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Antagonism of the anxiolytic effect of nicotine in the dorsal raphé nucleus by di-hydro-β-erythroidine

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Abstract

Nicotine has been reported to reduce anxiety in humans and in a number of animal tests. In the social interaction test of anxiety, administration of low doses of nicotine into the dorsal raphé nucleus (DRN) increases the time spent in social interaction without producing accompanying changes in locomotor activity, suggesting that nicotine acts specifically to reduce anxiety in this brain region. The present study examined the ability of the high-affinity competitive nicotinic receptor antagonist di-hydro-β-erythroidine hydrobromide (DHβE) to antagonise the anxiolytic effect of nicotine following intra-DRN infusion using the social interaction test. The increase in social interaction observed after administration of nicotine (5 ng) into the DRN was completely reversed by coadministration of 100 ng DHβE. DHβE (100 ng), when administered alone into the DRN, did not modify the time spent in social interaction. However, it did significantly increase locomotor activity, and this effect was not antagonised by coadministration of nicotine (5 ng) into the DRN. Because of the pharmacological profile of DHβE, our results suggest that the anxiolytic effect of nicotine in the DRN is mediated by the α4β2 nicotinic receptor subtype.

Keywords: Anxiety; Dorsal raphé nucleus; Di-hydro-β-erythroidine hydrobromide; Locomotor activity; Nicotine; Social interaction test

1. Introduction

Nicotine has been reported to reduce anxiety in both smokers (Gilbert, 1979; Gilbert et al., 1989; Pomerleau, 1986; Royal College of Physicians, 2000) and nonsmokers (Hutchinson and Emley, 1973; File et al., in press). Anxiolytic effects of nicotine have also been reported in several experimental models of anxiety, including the light–dark crossing test (Costall et al., 1989), mirrored chamber (Cao et al., 1993), fear-potentiated startle (Vale and Green, 1986) and the elevated plus maze (Brioni et al., 1993). In the social interaction test of anxiety, the anxiolytic effect of nicotine was found to be dose and time dependent (File et al., 1998; Irvine et al., 1999). Furthermore, the dorsal raphé nucleus (DRN) has been identified as an important neuroanatomical substrate mediating nicotine's effects in the social interaction test, and low doses of nicotine (2.5–10 ng) induced an anxiolytic effect when administered directly into this brain region (File et al., 1999; Cheeta et al., 2001).

Nicotinic acetylcholine receptors (nAChRs) are widely distributed in the CNS, and they exist in a variety of subtypes composed of genetically distinct subunits. The neuronal nicotinic acetylcholine receptor gene family is composed of eight α subunits (α2–α9) and three β subunits (β2–β4). In the rodent central nervous system, the most characterised forms of nicotinic receptors are the α4β2 receptor and an α-bungarotoxin-sensitive homopentameric receptor consisting solely of α7 subunits (Lena and Changeux, 1997; Lukas et al., 1999). Due to the development of a number of new compounds that show selective affinities for specific subtypes of the nicotinic receptor, and because of the wide ranging behavioural effects of nicotine (Decker et al., 1995; Holladay et al., 1997; Lena and Changeux, 1997), considerable research interest has focussed on trying to establish the in vivo functional roles of these nicotinic receptors. Accumulating evidence from a number of studies, which have evaluated the effects of nicotinic-receptor subunit antagonists, suggests that the

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α4β2 subtype may be responsible for mediating many of the behavioural effects of nicotine.

Infusion with the preferential α4β2 nicotinic receptor subunit antagonist di-hydro-β-erythroidine hydrobromide (DHβE) produced an attenuation of nicotine self-administration following both systemic administration (Stolerman et al., 1997; Watkins et al., 1999; Grottick et al., 2000) and direct administration into the ventral tegmental area or the pedunculopontine tegmental nucleus (Corrigall et al., 1994; Lanca et al., 2000). DHβE precipitates a withdrawal reaction as measured by elevations in the threshold for intracranial self-stimulation in nicotine dependent rats (Epping-Jordan et al., 1998) and has been implicated in mediating nicotine-induced locomotor activity (Grottick et al., 2000). DHβE also antagonises nicotine's discriminative stimulus and taste aversion effects (Stolerman et al., 1997; Shoaib et al., 2000; Gommans et al., 2000) and the enhanced cognitive performance with nicotine that has been demonstrated in the five-choice serial reaction time task (Blondel et al., 2000; Grottick and Higgins, 2000). As yet, the identity of the nicotinic receptor subtype or subtypes involved in mediating nicotine's anxiolytic actions has not been elucidated, but evidence points to a role for the α4β2 receptor subtype. A number of nicotinic agonists that bind to the α4β2 receptor configuration in vitro are known to have an effect on anxiety (Pomerleau, 1986; Gilbert et al., 1989; Brioni et al., 1993). The anxiolytic effect of nicotine in the social interaction test is mediated by the DRN, and evidence suggests that α4-containing nicotinic receptors are expressed within the DRN (Decker et al., 1998). Furthermore, only very low doses of nicotine are needed to induce an anxiolytic action of nicotine when administered both systemically and intra-DRN nicotine, and the α4β2 nicotinic receptor subtype is known to have a high affinity for nicotine (Buisson et al., 1996; Chavez-Noriega et al., 1997). The present experiment therefore examined the possibility that the anxiolytic effect of intra-DRN nicotine in the social interaction test is produced by the stimulation of the α4β2 nicotinic receptor subtype by coadministering a behaviourally inactive dose of DHβE with nicotine into this brain region. We aimed at the caudal region of the DRN because of evidence that this is the site of action on the 5-HT neurones of the anxiogenic neuropeptide CRF (Lowry et al., 2000).

2. Materials and methods

2.1. Animals

Male hooded Lister rats (Charles River, Margate, Kent, UK) weighing between 220 and 250 g were used in all experiments. Rats were housed singly following surgery and allowed to recover for 5 days prior to behavioural testing. The unoperated rats used in each experiment were housed singly for the same length of time as their operated test partners. Food and water were freely available for all animals. The room in which animals were housed was lit with dim light and maintained at 22 °C. Lights were on from 0700 to 1900 h. The experimental procedures carried out in this study were in compliance with the UK Animals (Scientific Procedures) Act 1986 (Home Office Project License Number 70/4041).

2.2. Apparatus

The social interaction test arena was a wooden box 60×60 cm, with 35 cm walls, and was lit by high light (300 lx). A camera was mounted vertically above the arena, and the rats were observed on a monitor in an adjacent room. The time spent in social interaction (sniffing, following, grooming the partner, boxing and wrestling) provided the measure of anxiety and was scored by an observer who was blind to the drug treatment. The interruption of infrared beams from photocells mounted in the walls 3.5 cm from the floor provided an automated measure of locomotor activity (File, 1980).

2.3. Drugs and chemicals

(−)-Nicotine hydrogen tartrate (Sigma, Poole, UK) and DHβE (RBI, St. Albans, Herts, UK) were dissolved in artificial cerebrospinal fluid (aCSF) of the following composition (in mM): 126.6 NaCl, 27.4 $NaHCO_3$, 2.4 KCl, 0.5 KH_2PO_4, 0.89 $CaCl_2$, 0.8 $MgCl_2$, 0.48 Na_2HPO_4 and 7.1 glucose, pH 7.4. Nicotine and DHβE were administered alone, but in order to perform the antagonism study, agonist and antagonist compounds were coadministered together in a single injection. All injection volumes were 0.5 μl. Injections were made over a period of 30 s using a CMA/102 microdialysis pump (Biotech Instruments, Stockholm, Sweden) and the needles were left in position for a further 30 s to allow drug diffusion; control animals received 0.5-μl infusions of aCSF. All doses are given as free base.

2.4. Surgery

In two rats, the stereotaxic coordinates were first verified histologically prior to the start of cannulations in the rest of the animals. Rats were anaesthetised by inhalation of 3% isofluorane (May and Baker, Dagenham, Essex, UK) in oxygen and positioned in the stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). The skull was exposed and the incisor bar adjusted such that bregma and lambda were at the same height. Four indentations were made in the skull to accommodate screws, which, together with the application of dental cement, held the cannulae in place. For unilateral cannulation of the DRN, 12-mm-long steel guide cannulae (23 gauge, Cooper's Needle Works, Birmingham, UK) were positioned at 7.4 mm posterior to bregma, 2.2 lateral and vertical −4.7 mm at an angle of 19°, thus siting them 2 mm

above the target area (according to the atlas of Paxinos and Watson, 1986). Cannulae were kept patent using 12-mm-long stainless-steel stylets (30 gauge, Cooper's Needle Works). On test days, rats were gently wrapped in a cloth and injected using needles constructed from 30-gauge steel tubing that extended 2 mm below the tip of the in-dwelling cannulae, into the DRN. In order to accustom the animals to handling and to keep the stylets patent, each day following surgery, the rats were gently wrapped in a cloth and the stylets were replaced.

2.5. Behavioural testing

2.5.1. Social interaction test

In the social interaction test, the light level and familiarity of the test arena can be varied in order to modify the level of anxiety generated by the test. A moderate level of anxiety is generated by testing in a brightly lit arena with which rats have been familiarised. This is the test condition selected for this experiment, since it has proved sensitive to the anxiolytic effects of systemic and intra-DRN nicotine (File et al., 1998, 1999; Irvine et al., 1999; Cheeta et al., 2001). Therefore, in order to familiarise rats to the social interaction test arena, each rat was placed individually in the test arena, under high light conditions of illuminance (300 lx) for a 5-min familiarisation trial on each of the 2 days prior to social interaction testing. In the experiment, rats were allocated to pairs, such that members of a pair did not differ in weight by more than 10 g. Three minutes after central injection, the operated rat was placed together with its unoperated and uninjected partner in the test arena and social interaction initiated by the treated rat was scored. Social interaction was scored for 4.5 min by an observer blind to the drug treatment. Rats were tested between 0900 and 1300 h in an order randomised for drug treatment, and the test arena was cleaned with a paper towel after each trial.

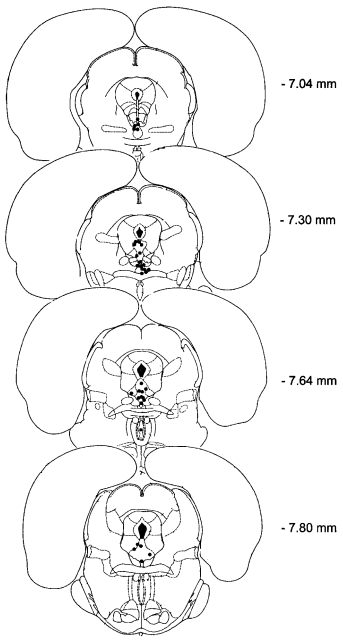

Fig. 1. Coronal sections of rat brain showing injection sites (filled circle) of the DRN. The single placement falling outside the target area is shown by a filled square marking the tip of the injection needle. Values give the distance in mm anterior to posterior to bregma, according to the atlas of Paxinos and Watson (1986).

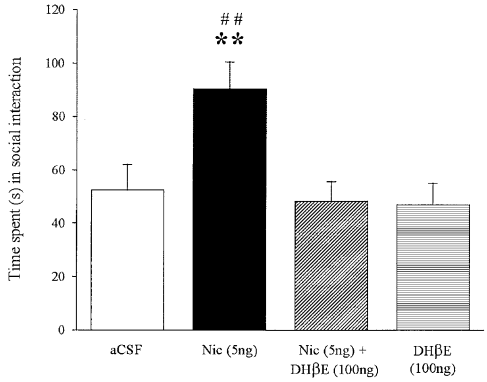

Fig. 2. Mean (± S.E.M.) time spent in social interaction by rats tested in the high light, familiar test condition following an injection into the DRN of artificial cerebrospinal fluid (aCSF, $n=9$), nicotine (Nic 5 ng, $n=11$), nicotine+DHβE (Nic 5 ng+DHβE 100 ng, $n=9$) or DHβE (DHβE 100 ng, $n=8$). $^{**}P<.01$ compared with aCSF control group, $^{\#\#}P<.01$ compared with Nic+DHβE group, Fisher's test after analysis of variance.

2.5.2. Reversal of the anxiolytic effect of nicotine by DHβE

Cannulated animals were divided into four experimental groups that were administered one of the following drug treatments, aCSF ($n=9$), nicotine 5 ng ($n=11$), nicotine 5 ng and DHβE 100 ng ($n=9$), and DHβE 100 ng ($n=8$). The numbers in parentheses indicate the numbers of rats in each group with verified cannulae placements. The dose of 5 ng nicotine was selected on the basis of previous studies (File et al., 1999; Cheeta et al., 2001). The dose of 100 ng DHβE was used in the antagonism study since it did not affect the time spent in social interaction when administered alone.

2.5.3. Histology

At the end of behavioural testing, all animals were killed, the brains removed and the injection sites verified histologically (according to the atlas of Paxinos and Watson, 1986) by a person blind to drug treatment.

2.5.4. Statistics

Data were analysed by two-way analyses of variance (ANOVAs). Comparisons between individual groups were then made with Fisher's least squares difference (LSD) test. To confirm that the effects of the drugs on social interaction were not due to changes in locomotor activity, analyses of covariance (ANCOVAs) were conducted in order to determine the independence of these two effects.

3. Results

3.1. Histology

As can be seen in Fig. 1, all placements were found to be within the anterior planes of −7.04 to −7.80 mm to bregma. Cannulae placements were generally located in the vicinity of the DRN, with most placements being directly in the DRN and a few of the placements were located just below in the DRN in the caudal linear raphé nucleus. Analysis of the behavioural data demonstrated that placements falling within the caudal linear nucleus raphé showed a very similar profile to placements falling directly within the DRN. Therefore, all these placements were included in the data analysis and are shown on Fig. 1 by filled circles. One animal was discarded from statistical analysis on the basis that its injection site was located well below the DRN in the region of the median raphé nucleus (filled square). The social interaction score of this animal which was injected with nicotine was 3.7 s.

3.2. Reversal of the anxiolytic effect of nicotine by DHβE

As can be seen in Fig. 2, nicotine (5 ng) significantly increased the time spent in social interaction [$F(1,33) = 4.72$, $P<.05$], and this effect was reversed by DHβE (100 ng), which had no effect on social interaction when administered alone [Nicotine×DHβE interaction, $F(1,33) = 4.1$, $P = .05$]. Nicotine when administered alone had no effect on locomotor activity [$F(1,33) = 1.0$], but there was a significant stimulatory effect of DHβE on locomotor activity [$F(1,33) = 8.5$, $P<.01$], and this was not antagonised by nicotine [Nicotine×DHβE interaction, $F(1,33) = 1.0$]. Thus, both the DHβE alone and in combination with nicotine differed significantly from the αCSF control group (Fisher's tests, $P<.05$), see Table 1. Analysis of covariance confirmed that the reversal of nicotine's anxiolytic effect by DHβE was independent of its effects on locomotor activity (adjusted means: nicotine = 91.6, nicotine+DHβE = 44.3, $P<.01$, Fisher's test).

Table 1
Mean (± S.E.M.) locomotor activity (beam breaks) of rats tested in the high light, familiar condition in the social interaction test following an injection into the DRN of aCSF, nicotine (5 ng), nicotine (5 ng) plus DHβE (100 ng) or DHβE (100 ng)

Drug treatment	Locomotor activity	*n*
aCSF	184±14.7	9
Nicotine 5 ng	215±7.39	11
Nicotine 5 ng and DHβE 100 ng	252±24.8*	9
DHβE 100 ng	253±17.3*	8

n, number of rats in each group.
* $P<.05$ compared with the aCSF control group, on Fisher's test after analysis of variance.

4. Discussion

The present results confirm that intra-DRN nicotine at a low dose (5 ng) induces an increase in social interaction, that is not accompanied by concomitant changes in locomotor activity, indicating an anxiolytic effect (File et al., 1999; Cheeta et al., 2001). The anxiolytic effect of nicotine (5 ng) was completely reversed by 100 ng DHβE, a dose that did not modify the social interaction score when administered alone into the DRN. Furthermore, this reversal was confirmed to be independent of the locomotor stimulant effect of DHβE. In the present study, it was also noted that administration of nicotine and DHβE, either alone or in combination, in injection sites that were located within the linear caudal raphe nucleus, which is adjacent to the DRN (see Fig. 1), produced behavioural effects that paralleled those found when the drugs were administered directly into the DRN. These data therefore suggest that either this neuroanatomical substrate also subserves an anxiolytic role or that the behavioural effects of 0.5-μl infusions of drug within the very close vicinity of the DRN could well have resulted from diffusion of nicotine and DHβE from the injection site to nicotinic receptors located within the DRN. The caudal region of the DRN has been identified as the site of action of the anxiogenic peptide CRF (Lowry et al., 2000), and our results suggest that this area may also be important for the nicotinic modulation of anxiety. The single score from an animal injected with nicotine into the median raphé nucleus was extremely low, suggesting that this region might mediate an anxiogenic effect. This is similar to the finding of an anxiogenic effect of flumazenil when injected into the periaqueductal grey (Gonzalez and File, 1997).

In vitro studies have shown DHβE to selectively target particular subtypes of nicotinic receptors. Studies have consistently demonstrated that *Xenopus* oocytes containing nicotinic receptors of the α4β2 subtype are more sensitive to DHβE than those of oocytes containing either α3β2, α3β4 or α2β2 subunit combinations (Luetje and Patrick, 1991; Harvey and Luetje, 1996; Harvey et al., 1996), suggesting DHβE acts primarily at α4 subunits, and probably at the α4β2 subtype. However, due to the lack of more selective antagonists, the present data do not make it possible to totally exclude the roles of other high-affinity nicotinic receptor subtypes in mediating the anxiolytic action of intra-DRN nicotine in the social interaction test. Furthermore, the use of a single dose of DHβE also places limitations on the interpretation of the results. However, it has also recently been demonstrated that α4 subunit knockout mice show anxiogenic effects in the elevated plus-maze test (Ross et al., 2000).

The neurochemical mechanism mediating nicotine's anxiolytic action in the social interaction test has been well characterised. It has been demonstrated that the anxiolytic effect of intra-DRN nicotine was completely antagonised by coadministration of a behaviourally inactive dose of the 5-HT_{1A} receptor antagonist, WAY 100635 (Cheeta et al.,

2001). Since nicotine stimulates the release of 5-HT in the DRN (Mihailsecu et al., 1998), and the anxiolytic effect of nicotine is reversed by WAY 100635, it would seem likely that nicotine indirectly stimulates the somatodendritic 5-HT_{1A} autoreceptors, leading to a reduction in 5-HT neuronal firing and a subsequent decrease in 5-HT release in terminal regions of the limbic system. A recent study may provide physiological evidence in support of this proposition, since it was demonstrated that intravenous nicotine administration reduced DRN firing in anaesthetised rats, an effect that could also be antagonised by WAY 100635 (Engberg et al., 2000). The reversal of the anxiolytic effect of nicotine by DHβE suggests that the nicotine-induced 5-HT release is mediated by presynaptic high-affinity nicotinic receptors, probably of the α4β2 subtype. An involvement of this receptor subtype in 5-HT release in the DRN would be consistent with the finding that nicotine-elicited currents could not be evoked from 5-HT neurones in the DRN in mice lacking both or either of the α4 and β2 subunits (Cordero-Erausquin et al., 2000). Although there are several reports of the presence of α7 receptors in various regions of the CNS, the present study did not examine the involvement of nicotinic receptors containing the α7 subunit in mediating the anxiolytic effects of nicotine. There are two main reasons for this. Firstly, in vitro evidence suggests that DRN neurones are insensitive to the specific α7 nicotinic receptor antagonist methyllycaconitine (Li et al., 1998). Secondly, α7 receptors have largely been implicated in the release of glutamate and ACh, and not in 5-HT release, which is thought to mediate nicotine's anxiolytic effects.

In the present study, while DHβE when administered alone did not change social interaction, it did significantly enhance locomotor activity. These findings are in contrast to the existing literature on the effects of DHβE on locomotor activity. Following systemic administration, DHβE does not affect locomotor activity and DHβE potently antagonised nicotine-induced hyperactivity (Stolerman et al., 1997; Grottick et al., 2000). Furthermore, an enhancement of locomotor activity was observed after systemic treatment with the selective α4β2 nicotinic receptor subtype agonist SIB 1765F (Menzaghi et al., 1997; Grottick et al., 2000). However, very few studies have investigated the effects of DHβE on locomotor activity following central drug administration, and the present findings may either result from higher drug concentration reaching the DRN following local injection, or because there are opposing stimulant and depressant effects mediated by different brain regions. The locomotor stimulant effect of DHβE was not antagonised by coadministration of nicotine, but this may well have been because an insufficiently high dose of nicotine was used.

In summary, the results from the present pharmacological study suggest that the anxiolytic effects of nicotine in the social interaction test are likely to be mediated by the α4β2 nicotinic receptor subtype in the DRN. These findings add to a growing body of evidence suggesting an important role for this nicotinic receptor subtype in mediating many of the most well-characterised behavioural effects of nicotine.

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