Differential Recovery Rates of Rat D₂ Dopamine Receptors as a Function of Aging and Chronic Reserpine Treatment Following Irreversible Modification: A Key to Receptor Regulatory Mechanisms

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The density of D₂ dopamine receptors identified by ³H-spiperone binding was significantly lower in the striatum of senescent (28-month-old) than in mature (4-month-old) Fischer 344 rats. The time course of recovery of ³H-spiperone binding to D₂ dopamine receptors following irreversible receptor modification by a single injection of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was significantly slower in senescent than in mature rats. The kinetics of D₂ dopamine receptor repopulation could be adequately described by a model assuming a constant rate of receptor production and a rate of degradation that was dependent on receptor concentration. Both the production rate and degradation rate constant of D₂ dopamine receptors were lower in the senescent rats compared to mature rats. The changes in receptor density that occur as a function of development and aging are the result of changes in both the receptor production rate and receptor degradation rate constant, which, while both are reduced, tend to maintain receptor density. That is, receptor density decreased by 26% from 4 to 28 months of age, although the receptor production rate and degradation rate constant decreased by 40–50%. Chronic treatment of rats with reserpine produced a 21% increase in the density of striatal D₂ dopamine receptors of mature, but not senescent, rats, accompanied by an increase in the initial recovery rate of ³H-spiperone binding sites following EEDQ treatment. It might have been expected that the reduced formation of agonist-bound receptors would have lead to a decrease in the receptor degradation rate constant. However, these data indicate that receptor upregulation is an active process resulting from an increase in the receptor production rate. These results may suggest that homologous receptor up-regulation is mediated by increased transcription of the gene(s) responsible for receptor production. The more general decrease in both receptor production rate and degradation rate constant seen in many neurotransmitter systems with aging suggests that de-

creases in posttranscriptional processes may prevent the homologous receptor up-regulation response in senescence.

D₂ dopamine receptor density in rat striatum is modulated by a number of conditions (Seeman, 1980; Creese and Sibley, 1981). For example, lesions of the nigrostriatal dopaminergic neurons (Creese and Snyder, 1979) produce an elevation of the density of postsynaptic striatal D₂ dopamine receptors. Similarly, chronic treatment of rats with D₂ dopamine receptor antagonists or the catecholamine depleter, reserpine (Burt et al., 1977; Creese and Sibley, 1981), produces reversible increases in striatal D₂ dopamine receptor density with no change in receptor affinity. In contrast to the increased density of D₂ dopamine receptors in response to chronic decreases in dopaminergic neurotransmission, a reduction in the density of D₂ dopamine receptors in the striatum of rodents and man occurs as a function of the natural aging process (Maggi et al., 1979; Finch et al., 1981; Roth and Hess, 1982). Furthermore, in aged rodents, relative deficits in the ability of dopamine receptors to increase their density in response to chronic dopamine receptor antagonist drug treatments have been described (Randall et al., 1981).

It is therefore evident that striatal D₂ dopamine receptors are not stable components of neuronal membranes. The receptor steady-state level, which is generally measured as the receptor B_{max} , is the product of the rates of receptor production and degradation. Therefore, alterations in the rate of receptor production and/or receptor degradation may be responsible for any observed changes in the steady-state level of receptors. Indeed, the steady-state levels of nicotinic cholinergic receptors and α_1 - and β -adrenergic receptors, for example, have previously been shown to be subject to such dynamic processes (Fambrough, 1979; Pitha et al., 1982; Sladeczek and Bockaert, 1983).

We have developed a technique to estimate D₂ dopamine receptor production and degradation rates *in vivo* by determining the rate of recovery of ³H-spiperone binding to D₂ dopamine receptors sites after the irreversible modification of D₂ dopamine receptors following a single injection of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (Hamblin and Creese, 1983). In a previous study, we demonstrated marked reductions in D₂ dopamine receptor "turnover" during development between rats 1 and 9 months of age, in spite of a significant increase in D₂ dopamine receptor density with maturity (Leff et al., 1984). In the present study we have investigated which of the processes determining receptor steady-state levels were altered as a function of senescence, which is associated with a decrease in D₂ dopamine receptor density. We also investigated whether ad-

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aptations in either the process of receptor production and/or degradation could account for the increased D_2 dopamine receptor steady-state levels observed following chronic reserpine treatment in mature rats.

Materials and Methods

In vivo drug treatments. Male Fischer 344 rats (4 and 28 months old), obtained from the colonies maintained by the National Institute on Aging, were housed in groups of 4 by age. EEDQ (Aldrich Chemical Co., Milwaukee, WI) was freshly dissolved in ethanol/water, 1:1 (vol/vol), and was injected i.p. in a volume of 1 ml/kg. Only rats displaying acute and marked catalepsy after EEDQ injections were included in this study, as we have previously shown that this behavior is associated with a maximal and consistent modification of D_2 receptors (Hamblin and Creese, 1983). Mature rats (4 months old) or senescent rats (28 months old) were injected with EEDQ (10 mg/kg) or vehicle and groups of 6 rats were sacrificed 6 hr later or at the same time of day, 1, 2, 4, 8, and 16 d after injection with either EEDQ or vehicle. An additional group of mature rats was injected with EEDQ or vehicle and was sacrificed 6 hr or 32 d later. Brains were rapidly removed and placed in ice-cold saline, and the striata were dissected, frozen in liquid nitrogen, and stored at -70°C until assayed. All rats were treated with EEDQ or vehicle between 8:00 and 10:00 a.m., as it was observed in previous studies that mortality increased if rats were treated with EEDQ later in the day.

Chronic reserpine treatment. Mature and senescent rats were injected daily with 0.25 mg/kg (s.c.) reserpine (Serpasil; Ciba-Geigy Corp., Summit, NJ) for 7 d and then were injected with 0.5 mg/kg (s.c.) every 2nd day. This drug treatment regimen was well tolerated by the mature (4-month-old) rats. However, there was a relatively high mortality (30%) among the senescent (28-month-old) rats. Untreated rats served as controls. On day 21 following the last reserpine injection on day 19, EEDQ (10 mg/kg i.p.) or vehicle was injected and rats were sacrificed 6 hr and 1, 2, 3, 4, 8, and 16 d later. Striata were dissected, frozen in liquid nitrogen, and stored at -70°C before ^3H -spiperone assays.

^3H -spiperone binding assays. After all the rats had been sacrificed, striata from individual rats killed at each of the time points and controls were homogenized using a Tekmar Tissumizer (setting 7, 10 sec) in 19 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.7 at 25°C) and centrifuged ($48,000 \times g$, 10 min, 4°C). The pellets were resuspended in 19 volumes of Tris-HCl buffer containing 5 mM MgSO_4 and incubated at 37°C for 10 min then washed twice by centrifugation and resuspended in Tris-HCl buffer. The final resuspension was into assay buffer consisting of 50 mM Tris-HCl, 5 mM MgSO_4 , 0.5 mM ethylenediaminetetraacetic acid (EDTA), and 0.02% ascorbic acid. All ^3H -spiperone (New England Nuclear Corp., Boston, MA; 20 Ci/mmol) saturation assays were conducted in a total volume of 3 ml of assay buffer containing 40 nM ketanserin (Janssen Pharmaceutica, N.V., Beerse, Belgium) to preclude binding of ^3H -spiperone to S_2 5-HT receptors. Tissue homogenates were added to duplicate plastic tubes containing ^3H -spiperone (0.03–0.8 nM) in the presence or absence of 1 μM (+)butaclamol (Ayerst Laboratories, Montreal) to determine nonspecific binding. The tubes were incubated at 37°C for 40 min before assays were terminated by rapid filtration over Whatman GF/C filters under vacuum using a modified Brandel cell harvester. Filters were washed 3 times with 5 ml of cold Tris-HCl buffer, and the radioactivity trapped on the filters was measured using a Beckman LS 7500 liquid scintillation spectrometer at an efficiency of 50%. B_{max} and K_D values for the binding of ^3H -spiperone to D_2 dopamine receptors was determined by Scatchard analysis (Scatchard, 1949) of ^3H -spiperone saturation data.

In vitro protection experiments. Striata from male Sprague-Dawley rats, 180–220 g (Simonsen Laboratories, Gilroy, CA), were homogenized and washed once in cold Tris-HCl buffer as described above. The pellet was resuspended in Tris-HCl buffer and divided into 3 aliquots. One aliquot received EEDQ to give a final concentration of 10 μM , a second aliquot received a final concentration of 100 nM haloperidol (Janssen Pharmaceutica, N.V.) and 10 μM EEDQ. The third aliquot received 100 nM haloperidol and served as the control. After incubation at 37°C for 15 min, the aliquots were diluted with cold Tris-HCl buffer and washed 3 times by centrifugation and resuspended in fresh buffer as described above. The final resuspension was into assay buffer consisting of 50 mM Tris-HCl buffer containing 5 mM MgSO_4 , 0.5 mM EDTA, and 0.02% ascorbic acid (pH 7.4 at 37°C). The tissues were then assayed for ^3H -spiperone binding and ^3H -(-)-N-propylnorapomorphine

Table 1. ^3H -spiperone binding to D_2 dopamine receptors in rat striatum as a function of aging and chronic reserpine treatment

Group	Mature (4 months)		Senescent (28 months)	
	B_{max} (pmol/g tissue)	K_D (pM)	B_{max} (pmol/g tissue)	K_D (pM)
Untreated	24.8 ± 0.5 ($n = 11$)	81 ± 5	18.3 ± 0.8^b ($n = 9$)	89 ± 9
Chronic reserpine	30.1 ± 2.5^a ($n = 6$)	84 ± 13	18.7 ± 2.2^b ($n = 6$)	81 ± 7

Male Fischer 344 rats were either not treated or were administered reserpine over a period of 21 d and were then decapitated and the striatum rapidly dissected. B_{max} and K_D values were determined from Scatchard analysis of ^3H -spiperone saturation data (in the presence of 40 nM ketanserin to preclude binding to S_2 5-HT receptors) as described in Materials and Methods. The concentration range of ^3H -spiperone was 0.03–0.8 nM and nonspecific binding was defined by 1 μM (+)butaclamol. Values represent the means $\pm$ SEM from the number of rats shown in parentheses.

^a Significantly different from untreated values ($p < 0.01$, 2-tailed t test).

^b Significantly different from mature values ($p < 0.01$, 2-tailed t test).

(NPA; New England Nuclear Corp.; 66.1 Ci/mmol) binding. ^3H -spiperone saturation assays were determined as described above except that the total assay volume was 2.5 ml. ^3H -(-)NPA binding was determined in an assay volume of 2.5 ml as described for ^3H -spiperone binding assays and using a concentration range of 0.1–1.5 nM ^3H -(-)NPA. Nonspecific binding of ^3H -(-)NPA was defined using 30 nM spiperone (Janssen Pharmaceutica, N.V.).

Results

As shown in Table 1, there was a significant 26% decrease in the B_{max} of ^3H -spiperone binding to D_2 dopamine receptors in the striatum of senescent (28-month-old) compared to mature (4-month-old) Fischer 344 rats. There was no significant difference in the K_D of ^3H -spiperone binding between senescent and mature control rats.

We investigated the recovery of D_2 dopamine receptors following *in vivo* administration of a single dose of EEDQ (10 mg/kg, i.p.). As shown in Figure 1, there were similar reductions (94 and 92%, respectively) in the mean B_{max} of ^3H -spiperone binding to D_2 dopamine receptors in the striatum of mature and senescent rats 6 hr after treatment with EEDQ. Repeated washing of the tissue did not increase the B_{max} . After an initial delay of 1 d, the B_{max} of ^3H -spiperone in the striatum of mature rats increased with time. Similarly, in the striatum of senescent rats there was a time-dependent increase in the B_{max} of ^3H -spiperone binding following treatment with EEDQ. However, the mean initial rate of recovery of ^3H -spiperone binding (between 1 and 4 d post-EEDQ) was lower in senescent rats (1.3 pmol/d) compared to mature rats (2.8 pmol/d). Although EEDQ produced a decrease in ^3H -spiperone B_{max} to similar levels in mature and senescent rats, the B_{max} of ^3H -spiperone binding was greater in mature than in senescent rats at days 2, 4, 8, and 16 after EEDQ treatment. A 2-way ANOVA indicated that the absolute levels of ^3H -spiperone binding sites were significantly greater in the striatum of mature rats than in senescent rats during the time course of recovery up to 16 d after treatment with EEDQ ($F = 24.6$, $df = 1,50$; $p < 0.001$). Furthermore, the ANOVA demonstrated that the differences in receptor recovery rate between mature and senescent rats were significant ($F = 3.1$, $df = 4,50$; $p < 0.05$). In both mature and senescent rats there was a small (~ 2 -fold) but significant ($p < 0.01$, 2-tailed t tests) increase in

Figure 1. Recovery of D₂ dopamine receptors in the striatum of mature and senescent rats following *in vivo* treatment with EEDQ. Rats were given a single injection of EEDQ (10 mg/kg, i.p.) or vehicle and decapitated 6 hr or 1, 2, 4, 8, 16, or 32 d later. The $B_{\max}$ of ³H-spiroperone binding to D₂ dopamine receptors was determined as described in Materials and Methods. The half-time for recovery of the recoverable D₂ dopamine receptors was 3.6 and 5.4 d for mature and senescent rats, respectively. The dashed lines represent the SEM $B_{\max}$ values for vehicle-treated controls; symbols represent the mean $\pm$ SE of 6 rats. Two-way ANOVA demonstrated that the overall receptor recovery rate was significantly different ($F = 3.1$, $df = 4,50$; $p < 0.05$) in mature versus senescent rats and that the absolute levels of ³H-spiroperone binding sites were significantly higher in mature versus senescent rats ($F = 24.6$, $df = 1,50$; $p < 0.001$) during recovery following EEDQ treatment.

the K_D of ³H-spiroperone binding at 6 hr, 1 d, and 2 d after EEDQ treatment; at subsequent time points, however, the K_D of ³H-spiroperone binding was not significantly different from control values (data not shown).

In senescent rats the $B_{\max}$ of ³H-spiroperone had returned to those values observed in untreated control rats 16 d after treatment with EEDQ (Fig. 1). In contrast, in mature rats, the $B_{\max}$ of ³H-spiroperone binding at this time point was significantly lower than control values ($p < 0.05$, 2-tailed t test), representing 84% of the control $B_{\max}$. The $B_{\max}$ of ³H-spiroperone binding was still maintained at a significantly lower value than that observed in control rats 32 d after EEDQ treatment (Fig. 1).

If receptor production rate is constant (zero order) and receptor degradation is dependent on receptor concentration (first order), then repopulation kinetics after irreversible blockade of receptors may be described by the equation

$$[R_t] = (r/k) (1 - e^{-kt}) \quad (1)$$

where $[R_t]$ is the receptor concentration at time t , r is the receptor production rate, and k is the rate constant for receptor degradation (Mauger et al., 1982; Sladeczek and Bockaert, 1983). As

Figure 2. Semilogarithmic plot of the time course of D₂ dopamine receptor recovery in the striatum of mature and senescent rats. $[R_{ss}]$, steady-state receptor concentration of recoverable receptors ($B_{\max}$ at day 16 and 32 after EEDQ treatment minus residual receptor levels at 6 hr following EEDQ treatment: 18.7 and 16.1 pmol/g tissue in mature and senescent rats, respectively); $[R_t]$, receptor density at time t minus receptor density at 6 hr. Rate constants for receptor degradation (k) were 0.0087 and 0.0051 hr⁻¹ for mature and senescent rats, respectively. Receptor production rates (r) were 0.164 and 0.085 pmol/g tissue/hr for mature and senescent rats, respectively. $t_{1/2}$ values were 79 hr (3.3 d) and 136 hr (5.7 d) for the D₂ dopamine receptors in the striatum of mature and senescent rats, respectively.

the time of repopulation proceeds to infinity, the term e^{-kt} approaches zero, $[R_t]$ approaches $[R_{ss}]$ (the concentration of receptors at steady state, or $B_{\max}$); therefore,

$$[R_{ss}] = r/k. \quad (2)$$

Logarithmic transformation of Eq. (1) (Sladeczek and Bockaert, 1983) gives

$$\ln [R_{ss}]/([R_{ss}] - [R_t]) = kt. \quad (3)$$

Plots of our receptor recovery data according to Eq. (3) are shown in Figure 2. The receptor degradation rate constants (k) for the recoverable receptors (defined as the $B_{\max}$ at days 16 and 32 after EEDQ ($[R_{ss}]$) minus the $B_{\max}$ at 6 hr after EEDQ, $[R_0]$) estimated from the slopes of these plots are significantly different for the 2 age groups. Substituting the values for k into Eq. (2), when t approaches infinite time and $[R_t] = [R_{ss}]$, the receptor production rate (r) can be calculated. For mature and senescent rats the respective degradation rate constants were 0.0087 hr⁻¹ and 0.0051 hr⁻¹ and the respective derived production rates were 0.164 and 0.085 pmol/g of tissue/hr. Thus, in senescent rats the values for both receptor production and degradation were 40–50% lower than those observed in mature rats. Receptor half-lives ($t_{1/2}$) of 79 hr (3.3 d) and 136 hr (5.7 d) for mature and senescent rats, respectively, can be calculated from substitution of k into the equation $t_{1/2} = 0.693/k$.

Effects of chronic reserpine treatment on recovery of ³H-spiroperone binding after EEDQ treatment

To determine whether D₂ dopamine receptor turnover rates could be modulated by a continuous reduction in dopaminergic neurotransmission in the striatum, rats were injected on a chron-

Figure 3. Recovery of D₂ dopamine receptors in mature rats following chronic treatment with reserpine. Rats were treated with reserpine for 21 d prior to a single injection of EEDQ (10 mg/kg, i.p.) or vehicle only. Rats were decapitated at the time points shown and the B_{max} of ³H-spiroperone binding to D₂ dopamine receptors was determined as described in Materials and Methods. Dashed lines represent the SEM B_{max} values for reserpine-treated and untreated control rats. Two-way ANOVA demonstrated that the absolute levels of ³H-spiroperone binding sites during recovery following EEDQ treatment were significantly higher in reserpine compared to non-reserpine-treated rats ($F = 9.7$; $df = 1,50$; $p < 0.01$). However, the overall receptor recovery rates were not significantly different in mature and senescent rats ($F = 0.2$; $df = 5,50$; $p > 0.05$). *, Significantly different from values observed in non-reserpine treated rats ($p < 0.02$, 2-tailed t test).

ic basis with the catecholamine-depleting agent reserpine. Chronic treatment with this drug produced a significant 21% increase in D₂ dopamine receptor-binding sites in the striatum of mature rats (Table 1). In contrast, this treatment had no significant effect on the B_{max} of striatal D₂ dopamine receptor binding sites in senescent rats (Table 1). Receptor recovery rates were not affected by the prior reserpine treatment in the senescent rats compared to nonreserpine-treated senescent rats (data not shown). There was no change in the K_d of ³H-spiroperone binding produced by these treatments in either age groups of rats (Table 1).

As shown in Figure 3, EEDQ appeared to be equally effective in irreversibly modifying ³H-spiroperone-binding sites in both control and reserpine-treated mature rats (i.e., receptor concentration at 6 hr was reduced to similar levels: control, 94%; reserpine-treated, 96%). The initial rate of recovery of ³H-spiroperone binding sites was greater in reserpine-treated mature rats compared to nonreserpine rats. This effect was especially evident at the 1 d time point, where there was a significant increase in the number of D₂ dopamine receptors in the striatum of mature rats chronically treated with reserpine ($p < 0.02$, 2-tailed t test), whereas in non-reserpine-treated rats, D₂ dopamine receptor levels were maintained at similar levels to those observed at the 6 hr time point. A 2-way ANOVA demonstrated that the absolute levels of D₂ dopamine receptors were significantly higher in reserpine compared to non-reserpine-treated rats for the time course of recovery from the EEDQ-induced reduction in ³H-spiroperone binding ($F = 9.7$, $df = 1,50$; $p < 0.01$). However, the overall rate of recovery of ³H-spiroperone binding sites was

Table 2. Effect of age on the initial rate of recovery of D₂ dopamine receptors in rat striatum following administration of EEDQ

Age (months)	Receptor recovery rate (pmol/g tissue/d)	r (pmol/g tissue/hr)	k (hr ⁻¹)
1	4.1 ± 0.6	0.391	0.0154
4	2.8 ± 0.2	0.164	0.0087
9	2.7 ± 0.2	0.173	0.0058
28	1.3 ± 0.2	0.085	0.0051

Rats of varying ages were injected with EEDQ (8–10 mg/kg, i.p.) and striata removed 6 hr or 4 d later. The values shown from 1- and 9-month-old rats are calculated from Leff et al. (1984). The values for r and k for these groups were calculated using the untreated control B_{max} levels as [R_{ss}] without subtracting [R₀]. The values for receptor recovery rate represent the mean ± SE daily increase in ³H-spiroperone binding between 1–1.25 and 4–4.25 d after EEDQ treatment.

not significantly greater ($F = 0.2$, $df = 5,50$; $p > 0.05$) in striatum from reserpine-treated rats than in control mature rats as the recovery curves were close to parallel over days 4–16. At 16 d after EEDQ treatment (and the termination of the reserpine treatment), D₂ dopamine receptor levels were still significantly greater in the previously reserpine-treated mature rats compared to nonreserpine-treated mature rats. However, receptor levels in the EEDQ-treated, reserpine-treated mature rats were significantly lower ($p < 0.05$, 2-tailed t test) than the B_{max} values observed in reserpine-treated mature rats that were not treated with EEDQ.

Recovery of ³H-spiroperone binding in rats of different ages

We have previously investigated D₂ receptor recovery in Sprague-Dawley rats of other ages (Leff et al., 1984). As shown in Table 2, the initial rate of recovery of ³H-spiroperone binding in the striatum following EEDQ treatment differs markedly as a function of the age of the rat. The rate of receptor recovery between 1–1.25 d and 4–4.25 d after EEDQ administration decreases by approximately 32% between 1 and 4 months of age and then remains relatively stable to 9 months of age. Between 9 and 28 months of age there is another 55% decrease in the initial rate of recovery of ³H-spiroperone binding. Thus, in senescent rats the initial receptor recovery proceeds at only one third of the rate observed in immature rats.

Table 3. Sensitivity of ³H-(–)NPA binding to Gpp(NH)p following *in vitro* treatment with EEDQ or protection with haloperidol

Treatment	³ H-(–)NPA binding (pmol/g tissue)
Control	19.2 ± 2.2
+ 100 μM Gpp(NH)p	4.0 ± 1.6 ^b
10 μM EEDQ	9.1 ± 1.8 ^a
+ 100 μM Gpp(NH)p	4.8 ± 1.9 ^b
10 μM EEDQ + 100 nM haloperidol	16.0 ± 2.0
+ 100 μM Gpp(NH)p	5.6 ± 1.1 ^b

Homogenates of rat striatum were divided into 3 aliquots and each aliquot was incubated for 15 min at 37°C with either 10 μM EEDQ, 10 μM EEDQ plus 100 nM haloperidol or 100 nM haloperidol (control). The tissues were then washed to remove EEDQ and haloperidol and the specific binding of ³H-(–)NPA (1–1.5 nM) in each aliquot was determined in the presence and absence of 100 μM Gpp(NH)p. Nonspecific binding was defined using 30 nM spiroperone. Values represent the mean ± SE of the specific binding of ³H-(–)NPA from 4 separate experiments.

^a Significantly different from control values ($p < 0.01$, 2-tailed t test).

^b Significantly different from values observed in the absence of 100 μM Gpp(NH)p ($p < 0.02$, 1-tailed t test).

Figure 4. *In vitro* protection by haloperidol from the reduction of ³H-spiperone binding to striatal D₂ dopamine receptors by EEDQ. Homogenates of rat striatum were divided into 3 aliquots, and each aliquot was incubated for 15 min at 37°C with either 10 μM EEDQ, 10 μM EEDQ plus 100 nM haloperidol, or 100 nM haloperidol (control). The B_{max} of ³H-spiperone binding was determined in each aliquot by Scatchard analysis of saturation data as described in Materials and Methods. Bars represent the means ± SE of B_{max} values from 4 separate experiments. *, B_{max} values significantly different from control ($p < 0.01$, 2-tailed *t* test).

In vitro protection experiments

As shown in Figure 4, incubation of striatal homogenates with 10 μM EEDQ produced a 48% decrease in the binding of ³H-spiperone; however, co-incubation with 100 nM haloperidol was able to completely protect the binding of ³H-spiperone from the irreversible reduction produced by EEDQ. Similarly, 10 μM EEDQ irreversibly reduced the B_{max} of the D₂ dopamine receptor agonist radioligand ³H-(−)NPA by 47%, and this reduction was also prevented by co-incubation with 100 nM haloperidol (Table 3). As shown in Table 3, 100 μM guanylyl-5'-imidodiphosphate [Gpp(NH)p; Sigma Chemical Co., St. Louis] reduced the binding of ³H-(−)NPA by 80% in tissue incubated with haloperidol (control). The binding of ³H-(−)NPA was reduced by 53% by prior incubation with EEDQ, and Gpp(NH)p produced a significant reduction in the remaining ³H-(−)NPA binding. Similarly, Gpp(NH)p produced a significant 65% reduction in ³H-(−)NPA binding following protection by haloperidol from the EEDQ-induced decrease in binding.

Discussion

The estimated rate constant for synthesis, *r*, must describe a multistep process that includes both the synthesis and insertion of receptors into the membrane. Equally, the degradation rate constant, *k*, must describe multiple processes, including the internalization and degradation of receptors. An important consideration in evaluating the present results is whether EEDQ affects only membrane surface receptor number or whether it can also modify intracellular proteins. EEDQ-induced alterations in receptor synthesis, receptor incorporation into the neuronal membrane and/or degradative machinery would thus lead to estimated values of *r* and *k* that would not be representative of the normal processing of receptors. One way of examining this possibility, albeit indirectly, is to determine whether EEDQ affects other cellular moieties necessary for transduction of neurotransmitter signalling.

First, that protection of ³H-spiperone binding from EEDQ modification is possible with D₂ dopamine receptor antagonists,

both *in vivo* (Meller et al., 1985) and *in vitro* (Table 3, Fig. 4), suggests that EEDQ modifies the D₂ dopamine receptor at the ligand recognition site on the external neuronal surface.

Second, the D₂ dopamine receptor has 2 affinity states for agonists that are interconvertible by divalent cations and guanine nucleotides (Sibley et al., 1982; Hamblin et al., 1984). These affinity states appear to be dependent on interactions of the receptor with a guanine nucleotide regulatory subunit (N_i), which may mediate inhibition of adenylate cyclase (Enjalbert and Bockaert, 1983; Onali et al., 1984; Battaglia et al., 1985, b). We have demonstrated that treatment of membranes with *N*-ethylmaleimide (Sibley and Creese, 1983) or heat treatment (Hamblin and Creese, 1982) has no effect on ³H-antagonist binding to D₂ dopamine receptors but does abolish the high-affinity agonist binding state of the D₂ dopamine receptor. Such data indicate that following these treatments all D₂ dopamine receptors were converted to the low-affinity agonist binding state, suggesting that the N_i subunit is more sensitive to such treatments than the D₂ dopamine receptor or that N_i can no longer interact with the receptor. In the present experiments it was possible to protect the high-affinity binding of the agonist ligand ³H-(−)NPA to the D₂ dopamine receptor from irreversible modification by EEDQ by coincubation *in vitro* with haloperidol. Furthermore, the ability of 100 μM Gpp(NH)p to decrease ³H-(−)NPA binding was maintained in the haloperidol-protected and EEDQ treated-tissue. This was also true for the remaining unmodified ³H-(−)NPA binding sites in unprotected EEDQ-treated tissue. Therefore, the functional interaction between the "protected" D₂ dopamine receptor and the N_i subunit would appear to be unaffected by EEDQ at a concentration that modifies greater than 50% of the D₂ dopamine receptor binding sites. Thus, the data presented here suggest that the ligand recognition site of the D₂ dopamine receptor is more susceptible to irreversible modification by EEDQ than are moieties distinct from the ligand recognition site present within or on the internal surface of the neuronal membrane.

Third, we have shown that both guanine nucleotide and sodium fluoride stimulation of adenylate cyclase *in vitro* are unaffected by prior *in vivo* treatment with EEDQ that abolishes 80% of D₁ and D₂ dopamine receptors (Hess et al., 1985, 1987; Battaglia et al., 1986).

Fourth, *in vitro* forskolin stimulation of striatal adenylate cyclase is also not affected by *in vivo* EEDQ treatment (Hess et al., 1985, 1987; Battaglia et al., 1986).

As has been found in previous studies (Maggi et al., 1979; Randall et al., 1981; Roth and Hess, 1982), there was a significant decrease in D₂ receptor density with senescence. The decrease in the B_{max} levels observed in senescent rats could be due to a loss of striatal cells containing D₂ dopamine receptors. However, if the reduction in the B_{max} of ³H-spiperone binding in senescent rats was due solely to a loss of cells containing D₂ dopamine receptors, then it would be expected that the time course of recovery of the remaining receptors would not be different from that observed in the mature rats. This is clearly not the case. The kinetics of the recovery of D₂ dopamine receptors following EEDQ treatment reveals significant differences between the processes regulating receptor steady-state concentrations in mature and senescent rats and, indeed, can explain the reduction in the number of D₂ dopamine receptors observed in senescent rats. Similarly, as we observed previously (Leff et al., 1984) when investigating D₂ dopamine receptor turnover in immature (1 month of age) and mature rats (9 months of age),

although D_2 receptor density *increased* developmentally, the receptor recovery rate was reduced by 34% (Table 2).

The raw data demonstrating a continual reduction in the initial D_2 dopamine receptor recovery rates with age over the complete life span of a rat are convincing (Table 2). However, it is important to remember that the derivation of the rate constants for receptor production and degradation are complex and depend on a number of assumptions. We will discuss the data derived from the senescent rats first, as EEDQ did not affect their final recovered $B_{\max}$. The logarithmic plot of the repopulation of 3H -spiperone binding sites shown in Figure 2 demonstrates that the overall process of receptor recovery can be adequately described by first-order kinetics since the recovery of D_2 dopamine receptors can be closely described by Eq. (3) as derived from Eq. (1). As demonstrated by Eq. (2), the ratio r/k determines the absolute *steady-state* level of receptors. Because there was no change in the recovered receptor steady-state level following treatment with EEDQ from that of the control senescent rats (i.e., the concentration of D_2 dopamine receptors returned to the original $B_{\max}$ level observed in rats not treated with EEDQ), there is no evidence for any change in r/k for D_2 dopamine receptors in senescent rats following recovery from EEDQ treatment. Although the overall r/k ratio is unaltered, it is possible that both r and k could be altered by equal proportions. This would maintain the original steady-state level of D_2 dopamine receptors; therefore, no effect on $B_{\max}$ would be observed, even though both r and k had been altered. However, as can be seen from Eq. (3), the *time to reach* any particular percentage of the steady-state level is dependent upon k and *not* r . Therefore, any alteration in k would alter the *time* to achieve any given percentage of the steady-state receptor level. As the time course of recovery is slower in senescent rats than in mature rats following EEDQ treatment, it can be inferred that k must be lower in the former than in the latter. Indeed, the derived values of receptor degradation rates show that k is approximately 40% lower in the senescent. That equivalent proportional changes in r and k do not alter the eventual steady-state level may have important consequences for studies of receptor adaptations to chronic drug treatments. It is evident that if any prolonged treatment produces no change in the $B_{\max}$ levels of a receptor population, it cannot necessarily be concluded that there was no effect on the kinetics of the processes determining steady-state receptor levels. Another obvious implication is that in studies of postmortem brain tissue from patients with various psychotic or neurological diseases that demonstrate control densities of receptors, the results in no way demonstrate that the kinetics of receptor turnover are not altered.

That chronic reserpine treatment does not alter the $B_{\max}$ (receptor steady-state level) in the senescent rats, as well as that there is no change in the rate of recovery of D_2 dopamine receptors in reserpinized senescent rats following an injection of EEDQ, demonstrates that no adaptations in either r or k occur in response to chronic reserpine treatment and the injection with EEDQ, as compared to senescent rats injected with EEDQ alone.

An interesting aspect of the recovery of D_2 dopamine receptors in mature rats following treatment with EEDQ is that the receptor concentration measured 16 and 32 d after EEDQ was significantly lower than the original steady-state concentration observed in untreated rats. It is therefore possible that a subpopulation of D_2 dopamine receptors in the mature rats is not subject to "turnover" and is lost following EEDQ treatment.

This observation could indicate that a population of 3H -spiperone binding sites, although present from at least as early as 4 months of age, is never subject to replacement. Alternatively, this population might be subject to very slow replacement that extends beyond the time course assessed in this study. It is also possible that EEDQ destroys a population of neurons containing these D_2 dopamine receptors. Whatever the explanation for this observation, it should be noted that this population of D_2 dopamine receptors does not appear to be present in senescent rats. This apparent subpopulation of 3H -spiperone binding sites may therefore be lost as a function of the natural aging process.

An intuitively more plausible explanation for the reductions in D_2 dopamine receptor steady-state levels observed in mature rats treated with EEDQ is that alterations in the kinetics of the processes determining receptor steady-state levels have been induced by the EEDQ treatment. The determination of the receptor production rate and the first-order rate constant for receptor degradation is critically dependent on the experimentally determined $B_{\max}$ values. However, as the D_2 dopamine receptors in mature rats did not return to the original $B_{\max}$ values, the derivation of the rate constants for their production and the degradation rate constants are complicated. As the mathematical model employed relates only to the *recoverable* receptors, we employed the $B_{\max}$ at days 16 and 32 as the steady-state level of receptors. In mature rats treated with EEDQ, r/k must have altered since the steady-state level (which is determined by this ratio) is different from that in the non-EEDQ-treated rats. A decrease in r or an increase in k , or a combination of both, must have occurred to account for the observed decrease in the receptor steady-state level from the original values. As the rate constants, by definition, were not measurable in non-EEDQ-treated rats, it is not possible to determine which of these possible adaptations occurred. Furthermore, if the reduction in the receptor steady-state level following EEDQ treatment was indeed the result of EEDQ-induced changes in r and/or k , rather than any of the other processes discussed above, it implies that this method of measuring receptor turnover has a major limitation in developmental studies since it is impossible to determine whether identical alterations occur at each age. Unfortunately, in our previous study (Leff et al., 1984) and other studies (Henry and Roth, 1984; Neve et al., 1985), sufficiently extended time courses of recovery were not used; thus, it cannot yet be determined whether original steady-state levels of D_2 dopamine receptors would have been achieved at these different ages. Bearing this important caveat in mind, it can be seen in Table 2 that while the receptor production rate constant (r) decreases by 78% from 1 month to 28 months of age, the receptor degradation rate constant (k) decreases by a lesser amount, 67%.

In contrast to the lack of effects of EEDQ and reserpine treatments on the steady-state levels and recovery of D_2 dopamine receptors in senescent rats, significant effects were observed in the mature rats. Chronic reserpine treatment of mature rats significantly increased the $B_{\max}$ of striatal 3H -spiperone binding. This change in D_2 dopamine receptor steady-state level implies that r/k is altered following chronic reserpine treatment, such that an increase in r , a decrease in k , or a combination of both must have occurred. As receptor degradation is assumed to be a first-order process, and the concentration of receptors is small at the *initial* time points, the rate of receptor degradation will also be very small. Therefore, the initial rate of recovery is a close approximation to r . It is thus important to note that in the reserpinized mature rats the initial rate of recovery following

EEDQ treatment is faster compared to the nonreserpinized rats. This implies that r must increase following chronic treatment with reserpine. A number of observations from the present study suggest that a significant reserpine-induced alteration in k is unlikely. If k was increased, the receptor steady-state level achieved should be reduced compared to the nonreserpinized rats. In fact, 16 d after EEDQ treatment, the $B_{\max}$ was significantly *higher* in the reserpinized rats than in the nonreserpinized rats, indicating that k is not likely to be greatly increased, at least in the long term. Furthermore, that the time course for receptor recovery in reserpinized and nonreserpinized rats closely paralleled each other between days 4 and 16 following EEDQ treatment indicates that the time to reach the new steady-state level was similar in these 2 groups. As the value of k determines the time to achieve any given percentage of the steady-state levels, irrespective of the absolute $B_{\max}$ level, it is evident that k cannot be markedly different. Similarly, Neve et al. (1985) concluded that k was unaltered following lesions of nigrostriatal dopaminergic neurons that elevated the $B_{\max}$ of striatal D₂ dopamine receptors.

In previous studies it has been shown that the reserpine-induced increase in $B_{\max}$ returns to control levels after withdrawal of drug treatment (Seeman, 1980). This implies that the ratios of r and k eventually return to control levels following withdrawal from reserpine treatment. However, this was not experimentally determined in this study. That the receptor $B_{\max}$ at day 16 was still significantly greater in the reserpinized rats than in the nonreserpinized rats indicates that r was still elevated at this time point (assuming k was similar for both groups of rats). It is interesting to speculate that if the time course of receptor recovery in the reserpinized rats was extended, then the eventual receptor steady-state level would be the same for both groups of rats. This would be the result of a decrease in receptor production rate with time, following withdrawal from the chronic reserpine treatment.

It is evident that there are a number of changes in the kinetics of the mechanisms determining D₂ receptor steady-state levels in the striatum following EEDQ treatment at various stages during development. In the striatum of immature rats (28 d of age), the receptor recovery rate following EEDQ treatment was faster than at 4 months of age. This transition from a fast to a markedly slower receptor "turnover" probably reflects a transition from the rapid receptor production rate during development necessary to increase the number of D₂ dopamine receptors from their low postnatal levels (Pardo et al., 1977) to the higher adult levels. Furthermore, a higher receptor production rate is necessary to overcome the rapid rate of degradation that occurs in these early postnatal periods, which probably reflects the high rate of neuronal involution and regression that occurs during this developmental period. Having achieved the adult steady-state concentration of receptors, the receptor production and degradation rate constants then alter to produce a receptor level that is maintained during the adult life of the animal. During this period the steady-state density of dopamine receptors in the striatum can be altered in response to changes in the level of neurotransmission. Finally, during senescence receptor production and the degradation rate constant are *both* lowered, a process that would tend to maintain the original steady-state level. However, r and k are reduced disproportionately, leading to a decline in steady-state receptor density.

It might be postulated that homologous receptor up-regulation in response to a decrease in the level of neurotransmission

results from a decrease in the rate of receptor internalization since one of the stimuli for this process, agonist-bound receptor (Harden et al., 1985), is reduced. Thus, receptor up-regulation could be viewed as a passive response to the decrease in agonist stimulation and a decrease in receptor internalization and subsequent degradation. However, our results indicate that this hypothesis is incorrect. Receptor up-regulation in response to chronic reserpine treatment is an "active" process dependent on an increase in receptor production rate rather than a decrease in the degradation rate. Intuitively, it might be expected that a neuron's response to a diminution in stimulation by a specific neurotransmitter would be reflected in homologous, in contrast to heterologous, receptor up-regulation initiated by an increase in transcription of the gene(s) responsible for the synthesis of a receptor for that particular neurotransmitter. On the other hand, k may represent the more general membrane processes involved in the global turnover of membrane constituents and not be amenable to such a specific regulatory response. Indeed, in similar studies of D₁ dopamine receptor turnover rates, we have found that despite the 2- to 3-fold higher density of D₁ dopamine receptors and their receptor production rate (r), the D₁ receptor degradation rate constant (k) is similar to that of D₂ dopamine receptors in the same brain region (G. Battaglia, A. B. Norman, and I. Creese, unpublished observations). Additionally, in senescent rats the mechanisms maintaining the steady-state levels of D₂ dopamine receptors are not able to adapt to a perturbation in the level of neurotransmission. Similarly, aging-related deficits in the ability of β -adrenergic receptors to exhibit up-regulation have also been described (Weiss et al., 1979). This could indicate that the mechanisms responsible for increasing the rate of gene(s) transcription are no longer functional. However, we have observed that receptor recovery, receptor production rate, and receptor degradation rate constants decrease in senescence for both D₁ (Norman et al., 1985) and D₂ dopamine receptors and S₂ serotonin receptors (Battaglia et al., 1985a, b), and this suggests that cellular processes in general may decrease in their absolute rates. Similarly, others have reported slower rates of recovery of α_1 -, α_2 - (Zhou et al., 1984) and β - (Pitha et al., 1982) adrenergic receptors after irreversible blockade in aged rats. Thus, the lack of receptor response to a decrease in neurotransmission may indicate that posttranscriptional deficit(s), rather than the lack of initiation of an increase in receptor gene transcription itself, is the reason for the lack of receptor up-regulation.

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