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TURNOVER OF SPECIFIC [^{3}H]SPIPERONE AND [^{3}H]*N*,*n*-PROPYLNORAPOMORPHINE BINDING SITES IN RAT STRIATUM FOLLOWING PHENOXYBENZAMINE ADMINISTRATION

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Abstract—Inclusion of phenoxybenzamine into incubates containing rat striatal preparations equipotently displaced specific striatal [^{3}H]spiperone and [^{3}H]NPA binding. Pre-incubation of striatal membranes with phenoxybenzamine followed by extensive washing equipotently inhibited the subsequent specific [^{3}H]spiperone or [^{3}H]NPA binding. In both displacement and pre-incubation experiments phenoxybenzamine caused complete inhibition of specific [^{3}H]spiperone binding to rat striatal membranes, but only partially inhibited specific [^{3}H]NPA binding. Following parenteral administration to rats, phenoxybenzamine caused a marked inhibition of *ex vivo* specific [^{3}H]spiperone binding in striatal tissue preparations from these animals which lasted approximately 24 hr following *in vivo* drug administration. In contrast, administration of phenoxybenzamine caused only a transient change in *ex vivo* specific [^{3}H]NPA binding. Phenoxybenzamine causes irreversible inhibition of [^{3}H]spiperone and [^{3}H]NPA binding *in vitro*. *In vivo* administration of phenoxybenzamine discriminates between [^{3}H]spiperone and [^{3}H]NPA in *ex vivo* studies suggesting that these binding sites have different turnover rates.

Multiple classes of dopamine receptor exist in brain [1–3]. The D-2 receptor sites, which act independently of adenylate cyclase, are labelled by both agonist and antagonist ligands [4–7]. However, there is dispute as to whether these ligands label the same or distinct sites [3, 8]. One means of distinguishing receptor sub-classes has been through the use of phenoxybenzamine. This drug causes irreversible inhibition of α-adrenergic receptors [9], but this action is not specific; phenoxybenzamine also binds to opiate [10], cholinergic [11] and dopamine receptors [12, 13].

Phenoxybenzamine irreversibly inhibits dopamine-sensitive adenylate cyclase [14, 15] and the specific binding of [^{3}H]neuroleptics to rat striatal membrane preparations [15, 12]. Phenoxybenzamine may distinguish dopamine agonist and antagonist binding sites by its ability to differentially inhibit [^{3}H]spiperone, [^{3}H]apomorphine and [^{3}H]dopamine binding [12, 16].

The irreversible interaction of phenoxybenzamine with dopamine receptors also may be useful in determining an index of dopamine receptor turnover *in vivo* and which may be different for agonist and antagonist binding sites. The ability of phenoxybenzamine to prevent ligand binding to dopamine receptors *in vitro* following its administration *in vivo* has not previously been investigated. We now compare the effect of *in vivo* phenoxybenzamine administration on *in vitro* binding of [^{3}H]spiperone and [^{3}H]NPA to striatal dopamine receptors to try to obtain some index of the turnover rate of the binding sites labelled by these ligands.

MATERIALS AND METHODS

Tissue preparation

Female Wistar rats (150 ± 10 g; Bantin & Kingman) were killed by cervical dislocation and decapitation. The brain was rapidly removed onto ice and the paired corpus striata dissected out. Pooled striatal tissue was homogenized in 50 vol. of ice-cold buffer using a Polytron homogenizer (setting 7 for 15 sec; Northern Media Supply Co.) and centrifuged in a Sorvall RC5 centrifuge at 40,000 *g* at 4° for 10 min. The supernatant was discarded and the pellet re-homogenised in 50 vol. of ice-cold buffer. Membrane preparations were pre-incubated at 37° for 10 min, re-centrifuged and washed twice more before final homogenisation in 100 vol. of incubation buffer. Tissue preparations were retained on ice until used.

Incubation procedure

Aliquots (1.0 ml) of striatal membrane preparations were incubated for 10 min at 37° following the addition of the appropriate ligand contained in 50 μl of incubation buffer and either a displacing drug dissolved in 50 μl of deionised water or deionised water alone. Each ligand concentration or concentration of displacing drug was examined in triplicate or quadruplicate. The assay was stopped by rapid filtration under vacuum through Whatman

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† Abbreviations: (±)-ADTN, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene; NPA, *N*,*n*-propylnorapomorphine.

GF/B glass fibre filters using a Millipore 3025 sampling manifold. Filters were washed twice with 5 ml ice-cold homogenising buffer, and then immersed in 4 ml of ES 299 scintillation cocktail (Packard) and radioactivity on the filter estimated by liquid scintillation spectroscopy using a Packard 460C scintillation spectrometer at an efficiency of approximately 40%.

Specific [^{3}H]spiperone binding to striatal membrane preparations

Striatal membranes were prepared in 50 mM Tris–HCl buffer (pH 7.6) before final homogenisation in 50 mM Tris–HCl buffer containing 120 mM sodium chloride (pH 7.55; Tris–Na$^+$). In saturation studies [^{3}H]spiperone (21 Ci/mmole; Amersham International) was added to the incubation mixture in concentrations between 0.1 and 4.0 nM. In displacement studies, a single ligand concentration (approximately 0.5 nM) was used throughout. Specific [^{3}H]spiperone binding was defined as the difference between total binding and that occurring in the presence of (−)-sulpiride (10^{-5} M; Delagrange).

Specific [^{3}H]N,n-propylnorapomorphine (NPA) binding to striatal membrane preparations

For the determination of [^{3}H]NPA binding a 15 mM Tris–HCl buffer containing EDTA 1 mM (pH 7.8; Tris–EDTA) was used throughout the tissue preparation procedure and for tissue incubations. Saturation studies with [^{3}H]NPA (60 Ci/mmole; New England Nuclear) were performed at concentrations between 0.05–2.0 nM and displacement studies were performed at a single concentration of approximately 0.25 nM. Specific [^{3}H]NPA binding was defined as the difference between total binding and that occurring in the presence of (±)-ADTN (10^{-6} M; Wellcome Research Laboratories).

In vitro *effects of phenoxybenzamine*

The ability of phenoxybenzamine (Dibenyline; Smith, Kline & French) to displace the specific binding of [^{3}H]spiperone or [^{3}H]NPA to striatal preparations was investigated by including a range of concentrations between 10^{-9} and 10^{-4} M into incubates. In other experiments, phenoxybenzamine (10^{-9}–10^{-4} M) was added to tissue preparations at the pre-incubation stage for 10 min at 37°. Free phenoxybenzamine was removed by centrifugation and the tissue was washed twice more by successive homogenisation in fresh buffer and re-centrifugation before use in binding assays.

In vivo *effects of phenoxybenzamine*

Two injections of phenoxybenzamine (4 mg/kg i.p.; 12 hr apart) in 1 ml 0.9% saline were administered to female Wistar rats (150 ± 10 g). These doses were based on those previously employed [17]. Control animals received 1.0 ml 0.9% saline alone on each occasion. Animals were killed at intervals from 0.5 hr to 14 days following the second injection of phenoxybenzamine and striatal tissue was used for ligand binding assays.

Data and statistical analysis

In saturation studies, ligand binding data was subjected to Scatchard analysis followed by linear regression analysis to determine the number of binding sites (Bmax; pmoles/g tissue) and the equilibrium dissociation constant (K_D; nM). Estimates of the binding parameters for each drug treatment were pooled and compared with those from saline-pretreated animals using an unpaired two-tailed Student's *t*-test. In inhibition studies, IC_{50} values were calculated from individual values obtained for the inhibition curves.

RESULTS

In vitro *effects of phenoxybenzamine*

Inclusion of phenoxybenzamine (10^{-9}–10^{-4} M) into tissue incubates inhibited equipotently specific [^{3}H]spiperone (0.5 nM) and specific [^{3}H]NPA (0.25 nM) binding to rat striatal membrane preparations (IC_{50} values: [^{3}H]spiperone, 1.7×10^{-7} M; [^{3}H]NPA, 1.6×10^{-7} M; Fig. 1). Phenoxybenzamine caused a total displacement of specific [^{3}H]spiperone binding (as defined by incorporation of (−)-sulpiride 10^{-5} M) but only caused a maximal displacement of 81% of specific [^{3}H]NPA binding (as defined using (±)-ADTN 10^{-6} M).

Pre-incubation of striatal membranes with phenoxybenzamine (10^{-9}–10^{-4} M) for 10 min caused a concentration related decrease in the subsequent specific binding of [^{3}H]spiperone and [^{3}H]NPA (Fig. 2). Pre-incubation with phenoxybenzamine did not affect the non-specific binding of either ligand.

Specific [^{3}H]spiperone binding was completely inhibited by pre-incubation with 10^{-4} M phenoxybenzamine. Specific [^{3}H]NPA was not inhibited completely by the concentrations of phenoxybenzamine up to 10^{-4} M (Fig. 2). Increasing the concentration of phenoxybenzamine from 10^{-6} M to 10^{-4} M caused no further inhibition of [^{3}H]NPA binding. Approximately 40% of specific [^{3}H]NPA binding was not inhibited by pre-incubation with phenoxybenzamine. Again the IC_{50} values showed inhibition of [^{3}H]spiperone and [^{3}H]NPA binding by phenoxybenzamine to be equipotent IC_{50} values: [^{3}H]spiperone, 1.7×10^{-7} M; [^{3}H]NPA, 1.8×10^{-7} M).

In vivo *action of phenoxybenzamine*

Administration of phenoxybenzamine (2×4 mg/kg i.p.) 0.5 hr to 14 days prior to death showed the number of specific [^{3}H]spiperone binding sites to be decreased up to 3 days following drug administration when compared to control animals receiving 0.9% saline (Fig. 3, Table 1). The dissociation constant (K_D) for [^{3}H]spiperone binding was increased over the same period (Fig. 3, Table 1). The $t_{\frac{1}{2}}$ for recovery of Bmax and K_D to normal levels was determined from the plot of Bmax or K_D against log time. From the graph $t_{\frac{1}{2}}$ for recovery of both Bmax and K_D was 8–9 hr.

In contrast, Bmax for specific [^{3}H]NPA binding was reduced 0.5 hr following phenoxybenzamine (2×4 mg/kg i.p.), but not thereafter (Table 1). K_D was decreased also at this time but not at later times. Indeed, at 1 hr following phenoxybenzamine treatment Bmax for [^{3}H]NPA was increased by 56% while K_D was increased by 50% (Table 1). However, these

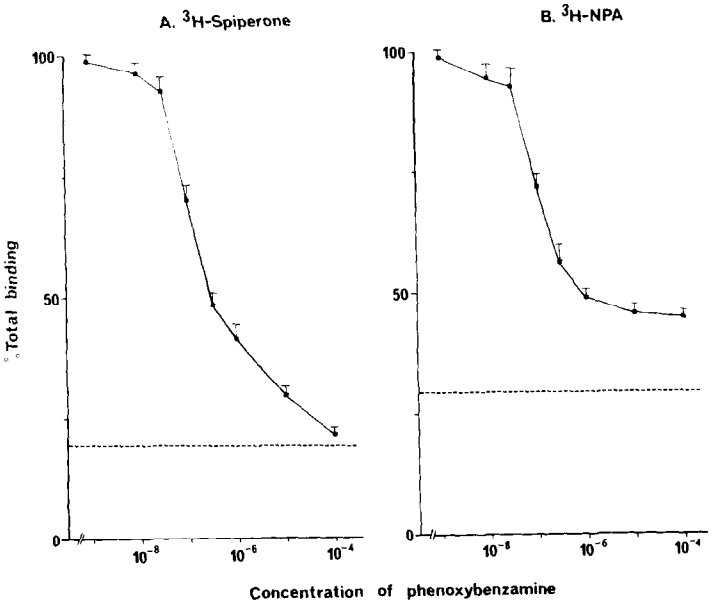

Fig. 1. Inhibition of the specific binding of [^{3}H]spiperone (A) and [^{3}H]NPA (B) binding to rat striatal membrane preparations *in vitro* by incorporation of phenoxybenzamine. Tissue preparations were incubated with [^{3}H]spiperone (0.54 nM) or [^{3}H]NPA (0.28 nM) and increasing concentrations of phenoxybenzamine (10^{-9}–10^{-4} M) added. Data represent the mean of two independent experiments performed in triplicate. Specific [^{3}H]spiperone binding was defined using (−)-sulpiride (10^{-5} M). Specific [^{3}H]NPA binding was defined using (±)-ADTN (10^{-6} M).

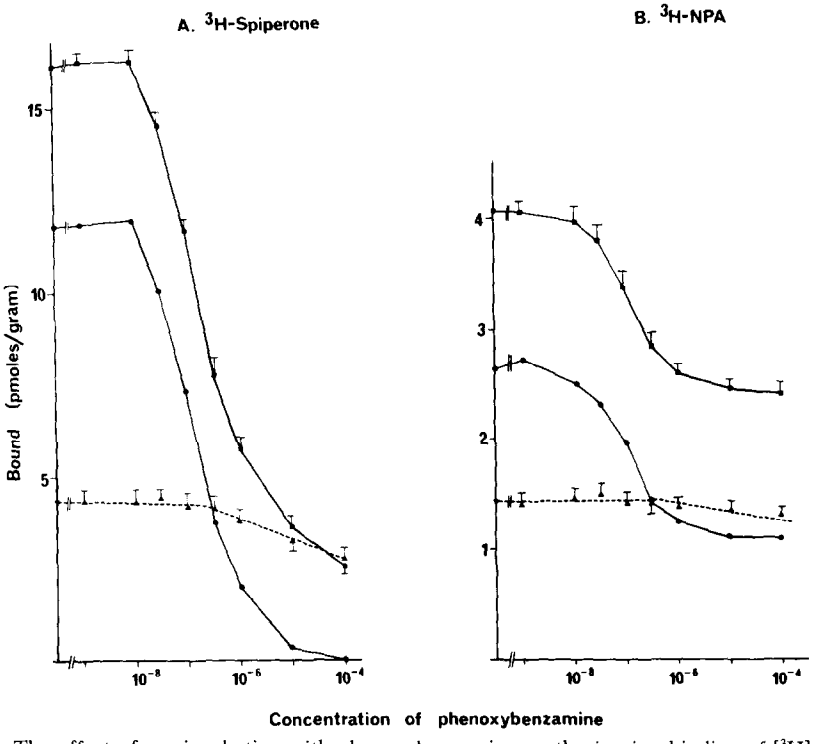

Fig. 2. The effect of pre-incubation with phenoxybenzamine on the *in vitro* binding of [^{3}H]spiperone (A) and [^{3}H]NPA (B) to rat striatal membrane preparations. Following the first centrifugation during the preparation of striatal membrane preparations pellets were resuspended in buffer, homogenised and then incubated with increasing concentrations of phenoxybenzamine (10^{-9}–10^{-4} M) for 10 min at 37°. Excess unbound phenoxybenzamine was removed during the remaining tissue preparation procedure. Data represents the mean (±1 S.E.M.) for 3 independent experiments performed in quadruplicate for total (—■—) and (—▲—) non-specific binding defined using (−)-sulpiride (10^{-5} M) for [^{3}H]spiperone (0.59 nM) and (±)-ADTN (10^{-6} M) for [^{3}H]NPA (0.31 nM) which were subtracted to give specific binding (—●—) at each concentration of phenoxybenzamine used.

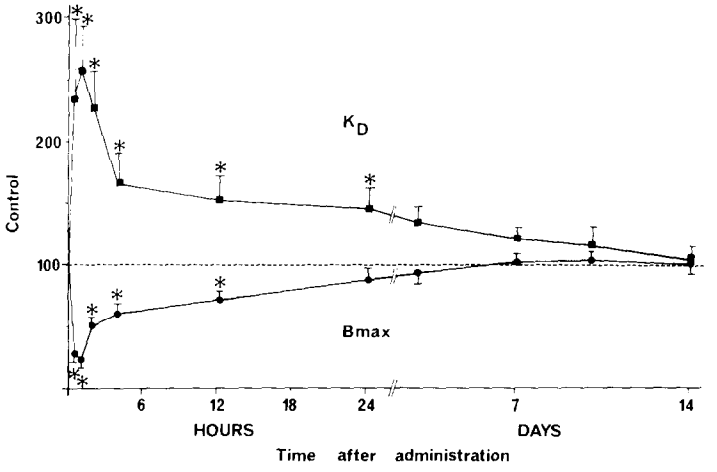

Fig. 3. Alterations in Bmax (—●—) and K_D (—■—) for (−)-sulpiride (10^{-5} M) specific [^{3}H]spiperone (0.1–4.0 nM) binding to rat striatal preparations up to 14 days following the second of two injections of phenoxybenzamine (4 mg/kg i.p.). Data represents the mean (±1 S.E.M.) as percentage of controls from 3 separate determinations using pooled tissue from 4 animals examined in triplicate at each ligand concentration. The data obtained were subjected to Scatchard analysis followed by linear regression to determine an estimate of the Bmax and K_D. *P < 0.05 compared to values from appropriate control animals.

parameters had returned to those observed in control animals 2 hr following phenoxybenzamine administration and remained at control levels to 14 days.

DISCUSSION

We have confirmed that phenoxybenzamine completely inhibits the specific binding of [^{3}H]spiperone to rat striatal preparations [12, 16]. This action was irreversible since preincubation of membranes with phenoxybenzamine, followed by extensive washing in fresh buffer to remove free drug again resulted in complete inhibition of specific [^{3}H]spiperone binding. In contrast, specific [^{3}H]NPA binding was partially inhibited by incorporation of phenoxybenzamine into striatal preparations. The pre-incubation of striatal membranes with phenoxybenzamine again suggested that inhibition was irreversible and confirmed that total inhibition of specific [^{3}H]NPA binding in rat striatum did not occur. The failure of high concentrations of phenoxybenzamine to cause total inhibition of specific [^{3}H]NPA suggests that at least part of the receptor population labelled by [^{3}H]NPA is distinct from that labelled by [^{3}H]spiperone. This

Table 1. Alterations in specific [^{3}H]spiperone (0.1–4.0 nM) and [^{3}H]*N*,*n*-propylnorapormophine (NPA) (0.05–2.0 nM) binding sites (Bmax; pmoles/g wet wt of tissue) and equilibrium dissociation constants (K_D; nM) in striatal tissue preparations from rats pretreated with two injections of phenoxybenzamine (4 mg/kg i.p.) compared to saline treated animals

		[^{3}H]Spiperone		[^{3}H]NPA	
		Bmax	K_D	Bmax	K_D
Control	24 hr	22.0 ± 2.1	0.16 ± 0.03	16.9 ± 2.3	1.09 ± 0.11
	7 days	19.3 ± 1.6	0.18 ± 0.03	14.7 ± 2.2	1.04 ± 0.14
	14 days	20.4 ± 1.3	0.18 ± 0.02	15.9 ± 1.9	1.08 ± 0.13
PBZ	0.5 hr	5.7 ± 0.9* (26%)	0.38 ± 0.10* (238%)	8.7 ± 0.7* (51%)	0.73 ± 0.08* (67%)
	1 hr	4.8 ± 0.4* (22%)	0.42 ± 0.05* (263%)	26.3 ± 2.7* (156%)	1.63 ± 0.15* (150%)
	2 hr	10.3 ± 0.8* (47%)	0.37 ± 0.03* (231%)	17.3 ± 1.9 (107%)	1.11 ± 0.12 (102%)
	4 hr	12.1 ± 1.1* (55%)	0.27 ± 0.03* (169%)	15.8 ± 1.9 (94%)	1.07 ± 0.14 (98%)
	12 hr	14.4 ± 0.9* (65%)	0.25 ± 0.02* (156%)	16.4 ± 2.2 (97%)	1.08 ± 0.13 (99%)
	24 hr	17.8 ± 1.6 (81%)	0.24 ± 0.02* (150%)	15.5 ± 2.0 (92%)	1.05 ± 0.12 (96%)
	3 days	19.1 ± 1.6 (98%)	0.21 ± 0.02 (117%)	14.9 ± 1.6 (101%)	1.07 ± 0.10 (103%)
	7 days	20.4 ± 0.9 (105%)	0.19 ± 0.01 (100%)	15.4 ± 1.9 (105%)	1.06 ± 0.12 (102%)
	10 days	21.1 ± 1.2 (103%)	0.19 ± 0.02 (106%)	15.7 ± 2.0 (99%)	1.07 ± 0.12 (99%)
	14 days	20.5 ± 1.7 (100%)	0.17 ± 0.01 (95%)	16.2 ± 1.7 (102%)	1.09 ± 0.09 (101%)

* P < 0.05 compared to values from saline treated controls. Animals receiving phenoxybenzamine up to 24 hr before death were compared to animals receiving saline 24 hr before death; phenoxybenzamine 3 and 7 days: controls 7 days; phenoxybenzamine 10 and 14 days: controls 14 days. Values are expressed as the mean (±1 S.E.M.) from 3 separate determinations from 5 animals examined in triplicate at each ligand concentration. Data was subjected to Scatchard analysis followed by linear regression to obtain estimates of Bmax and K_D.

is unlikely to represent pre-synaptic dopamine receptors located on the terminal of the nigro-striatal pathway since release of dopamine from pre-synaptic autoreceptors was completely inhibited by phenoxybenzamine in cat striatal slices [13].

The *in vivo* administration of phenoxybenzamine supported the existence of distinct agonist and antagonist binding sites. Pre-treatment of rats with phenoxybenzamine markedly reduced the number of [^{3}H]spiperone binding sites present in striatal tissue preparations up to 24 hr following phenoxybenzamine administration. The decrease in Bmax was accompanied by a large increase in the equilibrium dissociation constant. The changes observed in K_D for the reduced number of [^{3}H]spiperone binding sites might be due to altered receptor affinity for [^{3}H]spiperone, or competition with phenoxybenzamine with [^{3}H]spiperone for the binding sites. However, if we presume that the action of phenoxybenzamine is a truly irreversible process, then this action is non-competitive. Thus, the recovery in Bmax must represent a true indication of the turnover of receptor sites labelled by [^{3}H]spiperone.

In contrast, phenoxybenzamine pre-treatment caused a transient biphasic change in the Bmax and K_D for specific [^{3}H]NPA binding. Thus, 0.5 hr after administration of phenoxybenzamine, the number of [^{3}H]NPA binding sites present in striatal tissue preparation was decreased by 51%. However, by 1 hr the Bmax for [^{3}H]NPA binding was increased 56%. These changes in Bmax were accompanied by corresponding changes in K_D. Thus, 0.5 hr after administration of phenoxybenzamine, K_D was decreased by 33%, but was followed by a 50% increase 1 hr after phenoxybenzamine administration. While the decrease in Bmax might be attributed to a residual low affinity population of binding sites normally labelled by [^{3}H]NPA, the increase in number of binding sites is more difficult to explain. This might represent a rapid compensatory mechanism operating in response to phenoxybenzamine which results in a return to normal after 1 hr. If phenoxybenzamine binding is irreversible, this compensatory mechanism must represent a change in binding proteins.

The reasons for these changes in Bmax and K_D for both [^{3}H] spiperone and [^{3}H]NPA are complex. The rapid compensatory mechanism might involve synthesis of new receptors or the exposure of pre-formed but previously unavailable spare receptors. Metabolism and removal of phenoxybenzamine is unlikely, since this drug has been demonstrated previously to inhibit [^{3}H]prazosin binding to α-adrenergic receptors for up to 12 days [17].

Phenoxybenzamine irreversibly labels dopamine receptor binding sites. Using this drug *in vivo* to label dopamine receptors may provide an index of receptor turnover and suggests that agonist and antagonist binding sites have different turnover rates.

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