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Estrogen mediated inhibition of dopamine transport in the striatum: Regulation by $G\alpha_{i/o}$

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Abstract

In the current study, the interaction between estrogen priming and dopamine D2 receptor activation on dopamine uptake in the striatum of ovariectomized female rats was investigated. Basal ADP-[$^{32}P_i$]ribosylation of $G_{i/o}$ was examined in synaptosomal membranes prepared from ovariectomized, estrogen primed or *N-p-*(isothiocyanatophenethyl) spiperone (NIPS) treated rats. [$^{32}P_i$]-incorporation was significantly increased (141%) in tissue from NIPS treated animals but attenuated (57%) in tissue from estrogen primed animals. Dopamine uptake kinetics were measured in vivo following manipulation of the heterotrimeric G-protein by pertussis toxin (0.5 μg, 48 h). Pertussis toxin significantly inhibited dopamine uptake at all concentrations of dopamine examined. Co-treatment with estrogen and pertussis toxin resulted in a further attenuation of dopamine transport at high but not low dopamine concentrations. These data are consistent with an estrogen mediated alteration of G-protein activity and support the hypothesis that estrogen may alter transporter activity through a modulation of dopamine D2 autoreceptor/$G\alpha_{i/o}$ protein coupling.

Keywords: Dopamine transport; Steroid regulation; Dopamine autoreceptor; G-protein

1. Introduction

Estrogen alters nigrostriatal and mesolimbic dopaminergic activity. Specifically, estrogen attenuates the reuptake of both endogenous and exogenous dopamine in the striatum and nucleus accumbens as measured by voltammetry and synaptosomal uptake assays (Thompson and Moss, 1994; Thompson, 1999; Thompson et al., 2000), modifies 1-methyl-4-phenylpyridinium (MPP+, Callier et al., 2000), methamphetamine and 6-hydroxydopamine (6-OHDA) mediated neurotoxity, attenuates methamphetamine evoked dopamine release (Disshon and Dluzen, 1999; Myers et al., 2003; Dluzen, 1997) and alters dopamine transporter (DAT) binding properties in the striatum. The mechanism of steroid mediated attenuation of dopamine uptake is unclear. However, evidence suggests a steroid mediated alteration of dopamine D2 autoreceptor activation may explain the attenuation of dopamine uptake. Alterations in dopamine D2 receptor/G-protein coupling have been reported following estrogen treatment (Levesque and Di Paolo, 1999) and changes in striatal dopamine D2 receptor affinity have been reported over the estrous cycle (DiPaolo et al., 1988). Estrogen priming results in a decrease in agonist mediated [^{35}S]-guanosine 5′-(γ-thiotriphosphate) binding to striatal membranes consistent with a decrease in G-protein activation (Thompson et al., 2001). Chemical down regulation of the dopamine D2 receptor following treatment with *N*-(*p*-isothiocyanatophenethyl)spiperone (NIPS) is associated with a significant impairment of dopamine transport kinetics characterized by a decrease in rate and an increase in total clearance time (Thompson et al., 2001). Co-administration of estrogen and NIPS results in a further attenuation of uptake. Similar alterations in clearance have been reported in D2 (−/−) knock-out mice (Dickinson et al., 1999). These findings are consistent with estrogen mediated effects on dopamine clearance.

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The focus of the current study was two-fold: 1) to directly investigate the ability of estrogen to alter $G\alpha_{i/o}$ activation as measured by [$^{32}P_i$]ADP-ribosylation and 2) to indirectly investigate the role of $G\alpha_{i/o}$ in DAT activation following pertussis toxin treatment in vivo in ovariectomized and estrogen primed animals. The results support the hypothesis that estrogen can modulate dopamine availability indirectly through an alteration of dopamine D2 receptor responsiveness. Furthermore, they suggest a mechanism of steroid regulation which may have far reaching implications for neuronal excitation in general.

2. Materials and methods

2.1. Materials

Estradiol benzoate and NIPS (*N*-(*p*-isothiocyanatophenethyl) spiperone) were purchased from Sigma (St. Louis, MO). Pertussis toxin was purchased from List Pharmaceuticals (Campbell, CA).

2.2. Subjects

Adult (200–300 g) female Sprague–Dawley rats were used. All animals underwent surgical bilateral ovariectomies under metofane anesthesia. The animals were allowed to recover for at least 10 days. A portion of the animals were treated with estrogen (estradiol benzoate, 10 μg in oil, 48 and 24 h prior) or NIPS (10 mg/kg in ethanol) 24 h prior to experiment. Some animals received pertussis toxin (holotoxin, 0.5 μg/2.5 μl total in phosphate buffered saline (PBS), pH 7.4) infusion into one striatum and an equal volume of PBS into the opposite striatum 48 h prior to experiment. A subgroup of these animals was also administered estrogen (10 μg in oil, 48 and 24 h prior). Animals were housed under a 12 h light/dark cycle (lights on at 7:00 am) with free access to food and water. All animal procedures were preapproved by the Institutional Animal Care Advisory Committee at Mercer and in strict accordance with the National Institute of Health guidelines.

2.3. Synaptosomal membrane preparation and [$^{32}P_i$]ADP-ribosylation

Animals pretreated with estrogen, NIPS or vehicle were decapitated and striatal tissue rapidly dissected. Striata were homogenized in 40 volumes of 0.32 M sucrose, 5 mM HEPES, pH 7.4, and centrifuged at 1000×*g* for 5 min at 4 °C. The supernatant was centrifuged at 20,000×*g* for 20 min at 4 °C to sediment the P_2 fraction. The P_2 pellet was resuspended in 1 ml sonication buffer (50 mM Tris, 6 mM $MgCl_2$, 1 mM EDTA, 3 mM benzamidine, 1 mM dithiothreitol (DTT), 5% w/v sucrose, 1 μg/μl soybean trypsin inhibitor, pH 8.0) and sonicated on ice to disrupt synaptosomes. The suspension was centrifuged at 10,000×*g* for 10 min at 4 °C to sediment the membranes. Pellets were resuspended by sonication in 250 μl ADP-ribosylation buffer (100 mM Tris, 5 mM $MgCl_2$, 0.8 mM EDTA, 2,4 mM benzamidine, 2.8 mM DTT, 4% w/v sucrose, 0.8 μg/μl soybean trypsin inhibitor, 10 mM thymidine, 2.0 mM GTP, 10 mM isoniazid, 0.5% v/v Triton X-100, pH 8.0) and an aliquot taken for protein determination by the method of Lowry. Tissue was resuspended to a concentration of 2.5 μg/μl and aliquots frozen at −70 °C for future use.

Synaptosomal membranes prepared from ovariectomized, NIPS or estrogen treated animals (25 μg) were preincubated in ADP-ribosylation buffer (100 μl, final volume) for 15 min prior to the addition of pertussis toxin (1 μg) and [^{32}P]-NAD (504 pmol, 30 mCi/mmol), the incubation continued for an additional 60 min at room temperature. The reaction was terminated by the addition of ice-cold sonication buffer (750 μl) and centrifugation at 10,000×*g* for 15 min at 4 °C. Pellets were resuspended in buffer (40 mM Tris–HCl, 2% sodium dodecyl sulfate (SDS), pH 6.8 containing 1 mM DTT), incubated at 70 °C for 5 min and an aliquot of *N*-ethylmaleimaide (20 mM final concentration) was added and sample incubated for 15 min at room temperature. Samples were prepared for electrophoresis by the addition of 3× SDS-sample buffer, run on 10% polyacrylamide-SDS (PAGE) gels, stained, dried and [^{32}P]-incorporation quantified using a Molecular Dynamics Phosphor-imager.

2.4. Electrochemistry

Nafion coated multifiber carbon electrodes (three 30 μM fibers) were prepared as described previously (Thompson and Moss, 1994). Electrodes were calibrated prior to use in phosphate-buffered saline (pH 7.3) with increasing concentrations of dopamine in the presence of 250 μM ascorbic acid. Only electrodes that exhibited a linear slope with a correlation coefficient of r>0.997 and a sensitivity of dopamine over ascorbate >500:1 were used. Voltammetric measurements were made using an IVEC-10 high-speed chronomaperometric computer-controlled system (Harvard Apparatus, Holliston, MA, USA) as described previously.

2.5. In vivo uptake

Animals were anesthetized with urethane (1.75 g/kg, intraperitoneal) and placed in a stereotaxic apparatus on top of a heating pad to maintain body temperature. A portion of the skull was completely removed to expose the brain anterior to bregma (+3.00 mm anterior and ±3.5 mm lateral). A carbon fiber electrode was fixed to a glass pipette containing dopamine (400 μM dissolved in normal saline containing 100 μM ascorbic acid). The electrode-pipette assembly was lowered into the striatum (+1.5 mm anterior, ±1.5 mm lateral, −5.0–6.0 mm ventral) according to the atlas of Paxino and Watson (1982). A Ag+/AgCl reference electrode was placed just below the cortical surface anterior

to the microelectrode and fixed to the skull using sticky-wax (Kerr Brand, Emeryville, CA, USA). Dopamine clearance was determined by pressure injection of dopamine from the micropipette (12 psi, 0.1–10 s, Picospritzer; General Valve, Fairfield, NJ, USA). Various volumes of dopamine were injected to produce extracellular concentrations ranging from 1.0 to 11.0 μM. Clearance rates were determined from the slope of the decay curve. A second indicator of dopamine uptake, T_{50}, which is the time (in seconds) required for the signal to return to half-maximal amplitude was also determined. Dopamine infusions were made at 5 min intervals. At least two identical infusions were made at each concentration to insure signal stability. These values were averaged and data from each group (various pretreatments and dopamine concentrations) were averaged and are reported as the mean±S.E.M. At the completion of each experiment, histological methods were used to verify electrode placement. Only those animals having electrode tracks clearly placed within the striatum were included in data analysis.

2.6. Statistical analysis

In all cases ovariectomized, non-treated animals served as the controls. [$^{32}P_i$] incorporation data are expressed as percent ovariectomized control. Initial rates and T_{50} times are reported as the mean±S.E.M. Values represent the mean±S.E.M of at least four independent experiments. One-way analysis of variance followed by suitable multiple comparison tests or Student's *t*-test were used to determine statistical significance. A P value <0.05 was considered significant.

3. Results

3.1. [$^{32}P_i$]ADP-ribosylation

Basal $G\alpha_{i/o}$ activation was examined by pertussis toxin mediated [$^{32}P_i$]ADP-ribosylation of synaptosomal membranes prepared from ovariectomized, NIPS and estrogen primed animals. $G\alpha_{i/o}$ are substrates for pertussis toxin only in the heterotrimeric, inactivated form. A representative scan illustrates a single, poorly resolved doublet with a molecular weight of approximately 40 kDa (Fig. 1) which was verified by western blotting to consist of the $G\alpha_I$ (41 kDa) and $G\alpha_o$ (39 kDa) subunits (data not shown). NIPS treatment, which has been shown to functionally down-regulate dopamine D2 receptors, resulted in a significant increase in $G\alpha_{i/o}$ ribosylation (141%±2.9; N=5) consistent with a decrease in receptor-G-protein activation. In contrast, a significant attenuation of [$^{32}P_i$] incorporation (56.7%±3.0; N=9) into $G\alpha_{i/o}$ was observed in tissue from estrogen primed animals consistent with a basal activation of synaptosomal G-protein.

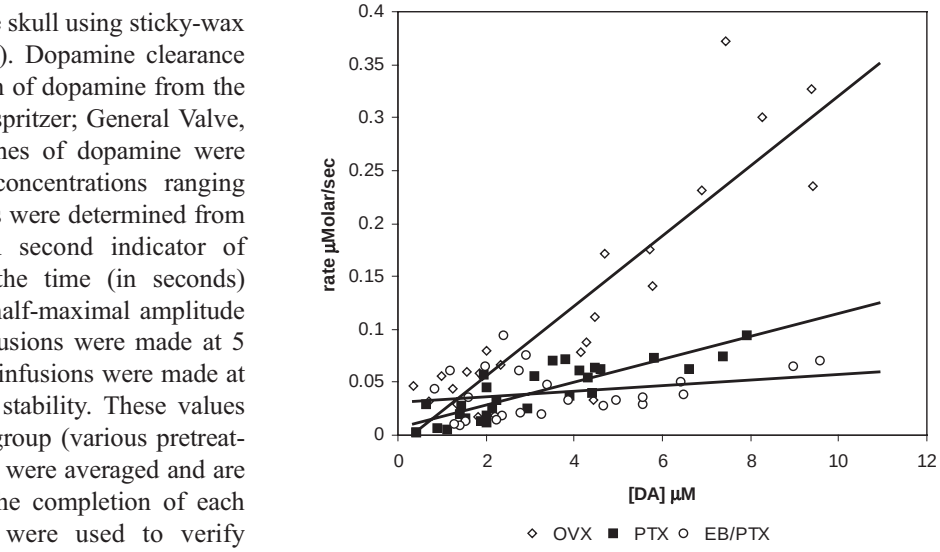

Fig. 2. Effect of treatment on concentration dependence of dopamine uptake. Dopamine (1–10 μM final extracellular concentration) was pressure ejected into the striatum of ovariectomized, pertussis toxin and estrogen/pertussis toxin treated animals as described. Each point represents the means of at least two measurements.

3.2. Effect of estrogen priming and pertussis toxin treatment on dopamine uptake and clearance

Dopamine uptake was measured in vivo following direct infusion of exogenous dopamine [approximately 1.0–10 μM extracellular concentration] into the striatum of ovariectomized, pertussis toxin treated and estrogen/pertussis toxin treated animals. In ovariectomized animals, there was a positive correlation between the rate (μM/s) of uptake and dopamine concentration (Fig. 2) as would be expected. Animals pretreated with pertussis toxin alone or in combination with estrogen had a significant attenuation of dopamine uptake at all dopamine concentrations examined. When data were grouped according to the two extreme extracellular dopamine concentrations, low [0.5–2.5 μM]

Fig. 1. Effect of NIPS and estrogen treatment on ^{32}P-ADP-ribosylation of the $Ga_{i/o}$ subunit. Ribosylated synaptosomal membranes (25 μg) were run on 10% SDS gels and [^{32}P]-incorportation visualized using the Molecular Dynamics Phospho-Imager. Lane 1: ovariectomized; Lane 2,3: NIPS; Lane 4,5: estrogen-primed.

Table 1
Effect of pretreatment on rate of dopamine uptake

Rate, μM/s	Ovariectomized	Pertussis toxin	Estrogen/pertussis toxin
Low [dopamine]	0.0471±0.005	0.0277±0.002[a]	0.0399±0.009
High [dopamine]	0.2545±0.031	0.0757±0.007[a]	0.0482±0.007[a]

Dopamine was pressure injected into the striatum and initial rate of uptake determined over three dopamine concentrations as described. Low [0–2.5 μM] and High [5.05–10.0 μM]. Data are expressed as the means±S.E.M of 4–14 placements. One-way ANOVA across [dopamine] revealed a significant effect of treatment: low, $F_{(2,33)}$=4.169, P<0.024; high, $F_{(2,14)}$=26.3, P<0.001. Pairwise multiple comparison (Tukey Test) was used to access individual differences. A P value <0.05 was considered significant; [a]Compared to concentration matched control. Number of animals: ovariectomized (7); pertussis toxin (7); estrogen/pertussis toxin (5).

and high [5.1–10 μM], pertussis toxin animals were significantly different from controls at both concentrations. In contrast, in animals treated with estrogen/pertussis toxin, uptake was not different from ovariectomized at low extracellular dopamine concentrations but was significantly reduced in comparison to both ovariectomized and pertussis toxin treated animals at high dopamine concentrations (P<0.05; Table 1).

Pertussis toxin pretreatment consistently prolonged T_{50} (1.5–2 fold) over all concentration ranges of dopamine examined (Table 2). As with rate, the effect of estrogen/pertussis toxin co-administration was additive at all but the lowest extracellular dopamine concentrations. There were no significant differences in T_{50} times between estrogen and pertussis toxin treated animals at the medium or high dopamine concentrations. Interestingly, the effect of co-treatment with estrogen and pertussis toxin was additive (2.3–3.7 fold increase) when dopamine concentrations were above 2.5 μM.

4. Discussion

Steroid regulation of mesolimbic, nigrostriatal and tuberoinfindubular dopaminergic activity is well documented. Estrogen priming down-regulates D2 but not D1 or D3 mRNA in the striatum and nucleus accumbens (Lammers et al., 1999); either increases or decreases D2 receptor density (Ferretti et al., 1992; Morissette et al., 1992) dependent on steroid priming conditions; inhibits autoreceptor potentiation of dopamine uptake in tissue from the nucleus accumbens (Thompson et al., 2000); functionally uncouples D2 receptors from its effectors in the pituitary (Munemura et al., 1989) and accumbens (Thompson et al., 2001); and alters the responsiveness of the dopamine transporter as measured behaviorally (Castner et al., 1993; Sell et al., 2000), in vivo in the accumbens and striatum (Walker et al., 2000; Thompson et al., 2001); and in vitro (Attali et al., 1997; Thompson et al., 2000; Demaria et al., 2000; Myers et al., 2003).

However, a precise understanding of the mechanism of steroid action on dopaminergic systems is complicated by the documented association between autoreceptor activation and potentiation of dopamine uptake (Meiergerd et al., 1993; Dickinson et al., 1999; Wu et al., 2002). It is conceivable that the primary site of steroid action may be at the level of the D2 receptor, resulting in a secondary modulation of transporter activity. Estrogen may modify receptor function: 1) externally, at the ligand binding site or 2) internally at the level of the receptor linked $G_{i/o}$ protein.

Attenuation of neuronal G-protein coupled receptor activation by estrogen treatment has been documented. Phosphorylation and subsequent G-protein uncoupling of α-adrenoceptors by estrogen exposure has been reported in hypothalamic tissue (Ansonoff and Etgen, 2000). Short, but not long-term exposure to estrogen has been shown to destabilize 5-HT_{1a}, 5-HT_{1b}, $GABA_b$ and cannabinoid receptor-$G\alpha_{i/o}$ protein interaction in brain tissue from female rats (Mize and Alper, 2000). Dopamine potentiation of [^{35}S]GTPγS binding is impaired in synaptosomal membranes and tissue sections prepared from estrogen primed ovariectomized rats (Thompson et al., 2001; Febo et al., 2003). In addition, one of these studies further suggests a link between G-protein activation and steroid mediated alteration of DAT activity (Thompson et al., 2001). Steroid priming conditions that result in impaired receptor mediated G-protein activation significantly attenuate dopamine uptake as measured in vivo in both the striatum and nucleus accumbens of ovariectomized female rats.

Previous studies have indicated that co-treatment with estrogen and NIPS impairs dopamine transport greater than

Table 2
Effect of pretreatment on T_{50}

Group		Low [dopamine]	Medium [dopamine]	High [dopamine]
Ovariectomized	[dopamine]	1.62±0.14	4.07±0.2	7.56±0.58
	T_{50} (s)	25.8±3.5	28.3±2.8	19.6±2.5
	(n)	(13)	(7)	(7)
Pertussis toxin	[dopamine]	1.68±0.14	3.91±0.17	6.93±0.5
	T_{50} (s)	41.2±3.8[a]	44.4±4.2[a]	42.5±1.4[a]
	(n)	(14)	(11)	(4)
Estrogen/ pertussis toxin	[dopamine]	1.67±0.17	3.57±0.3	7.09±0.71
	T_{50} (s)	49.4±8.4[a]	71.1±7.6[a,b]	87±6.5[a,b]
	(n)	(10)	(8)	(9)

Dopamine was pressure injected into the striatum and initial rate of uptake determined over three dopamine concentrations as described. Data are expressed as the means±S.E.M. of 7–13 placements. One-way ANOVA across [dopamine] revealed a significant effect of treatment. Low: $F_{(2,34)}$=P<0.01; Medium: $F_{(2,23)}$=P<0.001; High: $F_{(2,14)}$=P<0.001. Pairwise multiple comparison (Tukey Test) was used to access individual differences. A P value <0.05 was considered significant; [a]Compared to concentration matched control, [b]Compared to concentration matched pertussis toxin treated. Number of animals: ovariectomized (7); pertussis toxin (7); estrogen/pertussis toxin (5).

either agent alone (Thompson et al., 2001). These data suggest estrogen may interact at a site on the dopamine-receptor distinct from the ligand binding site which is altered by NIPS treatment (Xu et al., 1991). In the current study, we investigated the role of $G\alpha_{i/o}$ in estrogen mediated attenuation of dopamine uptake in the striatum. Chemical inactivation of the dopamine-D2 receptor by NIPS treatment resulted in an increase in pertussis toxin mediated [$^{32}P_i$] ribosylation of $G\alpha_{i/o}$, consistent with a receptor mediated impairment and subsequent inability to activate the hetero-trimeric form of the receptor linked G-protein. In contrast, estrogen priming resulted in an apparent decrease in ADP-ribosylation of synaptosomal $G\alpha_{i/o}$-protein which would be consistent with an increase in basal receptor activation and/or steroid mediated destabilization of the heterotrimeric G-protein. Estrogen has been postulated to cause a decrease in G-protein synthesis (Bouvier et al., 1991) which could also account for an apparent decrease in ribosylation.

We predicted that if estrogen was acting at the level of a pertussis toxin-sensitive G-protein, little difference would be detected in DAT activity when estrogen-primed, pertussis toxin treated and estrogen/pertussis toxin co-treated animals were investigated. This prediction was accurate in part. Infusion of pertussis toxin into the striatum significantly impaired dopamine uptake at all dopamine concentrations as measured by both a change in T_{50} and the rate of uptake. These data clearly suggest a role for $G\alpha_{i/o}$ activation in dopamine receptor mediated potentiation of DAT activity. Previous studies have demonstrated that pertussis toxin infusion into the striatum results in attenuation of dopamine D2 autoreceptor mediated cellular responses measured in vitro (Bean et al., 1998). It was not unexpected, therefore, that pertussis toxin treatment should also attenuate DAT activity in vivo. At low [1–2.5 μM] dopamine concentrations, estrogen treatment had little effect on the ability of pertussis toxin to alter dopamine uptake suggesting that pertussis toxin and estrogen are acting at similar sites; presumably the $G\alpha_{i/o}$ subunit associated with the D2 receptor. However, at higher extracellular dopamine concentrations, co-treatment with estrogen and pertussis toxin significantly enhanced the attenuation of dopamine transport detected in response to pertussis toxin alone. There are a number of possible explanations for this apparent dose dependent effect on dopamine uptake. The simplest explanation is that the pertussis toxin treatment was not sufficient to completely inhibit all of the available $G\alpha_{i/o}$ subunits. These subunits make up as much as 1% of the total membrane proteins in the brain and it is not unreasonable to assume that only a small fraction were ribosylated under the conditions used in this study. If that is the case, one might expect to see an additive effect of pertussis toxin and estrogen-treatment even if they were acting at the same site. This possibility can easily be addressed by conducting back phosphorylation/ribosylation experiments in membranes prepared from pertussis toxin treated animals.

Other possible explanations for the observed differences also need to be considered. The affinity of the dopamine D2 autoreceptor makes it particularly sensitive to low dopamine concentrations. Disruption of the receptor/G-protein complex by either pertussis toxin or estrogen apparently prevents the down-stream activation of effector molecules and therefore facilitation of dopamine transport. However, at higher dopamine concentrations, a greater number of dopamine D2 receptors might need to be recruited to mount the compensatory increase in cellular effectors necessary to stimulate DAT activity further. Estrogen pretreatment may inhibit the ability to 'recruit' additional dopamine receptors. One possible mechanism is that steroid treatment may facilitate phosphorylation of the dopamine D2 receptor by G-protein coupled receptor kinases (GRKs), resulting in an association of arrestins to the dopamine receptor and subsequent inhibition of heterotrimeric G-protein interaction with the receptor (Kim et al., 2001; Sterne-Marr et al., 2003; Nair and Sealfon, 2003). This would be consistent with the decrease in ADP-ribosylation which was observed in response to estrogen priming. In addition, estrogen may act to inhibit receptor recruitment by a mechanism independent of a pertussis toxin sensitive G-protein. Agonist specific activation of various intracellular pathways have been associated with the D2 receptor activation (Cordeaux et al., 2001; Nair and Sealfon, 2003) and these signaling mechanisms may also play a role in DAT modulation.

In addition to dopamine D2 receptors, various other neurotransmitter receptor subtypes are associated with pertussis toxin sensitive G-proteins (Ansonoff and Etgen, 2000; Selley et al., 2003). Inhibition of any of these receptors may be expected to alter neuronal excitability. In the current study, NIPS and estrogen ADP-ribosylation experiments, together with the results of in vivo pertussis toxin treatment, clearly suggest an interaction between dopamine D2 receptors, $G\alpha_{i/o}$ activation and facilitation of DAT activity. And while the physiological contribution of other receptor subtypes to the regulation of DAT activity would be predicted to be limited, the potential involvement of these $G\alpha_{i/o}$ linked receptors can not be ignored.

In conclusion, the results of the current study lend further support to the hypothesis that estrogen modulation of DAT activity is consistent with an alteration of dopamine D2/$G\alpha_{i/o}$ activation. In addition, these data suggest that estrogen may destabilize the D2/heterotrimeric G-protein association. One intriguing possibility is that estrogen modulates DAT activity through a phosphorylation dependent alteration of dopamine D2/G-protein receptor coupling. The ability of estrogen to selectively effect these types of signaling pathways is currently under investigation. This may contribute to the apparent ability of estrogen to modify responsiveness to psychostimulants (Sell et al., 2000; Myers et al., 2003) and to the reported sex differences in the expression of schizophrenia (Castle et al., 1995; Bartzokis, 2002).

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