Biochemical Pharmacology, Vol. 35, No. 7, pp. 1159–1162, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

POSTNATAL ONTOGENY OF DOPAMINE D2 RECEPTORS IN RAT STRIATUM

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(*Received* 4 *May* 1985; *accepted* 16 *September* 1985)

Abstract—Studies of the ontogeny of dopamine D2 receptors in rat striatum were carried out using [^{3}H]spiroperidol as ligand in the presence of 30 nM ketanserin. D2 receptors increased with age, and saturation studies indicated that this was due to an increase in receptor density (B_{max}) and not to a change in affinity (K_A). By 21 days of age, receptor density had reached adult levels. Hill plots indicated no cooperativity in the binding from 7 to 21 days of age. Pharmacologically, receptors at 7, 14 and 21 days of age were similar to those of adult rats, exhibiting characteristics of dopamine D2 receptors. These studies provide a basic analysis of dopamine D2 receptors in rat striatum during early postnatal development.

Dopamine (DA‡) receptors in the striatum (caudate-putamen) of many species have been characterized in depth, particularly by the use of membrane binding techniques [1, 2]. There appear to be two types of DA receptors in striatum, D1 receptors linked to adenylyl cyclase and D2 receptors which are not linked to adenylyl cyclase. Each of these receptors has a high affinity for a distinct group of antagonists or neuroleptic agents. In addition, each appears to have a high and low affinity state for agonists which is regulated by the linkage of the receptor to a macromolecule in the membrane via GTP.

Of these two, the D2 receptors have been studied and characterized much more extensively, primarily due to the availability of ligands with a high affinity and some selectivity for these receptors such as the butyrophenone neuroleptics [e.g. spiroperidol (SP), haloperidol, and domperidone]. Until very recently, a similar group of agents specific for D1 receptors has not been available. Studies on the characteristics of DA receptors during development have been relatively few. Initial studies demonstrated that striatal DA receptors increase in number with age through the first month postnatally [3]. Similar results were found in whole rat forebrain [4]. We here present a detailed analysis of dopamine D2 receptors in rat striatum during early postnatal development. Parts of this work have been presented in abstract form [5].

MATERIALS AND METHODS

Materials. [^{3}H]Spiroperidol (21–26 Ci/mmole) was obtained from the New England Nuclear Corp., Boston, MA. Drugs were obtained as follows: haloperidol, spiroperidol, domperidone, and ketanserin—Janssen Pharmaceutica, Beerse, Belgium; atropine—Sigma Chemical Co., St. Louis, MO; (+)- and (−)butaclamol—Research Biochemicals, Wayland, MA; chlorpromazine—Smith, Kline & French, Philadelphia, PA; cinanserin and fluphenazine—E. R. Squibb, Princeton, NJ; *cis*- and *trans*-flupenthixol—H. Lundbeck & Co., Copenhagen; mianserin—Organon, West Orange, NJ; naloxone—Endo Laboratories, Garden City, NY; sulpiride—Delagrange International, Paris; thioridazine—Sandoz, East Hanover, NJ. Tris-(hydroxymethyl)-aminomethane (Tris) buffers were research grade obtained from Research Organics, Cleveland, OH. All other chemicals were research grade and were obtained from Mallinckrodt, St. Louis, MO.

Membrane preparations. Striata from the brain of rat pups aged 2–40 days were dissected and weighed following decapitation. At younger ages, tissue was usually pooled to provide 100 mg tissue per preparation. Tissue was homogenized (Tissumizer, Tekmar Co., Cincinnati, OH, 70%, 20 sec) in ice-cold 0.05 M Tris buffer, pH 7.4, at 20°. Homogenates were centrifuged at 48,000 *g* for 15 min at 4°. Pellets were washed twice by resuspension in 5 ml buffer (Tissumizer, 10 sec, 70%) followed by centrifugation. The final pellets were frozen at −20° until use, always within 1 month.

Receptor binding assays. Most assays were carried out using the procedure of Creese *et al.* [6]. Tissue was resuspended in 0.05 M Tris buffer plus ions (120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4, at 37°) at 1.2 mg/ml except for studies at 2–7 days of age in which 2 mg/ml preparations were used. [^{3}H]SP was used at 0.5 nM for most studies. All assays except competition studies for determining IC_{50} values were run in the presence of 30 nM ketanserin to eliminate serotonin S2 receptor binding [7]. Blanks were usually generated by adding 1 μM (+)butaclamol, and use of 1 μM domperidone in a few studies produced identical results. Assay volume was 2.0 ml containing 1.8 ml of tissue

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‡ Abbreviations: DA, dopamine; and SP, spiroperidol or spiperone.

preparation. Incubations were for 10 min at 37° followed by vacuum filtration over Whatman GF/B glass fiber filters (Whatman, Inc., Clifton, NJ) followed by three 5-ml washes with ice-cold buffer. Bound radioactivity was determined by liquid scintillation counting with an average efficiency of 44%.

Association rate studies were carried out as described using [^{3}H]SP at 0.5 nM. A small number of tubes (usually six) were prepared with tissue and (+)butaclamol for blanks. [^{3}H]SP was added quickly, and the tubes were vortexed and placed in the water bath. Tubes were filtered as described above at 0, 1, 2, 3, 4, 6, 8, 10, 12, 15 and 20 min after addition of radioligand. Dissociation rate studies were carried out by incubating tissue preparations in the presence of 0.5 nM [^{3}H]SP for 15 min. At 15 min, unlabeled SP was added in 100 μl to bring the final SP concentration to 1 μM. At 0, 1, 2, 3, 4, 5, and 10 min after addition of unlabeled SP, samples were filtered as described above.

Saturation analysis studies were carried out with two protocols. The first protocol is described above and used eighteen spiroperidol concentrations of 0.1 to 6 nM combining labeled and unlabeled ligand. Blanks were determined by use of 1 μM unlabeled spiroperidol. The second group of studies examined the possibility of a high affinity site not detected in the first series of studies by using a final volume of 4 ml (3.7 ml of tissue preparation) and eighteen SP concentrations from 0.03 to 10.0 nM. The rest of the assay was identical to that described above. Saturation studies were initially analyzed by Scatchard analysis [8], and final analysis utilized LIGAND [9]. Hill plots [10] were carried out in a similar fashion using nine concentrations of [^{3}H]SP.

The IC_{50} values were determined by use of log-probit analysis. The method of Lowry *et al.* [11] was used for protein analysis. For statistical analysis one-way analysis of variance was used followed by Peritz' F test [12, 13].

RESULTS AND DISCUSSION

Age dependence. Analysis of [^{3}H]SP binding in striatum from 2 through 40 days of age postnatally indicates that there was a steady increase in DA receptor binding from day 2 to day 21, at which point receptor levels, expressed as pmoles/mg protein, reached adult levels (Fig. 1). These data are in agreement with the work of Pardo *et al.* [3] who expressed their data as pmoles/mg wet weight and with Bruinink *et al.* [4], who examined DA and serotonin receptors in whole forebrain. DA receptor development, a postsynaptic marker, paralleled the pattern seen with several markers associated with presynaptic dopaminergic terminals in striatum, including DA content and DA uptake [14], tyrosine hydroxylase activity [15, 16] and dopa decarboxylase activity [15, 17].

Association and dissociation rates. Analysis of association and dissociation rates in striata from 7- and 21-day-old rat pups yielded similar data at both ages. Graphic analysis of dissociation data as B/B_0 vs time (B_0 = specific binding at equilibrium and B = specific binding at t; [18]) gave dissociation rates of 0.0693 min^{-1} at 7 days of age and 0.0731 min^{-1} at 21 days of age. Analysis of association rate data as $\ln[B_e/(B_e\text{-}B)]$ vs time (B_e = specific binding at equilibrium and B = specific binding at t) to obtain k_{ob} and then deriving k_1, from the formula

$$k_1 = \frac{k_{ob} - k_{-1},}{L_T}$$

where L_T is total ligand concentration, yielded values of 8.614×10^8 l/moles-min at 7 days and 1.354×10^9 l/moles-min at 21 days.

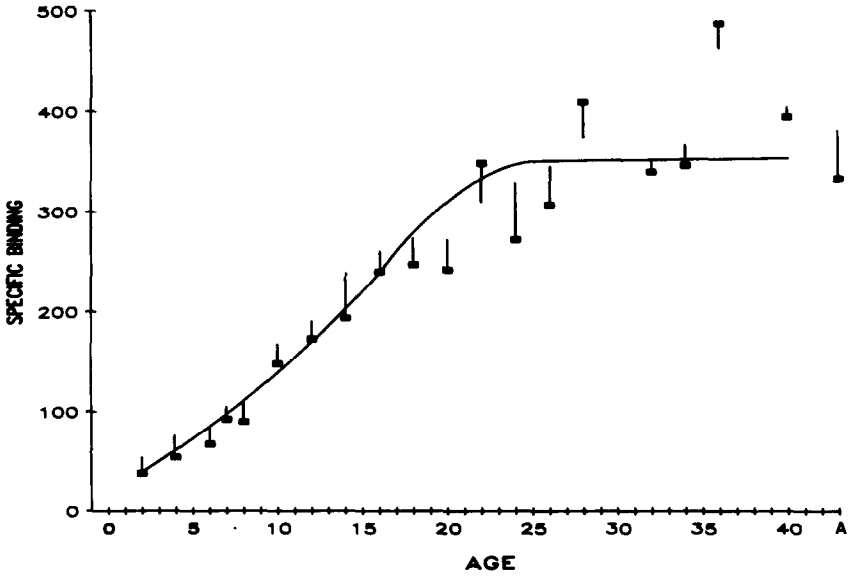

Fig. 1. Dopamine receptor binding in rat striatum with postnatal age. [^{3}H]SP was used as ligand at 0.5 nM in the presence of 30 nM ketanserin. Blanks contained 1 μM (+)butaclamol. See Materials and Methods for details. Data are the mean of at least three determinations with standard error bars. A = adult values.

Table 1. [^{3}H]Spiroperidol binding constants for D2 receptors in postnatal development

Age (days)	K_A ($\times 10^{-9}$)	B_{max} (fmoles/mg protein)	N
7	9.11 ± 3.05	26.2 ± 5.5	6
14	6.20 ± 1.28	44.0 ± 12.1	9
21	5.06 ± 1.12	155.6 ± 45.4*,†	7
28	5.91 ± 2.76	328.1 ± 30.7*,†	4

Data are mean ± S.E.M. See Materials and Methods for details on saturation studies. Data were analyzed using one-way analysis of variance followed by Peritz' F test. There were no significant differences between groups for K_A.

* Significantly different from 7-day group, $P < 0.05$.

† Significantly different from 14-day group, $P < 0.05$.

Saturation studies. An in-depth analysis of D2 receptors in striatum during development using saturation studies indicated that the increased binding of [^{3}H]SP with age was due to an increase in receptor density and not to changes in receptor affinity for the ligand (Table 1). Analysis of binding over a wide range of concentrations (0.03 to 10 nM; see Materials and Methods) using two protocols produced similar results. From 7 to 28 days of age there was no statistically significant alteration in the association equilibrium constant (K_A: Table 1). The large variability in the data from 7-day-old pups reflects the difficulty of carrying out a wide range saturation analysis at this age in which the specific to nonspecific radioligand binding ratio is about 1:1.

In contrast to data on receptor affinity, there was a clear-cut increase in receptor density, as measured by B_{max}, with increasing age (Table 1). Thus, the increase in DA receptor binding in striatum with increasing age that has been reported *in vitro* (Fig. 1, [31]) and *in vivo* [19] is due primarily to increases in receptor number. The B_{max} at 21 days of age was similar to the B_{max} found in adult rats both in this laboratory [20] and in many others [1]. The increase in B_{max} at 28 days of age (Table 1), while not statistically significant, corresponds to similar fluctuations in receptor density frequently seen in striatum during the fourth week postnatally (Fig. 1 and [3]). Eventually receptor number stabilized at adult levels. The explanation for these apparent rapid changes in receptor density is not known at present.

Hill plots of saturation studies [10] from 7-, 14- and 21-day-old animals (Fig. 2) indicated a lack of positive or negative cooperativity in D2 receptor binding. This is consistent with results found in adult animals [1, 2]. Hill coefficients (n_H) were 0.84, 1.04 and 1.07 for 7-, 14- and 21-day-old pups respectively. The coefficients of correlation (r^2) for these plots were 0.96 or greater.

Whereas there was a close parallel between the increase in D2 dopamine receptor number and DA concentration in striatum, as noted above, anatomically there is a clear distinction between the two dopaminergic markers up to 2 weeks postnatally. Dopamine terminals, as seen with fluorescence histochemistry, appear in the striatum of the rat in patches [21–23], termed "striosomes" [24]. In contrast, D2 receptors appear in a graded pattern, with the most dense area being the dorso-lateral striatum [19, 25]. Thus, whereas the biochemical studies indicate that presynaptic and postsynaptic elements of the dopaminergic neuronal system develop in parallel, anatomic studies suggest that there is no direct relationship between the two.

Pharmacology. Pharmacologically, D2 dopamine receptors in striatum were similar at all ages (Table 2) and displayed a pharmacology consistent with that found for dopaminergic neurons in adult animals [1]. Agents known to be active at dopamine D2 receptors

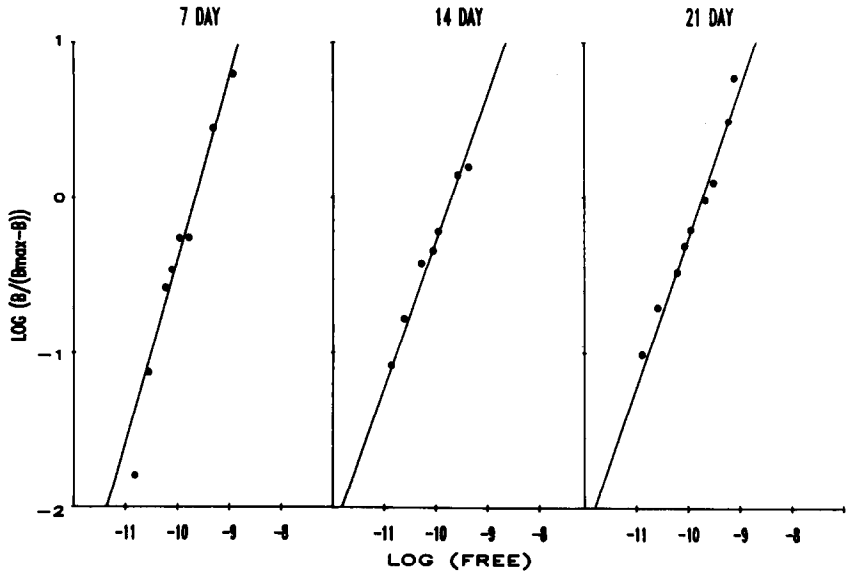

Fig. 2. Hill plots of [^{3}H]SP binding in rat striatum at 7, 14 and 21 days of age. See Materials and Methods for details. Hill coefficients (n_H) are 0.801, 1.17 and 1.033 for 7-, 14- and 21-day tissues, respectively, in this figure.

Table 2. Pharmacology of [^{3}H]spiroperidol binding in neonatal striatum

Agent	IC_{50} (nM)	
	7 Day	21 Day
Fluphenazine	3.2	4.2
cis-Flupenthixol	9.8	8.3
trans-Flupenthixol	1,250	970
(+)Butaclamol	9.8	7.3
(−)Butaclamol	>10,000	>10,000
Haloperidol	30	37.5
Thioridazine		58
Chlorpromazine	80	86
Sulpiride	450	1,925
Ketanserin	2,500	>10,000
Mianserin	6,500	9,500
Cinanserin	>10,000	>10,000
Phentolamine	>10,000	>10,000
Atropine	>10,000	>10,000
Naloxone	>10,000	>10,000

Data are the mean of at least two determinations, each carried out in quadruplicate. The IC_{50} values were determined by log probit analysis. [^{3}H]SP was used at 0.5 nM. See Materials and Methods for details. In these studies, ketanserin was not included in all tubes.

readily displaced [^{3}H]SP binding in neonatal rat striatum, whereas agents which are inactive or very weak at D2 receptors displaced [^{3}H]SP poorly. The binding to the receptors was stereospecific as reflected in the lack of potency of *trans*-flupenthixol and (−)butaclamol, the stereoisomers of the potent *cis*-flupenthixol and (+)butaclamol.

These studies provide a more complete analysis of the postnatal ontogeny of D2 dopaminergic receptors in the rat striatum than has been published previously. In addition, they focus more sharply on dopaminergic receptors by the routine inclusion of ketanserin to block serotonin S2 receptors, which are found in the striatum and to which [^{3}H]SP binds. They provide a basis for further studies on the ontogeny of striatal dopaminergic receptors.

Acknowledgements—We would like to thank Jeri Hitchcock for her excellent technical assistance and Tish A. Lindwall for her excellent secretarial assistance. This work was funded by a grant from the State of Nebraska (85–44) and by BNS-7921105.

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