

Acute Stimulatory Effect of Estradiol on Striatal Dopamine Synthesis

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Abstract: The acute effect of physiological doses of estradiol (E2) on the dopaminergic activity in the striatum was studied. In a first series of experiments, ovariectomized rats were injected with 17α or 17β E2 (125, 250, or 500 ng/kg of body weight, s.c.), and in situ tyrosine hydroxylase (TH) activity (determined by DOPA accumulation in the striatum after intraperitoneal administration of NSD 1015) was quantified. A dose-dependent increase in striatal TH activity was observed within minutes after 17β (but not 17α) E2 treatment. To examine whether E2 acts directly on the striatum, in a second series of experiments, anesthetized rats were implanted in the striatum with a push-pull cannula supplied with an artificial CSF containing [^3H]tyrosine. The extracellular concentrations of total and tritiated dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured at 20-min intervals. Addition of 10^{-9} M 17β (but not 17α) E2 to the superfusing fluid immediately evoked an ~50% increase in [^3H]DA and [^3H]DOPAC extracellular concentrations, but total DA and DOPAC concentrations remained constant. This selective increase in the newly synthesized DA and DOPAC release suggested that E2 affects DA synthesis rather than DA release. Finally, to determine whether this rapid E2-induced stimulation of DA synthesis was a consequence of an increase in TH level of phosphorylation, the enzyme constant of inhibition by DA ($K_{i\text{DA}}$) was calculated. Incubation of striatal slices in the presence of 10^{-9} M 17β (but not 17α) E2 indeed evoked an approximate twofold increase in the $K_{i\text{DA}}$ of one form of the enzyme. It is concluded that physiological levels of E2 can act directly on striatal tissue to stimulate DA synthesis. This stimulation appears to be mediated, at least in part, by a decrease in TH susceptibility to end-product inhibition, presumably due to phosphorylation of the enzyme. The rapid onset of this effect, and the fact that the striatum does not contain detectable nuclear E2 receptors, suggest a nongenomic action of the steroid. **Key Words:** Dopamine synthesis—Tyrosine hydroxylase—Push-pull perfusion—Dopamine feedback inhibition—Estradiol—Striatum.
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Since it was first reported (Bédard et al., 1977) that estrogens, administered orally, could improve the condition of patients suffering from tardive or DOPA-

induced dyskinesia, the biochemical and behavioral effects of these hormones on the female nigrostriatal dopaminergic system have been studied extensively. As a matter of fact, all the data from clinical and animal research clearly indicated that estrogens affect the neurochemistry of dopamine (DA) in the striatum as well as behaviors mediated by striatal DA (see Van Hartesveldt and Joyce, 1986, for review). So far, on the other hand, no consensus has been reached regarding either the locus, the direction, or the mechanism of estrogen actions. Actually, depending on the dose of estrogen administered, the duration of treatment, the time interval between estrogen treatment and testing, the behavior measured, and the part of the basal ganglia from which the behavior is elicited, estrogens appear either to enhance (Robinson et al., 1981; Di Paolo et al., 1985; Becker et al., 1987; Becker, 1990) or to suppress (Euvrard et al., 1980; Fields and Gordon, 1982) striatal dopaminergic transmission. This may be because (1) some of the effects observed after treatment with high doses of estrogen are not due to the steroid per se but are rather mediated either by prolactin (Euvrard et al., 1980; Hruska, 1986) whose secretion is increased by estrogens, or by catecholestrogens (Fishman, 1976); or (2) estrogens may have different, or sequential, effects on pre- versus postsynaptic elements of the nigrostriatal system (Gordon and Perry, 1983; Becker and Beer, 1986).

Thus, in an attempt to determine the nature of direct estrogen effects on the presynaptic component of the

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Abbreviations used: ANOVA, analysis of variance; BSA, bovine serum albumin; DA, dopamine; DOPA, L-3,4-dihydroxyphenylalanine; DOPAC, 3,4-dihydroxyphenylacetic acid; E2, estradiol; EBSS, Earle's balanced salt solution; $K_{i\text{DA}}$, enzyme constant of inhibition by DA; 6-MPH₄, DL-6-methyl-5,6,7,8,-tetrahydropterin; NSD 1015, 3-hydroxybenzylhydrazine dihydrochloride; PRL, prolactin; tDA and tDOPAC, total DA and total DOPAC; TH, tyrosine hydroxylase.

nigrostriatal dopamine system, the present study was performed using three different technical approaches. In particular, experiments were designed (1) to examine the effect of an acute physiological dose of estradiol (E2) on the *in vivo* rate of DOPA accumulation in the striatum after intraperitoneal administration of NSD 1015, (2) to determine *in vivo* whether E2 might act directly on the striatum to modulate DA synthesis and/or release, when delivered locally by means of a push-pull cannula, and (3) because short-term regulation of tyrosine hydroxylase (TH), the rate-limiting enzyme in the DA biosynthetic pathway, is effected mainly by phosphorylation (Zigmond et al., 1989), to assess indirectly the state of TH phosphorylation in control or E2-treated striatal slices, by determining the enzyme constant of inhibition by DA (K_{iDA}). This parameter of TH activity characterizes its sensitivity to end-product feedback inhibition and appears to be a sensitive index of the state of TH phosphorylation (Ames et al., 1978; Lazar et al., 1982; Albert et al., 1984; Fujisawa and Okuno, 1986).

MATERIALS AND METHODS

Animals

Female Wistar rats (200–220 g, Iffa Credo, Lyon, France) were housed under controlled temperature (22°C) and lighting (lights on from 0500 to 1900 h) and supplied with water and food *ad libitum*. All rats were bilaterally ovariectomized and used 11–13 days after surgery.

In vivo rate of DA synthesis

The *in situ* activity of TH was assayed between 1130 and 1330 h by determining the rate of L-3,4-dihydroxyphenylalanine (DOPA) accumulation in the striatum, 30 min after the intraperitoneal administration of 3-hydroxybenzylhydrazine dihydrochloride (NSD 1015), an inhibitor of brain DOPA decarboxylase activity (Carlsson et al., 1972), in a total of 70 rats treated with either vehicle or E2. Thus, 15 min before NSD 1015 injection, each rat received subcutaneously either the vehicle (0.1% ethanol in saline) or 17 α or 17 β E2 (125, 250, or 500 ng/kg of body weight in 0.2 ml). These doses were chosen, because earlier studies have revealed that a subcutaneous injection of the equivalent of 280 ng/kg of body weight 17 β E2 led to a transitory elevation in the plasma concentration of the steroid in the range of proestrus plasma levels (~60 pg/ml) between 15 and 30 min later, whereas plasma prolactin (PRL) levels remained unchanged (Morissette et al., 1990b). Thirty minutes later, the animal was decapitated and the dorsal striatum was immediately dissected out, homogenized in 150 μ l of 0.1 M perchloric acid containing 0.5 mM EDTA and finally centrifuged at 15,000 g for 15 min. The pellet was solubilized in 0.1 M sodium hydroxide and analyzed for protein content by the bicinchoninic acid protein microassay (Smith et al., 1985) using bovine serum albumin (BSA) as the standard. The content of L-DOPA in the supernatant was determined by HPLC with electrochemical detection as described previously (Pasqualini et al., 1991).

Differences between group means were evaluated by one-way analysis of variance (ANOVA) followed by *t* tests.

Local superfusion procedure

Striatum superfusion was performed as previously described (Leviel et al., 1989). In brief, an artificial CSF was continuously supplied to a push-pull cannula implanted in the anterior part of the caudate nucleus of halothane-anesthetized rats. After 1 h of superfusion (resting period) [3 H]tyrosine (80 μ Ci/ml) was added in the CSF (time = 0). Both the tritiated ([3 H]DA, [3 H]DOPAC) and total (tDA, tDOPAC) DA and 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations were then measured in serial 20-min superfusate fractions using HPLC analysis, electrochemical detection, and radioisotopic counting (Leviel et al., 1989). E2 was added to CSF 100 min after [3 H]tyrosine. For easier comparison, the results are presented after standardization to 100% of the spontaneous release. Statistical analysis was conducted on the percentages using a two-tailed Student's *t* test by comparing the mean of corresponding fractions of control (*n* = 10) and treated (*n* = 6) groups.

Inhibition studies with DA

For kinetic studies, TH activity was determined by measuring the amount of exogenous tyrosine converted to DOPA *in vitro* by striatal homogenates.

In this series of experiments, the dorsal striata were first dissected out and tissue slices of ~0.5 \times 0.5 \times 0.5 mm were prepared with a McIlwain tissue chopper. The tissue fragments were then equilibrated under an atmosphere of 95% O₂/5% CO₂ for 20 min at 37°C in 5 ml of Earle's balanced salt solution (EBSS). After removal of this preincubation medium, they were incubated in 5 ml of EBSS containing or not containing E2. Tissue slices were then homogenized in an ice-cold 20 mM potassium phosphate/Triton X-100 0.2% buffer (pH 6), containing sodium fluoride (5 mM), to inhibit phosphoprotein phosphatases (Yamauchi and Fujisawa, 1979; Bollen and Stalmans, 1988), and centrifuged at 15,000 g for 15 min. The pellets were solubilized in 0.1 M sodium hydroxide and analyzed for protein content as indicated above. TH activity in the supernatants was assayed immediately as described previously (Pasqualini et al., 1994). In brief, incubation was performed at 37°C for 15 min, with 0.1 or 0.5 mM DL-6-methyl-5,6,7,8-tetrahydropterin dihydrochloride (6-MPH₄; Sigma) and 20 μ M tyrosine, in the absence or presence of increasing concentrations of DA (1–50 μ M). The TH inhibitor constants, K_i values, were determined by the method of Dixon (1953), which was validated by nonlinear regression curve-fitting program (Kaleidagraph, version 2.0.2, 1992). Both methods provided similar results and demonstrated the biphasic pattern of the experimental curves.

RESULTS

In vivo rate of DA synthesis in the striatum after *in vivo* E2 treatment

In situ TH activity was evaluated by measuring DOPA accumulation in the striata 30 min after NSD 1015 injection in rats treated with either vehicle or E2. As shown in Fig. 1, *in vivo* administration of 17 β E2 (125, 250, or 500 ng/kg of body weight) 15 min before NSD treatment dose-dependently increased the drug-induced DOPA accumulation in the striatum by 20, 42.5, and 48.5%, respectively, compared with the con-

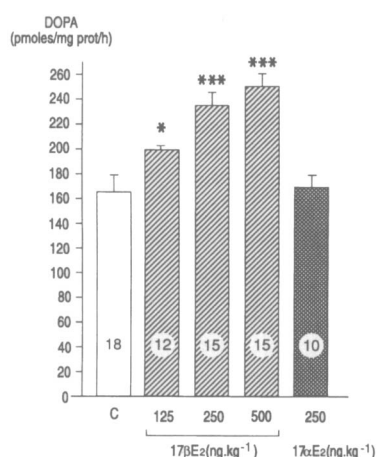


FIG. 1. Effect of physiological doses of 17β E2 on the in situ DOPA accumulation in the striatum. Ovariectomized rats were injected with vehicle (C) or vehicle containing 17α or 17β E2 and, 15 min later, with NSD 1015 to allow DOPA to accumulate for 30 min. Bars represent mean \pm SEM values; the number of rats used is at base of the bars (* $p < 0.05$, *** $p < 0.001$ vs. the control; ANOVA and Student's t test).

trols. Thus, although an in vivo treatment with 250 ng/kg 17α E2 had no effect (Fig. 1), the same dose of 17β E2 produced an already maximal increase in the in vivo rate of DOPA synthesis in the terminals of nigrostriatal neurons.

Local in vivo effects of E2 on DA and DOPAC release

In control conditions, the addition of [3 H]tyrosine to the superfusion medium led to the rapid appearance of [3 H]DA and [3 H]DOPAC in the extracellular space (Fig. 2). After a sharp increase during the first hour, the release was progressively stabilized during the following 160 min. E2 treatment was applied 100 min after the beginning of superfusion with [3 H]tyrosine.

Addition of (10^{-9} M) 17β (but not 17α ; data not shown) E2 to the superfusing fluid produced immediately an increase in [3 H]DA and [3 H]DOPAC extracellular concentrations, but total DA and DOPAC concentration remained constant. Thus, when applied directly into the striatum, 17β E2 produced an increased release of the newly synthesized amine leaving the tDA and tDOPAC release unchanged.

E2 effects on striatal TH susceptibility to inhibition by DA

The $K_{i\text{DA}}$ value of TH in control and E2-treated striatal slices was determined at pH 6.2 in the presence of 20 μ M tyrosine and either 100 or 500 μ M 6-MPH $_4$. Dixon plots of the data are presented in Fig. 3. Inhibition of TH activity by DA was found to be competitive with pterin cofactor, as judged by the convergence of

lines generated at the two 6-MPH $_4$ concentrations. The reported $K_{i\text{DA}}$ values were determined from the intersection of lines on these plots. In control striatal slices, the values obtained at 0.1 and 0.5 mM 6-MPH $_4$ displayed a biphasic character (which was also disclosed by the curve-fitting analysis performed using nonlinear regression procedures) and thus could be best resolved into two straight lines, as follows: (1) On the basis of the data obtained with the higher DA concentrations (extrapolated broken lines), a K_i value for DA was calculated to be 30 μ M. (2) On the basis of the data obtained with the lower concentrations of DA, a second K_i was calculated to be 2.5 μ M. Thus, as previously described in the median eminence of ovariectomized rats (Pasqualini et al., 1993, 1994), in the control striatal slices, TH existed as two kinetically different forms, with $K_{i\text{DA}}$ values of 30 ± 0.6 and 3.3 ± 0.5 μ M (both mean \pm SEM of six independent determinations). In striatal slices incubated in the presence of 17β E2 (10^{-9} M) for 30 min, two kinetically different forms of TH also coexisted; whereas the TH form exhibiting a $K_{i\text{DA}}$ value of 3 ± 0.4 μ M was still observed, the other one underwent an increase of approximately two

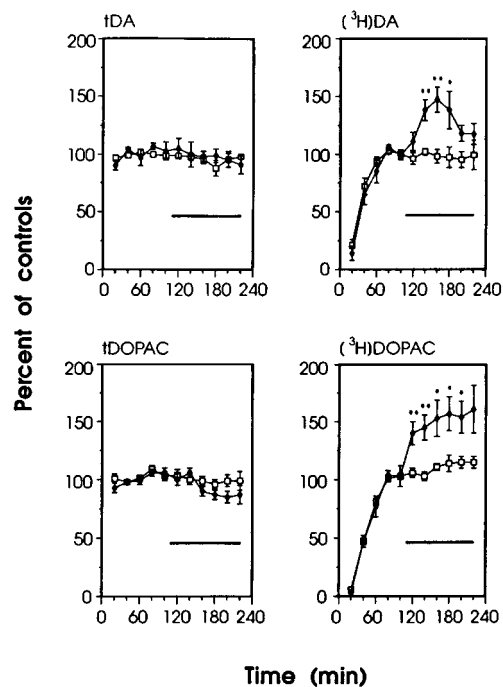


FIG. 2. Local effects of 10^{-9} M 17β E2 on the release of both the tritiated and unlabeled forms of DA and DOPAC in the rat caudate nucleus during a continuous labeling with tritiated tyrosine. Experiments and the treatment of results were performed as described in Materials and Methods. E2 was added to the superfusing CSF 100 min after [3 H]tyrosine (black bar). Results are expressed as percentages of the spontaneous release (100%). Each point is the mean \pm SEM obtained with 10 control (\square) and six treated (\blacklozenge) rats. Statistical analysis used the two-tailed Student's t test to compare the mean \pm SEM values of corresponding fractions (* $p < 0.05$, ** $p < 0.01$).

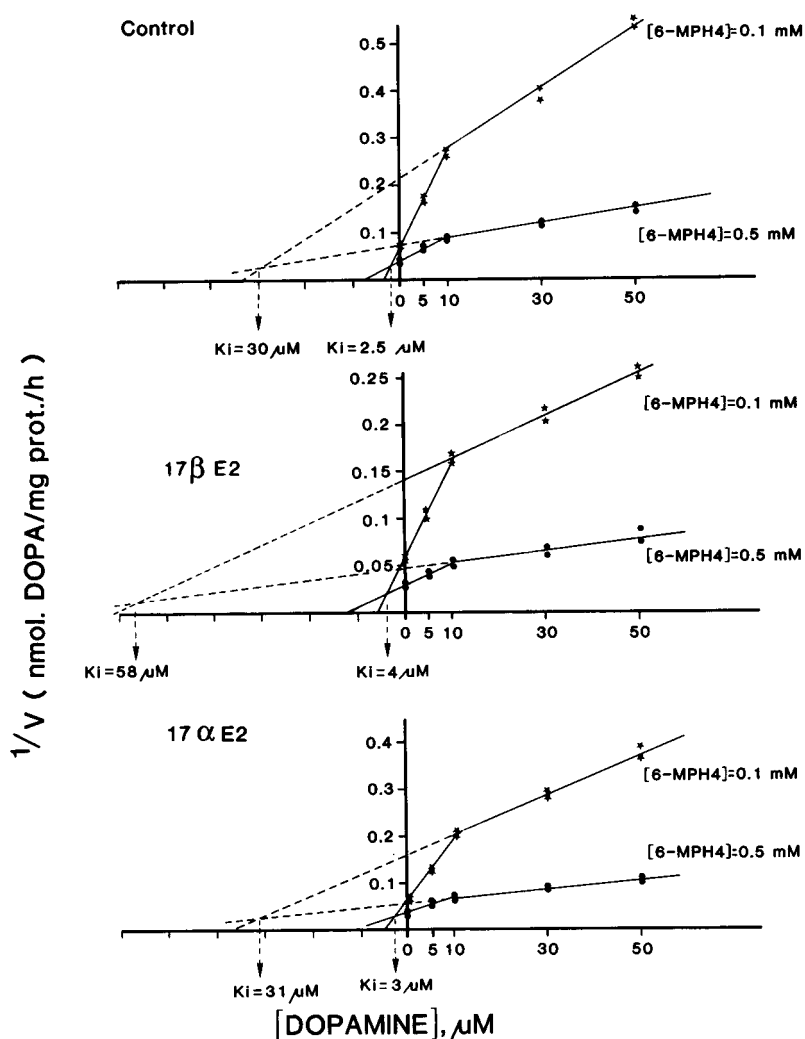


FIG. 3. Effect of E2 on TH susceptibility to DA inhibition. Dixon plots of TH activity as a function of DA and 6-MPH₄ concentrations, in striatal slices incubated for 30 min in the absence (top) and presence of 10^{-9} M 17β E2 (middle) or 17α E2 (bottom). Data are values for duplicate samples from a typical experiment replicated four times with similar results.

times its K_{iDA} value (from 30 ± 0.6 to 58.2 ± 0.6 μ M; both means of four independent determinations). On the contrary, when striatal slices were incubated in the presence of 17α E2 (10^{-9} M) for 30 min, no change in the K_{iDA} values of TH could be observed.

DISCUSSION

The present data provide the first *in vivo* evidence that physiological concentrations of E2, acting directly on striatal tissue, can within minutes stimulate DA synthesis, while leaving the amine-releasing process unchanged.

When injected systemically, 17β E2 induced an increase in TH activity measured in dissected striatal tissue, and when supplied locally, the steroid provoked a selective increase in the newly synthesized DA and DOPAC extracellular levels. These results suggest strongly that E2 can act directly on the striatum to enhance TH activity. Because this E2-induced increase

in DA biosynthesis occurred rapidly (15–20 min), it was unlikely to be due to a change in the number of TH molecules. Instead, an activation by phosphorylation of the preexisting enzyme molecules could be expected. Accordingly, an E2-induced increase in the K_{iDA} of TH was shown for the first time in the present study. Thus, after E2 stimulation, TH is relieved from end-product feedback inhibition, an effect that is characteristically brought about by phosphorylation (Ames et al., 1978; Mann and Gordon, 1979; Vrana et al., 1981; Albert et al., 1984; Fujisawa and Okuno, 1986). Indeed, not all TH molecules were affected in their catalytic properties by E2 treatment. However, it is known that TH exists as a soluble and a membrane-bound form, exhibiting a significantly different affinity for pterin cofactor and sensitivity to DA inhibition (Kuczenski and Mandell, 1972). Recently, we provided some evidence supporting that the low (~ 3 μ M) K_{iDA} form could correspond to nonphosphorylated membrane-bound TH, because its activity and sensitivity to

DA inhibition can be affected by protein kinases only after its solubilization (Pasqualini et al., 1994). Conversely, the high ($\sim 30 \mu\text{M}$) $K_{i, \text{DA}}$ TH form, which is phosphorylated and whose activity and sensitivity to DA inhibition can be readily modified by protein kinases, is likely to be the soluble and the more active form of this enzyme.

It is surprising that the release of DA was not altered by E2 treatment, as evidenced by the *in vivo* experiments. Such a dissociation between the modulation of DA synthesis and release had already been observed in certain other experimental situations—for instance, the local application of either the GnRH-associated peptide (Gobert et al., 1992) or glutamate at low concentrations (Leviel et al., 1990) also provided evidence that in striatal dopaminergic terminals these two metabolic steps are not strictly coupled. This was recently confirmed (Desce et al., 1994).

It thus appears that the continuous local perfusion with E2 does not affect basal dopaminergic transmission, but increasing the amount of cytosolic DA available for release enhances the capacity of these dopaminergic neurons to respond to any subsequent stimulus. This would also explain previous results from Becker's group who showed *in vitro* (Becker, 1990) and *in vivo* (Castner et al., 1993) a stimulatory action of physiological concentrations of E2 on striatal amphetamine-evoked DA release. Amphetamine is known to release preferentially newly synthesized cytosolic DA (Kuczenski, 1983). Thus, that a neuron previously exposed to E2 has an increased amount of readily releasable cytosolic DA likely explains its enhanced response to amphetamine stimulation.

The observed stimulation of striatal DA synthesis is likely to be an effect of E2 *per se*: during chronic treatment with high doses of estrogen, some of the observed E2 effects are mediated by PRL or locally synthesized catecholesterogen (Fishman, 1976; Euvrard et al., 1980; Hruska, 1986). This could be excluded in the present study, because (1) treatment of rats with such low doses of 17β E2 has been shown to leave plasma PRL unchanged for at least 1 h (Di Paolo et al., 1985); and (2) our study showed that the effects of E2 were stereospecific, because the 17α isomer of E2 was totally inactive. This result is important in at least two respects: First, it shows that a nonspecific action due to an alteration of membrane lipid composition is not involved. Second, it rules out the possibility that catecholesterogen formation would provide the biochemical link between estrogen and catecholaminergic function, because 17α E2 can form 2- and 4-hydroxy 17α as rapidly as the 17β epimer, and 2-hydroxy 17α E2 is known to modulate purified rat TH activity with a potency comparable to that of 2-hydroxy 17β (Hershey et al., 1982).

Discrimination between direct action of the steroid and its potential secondary effects, mediated for instance by PRL, may help to understand some seem-

ingly contradictory results of the literature. The idea that very low doses of E2, enhancing the response capacity of nigrostriatal DA neurons, are appropriate to improve DA transmission in this system fits well with the results of behavioral studies reporting, for instance, (1) an increased dopaminergic transmission in the striatum and nucleus accumbens after a similar E2 treatment (Di Paolo et al., 1985), or (2) the rapid improvement in sensorimotor performance produced by intrastriatal application of 17β E2 (Becker et al., 1987). Conversely, certain antidopaminergic effects observed after prolonged treatment with higher doses of E2 were not due to the steroid *per se*, but rather to the secondary stimulation of PRL release from the pituitary (Euvrard et al., 1980).

Where and how does E2 act in the striatum to modify the rate of DA synthesis? The E2-induced increase in TH phosphorylation may be related to the ability of the steroid to rapidly modulate DA action on the presynaptic terminals: within 15 min of injection, E2 can increase the number of striatal DA uptake sites (Morissette et al., 1990a) and decrease the density of the striatal D2 receptor high-affinity agonist sites (Lévesque and Di Paolo, 1988). Therefore, by stimulating DA degradation and reducing the sensitivity of D2 receptors, comprising also presynaptic autoreceptors, the steroid can reduce the inhibitory influence that these autoreceptors exert on TH phosphorylation and DA synthesis (Salah et al., 1989).

In any case, classic estrogen receptors are not found within this brain area. The observed rapid stimulation of TH by E2 would be best explained by the presence of a membrane receptor. That E2 is active at concentrations in the range of 10^{-10} to 10^{-9} M suggests that E2 binding to this receptor is of great affinity. Membrane receptors for steroid hormones have long been hypothesized, based on numerous neurochemical and electrophysiological evidences (see Schumacher, 1990, for review). However, biochemical evidence for such receptors is only now becoming more conclusive. Recently, the group of Ramirez, using ^{125}I -labeled BSA-linked steroids, isolated, purified, and characterized some of them. As an example, they purified from the hypothalamus and corpus striatum a membrane binding site with high affinity for progesterone, which is sexually dimorphic and corresponds to a 40–50-kDa protein (Tischkau and Ramirez, 1993). Besides, it has also been shown recently that in the striatum, independently of intracellular receptors, E2 can affect the (N type) calcium channels and decrease calcium currents within seconds of administration, via a G protein-mediated mechanism (Mermelstein et al., 1994). The observed stimulation of TH activity may thus be triggered by an E2-induced increase in the intracellular concentration of calcium, leading to activation of calcium-dependent protein kinases that ultimately will phosphorylate TH (Haycock, 1993). In support of this hypothesis, we observed (C. Pasqualini et al, unpublished

data) that the effect of E2 on the increase in the $K_{i, DA}$ of TH was selectively blocked by GF-109203X, a specific inhibitor of calcium/phospholipid-dependent protein kinase (Toullec et al., 1991). All together, these data are thus consistent with the view that E2 could act, through a membrane-linked mechanism, directly on DA terminals. On the other hand, because this evidence is still indirect, we cannot exclude that the pathway linking E2 action on calcium currents and phosphorylation of TH could also involve an interneuron within the heterogeneous striatal tissue.

In summary, the results of these experiments indicate that E2 has an acute stimulatory action on DA biosynthesis in striatal catecholaminergic terminals, which is based mainly on activation of TH by enhanced phosphorylation of the enzyme. Precise localization of the membrane receptor for E2 is now required to determine whether E2 acts primarily on the DA neurons and to finally elucidate the pathway linking E2 binding to the striatum and TH phosphorylation.

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