

The addition of five minor tobacco alkaloids increases nicotine-induced hyperactivity, sensitization and intravenous self-administration in rats

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Abstract

Several minor tobacco alkaloids have been found to exhibit properties pharmacologically relevant to the addictive profile of tobacco; however, little is known of their effects on a behavioural model of drug addiction. In this study we compared the locomotor and reinforcing effects of intravenous nicotine (30 µg/kg per infusion) *vs.* a cocktail of nicotine plus five minor alkaloids found in tobacco smoke (anabasine, nornicotine, anatabine, cotinine and myosmine). Rats were initially tested for their locomotor response to nicotine or nicotine plus the minor alkaloids with six intravenous injections over 1 h. We then assessed the spontaneous acquisition of intravenous self-administration with nicotine or nicotine plus the minor alkaloids, under a fixed-ratio 1 schedule followed by responding on a fixed-ratio 5 schedule, progressive-ratio schedule and a single within-session ascending dose–response test. The activity test was repeated following the progressive-ratio phase to assess locomotor sensitization. A second group of rats were then tested on the locomotor procedure to better clarify the role of each individual minor alkaloid in nicotine-induced locomotor activity. Compared to nicotine alone, addition of the minor tobacco alkaloids increased locomotor activity and increased locomotor sensitization following self-administration. During fixed-ratio 5, progressive ratio and the dose–response test, rats receiving nicotine plus the minor alkaloids responded significantly more than those receiving nicotine alone. Testing of each minor alkaloid in the second experiment indicated that anatabine, cotinine and myosmine individually increased nicotine-induced locomotor activity. These results suggest that the minor tobacco alkaloids, particularly anatabine, cotinine and myosmine, may increase the motivation for nicotine and thus facilitate smoking behaviour.

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Introduction

Tobacco continues to be the most commonly used drug of abuse, with an estimated 1 billion smokers worldwide (WHO, 2008). Nicotine is generally accepted as the chemical responsible for the addictive properties of tobacco (Harvey *et al.* 2004; Rose & Corrigall, 1997), primarily exerting its reinforcing effects via activation of the nicotinic acetylcholine receptor (nAChR) in the mesolimbic dopamine system

(Exley *et al.* 2008; Palmatier *et al.* 2007). Despite the clear reinforcing properties of tobacco, pure nicotine is not abused (West *et al.* 2000), nicotine replacement products are only moderately effective (Hajek *et al.* 1999), and denicotinized cigarettes are more satisfying and rewarding than nicotine received intravenously (Rose, 2006; Rose *et al.* 2000). In human and rodent studies of intravenous (i.v.) self-administration, nicotine presents a much less robust administration profile than other drugs of abuse such as cocaine or heroin, and thus has been considered a relatively weak reinforcer (Manzardo *et al.* 2002; Stolerman & Jarvis, 1995). Such findings are at odds with the high rate of tobacco use by humans and have led some researchers to suggest that other non-nicotine factors

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facilitate addiction to tobacco (Dar & Frenk, 2004; Rose, 2006).

With over 4000 chemicals present in cigarette smoke, it is plausible that some of these substances may augment the neurochemical action of nicotine, or indeed exert significant effects themselves. For example, acetaldehyde is a major component of tobacco smoke and has been found to increase responding for nicotine in rats, particularly in adolescent animals (Belluzzi *et al.* 2005). Several other non-nicotine tobacco derivatives act as monoamine oxidase inhibitors (MAOIs) (Castagnoli *et al.* 2002), and as shown in our laboratory, pre-treatment with MAOIs can markedly increase the motivation of rats to obtain nicotine and prolong the aversive state of nicotine withdrawal (Guillem *et al.* 2005, 2008). Such results have yet to be investigated in non-human primates or humans.

Nicotine is the primary alkaloid found in the tobacco leaf, accounting for 96–98% of the total alkaloid content, with the remaining portion composed of nornicotine, anabasine, anatabine, cotinine and myosmine (Huang & Hsieh, 2007). These five minor tobacco alkaloids exhibit a similar structure to nicotine and have been found to exert biologically relevant effects on the brain, with nornicotine and cotinine also playing a role as major nicotine metabolites (Crooks *et al.* 1997). Of the minor alkaloids, nornicotine is the most studied and independently has been shown to support i.v. self-administration in rats, albeit at very low levels and using much higher administration concentrations than that found in tobacco smoke (Bardo *et al.* 1999).

In addition to cotinine and nornicotine, the lesser known minor alkaloids anabasine and anatabine have also been identified as nAChR ligands through cellular membrane affinity chromatography screening of tobacco smoke (Maciuk *et al.* 2008). Interestingly, a tobacco extract containing all minor alkaloids produces greater nAChR-mediated inhibition of serotonergic neurons than nicotine alone, with nornicotine, anabasine and nicotine individually producing similar levels of inhibition (Touiki *et al.* 2007). Furthermore, anabasine, cotinine, and nornicotine also elicit a calcium-dependent increase in striatal dopamine release through activation of the nAChR, and similarly to nicotine, produce nAChR desensitization (Dwoskin *et al.* 1995; O'Leary *et al.* 2008). Such properties suggest a possible role of the minor alkaloids in modulating the reinforcing effects of nicotine, yet with the exception of nornicotine, this hypothesis has yet to be confirmed using a behavioural model of drug addiction.

Therefore, the aim of this study was to investigate whether the minor alkaloids are reinforcing in

themselves, and whether they interact with the reinforcing properties of nicotine at concentrations proportional to their presence in tobacco. The locomotor-activating effects of i.v. nicotine (Nic), nicotine plus a cocktail of the five non-nicotine tobacco alkaloids (Nic/CO), saline plus the five alkaloids (Sal/CO) or saline alone (Sal) were tested before and after the self-administration phase as a means of assessing locomotor sensitization and as an indicator of an altered sensitivity to reward. The reinforcing efficacy of i.v. infusions of the drugs was determined using spontaneous acquisition of self-administration under fixed-ratio (FR) schedules, followed by responding under a progressive-ratio (PR) schedule to assess the motivation to obtain the drug, and a within-session ascending dose-response (aDR) test for differences in sensitivity to the reinforcing effects of the drug. Finally, to better elucidate which minor alkaloid might be responsible for any effects obtained, we then tested the impact of each minor alkaloid individually on nicotine-induced locomotor activity.

Materials and methods

Subjects

Male Sprague–Dawley rats (175–200 g; France) were housed in groups of 2–3 rats per cage and maintained on a 12-h reversed light/dark cycle (lights off 09:00 hours).

Five days after arriving in the laboratory all rats were anaesthetized with a mixture of ketamine (100 mg/kg) and xylazine (12 mg/kg) and implanted with a chronic indwelling catheter as described previously (Guillem *et al.* 2005). When required, catheter patency was verified by infusing 0.1 ml of the short-acting non-barbiturate anaesthetic etomidate (Braun Medical, France), which elicits a rapid loss of muscle tone. Food and water were available *ad libitum* until recovery from surgery, and then restricted to 20 g of rat chow per day, delivered after the test session. All surgical and experimental procedures were performed in accordance with the European directive (86/609/EEC) and with the approval of the Centre National de la Recherche Scientifique.

Drugs

(–)-Nicotine hydrogen tartrate and the five non-nicotine tobacco alkaloids (±)-anabasine, (±)-anatabine, (–)-cotinine, (±)-myosmine and (±)-nornicotine were purchased from Sigma (USA). Concentrations of the minor alkaloids found in cigarette smoke extract

were provided by the Association pour la Recherche sur les Nicotianées (ARN, France; unpublished data), and were within the range of other published analyses of cigarette smoke alkaloid composition (Liu *et al.* 2008; Wu *et al.* 2002). The final drug concentrations for locomotor activity and self-administration were calculated relative to a standard dose of nicotine (30 µg/kg per infusion) to give: anabasine 0.9, nornicotine 0.9, anatabine 0.09, cotinine 0.09 and myosmine 0.09 µg/kg per infusion. All chemicals were dissolved in 0.9% NaCl and administered intravenously.

Expt 1: the effects of a minor tobacco alkaloid cocktail on nicotine-induced locomotor activity and i.v. self-administration

Locomotor response to i.v. drug infusion

After recovering from surgery (6–9 d), rats were assessed for their locomotor reactivity to i.v. drug infusions in self-administration chambers (40 × 36 × 30 cm; Imetronic, France) with levers withdrawn and equipped with infrared activity detectors. All chambers were housed within wooden sound-attenuation boxes that included a ventilation fan and a single diffuse white houselight. Illumination of this houselight signalled the onset of the 2-h test session. During all locomotor habituation and testing rats were attached to Tygon tubing protected by a spring connector and connected externally to a 10-ml syringe and pump.

One 2-h locomotor test procedure was repeated over three consecutive days: day 1 for habituation to the chambers, day 2 for i.v. saline infusion and day 3 for i.v. drug infusion. Activity recorded on day 2 was used to evenly assign rats to treatment groups for drug infusion (day 3) ($n=16$ for Nic and Nic/CO; $n=8$ for Sal/CO and Sal).

The session procedure was identical on each day. During the first hour no injections were made, thus establishing baseline activity. During the second hour syringe pumps were activated for 4 s every 10 min, producing six evenly spaced i.v. injections of 100 µl [$t(\text{min})=60, 70, 80, 90, 100, 110$] of nothing (day 1), saline (day 2) or drug (day 3). This pattern of administration aimed to replicate the frequency of infusions that we have previously observed during nicotine self-administration in the same experimental setting (Guillem *et al.* 2005).

The drug administration day was replicated on the day immediately following the 28th i.v. self-administration session. This final probe test assessed whether any locomotor sensitization occurred as a result of the self-administration procedure.

Beam breaks relating to ambulatory movement (entire cage crossings) and general activity (individual beam breaks) were recorded for each session.

Nicotine i.v. self-administration

The day after locomotor testing, rats began self-administration training in a room and chambers different to those used previously. Self-administration chambers (40 × 30 × 37 cm; Imetronic, France) were housed in sound attenuation boxes and illuminated by six white LEDs on the ceiling of the box. Chambers were equipped with two nose-poke apparatus: one in the centre of the left wall ('active') and another in the centre of the right wall ('inactive'). Activation of the active nose-poke resulted in the infusion of 100 µl Nic, Nic/CO, Sal/CO or Sal during 4 s, and was accompanied by the illumination of a white cue-light positioned above the nose-poke hole. A 20-s timeout followed each infusion whereby activation of the active nose-poke had no consequences. Inactive nose-poke responses were recorded but had no programmed consequences.

Fixed ratio and progressive ratio

The beginning of the 2-h self-administration session was indicated by illumination of the houselight and a single non-contingent infusion of the drug solution. Rats were first trained for the acquisition of self-administration under a fixed-ratio (FR) schedule of reinforcement (5 d, FR1; 3 d, FR2; 15 d, FR5).

All rats were then tested under a progressive-ratio (PR) schedule of reinforcement for 5 d whereby a single drug infusion was delivered contingent on an increasing number of active nose-poke responses. The sequence of required nose-pokes to receive each infusion was as follows: 1, 3, 6, 10, 15, 20, 25, 32, 40, 50, etc. (Depoortere *et al.* 1993). Sessions lasted a maximum of 3 h, or until 30 min elapsed without a drug infusion.

Within-session aDR test

Rats were returned to a FR5 schedule for 2 d before being submitted to a within-session aDR test (Deroche *et al.* 1999; Martin *et al.* 1996). Rats were exposed to four doses of their respective i.v. solution in ascending order: 0.5, 1, 2 and 4 times the training dose (corresponding to 15, 30, 60 and 120 µg nicotine/kg per infusion for Nic and Nic/CO). The doses were presented in ascending order to avoid any aversive effects that the higher doses of nicotine may have on subsequent responding. Each dose was available for 45 min,

Table 1. Concentrations of nicotine plus minor alkaloids tested in expt 2

Treatment	Nicotine (per infusion)	Minor alkaloid (per infusion)
Nic	30 µg/kg	–
Nic/anabasine	30 µg/kg	0.9 µg/kg
Nic/anatabine	30 µg/kg	0.09 µg/kg
Nic/cotinine	30 µg/kg	0.09 µg/kg
Nic/nornicotine	30 µg/kg	0.9 µg/kg
Nic/myosmine	30 µg/kg	0.09 µg/kg
Nic/all	30 µg/kg	All alkaloids
Sal/all	–	All alkaloids
Sal	–	–

Nic, Nicotine; Sal, 0.9% saline solution; All, a cocktail of all minor alkaloids at the concentrations described above.

separated by a 5-min time-out period during which time the house-light was extinguished and nose-pokes had no programmed consequences. Variation in dose was achieved by increasing the time of the injection and associated cue-light time from 2 s for the lowest dose to 16 s for the highest.

Expt 2: the effects of individual minor tobacco alkaloids on nicotine-induced locomotor activity

A second group of rats ($n=12$) were used to test the effect of each individual alkaloid on nicotine-induced activity. Using a within-subjects design, each rat received nicotine plus each minor alkaloid in a randomized order over days. The effects of each minor alkaloid alone (in the absence of nicotine) were not tested as results from expt 1 showed no significant effect of saline plus all minor alkaloids compared to saline alone. The nine alkaloid combinations used are presented in Table 1.

The surgery, apparatus and procedure for each session was identical to that used to test for the locomotor response to nicotine or nicotine plus the minor alkaloids in expt 1. On the first and the last day of drug infusions, all rats received nicotine alone. As repeated exposure to nicotine can produce locomotor sensitization (see expt 1), this double exposure to nicotine was used to detect the presence and magnitude of sensitization throughout the experiment. All alkaloid combinations were then administered in a random order over a 3-wk period. Each rat received drug infusions every Monday, Wednesday and Friday. On Tuesdays and Thursdays rats received infusions of saline under the same procedure. The saline infusions

were used to ensure adequate wash-out of each alkaloid as the metabolites can be detected up to 18 h post-administration (e.g. cotinine; Crooks & Dwoskin, 1997), and also to control for contextual conditioning to the locomotor activity chamber in the presence of nicotine.

Statistical analysis

For expt 1, locomotor data were condensed into 10-min bins and analysed separately for the first hour (baseline) and the second hour (drug infusion) of the activity session. Data were analysed using a three-way repeated-measures ANOVA with test day (before or after 28 d i.v. self-administration) and time as within-subjects factors, and group as the between-subjects factor.

Analysis of the acquisition and maintenance of nicotine self-administration was carried out using a three-way repeated measures ANOVA on each section of the experiment (FR1, FR5 or PR) with day and hole (active *vs.* inactive) as the within-subjects factors, and i.v. solution (Nic, Nic/CO, Sal/CO or Sal) as the between-subjects factor. For analysis of the aDR, a two-way repeated-measures ANOVA was used with dose as within-subjects factor and i.v. solution (Nic, Nic/CO, Sal/CO or Sal) as a between-subjects factor. Whenever main ANOVA effects were found, *post-hoc* analyses were performed using Fisher's protected LSD test. Eight rats were excluded from the experiment due to sickness or loss of catheter patency (one from the entire experiment, five from acquisition and two from PR onwards).

Correlations were conducted between locomotor response to novelty (habituation), locomotor activity before i.v. self-administration, locomotor activity after i.v. self-administration, percentage change in activity (sensitization), total number of infusions obtained during FR responding (acquisition), number of infusions obtained on the last 5 d of FR responding or break-point under a PR using a corrected p value of 0.007.

For expt 2, locomotor data were condensed into 10-min bins and activity scores for each rat at each drug dose were calculated as the average activity during the first hour (baseline) subtracted from average activity recorded during the second hour (drug infusion). This approach differed to expt 1 due to the difference in experimental design (within *vs.* between subjects). Data were analysed using repeated-measures ANOVA with *post-hoc* analysis when necessary. A probability factor <0.05 was considered statistically significant.

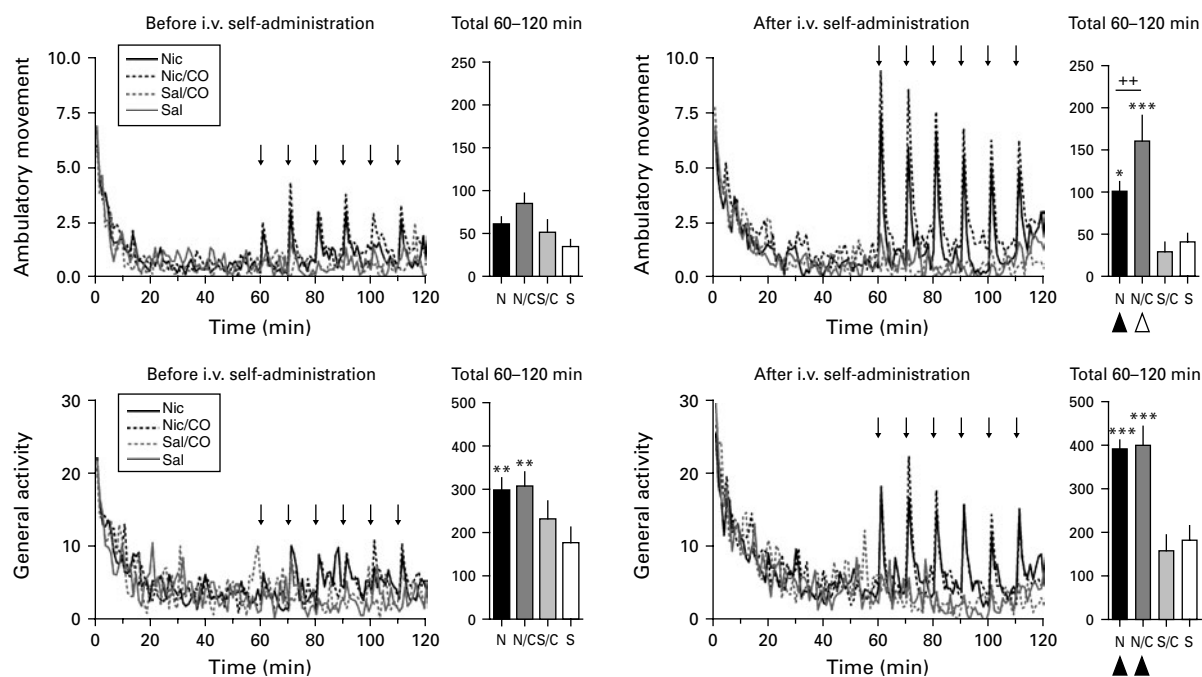


Fig. 1. Sensitization of the locomotor response to intravenous (i.v.) nicotine (N, Nic), nicotine plus a cocktail of five non-nicotine tobacco alkaloids (N/C, Nic/CO), saline plus the minor alkaloid cocktail (S/C, Sal/CO) or saline alone (S, Sal), as measured before and after 28 d i.v. self-administration. Line graphs indicate the mean levels of activity over the 2-h test session (0–60 min habituation only). Bar graphs indicate group mean \pm S.E.M. for the total activity recorded over the last 60 min of the session (when i.v. injections were administered). Small inverted arrows indicate time of i.v. injection. Asterisks indicate significant difference from Sal (* p < 0.05, ** p < 0.01, *** p < 0.001). Plus symbols indicate significant difference between Nic and Nic/CO (** p < 0.01). For reasons of clarity, differences between Nic and Nic/CO with Sal/CO are not shown; however, both Nic and Nic/CO were significantly higher than Sal/CO after i.v. self-administration for measures of ambulatory movement (p < 0.01, p < 0.001, respectively) and general activity (p < 0.001 for both). Triangles indicate significant difference between locomotor activity measured before *vs.* after i.v. self-administration (▲, p < 0.05; △, p < 0.001).

Results

Expt 1: the effects of minor tobacco alkaloids on nicotine-induced locomotor activity and i.v. self-administration

Locomotor response to i.v. drug infusion

Locomotor activity recorded during habituation sessions and during the first hour of the 2-h drug test session was equivalent for all groups (data not shown). Repeated i.v. infusion of Nic or Nic/CO resulted in discrete peaks of ambulatory movement and general activity corresponding to the six drug infusions (Fig. 1).

Comparison of the ambulatory response to each infusion in the drug-naïve rats before self-administration with that after 28 d i.v. self-administration, revealed significant effects of group ($F_{3,37}=7.26$, p < 0.01), day (before *vs.* after: $F_{1,37}=6.59$, p < 0.05), time ($F_{5,185}=$

8.01, p < 0.001), a day \times time interaction ($F_{5,185}=6.08$, p < 0.001) and a day \times group interaction ($F_{3,37}=4.90$, p < 0.01). *Post-hoc* analysis of the day \times treatment interaction revealed that in drug-naïve rats (before i.v. self-administration) the only significant group difference was between Nic/CO and Sal ($p=0.05$). Following 28 d self-administration, rats receiving Nic/CO now displayed markedly higher levels of ambulatory movement than rats receiving Nic alone (p < 0.01), with both Nic and Nic/CO demonstrating ambulatory movement significantly higher than Sal (p < 0.05, p < 0.001) or Sal/CO (p < 0.01, p < 0.001, respectively). Notably, for rats receiving Nic and the Nic/CO combination, there was a significant augmentation of ambulatory movement when comparing the locomotor response in drug-naïve rats, to that following the self-administration period (Nic p < 0.05, Nic/CO p < 0.001), indicative of sensitization to the locomotor stimulant properties of nicotine.

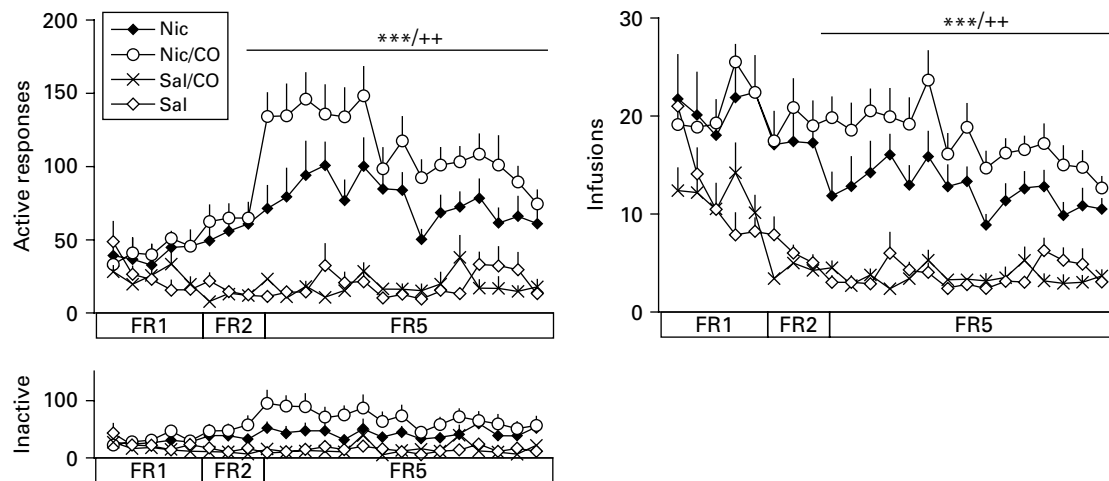


Fig. 2. Intravenous self-administration of nicotine (Nic), nicotine plus the minor alkaloid cocktail (Nic/CO), saline plus the minor alkaloid cocktail (Sal/CO) or saline alone (Sal) on fixed-ratio (FR) schedules of reinforcement. Data-points represent group mean \pm S.E.M. for number of active nose-pokes, inactive nose-pokes and infusions for each 2-h session. Asterisks indicate an overall significant difference between each nicotine group (Nic and Nic/CO) from each Sal and Sal/CO (** $p < 0.001$) over FR5 responding. Plus symbols indicate an overall significant difference between Nic and Nic/CO ($^{++}p < 0.01$) over FR5 responding.

A similar result was evident for generalized activity, with effects of group ($F_{3,37} = 9.73$, $p < 0.001$), time ($F_{5,185} = 2.55$, $p < 0.05$), a time \times group interaction ($F_{15,185} = 2.21$, $p < 0.01$), day \times time interaction ($F_{5,185} = 4.44$, $p < 0.01$) and a day \times group interaction ($F_{3,37} = 3.52$, $p < 0.05$). Prior to i.v. self-administration, Nic and Nic/CO both produced greater levels of activity than Sal ($p < 0.01$), and after i.v. self-administration both groups were significantly higher than both Sal and Sal/CO ($p < 0.001$ for all). Furthermore, rats receiving Nic and the Nic/CO combination exhibited a significant increase in generalized activity following the self-administration period ($p < 0.05$ for both), again indicating a sensitization to the effects of nicotine.

Nicotine self-administration

Fixed ratio. Rats receiving Nic and Nic/CO rapidly acquired i.v. self-administration during the FR schedules (Fig. 2) as evidenced by an effect of lever (active higher than inactive; $F_{1,36} = 10.07$, $p < 0.01$) and a group \times day interaction ($F_{12,144} = 3.89$, $p < 0.001$). A similar pattern was observed with the number of infusions received on the FR1 schedule as a significant group effect ($F_{3,36} = 3.04$, $p < 0.05$) and a group \times day interaction over the 5 d of FR1 ($F_{12,144} = 2.82$, $p < 0.01$).

Following transition to the FR5 schedule an overall effect of group ($F_{3,38} = 21.92$, $p < 0.001$), nose-poke hole ($F_{1,38} = 11.43$, $p < 0.01$), day ($F_{14,532} = 2.65$, $p < 0.05$) and a group \times nose-poke-hole interaction ($F_{3,38} = 3.00$,

$p < 0.05$) were evident. *Post-hoc* analysis revealed that rats receiving Nic/CO made significantly more responses on the active nose-poke hole than Nic alone ($p < 0.01$), Sal/CO ($p < 0.001$) and Sal alone ($p < 0.001$). Responding on the active hole was also greater for rats receiving Nic than Sal/CO ($p < 0.001$) and Sal ($p < 0.001$). Both Nic and Nic/CO successfully discriminated the active nose-poke hole ($p < 0.01$, $p < 0.001$, respectively), yet Sal and Sal/CO failed to show a significant preference for either hole. Rats receiving Nic alone responded significantly more on the inactive hole than Sal ($p < 0.05$), but not Sal/CO ($p = 0.06$), and Nic/CO responded slightly more at the inactive hole than the rats receiving Nic ($p < 0.05$), Sal ($p < 0.001$) and Sal/CO ($p < 0.001$).

The augmentation of responding in the Nic/CO group was reflected in the number of infusions received during FR5 self-administration. A significant group effect ($F_{3,38} = 26.67$, $p < 0.001$), but no group \times day interaction indicated that the overall level of responding was different with each infusion solution used, and that this difference was consistent over the 15 d of FR5 self-administration. *Post-hoc* analysis revealed that rats receiving the Nic/CO solution self-administered significantly more infusions than each of the other groups (Nic $p < 0.01$, Sal/CO $p < 0.001$, Sal $p < 0.001$). As expected the Nic group also received more infusions than either Sal group (Sal/CO $p < 0.001$, Sal $p < 0.001$).

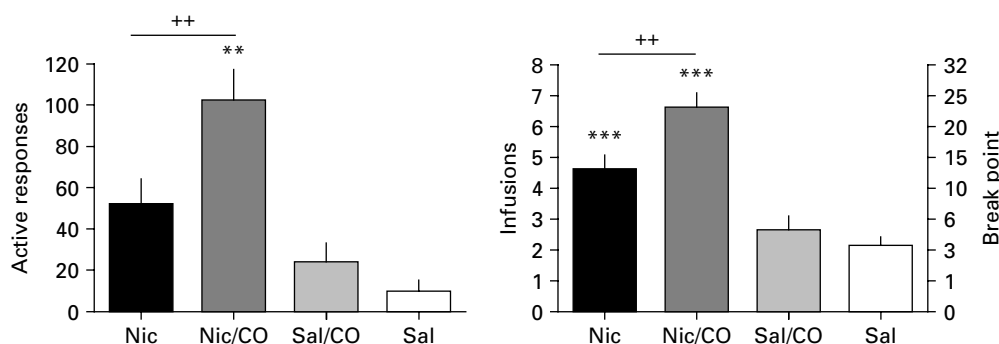


Fig. 3. Intravenous self-administration of nicotine (Nic), nicotine plus the minor alkaloid cocktail (Nic/CO), saline plus the minor alkaloid cocktail (Sal/CO) or saline alone (Sal) on a progressive-ratio (PR) schedule of reinforcement. Data represent the group mean \pm S.E.M. averaged over 5 consecutive days of PR for the number of active nose-pokes, infusions and the break-point achieved. Asterisks indicate a significant difference from Sal (** $p < 0.01$, *** $p < 0.001$). Plus symbols indicate a significant difference between Nic and Nic/CO (++) $p < 0.01$. In addition, Nic/CO was significantly different from Sal/CO for active responses ($p < 0.01$), and both Nic and Nic/CO were significantly higher than Sal/CO on the number of infusions received ($p < 0.01$, $p < 0.001$) and break-point achieved ($p < 0.05$, $p < 0.001$, respectively); however, for purposes of clarity these results are not indicated on the graph.

There were no differences between responding for Sal and Sal/CO at anytime during the FR schedules.

Progressive ratio. Responding under the more demanding PR schedule of reinforcement provided a further indication of the group differences seen using a FR schedule (Fig. 3). Responding over the 5 d of PR revealed an overall effect of group ($F_{3,36} = 8.11$, $p < 0.001$) and nose-poke hole ($F_{1,36} = 6.91$, $p < 0.05$). *Post-hoc* analysis indicated that rats receiving Nic/CO worked significantly harder for the infusion (made more responses) than all other groups ($p < 0.01$ for all). No other group differences were observed, although the difference between Nic and Sal approached significance ($p = 0.06$).

Analysis of the break-point (the final ratio achieved under the PR schedule) revealed an overall effect of group ($F_{3,36} = 15.02$, $p < 0.001$) over the 5 d of testing (Fig. 3). *Post-hoc* analysis indicated that rats receiving Nic/CO displayed a significantly higher break-point compared to all other groups (Nic $p < 0.01$, Sal and Sal/CO $p < 0.001$). In addition, rats responding for Nic alone also displayed a greater motivation for the drug than those receiving Sal ($p < 0.01$) or Sal/CO ($p < 0.05$). This corresponded to significantly more infusions (treatment effect: $F_{3,36} = 19.24$, $p < 0.001$) by rats receiving Nic/CO than all other groups (Nic $p < 0.01$, Sal and Sal/CO $p < 0.001$), and more infusions administered by rats receiving Nic than either Sal group (Sal $p < 0.001$, Sal/CO $p < 0.01$).

Again, there were no differences on any measure between Sal and Sal/CO groups at any time under the PR schedule.

Within-session dose-response

Within the single-session dose-response test, changing the amount of solution infused had little effect on the response to Sal or Sal/CO, yet had a dose-dependent effect on the amount of Nic or Nic/CO infused (Fig. 4). Statistical analysis revealed an overall effect of group ($F_{3,36} = 10.84$, $p < 0.001$), dose ($F_{3,108} = 12.65$, $p < 0.001$) and a group \times dose interaction ($F_{9,108} = 9.74$, $p < 0.001$). *Post-hoc* analysis indicated that the Nic/CO received significantly more infusions than all other groups except at the highest dose ($p < 0.05$ – 0.001), whereas rats receiving Nic alone administered more infusions than Sal at the two lowest doses ($p < 0.05$) and Sal/CO at the lowest dose ($p < 0.01$).

The amount of drug consumed increased with the increasing dose as indicated by a significant dose \times group interaction ($F_{3,108} = 3.88$, $p < 0.001$) (Fig. 4). *Post-hoc* analysis revealed that the Nic/CO group consumed significantly more drug at 15, 60 and 120 $\mu\text{g/kg}$ per infusion than Nic alone ($p < 0.05$, $p < 0.001$, $p < 0.00$, respectively).

Correlations of locomotor activity and self-administration

The increased locomotor sensitization could be due to the greater total nicotine intake during the self-administration phase in the Nic/CO group. However, for the Nic group there were no significant correlations between measures of the response to novelty, locomotor activity or self-administration ($p > 0.05$). For rats receiving Nic/CO, there was a significant correlation between the levels of ambulatory locomotion after i.v. self-administration with the total number of infusions

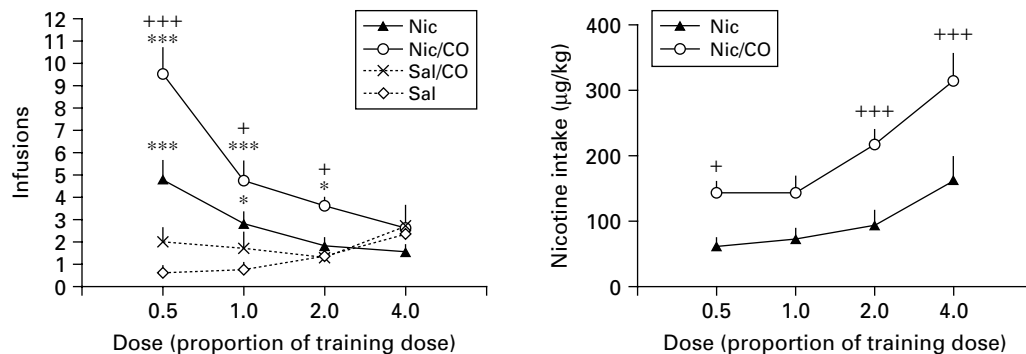


Fig. 4. Within-session ascending dose-response curve of intravenous nicotine (Nic), nicotine plus the minor alkaloid cocktail (Nic/CO), saline plus the minor alkaloid cocktail (Sal/CO) or saline alone (Sal). Data represent the group mean \pm S.E.M. of the number of infusions and the amount of drug received at each of four different infusions doses. Doses are proportional to the original training dose (see Materials and methods section for further details). Asterisks indicate a significant difference from Sal (* $p < 0.05$, *** $p < 0.001$). Plus symbols indicate a significant difference between Nic and Nic/CO (+ $p < 0.05$, +++ $p < 0.001$). In addition, the number of infusions of Nic/CO was significantly higher than Sal/CO at the three lowest doses (0.5, $p < 0.001$; 1.0, $p < 0.01$, 2.0, $p < 0.05$), and Nic was also higher than Sal/CO at the lowest dose (0.5, $p < 0.05$); however, for purposes of clarity these results are not indicated on the graph.

received over the acquisition phase ($r^2 = 0.71$, $p = 0.006$) and the last 5 d of acquisition ($r^2 = 0.78$, $p = 0.002$); however, this effect was due in the most part to a single rat with high levels of activity and high levels of self-administration.

Expt 2: the effects of individual minor tobacco alkaloids on nicotine-induced locomotor activity

Throughout the experiment, a single rat showed contextual conditioning to the experimental chambers as evidence by an increase in activity on Sal days, thus data from this rat were discarded. There was no significant difference between the locomotor response to nicotine alone at the beginning *vs.* the end of the experiment (no significant sensitization), nor was the total locomotor activity recorded on the first drug day in expt 2 significantly different from the first drug day in the Nic only group in expt 1.

Adding each minor alkaloid to nicotine revealed an overall effect of drug treatment on ambulatory movement ($F_{8,80} = 6.65$, $p < 0.001$; Fig. 5). *Post-hoc* analysis revealed no significant differences between nicotine and any of the nicotine/alkaloid combinations; however, all drug treatment sessions that included nicotine produced significantly greater levels of ambulatory movement than Sal/CO or Sal alone ($p < 0.01$ for all).

Analysis of general activity in response to each alkaloid drug treatment also produced an overall effect of drug ($F_{8,80} = 4.15$, $p < 0.001$; Fig. 5), indicating that the level of activity was dependent on the drug combination administered. *Post-hoc* analysis revealed that in comparison to nicotine alone, the addition

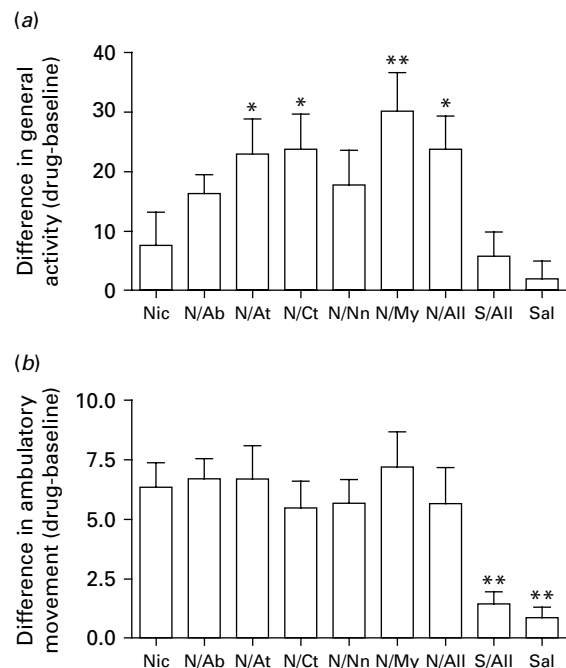


Fig. 5. The effect of individual minor alkaloids on general activity (a) and ambulatory movement (b) induced by nicotine or saline. Bars represent mean \pm S.E.M. for the difference in average levels of activity (in 10-min bins) during the drug infusion phase (last hour) minus baseline (50 min baseline) for each drug combination. Nic, nicotine only; N/Ab, nicotine + anabasine; N/At, nicotine + anatabine; N/Ct, nicotine + cotinine; N/Nn, nicotine + nornicotine; N/My, nicotine + myosmine; N/all, nicotine + all minor alkaloids; S/all, saline + all minor alkaloids; Sal, saline only. Asterisks indicate significant difference from nicotine alone (* $p < 0.05$, ** $p < 0.01$).

of anatabine ($p=0.03$), cotinine ($p=0.02$), myosmine ($p=0.001$) or a cocktail of all minor alkaloids ($p=0.02$) significantly increased levels of general activity.

Discussion

Non-nicotine tobacco products can play a significant role in enhancing the reinforcing effects of nicotine. Addition of acetaldehyde to nicotine facilitates initial acquisition of nicotine self-administration in adolescent rats (Belluzzi *et al.* 2005), whereas treatment with MAOIs significantly increases self-administration and prolongs the aversive state of nicotine withdrawal (Guillem *et al.* 2005, 2008). In the present study, we have shown for the first time that a cocktail of the minor tobacco alkaloids: anabasine, anatabine, cotinine, myosmine and nornicotine produces a similar effect. At concentrations relevant to those contained in cigarette smoke, addition of the minor alkaloids to the nicotine infusion solution significantly enhanced locomotor activity and sensitization, increased the reinforcing efficacy of nicotine over several doses and strengthened rats' motivation to obtain nicotine. We have also shown that of the minor alkaloids tested, myosmine, anatabine and cotinine individually increased nicotine-induced locomotor activity, highlighting a possible role for these specific minor alkaloids in tobacco addiction.

Using a novel locomotor-activity procedure, we report six discrete peaks of hyperactivity corresponding to the six i.v. nicotine infusions. These peaks of activity are similar in duration and strength to past reports following a single i.v. nicotine infusion (Samaha *et al.* 2005). A comparison of activity before *vs.* after 28 d self-administration demonstrated that locomotor sensitization can occur as a consequence of nicotine i.v. self-administration. This finding is consistent with previous reports of an increased sensitivity of the reward system after nicotine self-administration as measured by intracranial self-stimulation (Kenny & Markou, 2006) and increased nAChR density following continuous self-administration of nicotine (Parker *et al.* 2004). Interestingly, this effect of increased ambulatory movement following self-administration was accentuated in the rats receiving nicotine plus the minor alkaloids, suggesting a greater degree of neuroadaptive change in this group. Arguably, this increased sensitization could be a direct consequence of the greater drug intake over the self-administration phase; however, a lack of correlation between any measures of activity and self-administration in rats receiving nicotine alone suggests that this is unlikely.

Intravenous self-administration of nicotine in adult rats produced stable and robust acquisition of the nose-poke response that was sustained over a variety of reinforcement schedules and that is consistent with previous levels of nicotine self-administration reported by our group (Guillem *et al.* 2005, 2006) and others (Donny *et al.* 1998, 1999). Despite similar initial levels of responding during acquisition of the self-administration behaviour, group differences between Nic and Nic/CO emerged with increasing response requirement, an effect most evident following transition to the PR schedule. Because the break-point achieved under a PR schedule approximates the reinforcing magnitude of a given drug (Stafford *et al.* 1998), we suggest that the addition of the minor alkaloids to nicotine strengthens the effectiveness of nicotine as a reinforcer. This conclusion is further supported by the increased responding in Nic/CO rats over several doses, as evidenced by an apparent upward shift of the dose-response curve. It has been suggested that a vertical shift reflects an increased susceptibility of rats to the reinforcing effects of a drug (Piazza *et al.* 2000). Thus, the minor alkaloids may facilitate the transition of nicotine from a weak reinforcer to a moderately strong reinforcer, resulting in greater drug intake throughout the experiment.

We must also note here that the novel approach of using a within-session dose-response curve with nicotine is not without limitation. Used successfully in the past with both cocaine and heroin (Gerber & Wise, 1989; Martin *et al.* 1996), the within-session dose-response test produces similar results to those achieved using a classical dose-response approach. However, we cannot rule out the possibility of differential pharmacokinetics with increasing infusion time, or the possible interaction of the drug with the increasing cue exposure. Despite this, the reported differences between groups are clear and unambiguous, and under our conditions and for our purposes, the within-session dose-response test provided a rapid assessment of the sensitivity of rats to reinforcement over various drug doses.

The emerging difference in responding between rats administering Nic *vs.* Nic/CO may also reflect differential rates of neuroadaptive change occurring during the experiment. A similar pattern has been observed following pretreatment with MAOIs (Guillem *et al.* 2005), but contrasts with an immediate effect on reinforcing efficacy following the addition of acetaldehyde to nicotine (Belluzzi *et al.* 2005). It is possible that the divergence in responding following self-administration of nicotine or nicotine plus the minor alkaloids represents a differential transition of the underlying

neurobiology controlling the behavioural outcome, a theory supported by the finding that application of cigarette-smoke extract produces greater up-regulation of nAChRs than that observed following nicotine alone (Ambrose *et al.* 2007), where such variations in receptor density may translate into an altered motivation to seek nicotine (Le Foll *et al.* 2008). Importantly, this result, in combination with a significant discrimination of the drug source, suggests that the differences in nicotine self-administration are not simply a consequence of the greater ambulatory movement evident in the Nic/CO group. If this was the case, the group differences would have been especially evident under the lower requirement schedules whereby the infusions are less reliant on a discrete, directed response and are more susceptible to variations in locomotor activity.

Although the minor alkaloid cocktail appears to increase the reinforcing effects of nicotine, it is not clear whether one, or several, of the minor alkaloids are responsible for this effect. To further investigate this relationship, we examined the impact of each minor alkaloid individually on nicotine-induced activity. The addition of anatabine, cotinine or myosmine alone significantly increased nicotine-induced general activity, suggesting that these specific alkaloids may be particularly important in the effects reported here. Regarding expt 1, the addition of the minor alkaloid cocktail also significantly increased activity; however, under the conditions of expt 2 it was more evident as changes in general activity, rather than ambulatory movement. This discrepancy may reflect the differential exposure to the activity test procedure, the frequency of drug administration and/or the development of sensitization during the experiment. While anatabine and cotinine have been shown to interact with the nAChR to facilitate dopamine release in the striatum (Dwoskin *et al.* 1999; Middleton *et al.* 2007), there are currently no neuropharmacological studies of any kind involving myosmine, nor any other studies addressing the behavioural effects of these alkaloids. Whether the locomotor effects seen here are reliable over various doses and, more importantly, whether they translate into differential reinforcing efficacies remains to be determined.

Interestingly, the levels of activity induced by nicotine plus anatabine, cotinine or myosmine were equal to, or greater than, that recorded for the cocktail including all alkaloids, suggesting that the relationship of the minor alkaloids with nicotine is synergistic rather than additive. This conclusion is further supported by the lack of any locomotor or reinforcing effect of the minor alkaloids in the absence of nicotine

(Sal/CO) and a disparity between the increase in responding following addition of the minor alkaloid cocktail with that which could be predicted by adding an equivalent amount of nicotine (30 *vs.* 32 µg/kg per infusion; see Fig. 4). However, a full dose-response test for each alkaloid in the presence or absence of nicotine is required before any firm conclusions can be drawn about the behavioural effects of each individual minor alkaloid.

Pharmacologically, support for a synergistic relationship between nicotine and minor alkaloids comes from evidence that cotinine can act as an allosteric modulator of the nicotine receptor, allowing low concentrations of cotinine to result in partial desensitization of the nAChR (Buccafusco *et al.* 2007, 2009). Such an effect may help explain the discrepancy between nicotine and tobacco extract effects observed *in vitro* (see Ambrose *et al.* 2007; Touiki *et al.* 2007), but has yet to be investigated in respect of the other minor tobacco alkaloids.

Overall, these results demonstrate that the addition of the five minor tobacco alkaloids can significantly alter the stimulatory and reinforcing effects of nicotine over several behavioural procedures. Further studies are required to better elucidate the precise nature of the mechanisms underlying the changes seen in the presence of the minor alkaloids, both in combination with nicotine, and independently.

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Statement of Interest

None.

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