β2 nAChR subunit show no nicotine-induced reward (Grottick et al., 2000; Picciotto et al., 1998). High-affinity receptors, α4 and β2 subunits were upregulated after nicotine administration (Marks et al., 1992; Perry et al., 1999), at the low micromolar range (Parker et al., 2004). Ethanol acts as an allosteric activator of heteromeric nAChRs, including α4β2 (Cardoso et al., 1999; Covernton and Connolly, 1997; Criswell et al., 1993), α3, β2 and α6, in ethanol's stimulating effects (rewarding and locomotor activity) while α4-containing nAChRs modulate ethanol withdrawal (Butt et al., 2004). In contrast, α7 subunit functional activity is inhibited by ethanol (Bowers et al., 2005). Thus, nicotinic acetylcholine receptors subunits are likely to be one of the targets for the interaction between ethanol and nicotine.

Accumulating evidence suggests that glutamatergic neurotransmission is also involved in the dependence-producing effects of nicotine. Nicotine enhances the release of glutamate in the mesolimbic DA system while glutamate receptor antagonists inhibit the nicotine-induced release of dopamine in the nucleus accumbens (Nac) (Kenny and Markou, 2001). The NR2B subunit is potentiated by both acute and chronic nicotine administration and shows regional specificity (Kosowski, 2005; Kosowski et al., 2004). Both NR2A and NR2B subunit types are also upregulated after chronic ethanol administration (Nagy, 2004) and mediate many of the deleterious effects of ethanol, e.g., development of tolerance and dependence, as well as the acute and delayed signs of ethanol withdrawal dependence. Thus, glutamate-mediated neurotransmission is likely to play an important role in regulating the physiological and behavioral actions of both nicotine and ethanol with relevance to addiction.

Cessation from smoking induces an aversive withdrawal syndrome in humans, which is caused by the reduction in nicotine intake, exemplified by craving, depressed mood, dysphoria and anxiety. The severity of such withdrawal symptoms is proportional to the amount of nicotine exposure, particularly in rats, but not necessarily in man. A reduction in endogenous cholinergic tone, deficits in accumbal dopamine release, an inhibitory influence of somatodendritic 5-HT_{1A} autoreceptors, as well as enhanced glutamate release may all play a role in mediating such symptoms (reviewed in Kenny and Markou, 2001). It is important to note that nAChRs antagonists precipitate but do not alleviate the nicotine withdrawal symptoms in nicotine-dependent subjects.

Thus, previous studies have identified specific receptors subtypes which are involved in nicotine and ethanol addiction and withdrawal. However, there have been few studies of nicotine-induced changes in glutamate release, in vivo, after either an acute or chronic administration. Furthermore, it was of interest to ascertain what effect nicotine administration might have on glutamate release during ethanol withdrawal, specifically, whether it might modulate or exacerbate the neurotoxicity of drug withdrawal. Arginine, the substrate for nitric oxide synthase, was also analyzed in the NAc microdialysate, since previous studies had suggested that total nitric oxide products (nitrite + nitrate) was increased or decreased by intracerebellar nicotine or acute ethanol administration, respectively. This could indicate that nitric oxide is a factor involved in the cross-tolerance between nicotine and ethanol (Al Rejaie and Dar, 2006).

2. Results

2.1. *Basal levels of microdialysate amino acids*

There were no consistent significant changes in the basal concentrations of either glutamate or arginine after intraperitoneal nicotine injections when administered acutely, in combination with priming or withdrawal periods and daily for 28 days (data not shown). However, chronic oral administration of nicotine alone (0.3 mg/kg/day) increased glutamate release 4-fold [$F(3,36)=24.503$; $p<0.0001$]. When nicotine (0.15 mg/g/day) was administered in combination with chronic alcoholization the basal microdialysate content was reduced 2 fold [$F(3,36)=6.717$; $p=0.001$]. Chronic nicotine administration p.o., at all doses, significantly reduced the basal arginine content in the nucleus accumbens (Table 1). This dramatic reduction in arginine microdialysate content was further enhanced in rats which were chronically administered both nicotine and ethanol, particularly with the 0.15 and 0.6 mg/kg nicotine dose (Table 1).

2.2. *Acute intraperitoneal nicotine injection*

After an acute i.p. nicotine injection, glutamate microdialysate content was transiently significantly increased with the 0.3 mg/kg dose [$F(17,357)=2.278$; $p=0.0029$] (Fig. 1A). There were significant effects on arginine with all doses, [$F(4,41)=5.418$;

Table 1 – Basal concentration (μM) of glutamate and arginine in the nucleus accumbens at the commencement of the microdialysis studies after chronic nicotine p.o. ± chronic ethanol administration for 50 days

	Glutamate (μM)	Arginine (μM)
Chronic nicotine p.o.		
Control	3.6±0.6	14.8±2.0
0.15 mg/kg	3.8±0.4	3.9±0.7**
0.3 mg/kg	14.0±1.4**	9.1±1.2**
0.6 mg/kg	5.6±1.2	4.0±0.4**
Chronic co-administration of ethanol and nicotine		
Control	3.6±0.6	14.8±2.0
Chronically alcoholized	5.2±0.7	8.6±1.3*
0.15 mg/kg	2.5±0.5*	2.0±0.4**,††
0.3 mg/kg	4.2±0.5	8.4±2.0*
0.6 mg/kg	5.4±0.6	0.3±0.1**,††

Data are presented as mean±SEM. Significance is calculated either by comparison to the saline control, *$p<0.05$ and **$p<0.01$, or by comparison to the chronically alcoholized rats, †$p<0.05$ and ††$p<0.01$ (ANOVA 1 followed by Dunnett's procedure).

$p=0.0013$], the baseline level of arginine was maintained for each nicotine dose, while arginine levels in the nucleus accumbens of the control animals decreased during the microdialysis period [$F(17,204)=16.682$; $p<0.0001$] (Fig. 1B).

2.3. *Chronic nicotine intraperitoneal injection for 27 days followed by a further intraperitoneal nicotine injection at the commencement of microdialysis on day 28*

Both glutamate and arginine content were significantly modified by nicotine treatment; for the dose; glutamate [$F(3,47)=4.609$; $p=0.0066$], arginine [$F(3,40)=60.572$; $p<0.0001$] and the time; glutamate [$F(17,799)=1.674$; $p=0.0424$], arginine [$F(17,680)=10.041$; $p<0.0001$]. In addition there were significant interaction between the doses and time, glutamate [$F(51,799)=1.741$; $p=0.0013$], arginine [$F(51,680)=5.215$; $p<0.0001$]. An increase in glutamate microdialysate content commenced approximately 3 h after the i.p. 0.3 mg/kg nicotine injection and continued for the duration of the study (50% increased to the baseline value). A dramatic decrease in arginine content of the nucleus accumbens was evident in the rats which received 0.3 mg/kg dose. An 85% decrease in the concentration of this amino acid was assayed at the conclusion of the microdialysis study.

2.4. *Chronic saline intraperitoneal injection for 27 days followed by acute intraperitoneal nicotine injection*

There were significant effects of each nicotine dose on glutamate [$F(3,40)=11.509$; $p<0.0001$] and arginine [$F(3,29)=20.003$; $p<0.0001$]. There were also significant interactions between time and dose for glutamate [$F(51,680)=7.481$; $p<0.0001$] and arginine [$F(51,493)=2.890$; $p<0.0001$]. Glutamate release was significantly enhanced approximately 2–3 h after intraperitoneal injection of both 0.3 and 0.6 mg/kg nicotine; increasing by approximately 100% with the two lower doses and by almost 300% with the highest dose, 0.6 mg/kg (Fig. 2A). Arginine microdialysate content decreased significantly for the two higher doses of nicotine, 0.3 mg/kg (after 1 h) and 0.6 mg/kg (almost immediately), and continued for the duration of the experiment, 5 h (Fig. 2B).

2.5. *Chronic i.p. injection of nicotine for 27 days followed by a single dose of saline on day 28 at the commencement of microdialysis*

No consistent increases in glutamate were evident immediately after the injection of saline. This result is comparable to that of a single injection of nicotine i.p. (see 2.2). There were significant effect of the treatment on arginine microdialysate content [$F(3,35)=15.285$; $p<0.0001$] as well as an interaction between treatment and time [$F(51,595)=2.102$; $p<0.0001$]. The 0.3 mg/kg dose induced a significant decrease in the arginine microdialysate content, by 50%, commencing 1 h after the saline injection that was sustained for the duration of the study, 5 h (Fig. 3).

2.6. *Chronic i.p. nicotine for 27 days; followed by a period of abstinence for 7 days and the one acute i.p. nicotine dose at the commencement of microdialysis*

After injection of a single nicotine dose, which was comparable to that received for 27 days before the period of abstinence, no significant changes occurred in the glutamate release. Arginine significantly decreased 40 min after injection of 0.3 mg/kg, by 50%, and continued to decline throughout the experiment. [$F(3,27)=42.178$; $p<0.0001$] (Fig. 4).

2.7. *Chronic nicotine administration p.o. for 50 days*

During the first 12 h of withdrawal after oral administration of different nicotine doses for 50 days, there were significant enhancements in glutamate release ([$F(3,37)=36.208$; $p<0.0001$]) although at varying time points during the initial 12 h of withdrawal. The levels of the excitatory amino acid increased after 9 h for the 0.15 mg/kg dose and at 7 h for both 0.3 and 0.6 mg/kg doses (Fig. 5A). Glutamate levels had increased by 200%, in comparison to the initial baseline values, for the lowest dose, 0.15 mg/kg, at the termination of the microdialysis study, while the other two doses induced increases in glutamate content at 12 h postwithdrawal, by approximately 100–150%, in comparison to the baseline value at the beginning of the withdrawal stage. A significant decrease in arginine microdialysate content ([$F(3,35)=60.095$; $p<0.0001$]) was induced by each dose of nicotine, commencing 1 to 3 h from the start of withdrawal, after which there was a progressive decline throughout the 12 h of the study. At this time, the levels were 50% of the initial baseline value for the 0.15 mg/kg dose and only 20% of the initial baseline values for the other two doses (Fig. 5B).

2.8. *Chronic nicotine and alcohol administration*

At the conclusion of the alcoholization procedure, with or without nicotine administration, the blood alcohol levels were comparable (2.63±0.24; 2.26±0.13; 2.57±0.22 and 2.21±0.14 g/l respectively for control, 0.15, 0.3 and 0.6 mg/kg/day nicotine) [$F(3.53)=1.17$; $p=0.33$]. There were significant effects of chronic

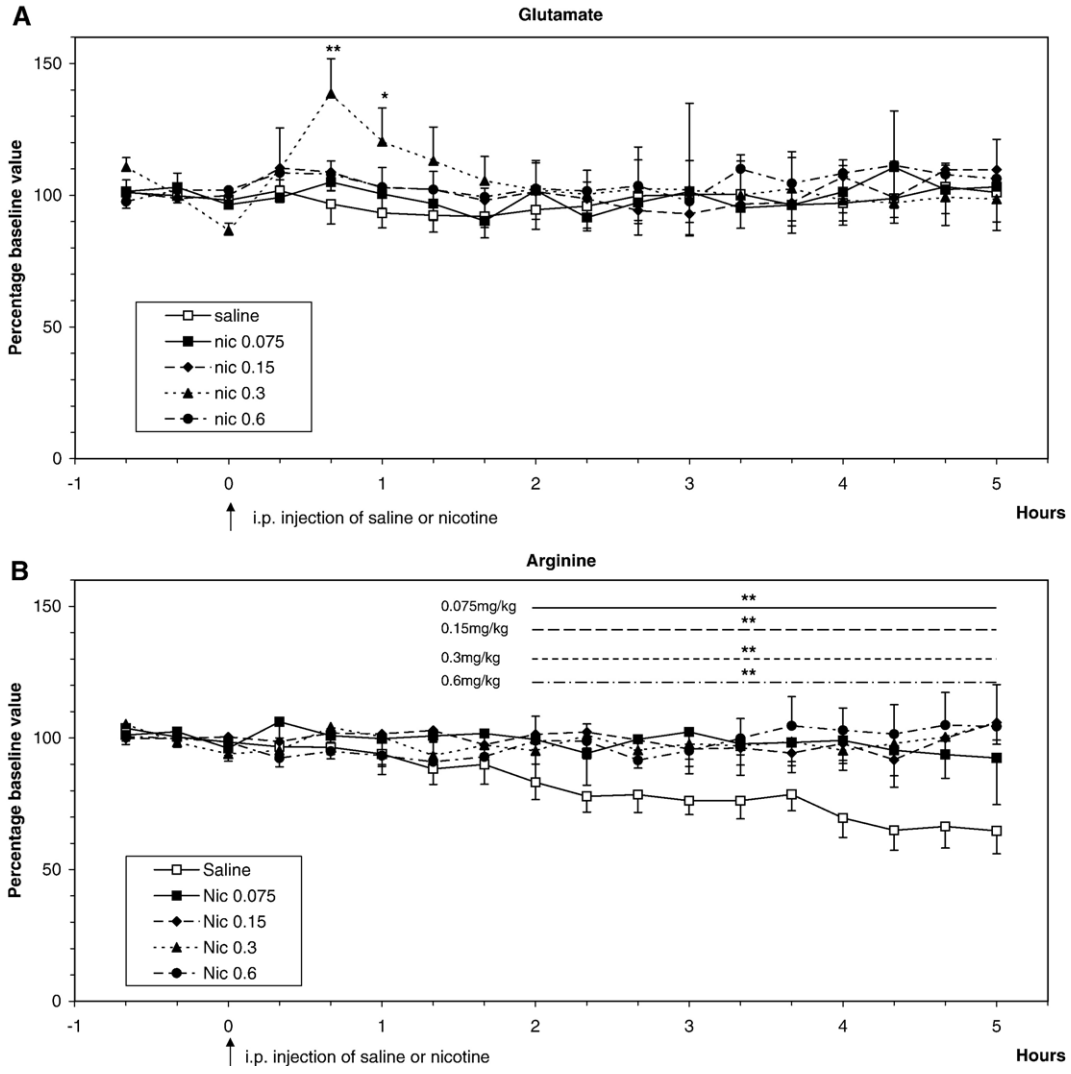

Fig. 1 – Changes in glutamate (A) and arginine (B) microdialysate content of nucleus accumbens after acute nicotine (0.075, 0.15, 0.3 and 0.6 mg/kg) or saline i.p. injection. Open squares = saline controls, nicotine doses are represented by black squares = 0.075 mg/kg, black diamonds = 0.15 mg/kg, black triangles = 0.3 mg/kg, black circles = 0.6 mg/kg. Significant time points between nicotine doses and saline are represented by $^{*}p < 0.01$. Results are presented as the mean of percentage of baseline value ± SEM.

nicotine and ethanol administration on glutamate [$F(3,45) = 51.914$; $p < 0.0001$] and arginine [$F(2,35) = 37.018$; $p < 0.0001$]. There were also significant interactions between time and dose for glutamate [$F(105,1575) = 11.774$; $p < 0.0001$] and for arginine [$F(70,1225) = 4.119$; $p < 0.0001$]. The rats that had been chronically alcoholized via the pulmonary alcoholization technique with no nicotine supplement for 4 weeks showed a typical alcohol withdrawal pattern. Glutamate release increased approximately 7 h after the beginning of withdrawal which continued for the subsequent 5 h [$F(1,30) = 34.630$; $p < 0.0001$] (Fig. 6), such that a 100% increase in its concentration in comparison to the initial baseline values was assayed. However when nicotine was administered orally during the chronic alcoholization procedure, such increases in glutamate release during this withdrawal period were not evident with the two lower doses of nicotine. The values were either unchanged (0.3 mg/kg) or decreased (0.15 mg/kg) in comparison with the initial baseline values. Furthermore, glutamate values decreased to approximately 25% of the initial baseline value with the lowest dose of nicotine (Fig. 7A). The highest dose of nicotine, 0.6 mg/kg, did not prevent the increase in glutamate release; the pattern of glutamate release was comparable to that of alcohol withdrawal. All doses of nicotine significantly diminished arginine release (Fig. 7B), by 80% with the 0.15 and 0.3 mg/kg doses while arginine was not detectable after the highest dose, 0.6 mg/kg.

3. Discussion

The nucleus accumbens and the ventral tegmental brain regions are thought to be important in the reinforcing effects of nicotine. These areas form the mesolimbic dopaminergic system and are critical regions that mediate the reinforcing actions of many drugs of addiction. In these present studies the

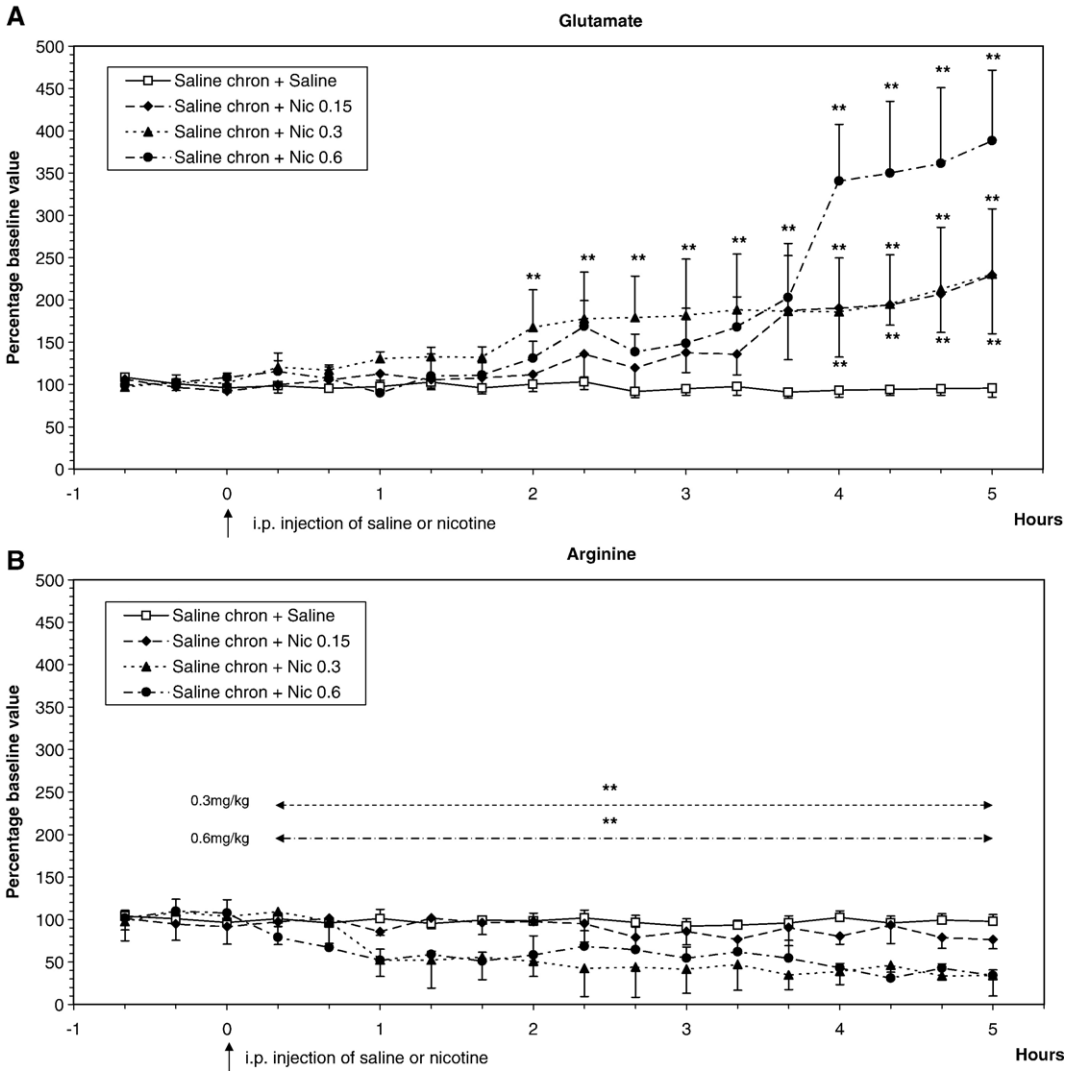

Fig. 2 – (A) Changes in glutamate release in the nucleus accumbens after 27 days of chronic saline i.p. injections followed by a single injection of 0.15, 0.3 or 0.6 mg/kg nicotine on day 28. (B) Changes in arginine microdialysate content in the nucleus accumbens after 27 days of chronic saline i.p. injections followed by a single injection of 0.15, 0.3 and 0.6 mg/kg nicotine on day 28. Open squares = saline controls. Nicotine doses are represented by black diamonds = 0.15 mg/kg, black triangles = 0.3 mg/kg, black circles = 0.6 mg/kg nicotine dose. Significant time points between nicotine doses and saline are represented by $^{*}p<0.05$ and $^{**}p<0.01$. Results are presented as mean of percentage of baseline value ± SEM.

action of nicotine, administered either acutely or chronically, on glutamate and arginine release in the nucleus accumbens has been investigated by microdialysis in freely moving rats. In addition, the release of these amino acids from nucleus accumbens has been studied during the initial 12-h withdrawal stage after co-administration of nicotine and alcohol for 4 weeks.

3.1. *Nicotine-induced changes in glutamate microdialysis content after an acute injection*

Nicotine rapidly traverses the blood brain barrier (15 s), has a half life of approximately 1–3 h, and stimulates a variety of neurotransmitter systems, including the cholinergic and dopaminergic systems, which play important roles in mediating, the rewarding and reinforcing actions of nicotine. Nicotine acts on α7nAChRs located on glutamate efferents (Mansvelder and McGehee, 2000; Pulvirenti and Diana, 2001; Kenny and Markou, 2001) to increase glutamate release in the VTA. In these present studies, acute nicotine, at a dose of 0.3 mg/kg, transiently increased glutamate release. However, when nicotine was administered acutely after a priming regime (i.e., 27 days of saline), each dose of nicotine elicited an increase in this excitatory amino acid. This might indicate that stress associated with 27 days of repeated saline injections may have sensitized glutamate release.

3.2. *Nicotine-induced changes in glutamate microdialysate after chronic administration*

Increases in microdialysate glutamate transmission after chronic nicotine administration, either i.p. for 28 days or

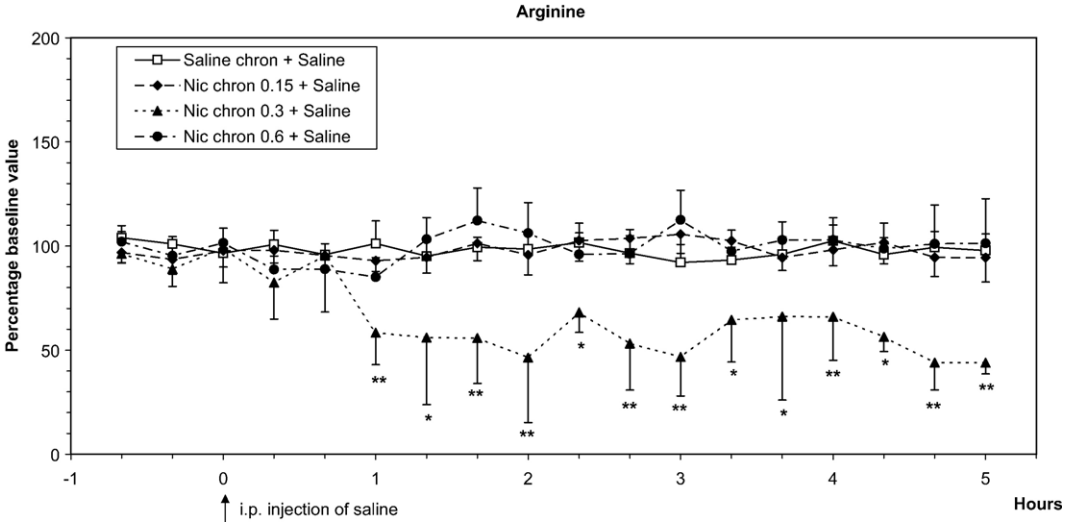

Fig. 3 – Changes in arginine microdialysate content in nucleus accumbens after chronic i.p nicotine injection (0.15, 0.3 and 0.6 mg/kg) for 27 days followed by a single i.p. saline injection on day 28. Open squares = saline controls. Nicotine doses are represented by black diamonds = 0.15 mg/kg, black triangles = 0.3 mg/kg nicotine dose, black circles = 0.6 mg/kg. Significant time points between nicotine doses and saline are represented by $^{*}p<0.05$ and $^{**}p<0.01$. Results are presented as mean of percentage of baseline value ± SEM.

orally for 50 days were evident, 3 and 5 h, respectively, after the commencement of microdialysis. Such results might indicate tolerance and withdrawal, respectively, both of which are mediated by this excitatory amino acid. In vitro studies have also identified increases in glutamate release after chronic nicotine administration, e.g., synaptosome preparations from hippocampus or striatum exposed to nicotine for 10 days (Risso et al., 2004). This was shown to be NMDA stimulated release, since the NMDA receptor antagonist CGP39551 inhibited the nicotine-induced increase in firing rate (Schilstrom et al., 2004). Although no changes in glutamate release were evident in the two experiments where chronic doses of nicotine were administered followed by either a single dose of saline or after a period of abstinence, a longer period of microdialysis might be necessary. In these experiments, the release of glutamate was not associated with mediating the rewarding effects of nicotine but in mediating the aversive aspects of nicotine withdrawal. It is unclear as to how this excitatory amino acid can act in such a diversive manner. However, this could be attributed to the selectivity of the brain region in which it is released or that other receptors, such as $mGluR_{2/3}$, are stimulated (Rasmussen et al., 1996) and

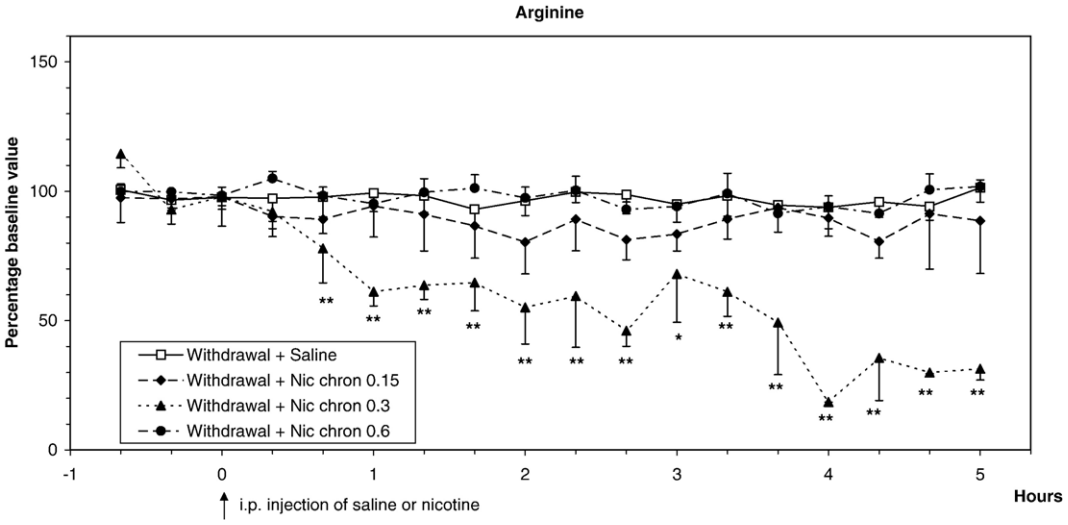

Fig. 4 – Changes in arginine microdialysate content of nucleus accumbens after chronic nicotine i.p. injection for 27 days followed by 7 days withdrawal and then a single i.p. nicotine injection (0.15, 0.3 and 0.6 mg/kg). Open squares = saline controls. Nicotine doses are represented by black diamonds = 0.15 mg/kg, black triangles = 0.3 mg/kg, black circles = 0.6 mg/kg. Significant time points between nicotine doses and saline are represented by $^{**}p<0.01$. Results are presented as mean of percentage of baseline value ± SEM.

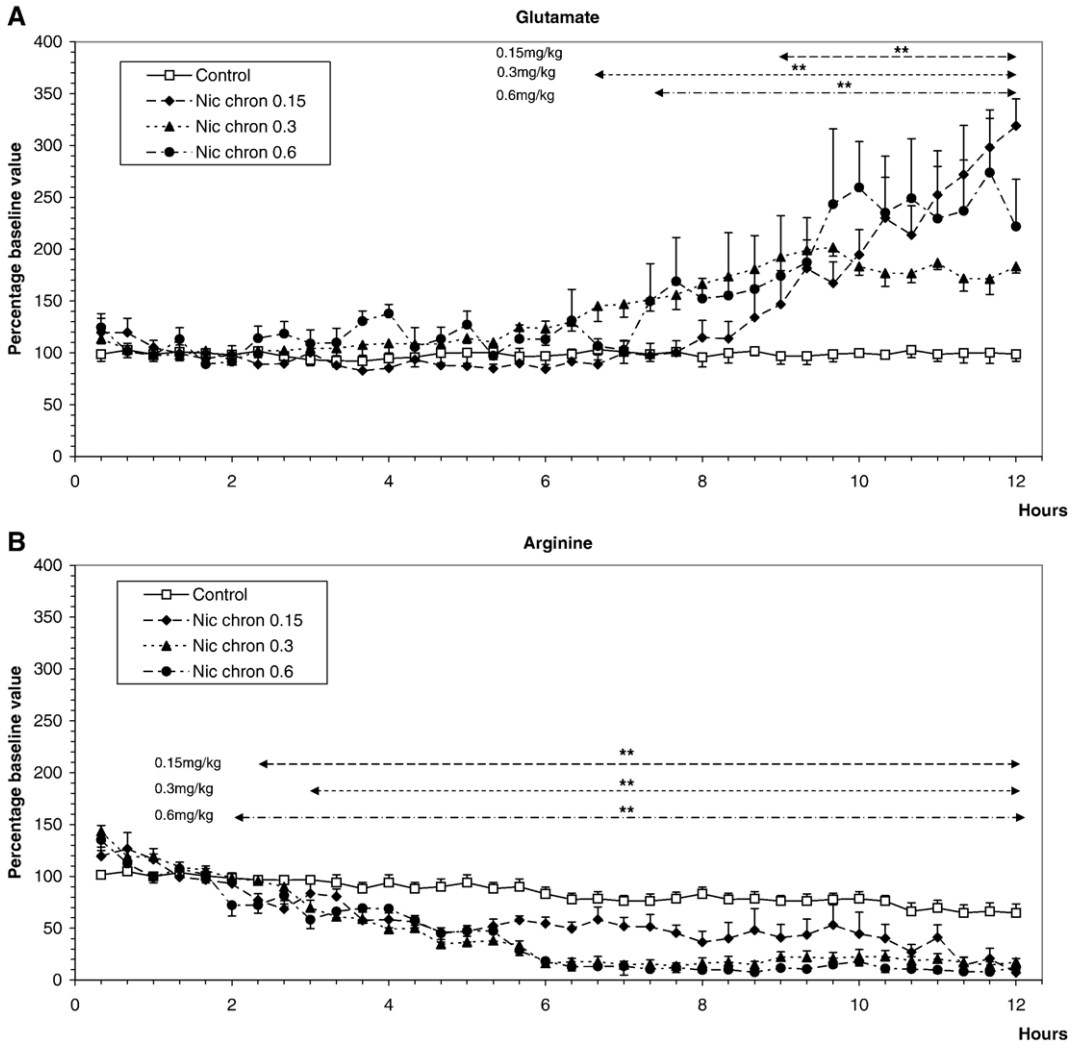

Fig. 5 – (A) Changes in glutamate microdialysate content during the initial 12-h period after withdrawal from oral nicotine administration (0.15, 0.3 and 0.6 mg/kg) for 50 days. (B) Changes in arginine microdialysate content during the initial 12-h period after withdrawal from oral nicotine administration (0.15, 0.3 and 0.6 mg/kg) for 50 days. Open squares = saline controls. Nicotine doses are represented by black diamonds = 0.15 mg/kg, black triangles = 0.3 mg/kg, black circles = 0.6 mg/kg. Significant time points between nicotine doses and saline are represented by $^{*}p<0.05$ and $^{**}p<0.01$. Results are presented as mean of percentage of baseline value ± SEM.

release other neurotransmitters which are involved in the withdrawal syndrome.

3.3. *Changes in glutamate release after chronic co-administration of nicotine and alcohol*

In previous microdialysis studies we have shown that there is an increase in glutamate release approximately 5–7 h after ethanol withdrawal (Dahchour et al., 1998) which is caused by changes in the sensitivities of the NMDA receptors. This ethanol-induced glutamate release could be prevented by the administration of taurine (Dahchour and De Witte, 2000a) or the taurine analogue acamprosate (Dahchour and De Witte, 2000b) during the alcoholization procedure. An interaction of acamprosate with the metabotropic glutamate receptor subtype 5 (mGluR5) was deemed to be responsible for changes in glutamate transmission (De Witte et al., 2005). In these present studies, it was shown that nicotine, when administered at either 0.15 or 0.3 mg/kg, also prevented the increase in glutamate release during the initial 12 h of ethanol withdrawal. Prendergast et al. (2000) reported that nicotine exposure protects against ethanol withdrawal-induced hippocampus damage in cell cultures that was dizocilpine maleate (MK801) sensitive (thereby indicating a role for NMDA receptors). Another study suggested that this protective effect might be via α7nAChRs (Prendergast et al., 2001a), when a calcium buffering effect occurred (Prendergast et al., 2001b). A partial agonist of α7nAChR, 3-[2,4-dimethoxybenzylidene] anabaseine also protected against neurotoxicity induced by ethanol in primary hippocampal neuron enriched cultures (de Fiebre and de Fiebre, 2003). It is also noteworthy that chronic ethanol administration inhibits α7nAChR (de Fiebre and de Fiebre, 2005) such that during withdrawal this inhibition may be reversed thereby enhancing protection. Furthermore, if

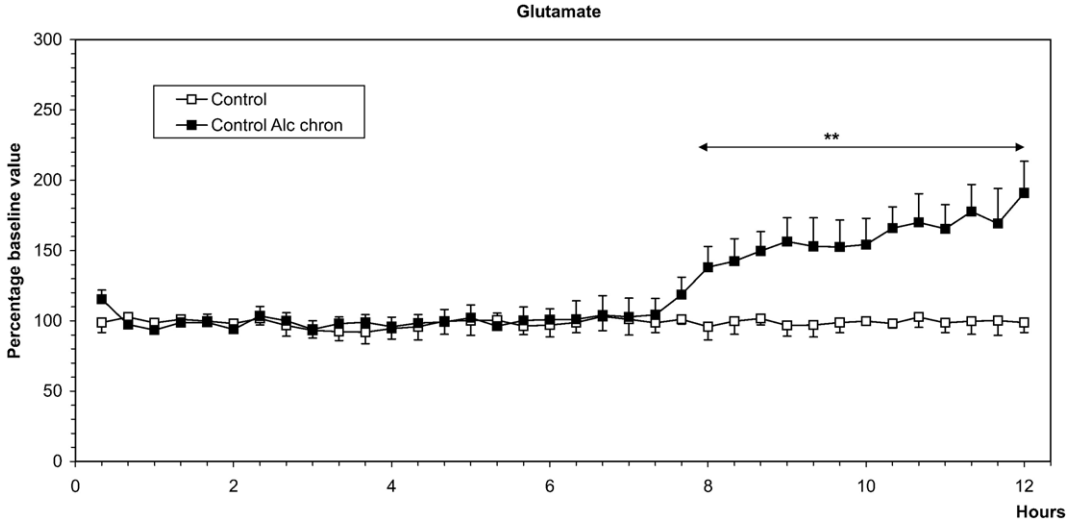

Fig. 6 – Changes in glutamate microdialysate content of nucleus accumbens during the initial 12-h withdrawal period compared to naive control rats. Groups are represented by Open squares = control non alcoholized rats, black squares = chronically alcoholized rats. Significant time points between control and control alcoholized rats are represented by $^{*}p<0.05$ and $^{**}p<0.01$. Results are presented as mean of percentage of baseline value ± SEM.

group II metabotropic glutamate receptors $mGluR_2$ and $mGluR_3$ were stimulated this would decrease glutamate transmission as these inhibitory receptors are located at presynaptic and postsynaptic locations (Kenny et al., 2003). Clearly further studies are required to identify the site of interaction between nicotine and ethanol which causes the inhibition of glutamate release during withdrawal from these two drugs. Although some studies have attempted to ascertain whether smoking during alcohol withdrawal might enhance or reduce withdrawal symptoms, as yet, no correlation has been reported (Schmidt and Smolka, 2001).

3.4. *Interaction of nicotine and arginine*

It was noteworthy in these present studies that nicotine administered either acutely or chronically evoked significant decreases in NAc arginine release. Arginine is an essential amino acid which can be synthesized and metabolized to urea in the liver. However in extrahepatic tissue, it is likely that the principle pathway for arginine utilization is constituted by its use in the synthesis of NO by NOS. Previously, the in vivo effects of nicotine on the nitric oxide synthase/cyclic GMP pathway of the adult rat hippocampus have been investigated by monitoring extracellular levels of GMP during microdialysis. IP administration of nicotine increased cyclic GMP levels which was totally abolished by infusion of a NO synthase inhibitor or partially (60%) by local administration of a NMDA receptor antagonist (Fedele et al., 1998). Whether such changes in arginine microdialysate were indicative of an increase or decrease in NOS synthesis remain to be elucidated. It is reported that NO synthase inhibitors can attenuate symptoms of the nicotine abstinence syndrome (Vleeming et al., 2002). Although Uzbay and Erden (2003) reported that high doses of L-arginine, 500–1000 mg/kg alleviated some behavioral signs of ethanol withdrawal this could have been due to a negative feed back process. In one previous study where chronically alcoholized rats were repeatedly withdrawn from ethanol withdrawal (Dahchour and De Witte, 2003), a significant reduction in arginine hippocampus microdialysate was also noted but only after the third withdrawal.

It remains clear that 80% of alcoholics smoke cigarettes. There is considerable genetic overlap, in terms of receptor subunit composition, between nicotine and alcohol addiction (Enoch, 2003). It is debatable whether smoking during alcohol withdrawal might reduce or even ameliorate the withdrawal symptoms (Friend and Pagano, 2005). Although our data might indicate some benefits of simultaneous withdrawal of animals from both drugs, i.e., alcohol and nicotine, the neurochemical processes involved remain to be thoroughly studied before the development of pharmacological compounds which may help and even prevent the neurotoxicity of withdrawal.

4. Experimental procedure

4.1. *Animal preparation*

Male Wistar rats, 200–250 g, were individually housed in standard plastic cages and maintained in a temperature (22 °C) and light controlled environment (12 light/12 dark light cycles, light on 8 am). They were given free access to commercial rat chow and either tap water or nicotine solution depending on the experimental study. All animal procedures were in strict accordance with the recommendations of EEC (86/609/CEF) and with the Belgian "projet de loi" (Moniteur Belge 19.02.1992, p. 3437) on the care and use of laboratory animals.

Rats underwent surgical procedures, as described previously (Dahchour et al., 2005). Briefly, under general anaesthesia (chloral hydrate 400 mg/kg, i.p.), the rats were fixed in a stereotaxic frame. Through a midline incision of skin and soft tissue, the skull surface was exposed and Bregma point was identified. A guide cannula (20-gauge stainless steel; Small

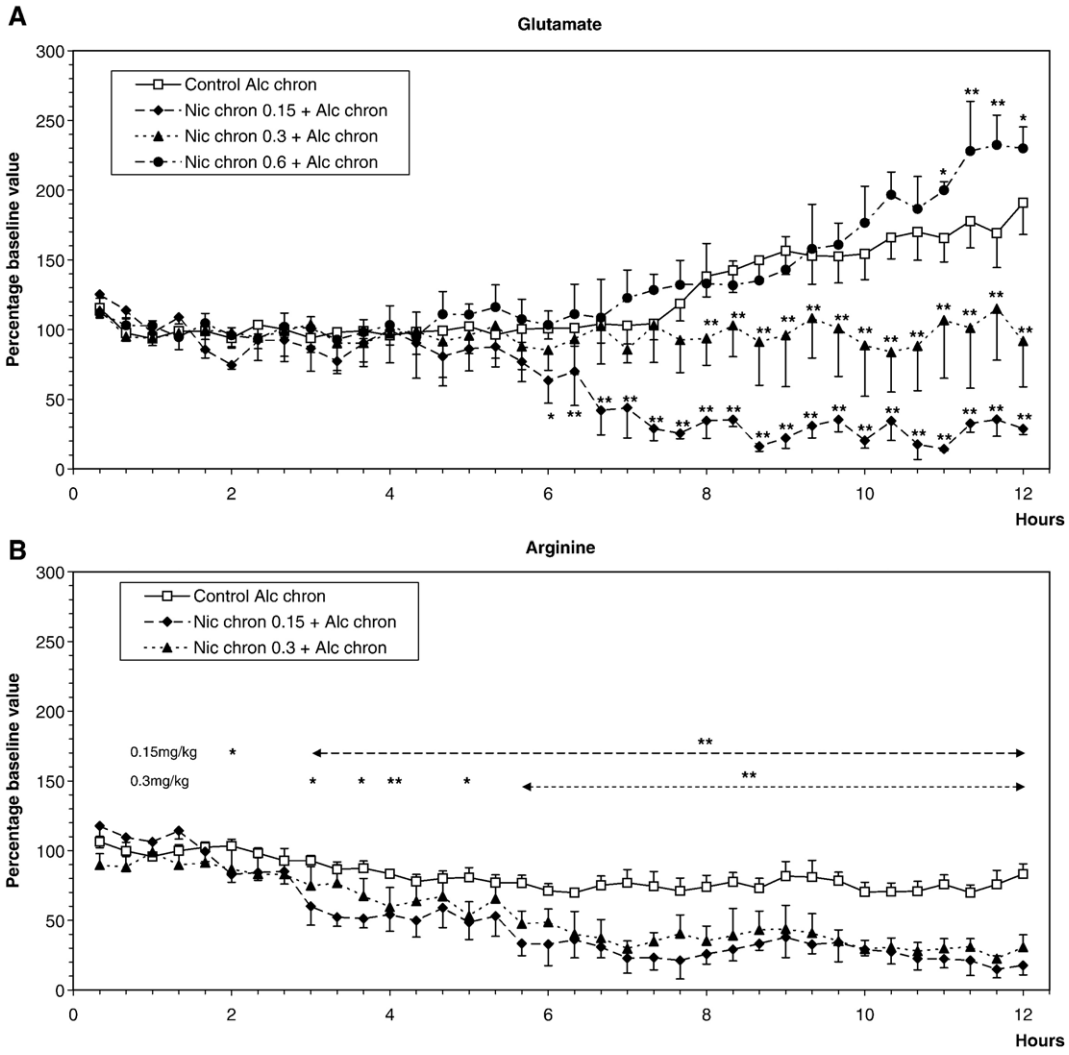

Fig. 7 – (A) Changes in glutamate microdialysate content of nucleus accumbens during the initial 12-h withdrawal period after chronic co-administration of nicotine (0.15, 0.3 and 0.6 mg/kg) p.o. and ethanol, 50 days of chronic pulmonary ethanol administration. (B) Changes in arginine microdialysate content of nucleus accumbens during the initial 12-h withdrawal period after chronic co-administration of nicotine (0.15, 0.3 and 0.6 mg/kg) p.o. and ethanol, 50 days of chronic pulmonary ethanol administration. Open squares = saline controls. Nicotine doses are represented by black diamonds = 0.15 mg/kg, black triangles = 0.3 mg/kg, black circles = 0.6 mg/kg. Significant time points between nicotine doses and saline are represented by $^{*}p<0.05$ and $^{**}p<0.01$. Results are presented as mean of percentage of baseline value ± SEM.

Parts, Miami, FL, USA) was inserted into the caudate putamen–nucleus accumbens (A/P1.2 mm; M/L 1.4 mm; D/V −4.4 mm) according to the atlas of Paxinos and Watson (1982). The guide cannula was secured to the skull with two steel screws and cranioplastic cement and kept patent with a 26-gauge stainless steel obturator (Small Parts Inc., FL, USA), Fig. 8.

Dialysis experiments commenced 72 h postoperation recovery period and was performed during the resting period. The dialysis probes had a molecular cut-off of 13 kDa and an inner membrane diameter of 0.2 mm (Spectrum Laboratories, Inc., USA). Dialysis tubing extended 3 mm beyond the tip of the probe. The probe was connected to a microinfusion pump (Infusion syringe pump 22, Harvard apparatus, South Natick, MA, USA) and continuously perfused at a flow of 1.1 μl/min with Ringers solution containing 145 mM NaCl, 4 mM KCl, 1.3 mM $CaCl_2$ (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) phosphate buffered to pH 7.2. Microdialysis tubing connected to probes was placed within a coiled metallic spring to allow the rat-free movement while protecting the tubing from damage.

HPLC grade water (resistivity = 18.2 MΩcm) from a Milli-Q water purification system (Millipore, Molsheim, France) was used in the preparation of the solutions).

4.2. *Microdialysis experiments with acute and chronic administration*

Nicotine tartrate (Sigma, St. Louis, MO, USA) was dissolved in isotonic saline. Doses, 0.15, 0.3, 0.6 mg/kg were prepared and administered intraperitoneally, i.p. in a volume of 1 ml/kg body weight. An equal volume of isotonic saline was used as a control solution. For the oral nicotine experiments, solutions

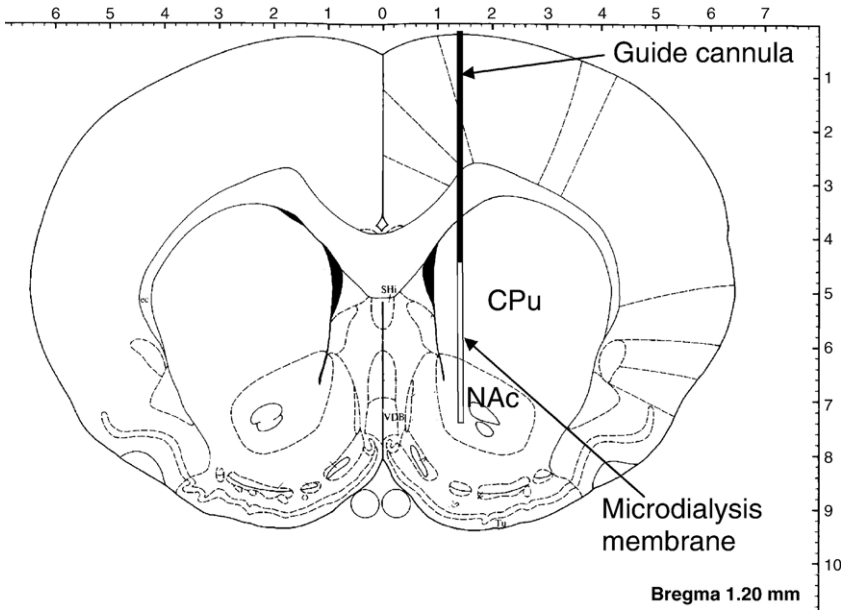

Fig. 8 – Diagrammatic figure showing location of dialysis tip in the Caudate putamen–nucleus accumbens (NAc: accumbens nucleus, CPu: caudate putamen, Shi: septohippocampal nu, Tu: olfactory tubercule, VDB nu: vertical limb diagonal band). Modified from Paxinos and Watson (1982).

were prepared at concentrations of 0.15, 0.3, 0.6 mg/kg in drinking bottle and adjusted every 3–4 days according to the mean liquid consumption and the mean rat body weight.

The designs of the experiments are shown in Fig. 9 and are briefly described.

4.2.1. *Acute nicotine intraperitoneal injection*

Seventy-two hours after surgery, the microdialysis probe was inserted into the guide cannula. During an initial period of 40 min, the perfusate amino acid content stabilized. Microdialysate samples were then collected every 20 min for 2 h into microcentrifuge tube which were connected to the outer cannula. The rats were administered a dose of nicotine intraperitoneally, 0.075 (n=8), 0.15 (n=7), 0.3 (n=9) or 0.6 mg/kg (n=8), or saline (n=13) and the microperfusates were collected every 20-min period for a further 5 h.

4.2.2. *Chronic nicotine i.p. injections*

The rats received daily i.p. injection of nicotine (0.15 (n=11), 0.3 (n=8), 0.6 mg/kg (n=14)) or saline (n=21) for 27 days prior to the

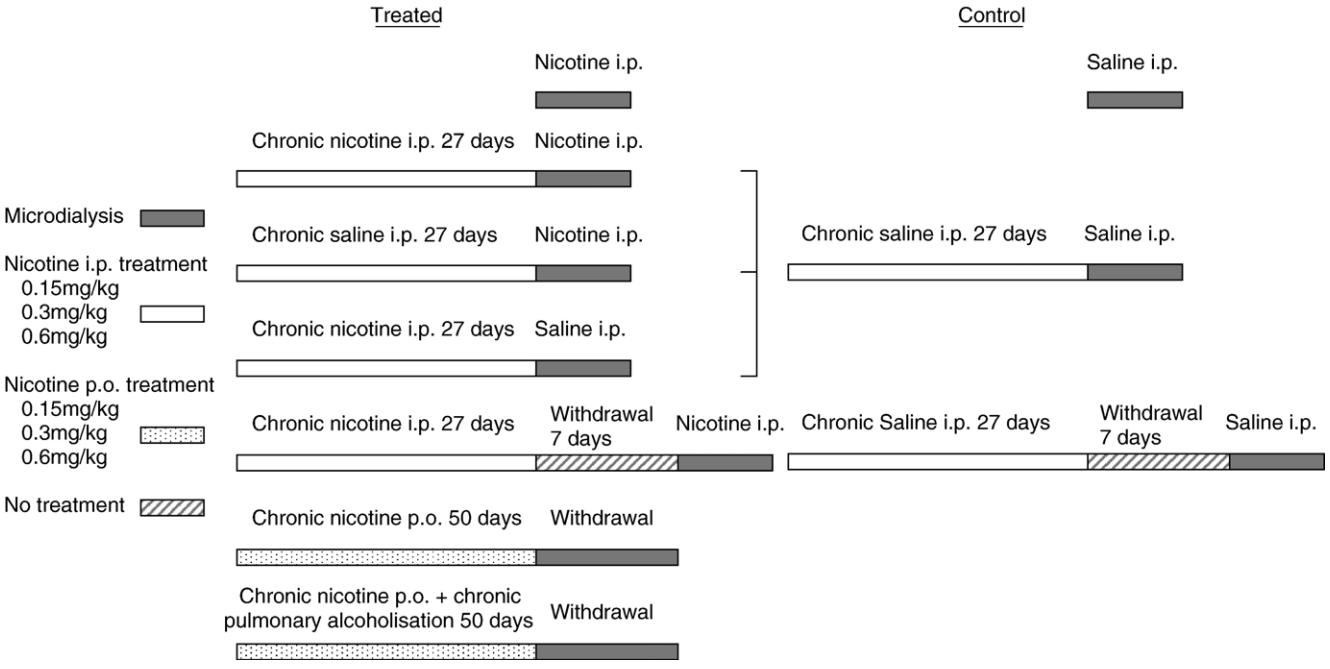

Fig. 9 – Schematic design of the different experiments of the study.

microdialysis experiment when a further i.p. injection of nicotine was administered at the same dosage received during chronic administration.

4.2.3. *Chronic saline i.p. injections and one nicotine i.p. injection*

The rats received daily i.p. injections of saline for 27 days prior to the microdialysis experiment when a single i.p. nicotine dose (0.15 (n=8), 0.3 (n=7), 0.6 mg/kg (n=8)) was administered.

4.2.4. *Chronic nicotine i.p. injections and one saline i.p. injection*

The rats received daily i.p. injection of nicotine (0.15 (n=11), 0.3 (n=8), 0.6 mg/kg (n=12)) or saline (n=21) for 27 days prior to the microdialysis experiment when a single i.p. dose of saline was administered.

4.2.5. *Chronic nicotine i.p. injections, 7 days withdrawal and one nicotine i.p. injection*

The rats received daily i.p. injection of nicotine (0.15 (n=13), 0.3 (n=14), 0.6 mg/kg (n=6)) or saline (n=10) for 27 days, prior to a withdrawal period of 7 days. On the 8th day, the microdialysis experiment commenced when the rats were administered a single nicotine i.p. injection at the same dosage received during chronic administration.

4.2.6. *Chronic nicotine p.o. and withdrawal*

The rats received nicotine in their drinking bottles (0.15 (n=10), 0.3 (n=9), 0.6 mg/kg (n=10)) for 50 days. The microdialysis experiment was carried out during the first 12 h of nicotine withdrawal.

4.3. *Chronic co-administration of nicotine and alcohol*

The animals (340–400 g) were individually housed in a plastic chamber (120×60×60 cm) and chronically intoxicated by inhalation of ethanol vapor during 7 weeks. Ethanol concentration was increased from 15 to 24 mg/l in air in successive steps of 1 mg/l every 2–3 days so that the average blood alcohol level (BAL) continued to rise (Aufrère et al., 1997; Le Bourhis, 1975). Blood was collected from the caudal portion of each rat's tail once per week during the alcoholization period, and then two times per week at the end of the chronic ethanol treatment period. The concentration of ethanol in the blood samples was assayed by the alcohol-dehydrogenase-based method (Boehringer-Mannheim, Germany). During the third week, the rats were removed from the chamber for surgery as described in the surgical procedure. The rats were allowed to recover from the anaesthetic and then returned to the appropriate environment within the chambers for 3–4 weeks.

Nicotine was administered as described in (vi) (0 (n=19), 0.15 (n=9), 0.3 (n=14), 0.6 mg/kg (n=13) nicotine) except that during the nicotine administration the rats were also chronically alcoholized with ethanol vapor. The microdialysis was performed during the initial 12 h of combined nicotine–ethanol withdrawal.

4.4. *HPLC analysis*

The fractions, 20 μl, were analyzed by HPLC with two channel electrochemical detector (Coulochem II, ESA, Bedford, Mass. USA) which consisted of a LDC ConstaMetric 3200 pump (LDC Analytical, Riviera Beach, FL, USA) delivering 1 ml/min, at a pressure of 5300 psi, of the mobile phase (118 mM Na_2HPO_4, 0.13 mM EDTA, 27% methanol, pH 6.4 adjusted with phosphoric acid). The mobile phase had been filtered through a 0.2-μm cellulose nitrate filter (Gelman Sciences, Ann Arbor, MI, USA) and degassed under vacuum before use in the HPLC system. Separation of compounds was achieved by reversed-phase column chromatography (125×3 mm, ODS Hypersil 3 μm) (VDS Optilab, Chromatographie Technik, GmbH) and detected coulometrically using three electrodes, a guard (0.5 V) (guard cell ESA 5020) placed between the pump and the autosampler (HPLC Autosampler 465, Bio-TeK Instruments, Milano, Italy), a preoxidation (E2: 0.380 V, Range: 100 μA) and a working (E1: 0.380 V, Range: 200 nA) electrode (analytical cell ESA5011). The position and height of glutamate and arginine peaks within the dialysates were compared to an internal standard, homoserine (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) which was added prior to the HPLC analyses, and the amino acid areas were quantified by a PC Integration pack (Kontron Instruments Spa, Milano, Italy). The baseline value in each experiment was determined either by meaning the last 3 fractions before the injection or by meaning the values in the first hour of withdrawal. The variation of concentrations in each perfusate was then expressed as a percentage of this baseline value.

4.5. *Histochemical analysis*

On completion of the experiments, rats were sacrificed and the brain fixed with 10% formalin. Coronal sections through the extent of the cannula tracks were cut (100 μm) with a vibratome (Polaron H 1200, Biorad, Cambridge, MA, USA). Dialysis probe placement was localized according to the atlas of Paxinos and Watson (1982). The data were discarded if the probe was incorrectly positioned.

4.6. *Statistical evaluation*

The results are presented as mean±standard error of the mean (SEM). Microdialysis data were analyzed by two-way analysis of variance (ANOVA) with repeated measures on time for each treatment group vs. their control to assess the significance of difference in glutamate and arginine levels in each study performed. Where appropriate, post hoc pair wise comparisons were analyzed by Fisher protected least significant difference test (GB-Stat 5.3 for Windows, Dynamic Microsystems, MD, USA). For the basal concentrations, significance was assessed by ANOVA 1 with completely randomized data and followed by the Dunnett's procedure. Criteria for significance was set at $p<0.05$ for all tests.

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