Biochemical Pharmacology, Vol. 31, No. 16, pp. 2643–2647, 1982.
Printed in Great Britain.

0006-2952/82/162643-05 $03.00/0

STRIATAL SYNAPTOSOMAL TYROSINE HYDROXYLASE ACTIVITY

A MODEL SYSTEM FOR STUDY OF PRESYNAPTIC DOPAMINE RECEPTORS

BRIAN A. MCMILLEN
Department of Pharmacology, University of Texas Health Science Center, Dallas, TX 75235, U.S.A.

(*Received* 8 *June* 1981; *accepted* 10 *February* 1982)

Abstract—Tyrosine hydroxylase activity determined in striatal synaptosomes by the formation of $[^3H]H_2O$ from [3, 5-^{3}H]tyrosine was used as a system for determining receptor affinities of neuroleptic drugs for the dopamine autoreceptor. Four agonists were tested for their abilities to inhibit tyrosine hydroxylation, and the order of potencies was apomorphine > dopamine > norepinephrine and buspirone was inactive. The abilities of different neuroleptic drugs to shift the IC_{50} of apomorphine (500 nM without additional drugs) were used to calculate K_B values for each drug. The butyrophenones and (+)butaclamol were the most potent of the drugs tested, while (−)sulpiride and thioridazine were very weak inhibitors of apomorphine. Clozapine, promethazine, (−)butaclamol and (+)sulpiride were all inactive. The order of potencies of antipsychotic drugs at the dopamine nerve-ending autoreceptor correlated closely with clinical dose, but it did not correlate with previous reports of displacement of radiolabeled dopamine or apomorphine striatal membrane binding. This observation combined with the fact that the IC_{50} of apomorphine at this functional receptor was 100 times the concentration needed to saturate its binding site(s) in radioligand receptor assays suggests that the dopamine autoreceptor is not labeled by nanomolar concentrations of apomorphine or dopamine.

Cessation of nigro-striatal impulse flow causes both a decrease in striatal dopamine metabolism [1] and an increase in striatal tyrosine hydroxylase activity [2, 3]. The activation of tyrosine hydroxylation is due to a loss of inhibition mediated by a presynaptic dopamine autoreceptor as a result of the decreased release of dopamine into the synaptic cleft [4]. This effect of dopamine on tyrosine hydroxylase can be demonstrated *in vitro* using striatal slices or synaptosomes. Dopamine or other direct acting agonists inhibit the rate of tyrosine hydroxylation, and antipsychotic drugs can partially restore the activity [5–7]. Disruption of the synaptosomes greatly reduces the inhibitory potency of apomorphine on tyrosine hydroxylase [6], and inhibition of dopamine uptake into nerve endings by benztropine has no [7], or a slight [6], effect on the inhibitory potency of dopamine, which suggests that recaptured amine is not of great importance for the presynaptic effect. Therefore, the presynaptic dopamine autoreceptor is an external membrane receptor which, when occupied by an agonist, reduces the rate of tyrosine hydroxylation. The rate of dopamine synthesis has important effects on the availability of dopamine for neurogenic release [8], and this system may form a short negative feedback loop that helps regulate dopaminergic neurotransmission in the corpus striatum [4].

Several laboratories have suggested that specific high-affinity apomorphine (or *N-n*-propylnorapomorphine) binding using striatal membranes is binding to the dopamine presynaptic autoreceptor and that specific spiperone binding is to postsynaptic receptors [9–11]. However, others [12–14] have suggested that both spiperone and apomorphine binding are to postsynaptic elements. On the basis of subcellular distribution and lesion studies with kainic acid or 6-hydroxydopamine, Leysen [13] concluded that agonist and antagonist sites are subunits of a single postsynaptic receptor. The difficulty in resolving these differences in the literature is that the criterion for receptor activity is binding affinity, not alteration or production of a response. Thus, half of the definition of a receptor is lost in ligand binding experiments. However, synaptosomal tyrosine hydroxylase activity represents an intact receptor system, with decreased tyrosine hydroxylation being the response to agonist stimulation of the dopamine autoreceptor. The inhibition curve for apomorphine or dopamine is shifted to the right when an antagonist is added to the incubation medium [6, 7]. Such a shift of receptor response can be used to calculate an affinity constant (K_B) for the antagonist at the receptor by the equation [15]:

$$K_B = \frac{[\text{antagonist}]}{X - 1}$$

where X equals the ratio of the IC_{50} values for apomorphine in the presence of antagonist and the absence of antagonist (IC_{50} is used instead of ED_{50} as the response is inhibition of activity).

Using this experimental approach, K_B values for dopamine antagonists at the autoreceptor can be compared to affinity constants determined by ligand binding experiments. It should be possible to determine if apomorphine or spiperone high-affinity binding is to the dopamine autoreceptor. To properly

interpret ligand binding data, it is important to know which receptor sites are labeled by spiperone or apomorphine. The data gathered with the functional dopamine autoreceptor system may allow a determination of whether striatal apomorphine binding is indeed to this presynaptic dopamine receptor.

METHODS

Female Sprague–Dawley rats (200–500 g, Holtzman) were used for all experiments. Animals were housed under a 12-hr off/on light cycle and maintained on standard rat chow. Striatal synaptosomal tyrosine hydroxylase activity was measured by the method of Nagatsu *et al.* [16] with minor modifications. L-[3-5-^{3}H]Tyrosine (New England Nuclear Corp., Boston, MA) was purified on a Dowex-50 anion exchange column (0.5 × 5.0 cm). After applying acidified [^{3}H]tyrosine, the column was washed with 20 ml of H_2O and 1.5 ml of 1.0 N NH_4OH, and the [^{3}H]tyrosine was eluted with 1.5 ml of 1.0 N NH_4OH. The purified [^{3}H]tyrosine was diluted so that 0.1 ml could be used for each daily experiment and then be divided into aliquots and frozen. A 0.1-ml aliquot was evaporated dry shortly before the start of incubation and taken up in Krebs–Ringer phosphate buffer (pH 7.2, containing 0.2% *d*-glucose, 0.02% ascorbic acid, and 0.005% EDTA, and gassed with O_2) with unlabeled tyrosine to give a final concentration of 8×10^{-5} M. The cell-free homogenate (1000 *g* × 10 min supernatant fluid) of the corpus striatum (in 10 vol. of 0.32 M sucrose) was used as the source of synaptosomes. Drugs were made up in 0.5 ml of Krebs–Ringer buffer and preincubated for 5 min with 0.2 ml of synaptosomal preparation. The reaction was started by addition of 50 μl of 8×10^{-5} M [^{3}H]tyrosine containing 200,000 cpm and allowed to proceed for 30 min at 37° under O_2. The reaction was stopped by addition of 50 μl of glacial acetic acid. After centrifugation (13,000 *g* × 10 min) in the cold, the supernatant fraction and a 1.0-ml H_2O rinse were each quantitatively transferred to Dowex 50 columns (0.5 × 4.0 cm) and 0.5 ml of H_2O was added to the columns. The eluate, containing tritiated H_2O, was collected directly into polyethylene scintillation vials. After mixing with 13 ml of Beckman Readisolv HP, the samples were counted in a Beckman LS 200 with external standardization. All determinations were in duplicate. The IC_{50} values of agonists were determined from log concentration–response plots of four to five concentrations of agonist repeated three to five times.

Drugs used were: apomorphine, dopamine, *l*-norepinephrine and pargyline–HCl (Sigma Chemical Co., St. Louis, MO); clozapine and thioridazine–HCl (Sandoz Pharmaceuticals, East Hanover, NJ); (+)- and (−)butaclamol (Ayerst Laboratories, Montreal, Canada); (+)- and (−)sulpiride (Ravizza, Milano, Italy); haloperidol and pimozide (McNeil Laboratories, Fort Washington, PA); benperidol and spiroperidol (Jansen Pharmaceatica, Beerse, Belgium); trifluoperazine–HCl, chlorpromazine–HCl and promethazine–HCl (Smith Kline & French Laboratories, Philadelphia, PA); buspirone–HCl (Mead/Johnson Pharmaceutical Division, Evansville, IN); and amfonelic acid (Sterling-Winthrop Research Institute, Rensselaer, NY).

RESULTS

Figure 1A shows the inhibition of tyrosine hydroxylase in striatal synaptosomes by apomorphine. The calculated IC_{50} from the log–concentration response regression was 0.52×10^{-6} M. Also, the shift in the response curve caused by 6×10^{-7} M spiperone is shown. This concentration of spiperone caused a 3-fold shift to the right of the apomorphine IC_{50}. Using the equation presented above, the K_B for spiperone at the autoreceptor was 2.9×10^{-7} M. Figure 1B shows the inhibition curves for dopamine, norepinephrine and buspirone, all of which were much weaker than apomorphine.

In Table 1, the IC_{50} values for several agonists are presented. Apomorphine was the most potent drug tested, and dopamine had an IC_{50} value 5-fold greater than apomorphine. Norepinephrine was slightly less potent than dopamine. Inhibition of dopamine uptake by 3×10^{-6} M amfonelic acid (AFA) caused a shift in the IC_{50} for dopamine from 2.52 to 3.68 μM. This concentration of AFA is 100 times its IC_{50} for inhibition of uptake of 10^{-7} M dopamine [17] and

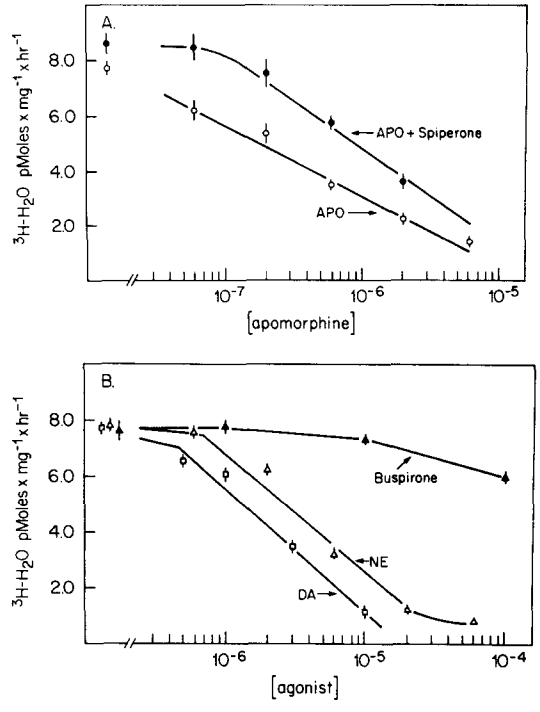

Fig. 1. Inhibition of striatal synaptosomal tyrosine hydroxylase activity. Enzyme activity was determined by the formation of [^{3}H]H_2O from [3, 5-^{3}H]tyrosine incubated with a cell-free homogenate of rat corpus striatum (see text for methods). Each point is the mean ± S.E.M. of three to five duplicate determinations. Spiperone (●) was added at 6×10^{-7} M to the apomorphine incubation. Incubations with dopamine or norepinephrine contained 10^{-6} M pargyline to protect the amines from degradation by monoamine oxidase. Note that the concentration scale in panel B starts higher than in panel A.

Table 1. Inhibition of striatal synaptosomal tyrosine hydroxylase by dopamine agonists*

Agonist	IC_{50} (μM)
Apomorphine	0.52
Dopamine	2.52
Dopamine + AFA	3.68
l-Norepinephrine	5.05
Buspirone	> 100

* Each IC_{50} was calculated from log concentration–activity plots. Formation of $[^3H]H_2O$ from [3, 5-^{3}H]tyrosine by tyrosine hydroxylase was used as the measure of the rate of enzyme activity. Each inhibition curve consisted of four to six concentrations of agonist repeated three to five times in duplicate. Dopamine uptake was inhibited by addition of 3×10^{-6} M amfonelic acid (AFA).

Table 2. Comparison of the presynaptic dopamine receptor affinity of antipsychotic drugs *

Drugs	K_B-Tyr-OH (M)
Benperidol	2.5×10^{-7} M
Spiroperidol	2.9×10^{-7} M
(+)Butaclamol	3.3×10^{-7} M
Trifluoperazine	4.5×10^{-7} M
Pimozide	5.7×10^{-7} M
Haloperidol	7.5×10^{-7} M
Chlorpromazine	1.2×10^{-6} M
(−)Sulpiride	3.8×10^{-6} M
Thioridazine	4.2×10^{-6} M

* The different neuroleptic drugs were added at 0.6 or 2.0×10^{-6} M to four different concentrations of apomorphine. Each K_B for the dopamine autoreceptor was calculated from the ratio (X) of IC_{50} for apomorphine plus drug divided by the IC_{50} for apomorphine alone (see text for equation). Correlation between log K_B and log clinical dose (as μmoles/kg) was $r = 0.809$. Clinical dose was taken from the literature [19, 21]. Other drugs tested and found to be inactive at 2.0×10^{-6} M were clozapine, promethazine, (−)butaclamol and (+)sulpiride.

actually potentiated the small inhibition of tyrosine hydroxylase activity caused by 5×10^{-7} M dopamine (27 vs 15% with dopamine alone). Iversen *et al.* [6] reported a similar effect using benztropine to inhibit dopamine reuptake, although others [7] found no effect of benzotropine on dopamine inhibition of tyrosine hydroxylase. Thus, a small intracellular component of inhibition may have occurred as the dopamine concentrations were increased. The new anxiolytic drug buspirone was suggested to be a presynaptic dopamine agonist because it has a higher affinity for the apomorphine binding site than for the spiperone binding site [18]. In this functional receptor model system, buspirone inhibited tyrosine hydroxylase by only 25% at a concentration of 100 μM. Thus, this drug does not behave as a potent agonist at the autoreceptor as originally suggested.

A series of antipsychotic drugs was tested for their abilities to inhibit apomorphine-induced decreased tyrosine hydroxylation. Most antipsychotic drugs significantly shifted the apomorphine response curve to the right at either 0.6 or 2×10^{-6} M. As noted in reports by others, many of the antipsychotic drugs reduced tyrosine hydroxylation at concentrations above the micromolar range (data not shown). The activity of tyrosine hydroxylase in the presence of antagonist alone was used for determining each new IC_{50} value for apomorphine. The values of K_B for each antagonist was calculated from its ability to shift apomorphine-induced reduction of tyrosine hydroxylation. Note that, from equation above, the concentration of antagonist that doubles the IC_{50} will equal the K_B.

Table 2 shows the calculated values for K_B of antipsychotic drugs at the striatal dopamine autoreceptor. The most potent of the antipsychotic drugs (e.g. butyrophenones or trifluoperazine) at the autoreceptor were those that are most potent clinically and most potent in their abilities to increase dopamine metabolism. (−)Sulpiride and thioridazine, which are used clinically in large doses, were very weak, and clozapine was without activity at a concentration of 2×10^{-6} M. Drugs that are inactive clinically for treatment of schizophrenia (promethazine or the inactive isomers of butaclamol and sulpiride) were inactive in this receptor test system. A log–log plot of K_B values for the presynaptic autoreceptor against clinical dose revealed a close correlation ($r = 0.809$). It seems reasonable that, as the abilities of antipsychotic drugs to increase dopamine metabolism increase, they would have higher affinities for pre- and postsynaptic receptors, with the latter site being the therapeutic site of action. The abilities of antipsychotic drugs to displace [^{3}H]haloperidol binding were reported previously to have a high correlation with clinical doses [19, 29]. However, comparing the present data with that of Creese *et al.* [19] on [^{3}H]haloperidol binding revealed a 2–3 order of magnitude difference in affinity constants. As the butyrophenone antipsychotic drugs have low affinities for [^{3}H]apomorphine [9, 10] binding, there would appear to be a poor relationship between affinity for the autoreceptor and for the agonist binding site. These observations suggest that the autoreceptor may be distinct from both of these ligand binding sites.

DISCUSSION

The results in the present report are in harmony with earlier work that the dopamine autoreceptor is demonstrable *in vitro* using slices or synaptosomes prepared from the corpus striatum. The dopaminergic nature of the effect is demonstrated by the greater potencies of apomorphine and dopamine compared to *l*-norepinephrine for inhibition of synaptosomal tyrosine hydroxylase. That an externalized membrane receptor is involved in the inhibitory action is confirmed by the weak effects of dopamine uptake inhibition with either benzotropine [6, 7] or AFA (Table 1) on the IC_{50} of dopamine for the autoreceptor. Additionally, Iversen *et al.* [6] demonstrated a 100-fold increase in the concentration of apomorphine needed to inhibit tyrosine hydroxylase if detergent-lysed synaptosomes were used instead of the usual cell-free homogenate. Thus, the data accumulated *in vitro* are in harmony with *in vivo* experiments [3, 4] and confirm that a presynaptic

membrane receptor mediates the regulation of tyrosine hydroxylase in the corpus striatum.

The data presented in Table 2 show that the neuroleptic drugs inhibit apomorphine at the autoreceptor with an order of potencies similar to their order of clinical potencies. Drugs inactive as antipsychotics, such as promethazine, (−)butaclamol and (+)sulpiride, are inactive in the autoreceptor test system. Thioridazine and (−)sulpiride have weak effects on this receptor *in vitro* which agrees with the large doses necessary to block apomorphine in the *in vivo* presynaptic receptor test [2, 22]. On the other hand, the butyrophenones and pimozide, which are very active *in vivo* [2, 23], are very active in this *in vitro* autoreceptor test. It is interesting to note that pimozide which has a delayed onset of action *in vivo* at pre- and postsynaptic receptors [23], has as rapid an effect *in vitro* on the autoreceptor as it does in ligand binding assays. The reason for the delayed onset *in vivo* remains obscure. If data with [^{3}H]apomorphine binding are used [9, 10] for comparison to the data in Table 2, there are marked differences in the order of potencies exhibited by the butyrophenones in these two receptor assay systems. For example, apomorphine binding not displaceable by domperidone (Class II site, [10]) is displaced by concentrations of (+)butaclamol 1/30 to 1/40 of haloperidol or spiroperidol (K_i values of 13.5, 332 and 522 nM respectively), whereas (+)butaclamol had a K_B value that was between those of spiroperidol and haloperidol at the autoreceptor (Table 2). Sokoloff *et al.* [10] reported that this apomorphine binding site is reduced by 6-OH-dopamine lesions. Although this binding site may be presynaptic, the much higher affinity for (+)butaclamol makes it unlikely to be the autoreceptor. Thus, it seems unlikely that ability to displace agonist binding represents affinity for the striatal dopamine autoreceptor.

What is striking about the data are the large concentrations of agonists and antagonists needed to produce the effects. Apomorphine binding to striatal membranes saturates around 5 nM [9, 10, 12], but the IC_{50} of apomorphine for the defined autoreceptor is 500 nM (Table 1, [5–7]). It is not possible using nanomolar concentrations of a ligand and vacuum filtration methods to detect binding in the 500 nM range [24]. Explaining how an agonist can saturate its receptor at 5 nM but have an IC_{50} of 500 nM seems very difficult. Thus, it would appear that the receptor which is labeled by small concentration of apomorphine is distinct from the dopamine autoreceptor which inhibits tyrosine hydroxylation in nerve endings. Whether the high-affinity apomorphine binding site is pre- or postsynaptic is unclear, but the binding site must represent a different receptor.

A binding site for which neuroleptics show a low affinity is on calmodulin [25, 26]. Binding to this regulatory protein occurs in a range similar to the K_B values in Table 2 (i.e. in the micromolar range). However, promethazine binds to Ca^{2+}-calmodulin as does the clinically inactive (-)butaclamol [25, 26], but both of these drugs are inactive in the presynaptic model system. Thus, inhibition of calmodulin by the neuroleptics is not the receptor site for neuroleptic reversal of presynaptic receptor stimulation.

The inhibition of synaptosomal tyrosine hydroxylase is a useful model for determining drug affinities for a defined receptor. The large affinity constants of antipsychotic drugs are consistent with the large amounts of drug needed to reverse apomorphine *in vivo* in the presynaptic receptor test [2]. Also, the large IC_{50} (500 nM) for apomorphine at this receptor is consistent with the doses of apomorphine needed to inhibit γ-butyrolactone (GBL)-induced activation of tyrosine hydroxylase [4]. This model system allows for the characterization of drugs as agonists or antagonists at the autoreceptor. Buspirone was tested as an agonist since it has a high affinity for the *N-n*-propylnorapomorphine binding site (IC_{50} = 20 nM) and is reportedly active as a dopamine agonist in the perfused rabbit ear artery system [18]. However, only a 25% decrease in tyrosine hydroxylase activity occurs at a buspirone concentration of 10^{-4} M. Preliminary experiments *in vivo* confirm that buspirone (10 or 30 mg/kg, i.p.) does not reverse GBL-induced activation of tyrosine hydroxylase (data not shown). These data further support the concept that the high-affinity apomorphine binding site on striatal membranes is different from the dopamine autoreceptor which regulates the rate of tyrosine hydroxylation. It remains unclear as to what functional receptor, if any, is labeled by nanomolar concentrations of apomorphine.

REFERENCES

1. R. H. Roth, L. C. Murrin and J. R. Walters, *Eur. J. Pharmac.* **36**, 163 (1976).
2. J. R. Walters and R. H. Roth, *Naunyn-Schmiedeberg's Archs Pharmac.* **296**, 5 (1976).
3. W. Kehr, A. Carlsson and M. Lindqvist, *Naunyn-Schmiedeberg's Archs Pharmac.* **297**, 111 (1977).
4. R. H. Roth, *Commun. Psychopharmac.* **3**, 429 (1979).
5. M. Goldstein, B. Anagnoste and C. Shirron, *J. Pharm. Pharmac.* **25**, 348 (1973).
6. L. L. Iversen, M. A. Rogawski and R. J. Miller, *Molec. Pharmac.* **12**, 251 (1976).
7. T. C. Westfall, N. A. Perkins and C. Paul, in *Presynaptic Receptors* (Eds. S. Z. Langer, K. Starke and M. L. Dubocovich), p. 243. Pergamon Press, New York (1979).
8. B. A. McMillen, D. C. German and P. A. Shore, *Biochem. Pharmac.* **29**, 3045 (1980).
9. M. Titeler, S. List and P. Seeman, *Commun. Psychopharmac.* **3**, 411 (1979).
10. P. Sokoloff, M-P. Martres and J-C. Schwartz, *Nature, Lond.* **288**, 283 (1980).
11. M. J. Bannon, E. B. Bunney, J. R. Zigun, L. R. Skirboll and R. H. Roth, *Naunyn-Schmiedeberg's Archs Pharmac.* **312**, 161 (1980).
12. I. Creese and S. H. Snyder, *Eur. J. Pharmac.* **56**, 277 (1979).
13. J. E. Leysen, *Commun. Psychopharmac.* **3**, 397 (1979).
14. M. Goldstein, J. Y. Lew, T. Asano and K. Ueta, *Commun. Psychopharmac.* **4**, 21 (1980).
15. H. O. Schild, *Br. J. Pharmac. Chemother.* **4**, 277 (1949).
16. T. Nagatsu, M. Levitt and S. Undenfriend, *Analyt. Biochem.* **9**, 122 (1924).
17. B. A. McMillen and P. A. Shore, *Pharmacology* **19**, 261 (1979).
18. H. C. Stanton, D. P. Taylor and L. A. Riblet, in *The Neurobiology of the Nucleus Accumbens* (Eds. R. B. Chronister and J. F. DeFrance), p. 316. Haer Institute, Brunswick (1981).

19. I. Creese, D. R. Burt and S. H Snyder, *Science* **192**, 481 (1976).
20. P. Seeman, *Biochem. Pharmac.* **26**, 1741 (1977).
21. E. Usdin and D. H. Efron, *Psychotropic Drugs and Related Compounds*, Publ. (HSM) 72-9074, Dept. Health, Education and Welfare, Washington, DC (1972).
22. T. Alander, N-E. Andén and M. Grabowska-Andén, *Naunyn-Schmiedeberg's Archs Pharmac.* **312**, 415 (1980).
23. B. A. McMillen, D. C. German, M. K. Sanghera, W. Warnack and P. A. Shore, *J. Pharmac. exp. Ther.* **215**, 150 (1980).
24. J. P. Bennett, in *Neurotransmitter Receptor Binding* (Eds. H. T. Yamamura, S. J. Enna and M. J. Kuhar), p. 57. Raven Press, New York (1978).
25. B. Weiss, R. Fertel, R. Figlin and B. Uzunov, *Molec. Pharmac.* **10**, 615 (1974).
26. J. A. Norman and A. H. Drummond, *Molec. Pharmac.* **16**, 1089 (1979).