Dopamine receptor turnover rates in rat striatum are age-dependent

(Spirperone/N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline/Parkinson disease/development/neuroleptics)

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ABSTRACT  The time course of recovery of [3H]spiperone binding in the rat striatum after a single injection of the irreversible antagonist N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) shows that a slower rate of regeneration/turover of D-2 dopamine receptors occurs in mid-life/mature versus young male rats. This slower receptor recovery reflects relatively slower rates of both receptor synthesis and degradation. Studies using cycloheximide indicate that protein synthesis plays a significant role in the reappearance of [3H]spiperone-binding sites. Other experiments indicate that chronic reserpine treatment, which produces dopamine receptor upregulation, also produces accelerated receptor recovery after EEDQ blockade. An age-related decline in dopamine receptor turnover, if present in humans and progressive into senescence, could be responsible for the increased risk of developing Parkinson disease and drug-induced parkinsonian-like extrapyramidal side effects with age. On the other hand, the more rapid receptor turnover rates seen in young rats may be a biochemical feature related to plasticity in the striatum during development.

Age-related differences in therapeutic dosages and extrapyramidal side effects have been reported for the clinical use of antipsychotic drugs (1, 2). Since these agents are hypothesized to exert their therapeutic as well as extrapyramidal side effects by blocking dopamine receptors in the brain, one might expect the regulation of functional levels or other properties of these dopamine receptors to exhibit age-dependent differences. Indeed, dopamine receptor levels in rat striatum have been observed to increase over the first month postnatally (3), and age-correlated losses of dopaminergic receptor-binding activity and function have been noted in humans and rodents (for review, see refs 2 and 4). In aged rodents relative deficits in the ability of dopamine receptors to exhibit up regulation in response to chronic dopamine receptor antagonist (antipsychotic) drug treatments have been described (5), although other researchers have not observed such aging-related deficits in dopamine receptor up regulation when more robust (lesion-induced) decreases in agonist input were used (6-9).

Aging-related deficits in the ability of β-adrenergic receptor to exhibit up regulation have also been described (10-12). A recent report described aging-related decreases in the regeneration of visceral beta-adrenergic receptors after their irreversible blockade (13), thus suggesting one possible mechanism for the observed age-related deficits in receptor up regulation. A technique has recently been developed to determine D-2 dopamine receptor regeneration rates by determining the in vivo recovery of [3H]spiperone-binding activity after acute treatment with the irreversible dopamine receptor alkylating agent N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (14). The production by EEDQ of an accompanying long-term catalepsy (14-16) and inhibition of apomorphine-induced stereotyped motor behavior provides behavioral evidence for the blockade of D-2 receptors (17, 18). We report here that the rate of reappearance of striatal D-2 dopamine receptors, as measured by the recovery of [3H]spiperone-binding sites following EEDQ injection, is slower in mature compared with young rats. These findings are discussed in relation to models of receptor turnover, the emergence of extrapyramidal side effects with antipsychotic treatment, and previously reported age-related declines in other dynamic parameters of dopamine receptor function.

MATERIALS AND METHODS

Animal Treatments and Drug Preparation. EEDQ (Aldrich) freshly dissolved in ethanol/propylene glycol, 1:1 (vol/vol) or vehicle alone was injected i.p. in a volume of 2 ml/kg. Male Sprague-Dawley rats were divided into two groups by age (22-30 days or 9-12 months old; Charles River Breeding Laboratories), injected with EEDQ (8 mg/kg) or vehicle and decapitated at various times. For time-course experiments, rats were sacrificed at the same time of day 6, 28-30, 52-54, 100-102, 148-150, and 172-174 hr after injection with either EEDQ or vehicle. Their brains were rapidly removed and placed in ice-cold saline, and their striata were dissected and stored frozen at −70°C for up to 6 days. An EEDQ dose of 8 mg/kg was chosen because it produced near maximal receptor blockade but only a moderate mortality rate (10-15%), as noted by Hamblin and Creese (14). These deaths may be related to the potent and long-lasting hypotensive effects of EEDQ combined with the hypotensive actions of both components of the vehicle. In addition, a significant portion (=20%) of the EEDQ-injected rats did not become cataleptic and concomitantly exhibited much lower or no inhibition of [3H]spiperone binding. Thus, these animals were excluded from further analysis. The failure of EEDQ to block D-2 receptors in these rats may be related to the known instability of this compound in aqueous solution (refs. 15, 16, and 19; also see Discussion).

Cycloheximide Treatment. Cycloheximide (360 μg in 30 μl saline) or saline only was injected under ether anesthesia into the left lateral ventricle (1.5 mm left of the midline, 0.5-1.5 mm caudal of the bregma suture) with a 27-gauge needle fitted with a shrink tip stop 4.0 mm from the tip (20). Dye injections confirmed a rapid and complete ventricular spread of solutions injected in this manner. Beginning 12 hr after EEDQ injection (8 mg/kg), rats (35-40 days old) were injected intracerebrally ventricularly (i.e., v.) with cycloheximide or saline every 3 hr for 11 hr (four injections). [3H]Leucine

Abbreviations: EEDQ, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; ANOVA, analysis of variance; i.e.v., intracerebral ventricularly.

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Analyses show described in detail determined were trations samples serin, and er.

Final earlier and mixtures pension were Serum was washed min at 37°C concentration of 1.35 HCl before reaction quenched was injected 0.5 either ascorbic acid 2 ml and a final volume of 2 ml and a final tissue concentration of 1.35 mg/ml. Tubes were incubated 15 min at 37°C before filtration over GF/C glass fiber filters (Whatman) under vacuum using a Brandel automated cell harvester modified for receptor-binding assays. Filters were rapidly washed with 10 ml of ice-cold Tris buffer and trapped radioactivity was determined by standard scintillation spectroscopy.

Radioreceptor Assays. Blood samples from EEDQ- or vehicle-injected rats were collected by exsanguination and allowed to clot before centrifugation for 5 min at 400 g at 4°C. Serum samples were reserved on ice and serum from untreated rats was used as diluent for serial dilution of EEDQ. Membrane preparations of rat striata were washed once with 50 mM Tris/Cl (pH 7.7 at 25°C) and then suspended in 40 vol of Tris buffer containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, and 1 mM MgCl₂. To 1.0-ml volumes of this suspension were added 1.0 ml of serum samples containing standard or unknown concentrations of EEDQ. These reaction mixtures were allowed to incubate for 30 min at 37°C at which time the reaction was quenched with 10 ml of ice-cold Tris buffer. Membranes were subsequently centrifuged twice more with an intervening suspension in cold Tris buffer. Final homogenates were made in the assay buffer described earlier and placed in triplicate tubes containing (final assay concentrations) 0.5 nM [³H]spiperone, 50 nM ketanserin, and either ascorbic acid (total determinations) or (+)-butaclamol (1 μM; nonspecific determination). Unknown serum samples were compared with standards, and concentrations of “EEDQ activity” were defined in terms of μg equivalents per ml.

Data Analysis. Receptor densities and affinity estimates were determined by computer analysis using LIGAND, a weighted nonlinear least-squares curve-fitting program (23) as described in detail (24). For convenience, saturation data are graphically displayed in the form of Scatchard plots (25). Tabulated values are given as mean ± SEM values from multiple experiments unless otherwise indicated.

RESULTS

Receptor Regeneration Rates Differ with Age. Saturation analyses show that in vivo EEDQ treatment produces a marked decline in the number of D-2 receptor sites available for binding, with an 80–95% inhibition in the Bmax of D-2 receptor-specific [³H]spiperone binding, as measured 4–6 hr later, in both young (Fig. 1) and mature rats (data not shown). The recovery of [³H]spiperone binding activity after EEDQ blockade (Figs. 1 and 2) was faster for the young rats (t½ = 2.5 days) compared with older rats (t½ = 4.5 days). Comparison of the Bmax values of EEDQ- and vehicle-treated, young and mature, rats over recovery time using a three-way analysis of variance (ANOVA) indicates that this age-related difference in receptor recovery rate is significant (F = 3.30; df = 3; P < 0.025). In three independent replications of these experiments, young rats exhibited similar faster rates of receptor recovery compared with mid-life/mature rats.

[³H]Spiperone Kd values for young control rats (139 ± 11 × 10⁻¹¹ M) were significantly greater than observed for mature rats (46 ± 4 × 10⁻¹² M) [t(22) = 7.94, P < 0.001]. This difference was observed regardless of drug treatment over time (ANOVA; F = 99, P < 0.001). In addition, EEDQ treatment in both age groups collapsed over time produced a small but significant increase in Kd for [³H]spiperone (ANOVA; F = 13.4, P < 0.001). This effect appeared most marked at the earliest time point but amounted to a less than 2-fold increase. Control [³H]spiperone Bmax values for mature animals (29.8 ± 1.2 fmol/mg of tissue) were greater than those observed in young animals (25.4 ± 1.1 fmol/mg of tissue) [t(22) = 2.72, P < 0.02].

Repopulation kinetics after irreversible blockade of a receptor can be described by the equation

\[ R(t) = r/k(1 - e^{-kt}) \]

where \( R(t) \) = receptor concentration at time t, \( r \) = receptor production rate, and \( k \) = rate constant for receptor degradation (26, 27). As the time of repopulation proceeds to infinity, \( R(t) \) approaches the value \( r/k \) and therefore approaches \( R_{inf} \), the concentration of receptor at steady state. Logarithmic transformation (27) of Eq. 1 gives

\[ \ln(R_{inf})/\{R_{inf} - R(t)\} = kt \]

![Scatchard analysis of [³H]spiperone binding to striata of young rats at different times after EEDQ blockade of D-2 dopamine receptors. □, Control; △, 6 hr; ×, 54 hr; ○, 102 hr. Representative saturation analyses from single young animals show that in vivo EEDQ treatment produces a marked decline in the number of D-2 receptor sites available for binding that shows a time-dependent recovery. [³H]Spiperone affinities at each time point were similar to affinities in control animals except for the earliest time point, at which the affinity was decreased from \( K_d \) of 140 to 260 × 10⁻¹⁰ M. Similar saturation experiments conducted at each time point after EEDQ injection in rats show a similar loss and time-dependent recovery in [³H]spiperone-binding site density. Data from mature rats were qualitatively similar.](image-url)
Fig. 2. Time course of D-2 dopamine receptor recovery in striatum after EEDQ alkylation in young (○) and old (■) rats. Ordinate values represent mean ± SEM. Values shown are based on means for 6-9 treated animals and 12 control animals for each age group. Points from days 6 and 7 after injection were from separate experiments. Three-way ANOVA of raw $B_{\text{max}}$ data indicates a significant three-way interaction between age group and drug treatment over time ($F = 3.30, \text{df} = 31; P < 0.025$), while no significant two-way interaction between age group and recovery time alone was found. Analysis by Student’s $t$ test (one tail) comparing percent control receptor density for young and old rats at each time point gave results as follows: * $P < 0.025$; † $P < 0.0025$; ‡ $P < 0.001$. Control receptor densities ($R_0$) and apparent $K_d$ values were young, $R_0 = 25.4 \text{ fmol/mg of tissue}$, $K_d = 139 \pm 11 \times 10^{-12} \text{ M}$; old, $R_0 = 29.8 \text{ fmol/mg of tissue}$, $K_d = 46 \pm 4 \times 10^{-12} \text{ M}$.

Plots of our receptor recovery data according to Eq. 2 are shown in Fig. 3. The slopes of these plots, which are estimates of their respective receptor-degradation rate constants ($k$), are significantly different for the two age groups ($F = 3.6, \text{df} = 54, P < 0.001$; two-way ANOVA). Substituting these values for $k$ into Eq. 1 when $t$ approaches infinite time and $[R] = [R_0]$ allows the receptor production rate ($r$) to be calculated. For young and mature animals the respective production rates were 0.391 and 0.173 $\text{fmol/mg of tissue per hr}$ and the respective degradation rates were 0.0154 and 0.0058 $\text{hr}^{-1}$. Thus, mid-life/mature rats were 50-60% lower for both receptor production and degradation. Substitution of $k$ into Eq. 2 yields receptor half-lives of 45 and 119 hr for young and mature rats, respectively.

**D-2 Dopamine Recovery Is Dependent on Protein Synthesis.** To determine whether the recovery of D-2 receptor-binding sites is dependent on protein synthesis, the protein synthesis inhibitor cycloheximide was administered to a group of young rats. Independent estimates of relative protein synthesis based on the incorporation of $[^{3}H]$leucine into trichloroacetic acid-precipitable material indicated that, 1.5 hr after cycloheximide treatment, protein synthesis was inhibited by 87 ± 2.5%. Table 1 shows that cycloheximide treatment significantly impaired recovery of receptor concentration when compared with controls ($P < 0.004$, Mann–Whitney $U$ test).

**EEDQ Metabolism.** In a separate experiment, the presence of EEDQ in sera of treated mature rats was determined by a radioreceptor assay. Inhibition of $[^{3}H]$spiperone binding to brain membranes preincubated with serum from EEDQ-injected rats showed that moderate levels of EEDQ activity (2 $\mu$g equivalent per ml) were present in serum 12 min after i.p. injection of EEDQ at 8 mg/kg. This value declined rapidly such that 30 min after injection only 0.15 $\mu$g EEDQ equivalent per ml was present in serum 12 min after i.p. injection of EEDQ at 8 mg/kg.

**Fig. 3.** Semilogarithmic plot of time course of D-2 dopamine receptor recovery in old (■) and young (○) rats. $[R_0] = \text{steady-state receptor concentration (25.4 and 29.8 fmol/mg of tissue in young and old rats, respectively, } [R] = \text{receptor density at time } t, \text{ and } t_{1/2} = \text{half-life calculated from Eq. 1. Degradation constants } k \text{ and synthesis rates } r \text{ in young and old rats were } k(\text{young}) = 0.0154 \text{ hr}^{-1}, r(\text{young}) = 0.391 \text{ fmol/mg of tissue- hr}^{-1}, k(\text{old}) = 0.0058 \text{ hr}^{-1}, \text{ and } r(\text{old}) = 0.173 \text{ fmol/mg of tissue- hr}^{-1}. \text{ Values for } k \text{ (old) and } k \text{ (young) differed significantly } (P < 0.001, \text{two-way ANOVA}). t_{1/2} \text{ values were as follows: young, } 45 \text{ hr; old, } 119 \text{ hr.}\)**

**Table 1.** Effect of i.c.v. injection of cycloheximide on recovery of $[^{3}H]$spiperone binding in rat striatum after EEDQ blockade of D-2 receptors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$n$</th>
<th>$K_d$ (x 10^{-12})</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEDQ + saline, i.c.v.</td>
<td>6</td>
<td>5.96 ± 0.74</td>
</tr>
<tr>
<td>11 hr</td>
<td>39 ± 7</td>
<td></td>
</tr>
<tr>
<td>EEDQ + cycloheximide, i.c.v.</td>
<td>6</td>
<td>4.33 ± 0.72*</td>
</tr>
<tr>
<td>11 hr</td>
<td>48 ± 3</td>
<td></td>
</tr>
<tr>
<td>Saline + saline, i.c.v.</td>
<td>20 min</td>
<td>26.19 ± 1.62</td>
</tr>
<tr>
<td>45 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline + cycloheximide, i.c.v.</td>
<td>20 min</td>
<td>24.16 ± 2.34</td>
</tr>
</tbody>
</table>

Results represent mean ± SEM. Acute cycloheximide treatment had no significant effect on $[^{3}H]$spiperone binding in control rats, but long-term cycloheximide treatment impaired receptor recovery when started 12 hr after EEDQ treatment.

* $P < 0.004$ by Mann–Whitney $U$ test, one-tailed.
Table 2. Effects of chronic reserpine treatment on recovery of [3H]spiperone binding after EEDQ

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time of sacrifice after EEDQ or vehicle, hr</th>
<th>Receptor, fmol/mg of tissue</th>
<th>K_d (x10^-12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reserpine + EEDQ</td>
<td>6</td>
<td>5</td>
<td>1.31 ± 0.22*</td>
</tr>
<tr>
<td>Saline + EEDQ</td>
<td>6</td>
<td>5</td>
<td>2.47 ± 0.40</td>
</tr>
<tr>
<td>Reserpine + EEDQ</td>
<td>54</td>
<td>5</td>
<td>10.61 ± 0.89†</td>
</tr>
<tr>
<td>Saline + EEDQ</td>
<td>54</td>
<td>5</td>
<td>8.07 ± 1.04</td>
</tr>
<tr>
<td>Reserpine + vehicle</td>
<td>54</td>
<td>7</td>
<td>33.8 ± 1.2</td>
</tr>
<tr>
<td>Saline + vehicle</td>
<td>54</td>
<td>7</td>
<td>28.7 ± 1.5</td>
</tr>
</tbody>
</table>

Chronic reserpine treatment (19 days) produced D-2 dopamine receptor up regulation and accelerated D-2 receptor turnover as measured by recovery from EEDQ blockade. Daily reserpine (Serpasil) or saline injections (0.25 mg/kg, s.c.) were administered throughout the experiment. On day 17, EEDQ or vehicle was injected and rats were sacrificed at times indicated. Results represent mean ± SEM and were analyzed by paired t test (two-tailed).

*Not significant.
†P < 0.01.
‡P < 0.001.

concentrations were 31.5% higher in reserpine versus salinetreated rats 54 hr after EEDQ treatment (P < 0.01, two-tailed paired t test). This may reflect an acceleration in D-2 dopamine receptor turnover or synthesis rate in these rats.

DISCUSSION

We have shown that the rate of recovery of D-2 dopamine receptor-binding activity after irreversible blockade in the neostriatum is slower in mid-life/mature than in young rats. The receptor recovery curves for both age groups were analyzed according to a simplified receptor repopulation model previously described for nicotinic cholinergic (29), insulin (30, 31), and a2-adrenergic receptors (26, 27). This model assumes a linear receptor production rate (r) and an exponential receptor degradation rate constant (k). Although more complex models might also fit the data, the relatively linear relationships shown in Fig. 3 (correlation coefficient = 0.75 and 0.935 for young and old rats, respectively) suggest that Eq. 1 is a valid first approximation of D-2 receptor repopulation kinetics in striatum after EEDQ blockade.

We attempted to determine whether the recovery of D-2 receptor-binding sites is dependent on protein synthesis and thus, perhaps, de novo receptor synthesis. Table 1 shows that administration of cycloheximide during recovery from EEDQ-induced receptor loss significantly lowered densities of striatal [3H]spiperone-binding sites. These data are consistent with the hypothesis that protein synthesis of de novo receptor is involved in the regeneration of D-2 dopamine receptors after irreversible blockade. However, it is possible that ongoing protein synthesis supports some other function, such as insertion of already existing receptors into the membrane, rather than simply de novo synthesis of D-2 dopamine receptors. While it cannot be ascertained that these effects of cycloheximide are specific to its activity as an inhibitor of protein synthesis, controls indicated that cycloheximide exerted no direct inhibition of [3H]spiperone binding.

It was considered that differential rates of EEDQ metabolism, should they occur in young and old rats, might account for their respective differences in receptor recovery. Several lines of evidence argue against this possibility. First, the slopes of the receptor recovery curves over time (Fig. 2) differ for the two age groups of rats studied. A simple right shift of the curve for mature versus young rats might be expected if slower EEDQ metabolism was the primary factor in their slower recovery rates. Second, EEDQ is unstable in aqueous solution (15, 16, 19) and probably undergoes rapid catalysis due to hydrolysis and reaction with serum and tissue proteins. Radioreceptor assays suggest that serum EEDQ declines to insignificant levels by 30 min after injection. Since this rapid catalysis (measured in minutes) was observed in mature rats, which might be predicted to metabolize EEDQ more slowly, it is unlikely that different rates of EEDQ metabolism are the explanation for the slower D-2 dopamine receptor regeneration rate (measured in days) observed in older rats. Evidence that similar intracerebral EEDQ levels were achieved in both age groups of rats after injection derives from the observation that the initial inhibition of [3H]spiperone binding in both age groups was similar (85–90%; Fig. 2).

The half-lives of D-2 dopamine receptors estimated in the present study for either young or old male rats differ considerably from the t<sub>1/2</sub> of 8–9 hr recently reported by Hall et al. (32). The reason for this discrepancy is unclear, but it may be related to a number of differences in our studies. First, Hall et al. used a different alkylation agent (phenoxybenzamine), reported a lesser maximal inhibition (75%) of specific [3H]spiperone binding than we observed. It remains to be determined whether a relationship exists between completeness of blockade and t<sub>1/2</sub> of receptor recovery. However, in another study McKernan and Campbell (33) found a relatively short t<sub>1/2</sub> value (12.5 hr) for recovery from a 25% phenoxybenzamine blockade of rat cortical [3H]Clonidine binding while the t<sub>1/2</sub> was 5–6 days after a 95% maximal blockade of [3H]Prazosin binding. Second, Hall et al. measured [3H]spiperone binding in the absence of a 5HT<sub>2</sub> receptor antagonist. Thus some nondopaminergic [3H]spiperone binding may have affected their results. Third, their protocol utilized two injections of phenoxybenzamine given 12 hr apart, compared with our single injection of EEDQ. Last, their study was conducted on 150-g female rats. It should also be considered that the alkylation of receptors may influence receptor turnover.

The significance of the age-related differences we observed in K_d values for the antagonist [3H]spiperone is unclear. Indeed the control animals from our studies using cycloheximide or reserpine were only 30–55 days old and exhibited K_d values ranging from 40 to 100 x 10^-12 M (Tables 1 and 2). Thus absolute K_d values can be variable at any age when measured in different experiments. The slight but significant effects of EEDQ treatment on [3H]spiperone K_d values are equally unclear. It would be premature to suggest that residual nonalkylated or regenerated D-2 receptors represent pharmacologically or biochemically distinct receptor subtypes.

The functional significance of the more rapid receptor turnover rate we observed in young rats is unknown. One might speculate that the faster receptor synthesis and degradation rates we observed in young rats may reflect the presence of a greater degree of plasticity in the striatum at this age. Nevertheless, the more rapid turnover rate in young rats is consistent with previous ontogenetic findings indicating that D-2 dopamine receptor levels are terminating their rapid increase from neonatal to adult levels (3). Our observation that control young rats exhibited lower B<sub>max</sub> values than mature rats suggests that their receptor levels were below the maximum levels observed in young adults because B<sub>max</sub> values are reported to be lower in mature rats when compared with young adults (reviewed in refs. 2 and 4). However,
er, $B_{\text{max}}$ values in control young rats did not increase in the course of our turnover experiments.

The slower recovery rate we have observed in older rats may be consistent with a reported aging-related deficit in dopamine receptor up regulation after chronic antagonist treatment in mice (5). Our results with chronic reserpine treatment indicate that increases in receptor recovery accompany receptor up regulation. It should be noted that the aging-related deficit observed by Randall et al. (5) was seen only in senescence while, in the present study, differences in receptor turnover between immature and mid-life/mature rats were observed. It will be of interest to determine whether even greater reductions in receptor turnover occur in senescent rats.

Our findings are consistent with clinical observations that older patients show an increasing tendency to develop and exhibit the symptoms of Parkinson disease (1, 2). Concretely, older patients generally require lower doses of antipsychotic drugs for therapeutic effect and present a higher risk for the development of drug-induced parkinsonian-like extrapyramidal side effects (1, 2), although this may be related to a general tendency toward overdosage in these patients. Taken together, this evidence suggests that age reduces the capacity for functional adaptation of dopaminergic mechanisms in the central nervous system. We further suggest that age-dependent changes in receptor turnover should be taken into consideration in the interpretation of studies of human neurotransmitter receptors in aging, diseased, or drug-treated brains. Determination of absolute levels of receptor number alone may not provide adequate appreciation of the dynamics of receptor regulation and their relationship to changes in behavior.

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