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Locomotor activation and dopamine release produced by nicotine and isoarecolone in rats

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1 Isoarecolone was approximately 250 times less potent than nicotine as an inhibitor of [^{3}H]-nicotine binding to rat brain membranes. Isoarecolone failed to inhibit the binding of the nicotinic ligand [^{125}I]-α-bungarotoxin or of the muscarinic ligand [^{3}H]-QNB.

2 Nicotine (0.01-30 μM) evoked the release of [^{3}H]-dopamine from striatal and frontal cortex synaptosomes, with EC_{50} values of approximately 0.5 μM in each case. This release was largely mecamylamine-sensitive.

3 Isoarecolone (1-200 μM) evoked predominantly mecamylamine-sensitive dopamine release from both striatal and cortical synaptosomes, with a potency at least 20 times less than that of nicotine. The maximum effect of isoarecolone was less than that of nicotine, particularly in the frontal cortex preparation.

4 In control rats treated chronically with saline, neither nicotine nor isoarecolone had clear effects on locomotor activity at the doses tested. Chronic treatment with nicotine clearly sensitized rats to the locomotor activating effect of nicotine; there was only weak cross-sensitization to isoarecolone. The maximum effect of isoarecolone was seen at a dose about 40 times larger than that of nicotine.

5 The low potency and efficacy of isoarecolone in facilitating sensitized locomotor activity resembled its lower potency and efficacy, compared with nicotine, in evoking dopamine release *in vitro*. The agonist profile of the nicotinic receptor population mediating dopamine release may determine the pharmacological characteristics of consequent locomotor behaviour.

Keywords: Nicotine; isoarecolone; locomotor activity; striatum; frontal cortex; dopamine

Introduction

Nicotine has a broad spectrum of behavioural effects, including the ability to serve as a positive reinforcer and as a discriminative stimulus, and to promote changes in rates of both conditioned and unconditioned behaviour (Clarke, 1987; Stolerman, 1990). Recent investigations have compared the behavioural effects of (-)-nicotine with those of other nicotinic agonists to determine whether each agonist exhibits the whole spectrum of effects shown by nicotine. Some agonists, such as N-(3-pyridylmethyl)pyrrolidine (PMP), were indistinguishable from nicotine in the tests used; the nicotine discriminative stimulus generalised to these compounds which also decreased locomotor activity in experimentally naive rats and increased locomotion under different experimental conditions (i.e. after repeated exposure to nicotine and to the test apparatus). Certain other compounds, such as isoarecolone and 1-acetyl-4-methylpiperazine, were generalized with nicotine and decreased locomotor activity in naive rats, but failed to increase locomotion under the conditions where nicotine had such an effect (Reavill *et al.*,1987; 1990; Garcha *et al.*, 1993). These results indicate that not all nicotinic agonists produce the full spectrum of behavioural effects of (-)-nicotine, suggesting possible differences in mechanisms at the receptor level. However, all the agonists totally inhibited the binding of [^{3}H]-nicotine to rat brain membranes, so a differential interaction with this particular nicotinic receptor site is unlikely to be the explanation of the differences in behavioural effects.

The mechanisms underlying these differences in behavioural effects have not been defined and the present study aims to provide novel neurochemical data that shed some light on the differences between two of the nicotinic agonists. Microinjections of nicotine into the ventral tegmental area that contains the cell bodies of the mesolimbic dopamine system can increase locomotor activity in rats (Pert & Chiueh, 1986; Reavill & Stolerman, 1990; Leikola-Pelho & Jackson, 1992). Nicotine, given systemically, can also increase the extracellular concentration of dopamine in the nucleus accumbens, with some selectivity as compared with the striatum (Imperato *et al.*, 1986; Damsma *et al.*, 1989; Mifsud *et al.*, 1989; Brazell *et al.*, 1990). Neurotoxin lesions of the mesolimbic dopamine pathway can attenuate or abolish the locomotor activating and positive reinforcing effects of nicotine (Clarke *et al.*, 1988; Corrigall *et al.*, 1992). However, it is not known whether different effects of nicotinic agonists in releasing dopamine from ascending dopamine pathways might account for variations in their locomotor activating effects. Chronic exposure to nicotine produces a striking upregulation of the numbers of nicotinic receptors, as well as sensitization to the ability of nicotine to increase extracellular dopamine (Benwell & Balfour, 1992); different effects of nicotinic agonists on these adapted receptors may contribute to differences in their behavioural effects.

Nicotine can act directly on presynaptic nicotinic receptors localized on dopaminergic nerve terminals (Wonnacott *et al.*, 1990) and at nicotinic receptors on dopaminergic cell bodies in the ventral tegmental area (Nisell *et al.*, 1994) and substantia nigra (Clarke *et al.*, 1985). Although it is possible that all of these loci play a significant role in mediating the behavioural effects of nicotine, for the present experiments, the presynaptic modulation of dopamine release was used as an *in vitro* model for studying the functional interactions of drugs with nicotinic receptors. The stimulation of dopamine release from striatal synaptosomes by activation of nicotinic receptors has been well documented (e.g. Rapier *et al.*, 1990; Grady *et al.*, 1992; El-Bizri & Clarke 1994), and provides a system for comparing the activities of nicotinic agonists. Although nicotine-evoked dopamine release from minced nucleus accumbens *in vitro* has been described (Rowell *et al.*, 1987), the tiny size of this brain region precludes its use for most studies. The corpus striatum used here encompasses accumbens as well as striatum (Heimer *et al.*, 1985). Like the mesolimbic pathway, the mesocortical dopamine system projecting to the frontal cortex arises from

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cell bodies in the ventral tegmental area. Despite its importance in mediating drug effects on motivated action and movement (Koob, 1992) this pathway has been relatively neglected in neurochemical studies. Therefore dopamine release from frontal cortex terminals has also been examined for comparison with striatal preparations.

Experiments have, therefore, been carried out to compare the effects of nicotine and isoarecolone on these dopamine systems *in vitro* and on behaviour. In particular, the hypothesis was tested that isoarecolone failed to increase locomotor activity in earlier studies (Reavill *et al.*, 1990) because it was less potent or less efficacious than nicotine as a releaser of dopamine. For the earlier studies, isoarecolone was prepared according to the original synthesis and was found to be 50 times less potent than (–)-nicotine as an inhibitor of [^{3}H]-(–)-nicotine binding (Spivak *et al.*,1986; Reavill *et al.*, 1987). For the present work, isoarecolone was prepared according to the more efficient synthesis of Ward & Merritt (1990) and the effects of this material on binding sites for nicotinic and muscarinic ligands have been determined. In addition, the effects of nicotine and isoarecolone on locomotor activity were directly compared in a wide range of doses in rats chronically exposed to nicotine or saline, with the amount of previous exposure to the test apparatus held constant. It was not possible, in previous studies, to distinguish the role of chronic nicotine exposure from the other factors, such as the apparatus exposure and test duration, that may have influenced responses to nicotine and isoarecolone (Reavill *et al.*, 1990).

Previous studies have shown that the locomotor activating effect of nicotine becomes more marked upon repeated administration of the drug (Clarke & Kumar, 1983; Ksir *et al.*, 1985; 1987). Similarly, the locomotor stimulant effect of amphetamine, which is also mediated through increases in extracellular dopamine in the accumbens, can show sensitization after amphetamine is administered repeatedly (Robinson & Becker, 1986). In view of the possible similarity between the dopaminergic mechanisms through which amphetamine and nicotine increase locomotion, behavioural tests for cross-sensitization from nicotine to amphetamine have been carried out.

Methods

Animals

For behavioural experiments, male Lister hooded rats (Olac, Bicester) were housed individually at a temperature of 21 ± 1°C with a regular light-dark cycle (light from 07 h 30 min – 19 h 30 min). Initially the animals weighed 210–280 g. For ligand-binding and monoamine release experiments, preparations of rat brain were made from male Sprague-Dawley rats (Bath Animal House breeding colony) weighing 250 g. All animals had unlimited access to food and water at all times.

Preparation of synaptosomes

Rats were killed by cervical dislocation, the brains removed and cut into rostral and caudal sections at the level of the hypothalamus. The exposed striatal tissue was removed from both sides of the cut with a microspatula. The remaining rostral section of the brain was used as frontal cortex tissue. Tissue (10% w/v) in 0.32 M sucrose, pH 7.4 was homogenized at 4°C with 12 strokes in a rotary homogenizer (glass mortar, PTFE pestle, 300 r.p.m., 0.3 mm clearance). The resulting homogenate was centrifuged (3000 *g*, 10 min). The supernatant was recovered with a Pasteur pipette and recentrifuged (20000 *g*, 20 min), while the pellet (P_1) was discarded. Following recentrifugation the supernatant was discarded. The pellet (P_2) was then resuspended and used for release studies (see below). For ligand-binding studies, a P_2 membrane fraction was prepared from rat brain essentially as above, but with the addition of protease inhibitors (Rapier *et al.*, 1990).

Ligand-binding

For inhibition of [^{3}H]-(–)-nicotine binding, 10 μl of drug was incubated for 10 min at 20°C with the prepared membranes (250 μl; approximately 2 mg protein ml^{-1} in Krebs-Ringer HEPES buffer containing NaCl 118 mM, KCl 4.8 mM, $CaCl_2$ 2.5 mM, HEPES 20 mM, Tris 200 mM, pH 7.4). This was followed by addition of [^{3}H]-(–)-nicotine (10 nM) and incubation at 20°C for a further 1 h, cooling to 4°C and filtration using a Brandel Cell Harvester. Non-specific binding was determined by including 10^{-3} M (–)-nicotine in the initial incubation. Filters were counted for tritium in 5 ml Optiphase 'Safe' scintillant in a Packard Tricarb 1600TR scintillation spectrometer. Counting efficiency (≈40%) was determined for each experiment by reference to triplicate tritium standards.

For inhibition of [^{125}I]-α-bungarotoxin binding, drug (20 μl) was incubated at pH 7.5 for 10 min at 20°C with rat brain P_2 membranes (500 μl; approximately 1 mg protein ml^{-1} in 50 mM potassium phosphate buffer). This was followed by addition of [^{125}I]-α-bungarotoxin (1 nM) and incubation at 37°C for a further 2 h. Non-specific binding was determined by including 10^{-6} M α-bungarotoxin in the initial incubation. Bound ligand was precipitated by centrifugation (MSE Microfuge, 2 min at 10,000 *g*) and the membrane pellets were washed by resuspension in phosphate-buffered saline (2 mM KH_2PO_4, 8 mM K_2HPO_4, 150 mM NaCl, pH 7.4) at 4°C, followed by recentrifugation. Radioactivity was quantitated in a Packard Cobra II gamma spectrometer.

For inhibition of [^{3}H]-QNB binding, drug (10 μl) was incubated for 10 min at 20°C with P_2 membranes (250 μl, approximately 0.2 mg protein ml^{-1} in Krebs-Ringer HEPES buffer containing NaCl 118 mM, KCl 4.8 mM, $CaCl_2$ 2.5 mM, HEPES 20 mM, Tris 200 mM, pH 7.4). This was followed by the addition of 10 μl [^{3}H]-QNB (0.2 nM) and incubation for a further 1 h, cooling to 4°C and filtration using a Brandel Cell Harvester. Non-specific binding was determined by including 10^{-5} M atropine in the initial incubation. Filters were counted for tritium in 5 ml Optiphase 'Safe' scintillant in a Packard Tricarb 1600TR scintillation spectrometer (counting efficiency ≈40%, as above).

[^{3}H]-dopamine release

[^{3}H]-dopamine release was recorded in an open chamber superfusion apparatus similar to that described previously (Rowell & Wonnacott 1990; Grady *et al.*, 1992). Briefly, P_2 synaptosome preparations (150 μl, 5 mg protein ml^{-1}), were loaded with 0.11 μM [7,8-^{3}H]-dopamine in modified Krebs-Ringer bicarbonate buffer (NaCl 118 mM, KCl 2.35 mM, $CaCl_2$ 2.4 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25 mM, glucose 10 mM, (–)-ascorbic acid 2 mM and pargyline 100 μM, pH 7.4). Uptake values were 28.2 ± 5.1 pmol ([^{3}H]-dopamine) mg^{-1} (protein) for striatal preparations (mean ± s.e.mean for 10 experiments) and 15.1 ± 4.8 pmol ([^{3}H]-dopamine) mg^{-1} (protein) for frontal cortex preparations (mean ± s.e.mean for 10 experiments). The synaptosomes were layered onto Gelman A/E filter discs in chambers made from the bases of Bio-Rad polyprep columns. Eight chambers were run in parallel. Assay buffer (with the addition of 5 μM nomifensine) was dripped onto the samples at a rate of 0.5 ml min^{-1}, and removed at a rate of 0.52 ml min^{-1} with a pair of Gilsen 8 channel peristaltic pumps. The apparatus was maintained at 37°C with a Brandel temperature control hood. Superfusates were collected from each chamber at intervals of 2 min and counted for tritium in 5 ml Optiphase 'Safe' scintillant in a Packard Tricarb 1600TR scintillation spectrometer (counting efficiency ≈40%). Agonist solutions (40 s pulses) were introduced into the apparatus *via* the input pump, separated from the main flow by 10 s air gaps. In studies with mecamylamine (20 μM), the antagonist was included in the perfusion buffer throughout the superfusion to determine the receptor-mediated component of the response.

Locomotor activity

Locomotor activity was recorded in cages equipped with photocells and two beams of infra-red light as described previously (Reavill *et al.*, 1990). During each session, interruptions of either beam of light that followed interruptions of the other beam were recorded as 'cage crosses', the main measure of activity that was intended to reflect locomotion rather than repetitive movements in the same place. Successive interruptions of the same beam were called 'repeated moves'; this supplementary measure may reflect rearing onto the hind legs, grooming and small locomotor movememts falling short of crossing over to the other side of the cage (plus other stereotyped behaviours). Fourteen rats were allocated to 2 groups by a random method ($n=7$). One group of rats received daily injections of (–)-nicotine at a dose of 0.4 mg kg^{-1}; subsequently, these animals are called 'chronic-nicotine' rats. The second groups ('chronic-saline' rats) received saline injections whenever rats in the first group received nicotine; all animals were placed in the activity cages for 60 min beginning immediately after injection. These procedures continued 5 days a week for 8 weeks (Clarke & Kumar, 1983; Reavill *et al.*, 1990).

Two experiments were then carried out with the 14 rats. In the first of these experiments, four doses of (–)-nicotine and six doses of isoarecolone and saline were tested once in each rat over a period of 5 weeks. A different random sequence of testing was used for each rat. The doses of nicotine were 0.04, 0.13, 0.4, and 0.8 mg kg^{-1} (s.c.) and the doses of isoarecolone were 0.4, 1.3, 4.0, 8.0, 16 and 32 mg kg^{-1} (s.c.). In the second experiment similar tests were carried out over a period of 3 weeks after injections of (+)-amphetamine (0.1, 0.32, and 1.0 mg kg^{-1} (s.c.)) or saline. All test sessions began immediately after administrations of drugs and lasted for 1 h within which data were collected for 6 consecutive periods of 10 min each. Tests took place twice weekly and on the intervening days the rats continued to be placed in the photocell cages and to receive regular injections of (–)-nicotine or saline.

Data analysis

Competition binding assays were analysed by non-linear least squares curve fitting to the Hill equation to determine IC_{50} values. K_i values were calculated using the Cheng and Prusoff relationship (Cheng & Prusoff, 1973); in the case of α-bungarotoxin binding the derived K_i value is designated K_{iapp} to acknowledge the pseudo-irreversibility of binding with this ligand.

Most of the agonist-evoked [^{3}H]-dopamine release occurred within a single fraction (2 min), with a small 'tail' of release in the following fraction. For this reason, release was quantitated by summing the activity in these two samples followed by subtraction of the activity of the samples immediately before and after the peak. The peak of release was calculated as a fraction of the total [^{3}H]-dopamine present in the perfusion chamber, and in order to correct for variability in release between experiments, it was expressed as a percentage of the release induced by a 1 μM nicotine pulse, included as a standard in each experiment. Specific nicotine-evoked [^{3}H]-dopamine release was calculated as the difference between total and mecamylamine-sensitive release at each nicotine concentration. Where specific release attained a clear maximum, data were fitted to the Hill equation by non-linear least squares curve fitting, using the programme SigmaPlot for Windows.

Results for locomotor activity were examined by one- and two-factor analyses of variance for repeated measures, and by *t* tests, with the Unistat statistical package. The Tukey-B test was used for multiple comparisons.

Drugs

For ligand-binding studies, (–)-[N-methyl-^{3}H]-nicotine (80 Ci $mmol^{-1}$), [benzilic-4,4'-^{3}H(N)]-L-quinuclidinyl benzilate ([^{3}H]-QNB, 30–60 Ci $mmol^{-1}$), and [^{125}I]-Na were purchased from NEN DuPont, Stevenage, Herts. α-Bungarotoxin (Sigma, Poole, Dorset) was iodinated to a specific activity of 700 Ci $mmol^{-1}$. Isoarecolone oxalate was synthesized by SmithKline Beecham Pharmaceuticals, Harlow, Essex according to Ward & Merritt (1990); its purity was confirmed by ^{1}H and ^{13}C nuclear magnetic resonance (n.m.r.). (–)-Nicotine bitartrate was purchased from BDH (Poole, Dorset). Serial dilutions of drugs for competition assays were made in assay buffer.

For release studies [7,8-^{3}H]-dopamine (50 Ci $mmol^{-1}$) was purchased from Amersham International plc., Little Chalfont, Bucks. Stock solutions of isoarecolone oxalate and (–)-nicotine bitartrate (as above), mecamylamine (Sigma, Poole, Dorset), and nomifensine maleate (gift from Merck, Sharp & Dohme, Harlow, Essex) were prepared in Krebs-Ringer bicarbonate buffer and aliquots were stored at −20°C. Dilutions of drugs were made in assay buffer immediately before use. Pargyline (Sigma, Poole, Dorset) was stored desiccated in crystal form at −20°C and added directly to the assay buffer on the day of use.

For behavioural experiments, (–)-nicotine bitartrate was dissolved in saline and the pH was adjusted to 7.0 with dilute NaOH; isoarecolone oxalate and (+)-amphetamine sulphate (Smith Kline and French, Welwyn, Herts.) were dissolved in saline. All injections were subcutaneous in volumes of 1.0 ml kg^{-1} and the control was 0.9% saline. All doses were calculated as those of the bases.

Results

Potency of isoarecolone in nicotinic ligand binding assays

Isoarecolone oxalate was evaluated for activity at binding sites for muscarinic and nicotinic ligands in competition binding assays (Table 1). Isoarecolone competed for [^{3}H]-nicotine binding sites in rat brain membranes with a K_i of 4 μM and was approximately 250 times weaker than (–)-nicotine. At the nicotinic binding site defined by [^{125}I]-α-bungarotoxin, isoarecolone failed to inhibit binding. A similar lack of effectiveness was observed in competition assays with the muscarinic ligand [^{3}H]-QNB for sites on rat brain membranes.

[^{3}H]-dopamine release; nicotine and isoarecolone

The abilities of nicotine and isoarecolone to stimulate [^{3}H]-dopamine release from striatal synaptosomes were compared (Figure 1). Nicotine elicited little release at the lowest concentration studied (0.01 μM), but release increased steadily with higher concentrations (Figure 1a). Release evoked by the 1 μM nicotine standard in striatum was 291 ± 37 fmol [^{3}H]-dopamine mg^{-1} protein (mean ± s.e.mean). This was approximately double the basal release. The mecamylamine-insensitive (non-specific) component of release was low at all nicotine concentrations examined. Subtraction of mecamylamine-insensitive release from total release yielded mecamylamine-sensitive (specific) release (Figure 1c). This was slight at the lower nicotine concentrations (0.01–0.1 μM), rising to a sustained maximum at 10 μM nicotine and above.

Table 1 Nicotine and isoarecolone: comparison of potencies at cholinoceptor binding sites

Binding site	*(–)-Nicotine* K_i (M)	*Isoarecolone* K_i (M)
[^{3}H]-nicotine	$1.6 \pm 0.3 \times 10^{-8}$	$4.0 \pm 0.9 \times 10^{-6}$
[^{125}I]-Bgt	$2.4 \pm 1.6 \times 10^{-6}$	$> 10^{-3}$
[^{3}H]-QNB	ND	$> 10^{-3}$

[^{125}I]-Bgt: [^{125}I]-α-bungarotoxin; [^{3}H]-QNB = [^{3}H]-quinuclidinyl benzilate.

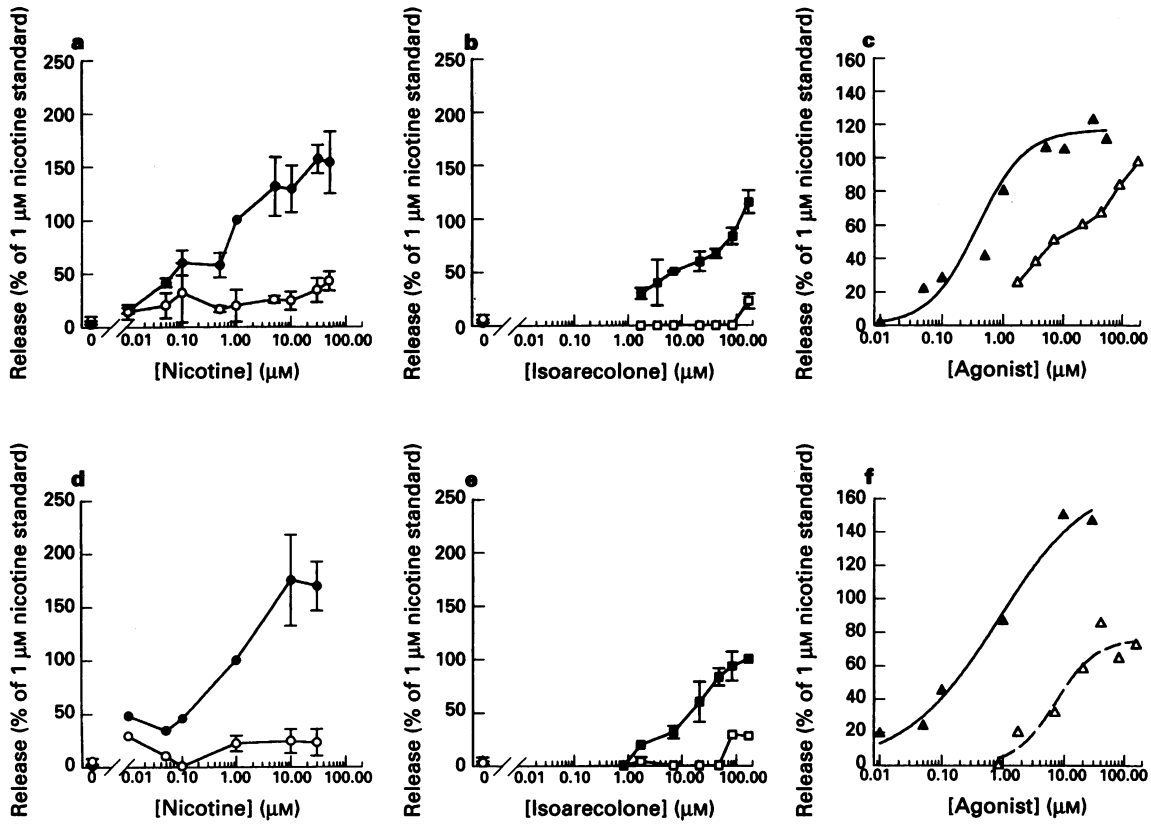

Figure 1 Dose-dependence of *in vitro* [^{3}H]-dopamine release from P_2 synaptosomes from rat striatum (upper panels) and frontal cortex (lower panels). (a,d) Nicotine-evoked total (●) and mecamylamine-insensitive release (○); (b,e) isoarecolone evoked total (■) and mecamylamine-insensitive release (□). Abscissae, concentration of agonists; ordinates, [^{3}H]-dopamine release expressed as a percentage of the reference standard (1 μM nicotine). Each point represents the mean ± s.e.mean of at least three independent experiments. (c,f) Mecamylamine-sensitive release induced by nicotine (▲) and isoarecolone (△). Points are derived from the preceding data.

For isoarecolone, [^{3}H]-dopamine release from striatal synaptosomes (Figure 1b) was studied over the concentration range 2–200 μM. At the lower concentrations, isoarecolone produced only a weak response. As the concentration of agonist was increased, the evoked release also rose; no peak was observed over the dose-range tested. Except at the highest concentration, all of the release of [^{3}H]-dopamine induced by isoarecolone was mecamylamine-sensitive (Figure 1b), in contrast to nicotine. As a result, specific isoarecolone-induced release exhibited the same characteristics as total release, rising steadily in tandem with the concentration of isoarecolone (Figure 1c).

[^{3}H]-dopamine release from the frontal cortex was also examined. Nicotine evoked the release of smaller amounts of [^{3}H]-dopamine than in the striatum (release evoked by the 1 μM nicotine standard in frontal cortex was 128 ± 24 fmol [^{3}H]-dopamine mg^{-1} protein (mean ± s.e.mean)). Similarly to striatal tissue, nicotine-induced release increased with nicotine concentration (Figure 1d), reaching a maximum at a nicotine concentration of 10 μM. Non-specific release was consistently low at all concentrations tested. Specific release induced by nicotine thus followed the same pattern as total release, increasing with nicotine concentration until it reached a plateau at 10 μM nicotine.

Total and non-specific isoarecolone-evoked [^{3}H]-dopamine release gave similar concentration-response profiles to nicotine in this tissue (Figure 1e). Total release was zero at the lowest concentration tested (2 μM), but rose steadily with increasing agonist concentration before levelling off at concentrations exceeding 20 μM. Only specific release was seen at isoarecolone concentrations below 90 μM and its concentration-dependence conformed to a sigmoid shape, rising from zero at 2 μM isoarecolone and reaching a plateau at 20 μM (Figure 1f).

In striatal and cortical tissues, the dose-response curves for isoarecolone were shifted to the right of those of nicotine, indicating the lower potency of isoarecolone as a dopamine releasing agent (approximate EC_{50} values were 0.5 μM for nicotine-evoked [^{3}H]-dopamine release from both tissue preparations, and ⩾10 μM for isoarecolone-induced release). In frontal cortex, the maximum specific release induced by nicotine was almost double that evoked by isoarecolone, suggesting that isoarecolone was also less efficacious than nicotine. In striatal tissue, no peak of release was observed over the concentration-range of isoarecolone that was tested, and the highest concentration studied (200 μM) evoked only 80% of the maximum release induced by nicotine.

Locomotor activity: nicotine and isoarecolone

Locomotor activity was recorded during tests of 60 min duration both in 'chronic-saline' rats and in 'chronic-nicotine' rats. In tests beginning immediately after injection of saline, the chronic-saline rats registered totals of 39.1 ± 6.4 cage crosses (mean ± s.e.mean) and 230 ± 17 repeated moves over the 60 min period of testing. The corresponding baseline scores for the chronic-nicotine rats were 47.6 ± 5.8 cage crosses and 277 ± 41 repeated moves; these scores did not differ from those for the chronic-saline rats ($t(12) = 0.97$ and 1.07, for cage crosses and repeated moves respectively).

The data for nicotine were analysed with a two-factor analysis of variance for repeated measures, the factors being the chronic treatment (nicotine or saline) and the test treatment (dose of nicotine). This analysis showed that when tested with nicotine, the chronic-nicotine rats yielded much larger numbers of cage crosses than the chronic-saline rats ($F(1,12) = 10.5$, $P < 0.01$); cage crosses were also influenced by the dose of nicotine ($F(3,36) = 7.9$, $P < 0.001$) but there was no group × dose interaction ($F(3,36) = 1.7$). Tukey tests confirmed

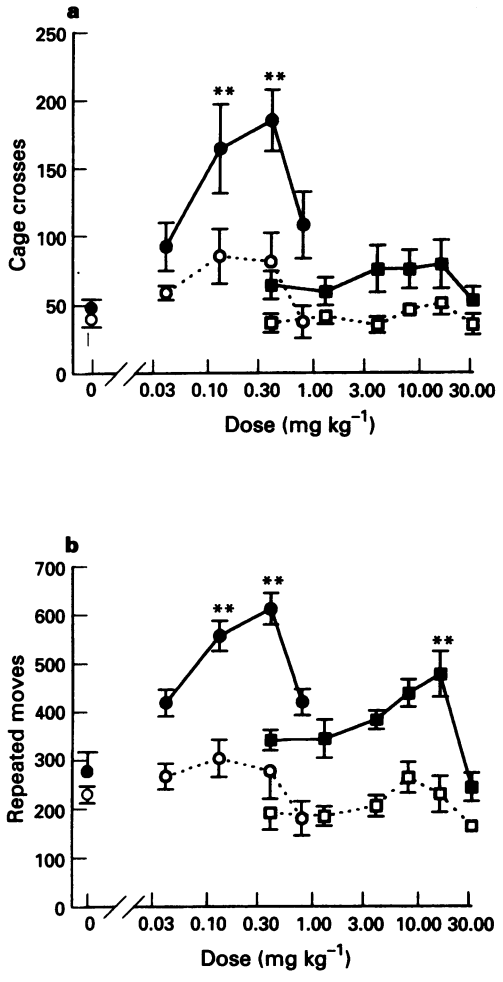

Figure 2 Effects of (-)-nicotine and isoarecolone on locomotor activity of rats treated chronically with saline or nicotine during 60 min periods beginning immediately after injections of nicotine or isoarecolone ($n=7$). Abscissae, doses; ordinates, movements between beams of infra-red light (a) or repetitive interruptions of same beam (b). Each point represents mean ± s.e.mean for doses shown of nicotine (○) and isoarecolone (□) in chronic-saline rats or in chronic-nicotine rats (●, ■). Significant differences from saline controls are marked (**$P<0.01$ according to Tukey tests).

that nicotine (0.13 and 0.4 mg kg^{-1}) increased cage crosses in the chronic-nicotine rats but not in the chronic-saline rats (Figure 2a). Two-factor analysis of variance of the results shown in Figure 2b also indicated that chronic-nicotine rats yielded larger numbers of repeated moves than chronic-saline rats ($F(1,12)=48.2$, $P<0.001$) and the effects due to the test dose of nicotine ($F(3,36)=11.3$, $P<0.001$) and the group × dose interaction were also significant ($F(3,36)=3.2$, $P<0.05$). Thus, in the chronic-nicotine rats, the maximum number of cage crosses after administration of nicotine (at a dose of 0.4 mg kg^{-1}) was 3.9 fold greater than that after saline, whereas the number of repeated moves was increased only 2.2 fold.

In contrast to the findings with nicotine, isoarecolone did not influence cage crosses in either the chronic-saline or the chronic-nicotine groups (Figure 2a); the overall scores were slightly greater after administering isoarecolone to the chronic-nicotine than to the chronic-saline group ($F(1,12)=5.73$, $P<0.05$), but there was no effect associated with the dose of isoarecolone ($F(5,60)=2.1$) or the group × dose interaction ($F(5,60<1$). No single dose of isoarecolone increased cage crosses above control levels (Figure 2a). The numbers of repeated moves were much greater after administering isoarecolone to chronic-nicotine than to chronic-saline rats ($F(1,60)=35.4$, $P<0.001$), and there was an effect attributable to the dose of isoarecolone ($F(5,60)=10.2$, $P<0.001$) but not to the group × dose interaction ($F(5,60)=2.3$). In the chronic-nicotine rats, isoarecolone increased repeated moves above control levels at a dose of 16 mg kg^{-1} only (Figure 2b) and the maximum numbers of cage crosses and repeated moves after administration of isoarecolone (16 mg kg^{-1}) were 1.6 and 1.7 fold larger than those after saline. Thus, the maximum number of cage crosses after administration of isoarecolone was significantly less than the number after any dose tested of nicotine ($P<0.01$, Tukey tests), but this was not the case for repeated moves.

The temporal pattern of cage crossing within the 60 min tests was examined to determine whether any differences between the effects of nicotine and isoarecolone on overall levels of activity may have reflected differences in duration of action rather than in maximal effects. There was a general decline in both cage crosses and repeated moves for control and treated rats as the session proceeded (Figure 3). To maintain clarity, Figure 3 shows only the results for saline and the doses of nicotine or isoarecolone that produced the largest overall scores.

In chronic-saline rats, neither nicotine (0.4 mg kg^{-1}) nor isoarecolone (16 mg kg^{-1}) had striking effects in any time period (Figure 3a and c). Analyses of variance for these data alone showed that the small increases in overall numbers of cage crosses after administering nicotine ($F(2,12=4.28$, $P<0.05$) was distributed fairly evenly over the whole 60 min session (drug × time interaction $F(10,60)<1$). Although there was no overall effect of either nicotine or isoarecolone on repeated moves ($F(2,12)<1$), there was a drug × time interaction with this measure ($F(10,60)=2.95$, $P<0.01$). Inspection of Figure 3c suggests that this was associated with reduced activity at the beginning of the session after administration of nicotine, followed by an increase above control levels.

In chronic-nicotine rats, there were substantial effects of the drugs on both cage crosses ($F(2,12)=42.7$, $P<0.001$) and repeated moves ($F(2,12)=31.5$, $P<0.01$). Nicotine (0.4 mg kg^{-1}) markedly increased both cage crosses and repeated moves throughout the 60 min test sessions. Isoarecolone (16 mg kg^{-1}) had no effect on cage crosses at any time (Figure 3b) but it increased the overall numbers of repeated moves (Figure 3d). Although isoarecolone appeared to have had its most striking effect early in the test session, there was no drug × time interaction ($F(10,60)=1.23$).

Locomotor activity: amphetamine

Tests for cross-sensitization to amphetamine were carried out with the chronic saline and chronic nicotine rats. In tests beginning immediately after injection of saline, the chronic-saline and chronic-nicotine rats registered totals of 65.7 ± 13.9 cage crosses and 98.6 ± 7.7 cage crosses respectively, over the 60 min period beginning immediately after injection; these scores did not differ ($t(12)=2.07$). However, the chronic-nicotine rats registered 493 ± 51 repeated moves in the test with saline, as compared with 267 ± 39 repeated moves for the chronic-saline rats ($t(12)=3.52$, $P<0.01$).

The results for tests with saline and amphetamine were examined with two-factor analyses of variance, with groups as one factor and dose of amphetamine as the other factor. Amphetamine increased the numbers of cage crosses in a dose-related manner ($F(3,36)=30.6$, $P<0.001$); there was no overall difference between the groups ($F<1$), but there was a group × dose interaction ($F(3,36)=3.20$, $P<0.05$). Inspection of Figure 4a suggests that this interaction was associated with a greater increase of cage crosses in the chronic-saline rats than in the chronic-nicotine rats. Figure 4b shows that amphetamine also increased the number of repeated moves ($F(3,36)=12.2$, $P<0.001$), but there was no overall difference between groups and no groups × dose interaction.

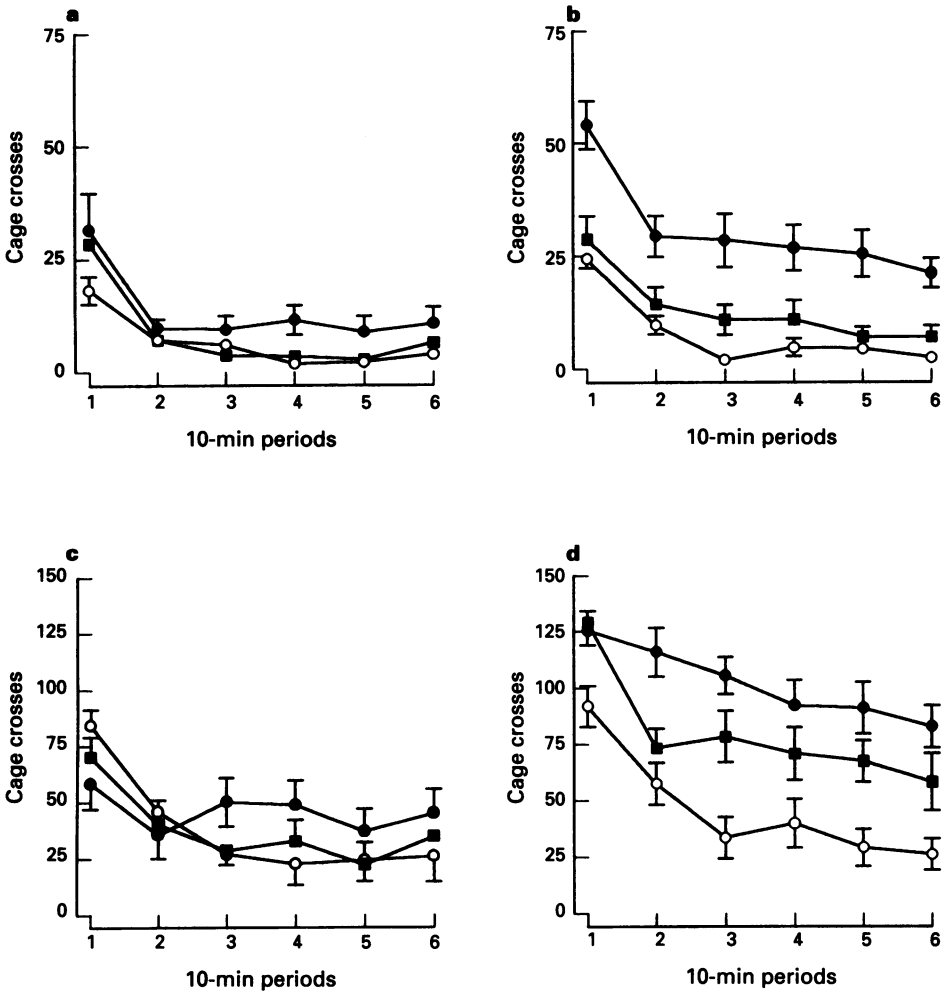

Figure 3 Time-courses for (-)-nicotine (0.4 mg kg^{-1}) and isoarecolone (16 mg kg^{-1}); (a,b) show changes in cage crosses of rats treated chronically with saline and nicotine respectively, whereas (c,d) show the corresponding data for repeated moves. Each segment shows results obtained after administration of (-)-nicotine (●), isoarecolone (■) and saline (○). All data were obtained in test sessions of 60 min duration and each point represents mean ± s.e.mean ($n = 7$).

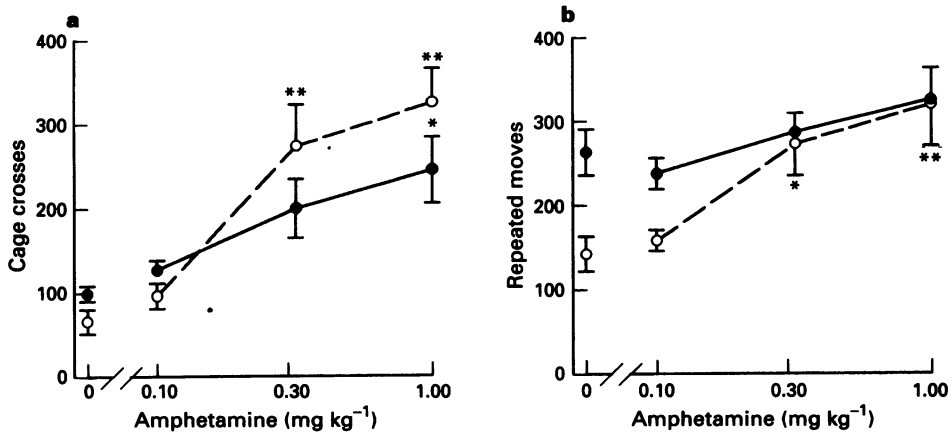

Figure 4 Effects of (+)-amphetamine on locomotor activity of rats treated chronically with saline (○) or nicotine (●) during 60 min periods beginning immediately after injections ($n = 7$). Abscissae, doses; ordinates, movements between beams of infra-red light (a) or repetitive interruptions of same beam (b). Each point represents mean ± s.e.mean; significant differences from saline are marked (*$P < 0.05$; **$P < 0.01$ according to Tukey tests).

Discussion

Advances in understanding the psychopharmacology of nicotine have been constrained by the limited numbers of nicotinic agonists and antagonists available for study. The present work, combining behavioural and neurochemical observations, indicates that some intriguing observations are possible despite this constraint. The findings confirm and extend the previously reported dissociation between the locomotor activating effects of nicotine and isoarecolone (Reavill *et al.*, 1990),

and it can be suggested that the failure of isoarecolone to produce full nicotine-like increases in locomotion may be related to its lower efficacy as a releaser of dopamine.

Comparative binding assays showed that in mammalian brain, isoarecolone, like nicotine, did not interact with binding sites for muscarinic ligands, and confirmed that isoarecolone competed at binding sites for nicotinic ligands (Ward *et al.*, 1994). Thus, studies of [^{3}H]-nicotine binding to brain membranes showed that isoarecolone was capable of competing fully for this site, but that compared with nicotine it had approximately 250 fold lower affinity. Previous binding assays reported a 50 fold difference (Reavill *et al.*, 1987). Possibly as a consequence of its generally lower nicotinic potency, isoarecolone did not interact with the neuronal α-bungarotoxin site, in contrast to nicotine. This difference could contribute to the differences in the behavioural actions of the two drugs, although the physiological significance of α-bungarotoxin-sensitive nicotinic receptors in the brain is still poorly defined (Clarke, 1992).

Isoarecolone and nicotine were both capable of eliciting concentration-dependent release of dopamine from synaptosomes preloaded with [^{3}H]-dopamine. This release was largely sensitive to the nicotinic antagonist, mecamylamine, supporting its mediation by nicotinic acetylcholine receptors as previously documented (Wonnacott *et al.*, 1990; Grady *et al.*, 1992; El Bizri & Clarke, 1994). Dose-response profiles were similar in the two brain regions examined, although the magnitude of the responses was greater in striatal preparations than in frontal cortex, reflecting the higher proportion of dopaminergic terminals in striatum. In this tissue, the EC_{50} for nicotine-evoked dopamine release of approximately 0.5 μM was similar to values previously reported using the same technique (Rapier *et al.*, 1988; Grady *et al.*, 1992). The proposition that binding assays measure the affinity for the high affinity desensitized form of the receptor (Grady *et al.*, 1992) may explain the lack of concordance between the EC_{50} concentration for receptor-mediated function and the IC_{50} value for binding to [^{3}H]-nicotine binding sites. However, in view of the heterogeneity of nicotinic receptor subtypes in the CNS, and the lack of identifying ligands for subtypes, it is still a matter of debate whether [^{3}H]-nicotine binding sites correspond to presynaptic nicotinic receptors that mediate dopamine release (Wonnacott *et al.*, 1990).

Although isoarecolone was able to evoke mecamylamine-sensitive dopamine release in a concentration-dependent manner in both tissues, its effects were qualitatively different from those of nicotine. Not only was it more than 20 times less potent than nicotine, but it was clearly less efficacious in frontal cortex: the maximum response seen with isoarecolone was approximately 50% of the maximum response to nicotine in this tissue. These *in vitro* measures were paralleled by differences between nicotine and isoarecolone in the behavioural studies.

The finding that nicotine produced little or no increase in locomotor activity of rats previously exposed to the test apparatus but not to the drug is compatible with earlier observations (Ksir *et al.*, 1985; 1987; Shoaib & Stolerman 1992). Similarly, many studies have shown that previous exposure to nicotine can sensitize rats to its locomotor-activating effect (Clarke & Kumar 1983; Ksir *et al.*, 1985; 1987; Shoaib & Stolerman 1992). The results with isoarecolone suggested that rats sensitized to nicotine did not show cross-sensitization to isoarecolone as far as cage crosses, the main measure of locomotion, were concerned (Figure 2a). There was, however, some degree of cross-sensitization in the case of the index called repeated moves, although this appeared less striking than the sensitization to nicotine itself (Figure 2b). Repeated moves may be sensitive to drug-induced repetitive movements such as the stereotyped behaviours induced by large doses of dopamine agonists or by, for example, 5-HT_2 agonists; in order to clarify the interpretation of the changes in repeated moves, additional studies with direct observations of animals treated with isoarecolone have been carried out and these will be described separately.

It follows that the present studies support previous observations that isoarecolone failed to increase locomotor activity above control levels in rats that were exposed previously to both nicotine and the test apparatus (Reavill *et al.*, 1990). The earlier work did not test directly for sensitization and used smaller doses of isoarecolone, whereas the present studies included tests up to 32 mg kg^{-1}; the pattern of results in Figure 2 suggests that it was unlikely that the failure to see full cross-sensitization to isoarecolone was due to testing of insufficiently large doses. In the studies of Reavill *et al.* (1990), the acute effects of nicotine and isoarecolone were examined in rats that had had no previous exposure to the test apparatus and under those conditions, both drugs descreased locomotor activity. These effects were not seen in the present experiments where all rats had extensive previous exposure to the test apparatus; in addition, the repeated testing of each drug in the same rats may have produced tolerance to depressant effects in the chronic-saline group. Consideration of the time-courses for the effects of the drugs also suggested that the efficacy of isoarecolone was not masked by an unusually short duration of action (Figure 3). These results are suggestive of a difference between the mechanisms of action of nicotine and isoarecolone. Stolerman *et al.* (1995) have reported upon several other nicotinic agonists that produce only weak or no nicotine-like activating effects in rats.

Sensitization to nicotine was not accompanied by cross-sensitization to amphetamine (Figure 4); if anything, the response to amphetamine was slightly weakened, but this subtle difference was confounded by a change in the baseline amount of locomotor activity. The lack of cross-sensitization was unexpected in view of evidence that both nicotine and amphetamine increase locomotor activity by actions on the mesolimbic dopamine system, and in the light of previous reports of cross-sensitization from nicotine to cocaine and methamphetamine (Horger *et al.*, 1992; Suemaru *et al.*, 1993). However, nicotine may increase locomotion primarily by an action on nicotinic receptors in the somatodendritic region (ventral tegmental area) rather than on terminals in the NAc (Reavill & Stolerman 1990; Leikola-Pelho & Jackson, 1992; Nisell *et al.*, 1994); in contrast, amphetamine and cocaine can increase the synaptic availability of dopamine by their actions in the terminal area. Cross-sensitization between nicotine and illicit drugs such as amphetamine and cocaine is a potentially important factor in drug abuse. Reports suggest that nicotine produces cross-sensitization to the positive reinforcing effect of cocaine but not to its effect on locomotor activity (Schenk *et al.*, 1991; Horger *et al.*, 1992) and further investigation is warranted.

The inability of isoarecolone to mimic nicotine with respect to effects on cage crosses in nicotine-sensitized rats, whereas it did increase repeated moves, is consistent with the lower efficacy of isoarecolone revealed in the dopamine release assays. The larger doses of isoarecolone, compared with nicotine, required for these effects on activity were also paralleled by a similar difference in potency between the two agonists in the *in vitro* assays. If nicotinic receptors in the somatodendritic regions of ascending dopamine pathways have similar sensitivities to these agonists as presynaptic receptors, they may be the major determinants of the locomotor activating effects of nicotine *in vivo*. Thus, this comparative study was consistent with the proposition that the ability of these agonists to elicit dopamine release could account for their different effects on locomotor behaviour. The findings with locomotor activation may be contrasted with those for locomotor depressant and discriminative stimulus effects, where minimal differences between isoarecolone and nicotine have been detected (Reavill *et al.*, 1987; 1990). The different effects of nicotine and isoarecolone may reflect either actions on different sub-populations of nicotinic receptors or different actions, such as full and partial agonism, on the same receptors.

How do these functional measures compare with the binding site data? Partial agonists can compete fully for the binding site; indeed competition binding assays fail to distinguish between agonists and competitive antagonists. Therefore the

ability of isoarecolone to displace [^{3}H]-nicotine binding to brain membranes is not incompatible with its low efficacy in assays of nicotinic function. The potency of isoarecolone is 250 times lower than that of nicotine in binding to [^{3}H]-nicotine binding sites, compared with a 10–30 fold difference in releasing dopamine and stimulating locomotor activity. A comparison of a series of agonists for their abilities to evoke dopamine release from mouse striatal synaptosomes and their affinities for [^{3}H]-nicotine binding sites revealed no simple correlation (Grady *et al.*,1992). This could mean that the [^{3}H]-nicotine binding site does not correspond to the presynaptic nicotinic receptor that mediates dopamine release from synaptosomes, or that there is no simple relationship between the affinities with which an agonist binds to the active and desensitized conformations of the receptor. The situation may be further confounded by the participation of two or more receptor subtypes (each having different agonist affinities) in behavioural responses. For example, if the receptor characterized by [^{125}I]-α-bungarotoxin binding had a role in the present experiments, it would be more sensitive to nicotine than to isoarecolone. However, this particular subtype is not considered to participate in nicotine-evoked dopamine release (Rapier *et al.*, 1990; Grady *et al.*, 1992).

In conclusion, these results demonstrate that isoarecolone is a nicotinic agonist in the brain, but its profile of effects differs from that for nicotine; its low efficacy and weak potency in stimulating locomotor activity (compared with nicotine) in sensitized rats are mirrored by *in vitro* assays of dopamine release. This comparison of *in vivo* and *in vitro* function has proved to be more informative than comparison with ligand-binding data.

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