

Viral Vector-Mediated Overexpression of Estrogen Receptor- α in Striatum Enhances the Estradiol-Induced Motor Activity in Female Rats and Estradiol-Modulated GABA Release

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Classical estrogen receptor-signaling mechanisms involve estradiol binding to intracellular nuclear receptors [estrogen receptor- α (ER α) and estrogen receptor- β (ER β)] to promote changes in protein expression. Estradiol can also exert effects within seconds to minutes, however, a timescale incongruent with genomic signaling. In the brain, estradiol rapidly potentiates stimulated dopamine release in the striatum of female rats and enhances spontaneous rotational behavior. Furthermore, estradiol rapidly attenuates the K⁺-evoked increase of GABA in dialysate. We hypothesize that these rapid effects of estradiol in the striatum are mediated by ER α located on the membrane of medium spiny GABAergic neurons. This experiment examined whether overexpression of ER α in the striatum would enhance the effect of estradiol on rotational behavior and the K⁺-evoked increase in GABA in dialysate. Ovariectomized female rats were tested for rotational behavior or underwent microdialysis experiments after unilateral intrastriatal injections of a recombinant adeno-associated virus (AAV) containing the human ER α cDNA (AAV.ER α) into the striatum; controls received either the same vector into areas outside the striatum or an AAV containing the human alkaline phosphatase gene into the striatum (AAV.ALP). Animals that received AAV.ER α in the striatum exhibited significantly greater estradiol-induced contralateral rotations compared with controls and exhibited behavioral sensitization of contralateral rotations induced by a low-dose of amphetamine. ER α overexpression also enhanced the inhibitory effect of estradiol on K⁺-evoked GABA release suggesting that disinhibition of dopamine release from terminals in the striatum resulted in the enhanced rotational behavior.

Key words: estradiol; dopamine; rotational behavior; microdialysis; striatum; adeno-associated viral vector; estrogen receptor

Introduction

Estradiol rapidly enhances stimulated dopamine (DA) release from striatum *in vitro* (Becker, 1990a; Xiao and Becker, 1998;

Xiao et al., 2003) or *in vivo* (Becker, 1990b; Castner et al., 1993). This rapid effect of estradiol on striatal DA is thought to contribute to the enhanced acquisition of cocaine self-administration and enhanced motivation to take cocaine found in female rats after estradiol treatment (Jackson et al., 2006; Becker and Hu, 2008; Hu and Becker, 2008).

DA release in striatum is inhibited by intrinsic GABA input (Smolders et al., 1995; Whitehead et al., 2001), so we hypothesized that estradiol enhances DA release in the striatum indirectly via inhibition of GABA input onto DA terminals. In whole-cell clamp recordings, application of physiological concentrations of 17 β -estradiol decreases Ca²⁺ current mediated by L-type Ca²⁺ channels in acutely dissected medium spiny neurons within seconds of application (Mermelstein et al., 1996). Since inhibition of L-type Ca²⁺ channels on cell bodies decreases neurotransmitter release from neurons (Vigh and Lasater, 2004), and striatal me-

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dium spiny neurons are GABAergic, this would suggest that estradiol can inhibit GABA release. In microdialysis experiments estradiol significantly attenuates the K^+ -evoked increase in GABA in dialysate from the striatum (Hu et al., 2006) supporting this hypothesis.

Activation of membrane-associated estrogen receptor- α (ER α) and/or estrogen receptor- β (ER β) may mediate some of the rapid effects of estradiol in striatum, and other brain regions, through a novel mechanism (Boulware et al., 2007; Micevych and Mermelstein, 2008). ER α and/or ER β can associate with caveolin in the extracellular membrane of cells to mediate rapid responses to estradiol. For example, extracellular estradiol can rapidly activate the mitogen-activated protein kinase pathway, one of the primary mediators of the intracellular signal transduction cascade triggered by estradiol binding to ER α at the membrane (Razandi et al., 2003; Wade and Dorsa, 2003). Whether estradiol acts in the striatum via ER α to influence behavior or GABA release remains to be determined.

These experiments investigate the effects of overexpression of ER α in the striatum. Female rats have an endogenous asymmetry in the ascending DA system, so they turn in circles away from the striatum with greater DA activity (Jerussi and Glick, 1976; Becker et al., 1982), and turn more during behavioral estrus than on other days of the cycle (Becker et al., 1982). We took advantage of this estradiol-modulated behavioral and neurochemical asymmetry to investigate whether overexpression of ER α in striatum would enhance the effect of estradiol on this behavior. We also investigate whether ER α overexpression would enhance the effect of estradiol on the K^+ -evoked increase in GABA in dialysate from striatum. The results demonstrate that increasing expression of ER α unilaterally in the striatum enhances the rapid behavioral effect of estradiol and induces greater attenuation of K^+ -evoked GABA release after estradiol treatment compared with control animals. Furthermore, we demonstrate that in the striatum of adult ovariectomized rats there is low-level expression of ER α protein. These results indicate that endogenous ER α may mediate rapid effects of estradiol on GABA activity and striatal-mediated behaviors observed in the female rat.

Materials and Methods

Animals

Adult female Sprague Dawley rats (Harlan) were maintained on a 14:10 light/dark cycle with soy-free rat chow (Teklad #2014, Harlan rat chow) and water available *ad libitum*. Rooms were maintained at a constant temperature of 20–21°C. For the behavioral experiments animals were housed in groups of 2–3 with lights on at 6:30 P.M. For the microdialysis experiments, animals were individually housed after guide cannula implantation, and lights in the colony room were on at 5:30 A.M. All experiments were conducted in accordance with the National Institute of Health guidelines on a protocol approved by the University of Michigan Committee for Use and Care of Animals.

Rats were ovariectomized (OVX) under isoflurane (Baxter Healthcare Corporation) anesthesia (Hu and Becker, 2003), and those undergoing behavioral testing were tested for rotational behavior 2 weeks later with 0.85 mg/kg D-amphetamine (AMPH) sulfate (1 h test on 3 consecutive days). Rats then received intrastriatal injections of the viral vector [adeno-associated virus (AAV) with ER α ; AAV.ER α ; $N = 14$; see below for details] or control construct (adeno-associated virus with the enzyme alkaline phosphatase; AAV.ALP; $N = 9$) into three sites, unilaterally in the striatum contralateral to preferred direction of turning (i.e., into the dominant striatum). On histological examination, four of the animals in the AAV.ER α group did not have ER expression in striatum (the expression was only in cortex) and data from these animals are included with the control group (final groups AAV.ER α ; $N = 10$, AAV.ALP; $N = 13$).

Adeno-associated viral vectors

Experimental animals received an AAV containing the human ER α cDNA (AAV.ER α). Control animals received an AAV harboring the human placental alkaline phosphatase gene (AAV.ALP). The expression of the transgenes was driven by the chicken β -actin/cytomegalovirus hybrid promoter (CAG). In addition, an internal ribosomal entry site element provides a bicistronic expression of enhanced green fluorescent protein (GFP) from both constructs to permit visualization of transduced neurons. The expression cassettes were flanked by AAV inverted terminal repeats derived from psub201 (Samulski et al., 1987).

Preparation of adeno-associated viral vector stocks. To prepare virus stocks, the vector plasmids were packaged into AAV-2 particles using helper-free plasmid transfection system in 293 cells. The vectors were purified using heparin affinity chromatography (Clark et al., 1999) and dialyzed against PBS. Genomic titers were determined by quantitative PCR (Veldwijk et al., 2002) and adjusted to 10^{12} particles per ml.

Injection of viral vector unilaterally into striatum. OVX female rats underwent stereotaxic surgery under ketamine (75 mg/kg, i.p.) and medetomidine hydrochloride (0.5 mg/kg, i.p.) anesthesia. Small burr holes were drilled through the skull immediately above the dominant striatum (determined by AMPH-induced behavior as described above) with a drill mounted on a stereotaxic arm. Three holes were drilled and a microsyringe was slowly lowered over 2 min to the following coordinates: (1): Ant + 1.0, Lat \pm 2.0, Ventral 2.7; (2) Ant + 0.15, Lat \pm 3.5, Ventral 3.0; (3) Ant - 0.75, Lat \pm 3.8, Ventral 3.0. At each site, 1 μ l of the AAV was injected over 7–10 min, the needle was left in place for an additional 2 min and then the needle was slowly raised.

Behavioral testing

Animals were tested repeatedly for rotational behavior. As discussed above, in unlesioned female rats there is an endogenous asymmetry in turning behavior seen during the dark phase of the cycle or with a low dose of AMPH (Jerussi and Glick, 1976; Becker et al., 1982). We hypothesized that enhanced expression of ER α unilaterally in the striatum would induce a greater asymmetry in the striatum, and we would see greater rotational behavior after estradiol or in response to AMPH in animals with enhanced expression of the ER α transgene. Thus, animals were repeatedly tested for rotational behavior after acute estradiol, oil, and/or AMPH injection.

Estradiol-induced turning. Three weeks after the viral vector had been injected into the brain, animals were repeatedly tested in automated rotometers for turning behavior (Hu and Becker, 2003). Testing was initiated during the first hour after lights off in the colony. There was a 1 h habituation session, then animals received 5 μ g estradiol benzoate (EB) in 0.1 ml peanut oil or 0.1 ml peanut oil by subcutaneous injection, followed by a 1 h test session. Rotational behavior was recorded by computer at 15 min intervals.

Animals received 3 tests/week (2 with EB, 1 with oil) every other day for 2 weeks, then had 1 week off when they were tested with AMPH (see below), followed by 1 additional week of testing with EB and oil for a total of 6 EB tests and 3 oil tests. The first 15 min of the habituation periods and postinjection test periods were used for analyses, since animals exhibited the greatest activity during these periods.

AMPH-induced turning. On 3 consecutive days, 5 weeks after the viral vector had been injected into the striatum, animals received EB or oil (EB on days 1 and 3, oil on day 2). Animals were injected with EB or oil, placed in the automated rotometers, and 30 min later animals received 0.85 mg/kg AMPH (i.p.); rotational behavior was recorded for 1 h.

Serum concentrations of estradiol at the time of the test were determined in independent groups of OVX animals of the same size using an immunofluorescent assay: 184.5 ± 38.3 (17 β -estradiol in pg/ml \pm SEM; day 1 and 3) and 109.5 ± 19.2 (day 2). These values were not significantly different from each other, and were within the range of values reported for this strain of rat during proestrus (Butcher et al., 1974). Thus, on all 3 d animals had elevated serum estradiol concentrations relative to OVX animals, which we measured as 23.6 ± 2.5 pg/ml (Hu et al., 2004).

Guide cannula and microdialysis probe implantation

At least 4 weeks after insertion of the viral vector, a separate group of rats were anesthetized using a combination of ketamine and medetomidine

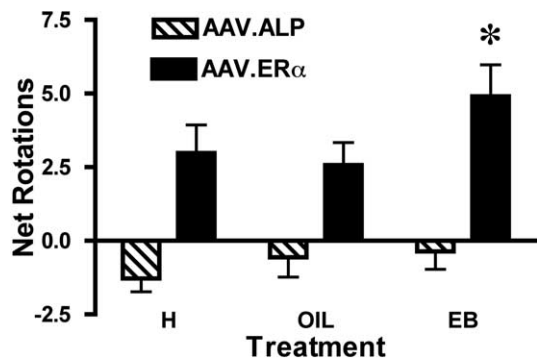


Figure 1. Full turns (360°) made contralateral to the striatum with the AAV.ER α or AAV.ALP transgene minus turns in the opposite direction during the first 15 min of the habituation period (H), the first 15 min after animals received 0.1 ml peanut oil (OIL; s.c.) or the first 15 min after 5 μ g EB in oil (s.c.). *Animals with the AAV.ER α transgene expressed unilaterally in the striatum turned significantly more ($p < 0.01$) after EB than during the habituation period or after OIL and turned more than the control animals ($p < 0.01$). Bars indicate the mean \pm SEM.

hydrochloride anesthesia as outlined previously. Using aseptic surgical techniques, guide cannulas were implanted stereotaxically through the skull (from Bregma skull flat in mm, AP: +0.2 mm; ML: \pm 3.2 mm; DV: -2.25 mm) and secured with cranioplastic cement. A stylet was placed in the guide cannula to keep the cannula patent.

After a minimum 1-week recovery period, the animal was lightly anesthetized with isoflurane (Baxter Healthcare Corporation), and a microdialysis probe (4 mm active length CMA/11, CMA/Microdialysis AB) was inserted into the guide cannula. The animal was placed inside a Rarun (BioAnalytical Systems) bowl and artificial cerebral spinal fluid (aCSF: 145 mM NaCl, 2.68 mM KCl, 1.01 mM MgSO $_4$ ·7H $_2$ O, 1.22 mM CaCl $_2$, pH 7.3) was perfused through the probe at 0.1 μ l/min overnight.

Microdialysis and determination of GABA concentrations in dialysate

Experiments began 15–18 h after implantation of the dialysis probe. aCSF was perfused through the probe at 1 μ l/min, and on-line analysis of the dialysate was performed using capillary electrophoresis with laser-induced fluorescence detection, which has been described previously (Bowser and Kennedy, 2001). Briefly, dialysate was mixed online with a derivatization solution (10 mM *o*-phthalaldehyde, 40 mM β -mercaptoethanol, 36 mM sodium borate, 0.81 mM hydroxypropyl- β -cyclodextrin (HP β CD), and 10% methanol (v/v) at pH 9.5) and allowed to react for 90 s. The derivatized dialysate was then electrokinetically injected onto the separation capillary (10 μ m, i.d.; 150 μ m, o.d.; and 9.5 cm in length) with a flow-gated interface. Cross flow buffer (40 mM sodium tetraborate with 0.9 mM HP β CD at pH 9.5) prevented leakage of dialysate onto the separation capillary during each separation. The applied voltage for separation equated to an electric field of 2.22 kV/cm. Fluorescence detection was performed off-column with the aid of a sheath-flow cuvette. The sheath flow buffer consisted of 40 mM sodium tetraborate at pH 9.5. Fluorescence was induced with the 351 nm laser line of an argon ion laser (Enterprise 622 argon ion laser; Coherent), and fluorescence emission (450 nm) was collected orthogonally to the incident beam. Data were collected using software written in Labview 5.0 (National Instruments). The detection limit for GABA in these assays was 15.1 \pm 0.7 nM.

For each experiment, 50 electropherograms were collected at 15 s interval to establish basal levels before a subcutaneous injection of 5 μ g EB ($N = 19$) or vehicle (peanut oil, 0.1 ml, $N = 17$). Electropherograms were collected for 30 min after vehicle/EB administration to determine the effect of EB on basal GABA release. Then high potassium aCSF (75 mM K $^+$) was perfused through the probe for 10 min. The ionic strength of the aCSF was maintained by lowering the sodium concentration to 72.7 mM. Electropherograms collected during the 10 min stimulation and 30 min poststimulation period were used to determine effects of estradiol on stimulated GABA release.

Once the dialysis experiment was complete, Flurogold (Molecular Im-

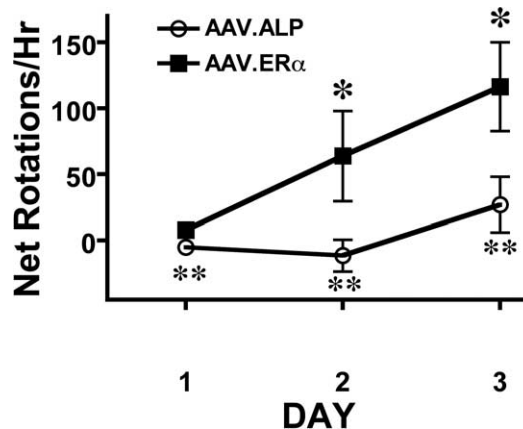


Figure 2. Animals with the AAV.ER α transgene expressed unilaterally in the striatum exhibited sensitization of asymmetrical rotational behavior when treated with 5 μ g EB followed 30 min later with 0.85 mg/kg *D*-amphetamine (AMPH). Rotational behavior contralateral to the side with the ER α transgene (minus turns in the other direction) was recorded for 1 h after animals received AMPH. *In the animals with AAV.ER α transgene, asymmetrical rotational behavior induced by AMPH was greater on day 3 than on day 1 ($p < 0.008$) or 2 ($p < 0.03$). **In the animals with AAV.ER α transgene, asymmetrical rotational behavior induced by AMPH was greater than the control animals on all days ($p < 0.015$). Data are depicted as the mean \pm SEM.

Table 1. Mean number of rotations in both directions induced by 0.85 mg/kg AMPH during 1 h of rotational behavior testing

Group	Total rotations day 1 of AMPH	Total rotations day 2 of AMPH	Total rotations day 3 of AMPH
AAV.ER α	14.6 \pm 5.2 ^a	172.3 \pm 44.2**	141.7 \pm 26.1*
AAV.ALP	16.6 \pm 7.4	128.2 \pm 27.1**	93.2 \pm 12.4*

* $p < 0.05$ compared with day 1; ** $p < 0.001$ compared with day 1.

^aMean \pm SEM.

aging Products) was perfused through the probe for 10 min to mark the location of the dialysis probe in the tissue. After explant the probe was placed in ethanol for 5 min and then in aCSF. The probe was then calibrated using GABA standards that were heated to 37°C.

Immunocytochemistry

At the completion of testing, animals were intracardially perfused with 4% paraformaldehyde and brains were stored in 30% sucrose at 4°C. Sections were cut at 35 μ m and stored in cryoprotectant solution (30% ethylene glycol, 30% sucrose in 0.1 M PBS, pH 7.2) at -20°C until immunocytochemistry. Then, sections were washed 4 \times 5 min w/PBS, followed by NaBH $_4$ (1%) in PBS for 30 min. After additional washes, sections were incubated in blocking serum with 4% normal goat serum (NGS), 0.2% Triton X-100 for 15–60 min, then transferred to incubate with the primary antibody at 4°C for 48 h (ER α H222 antibody; courtesy Dr. G. Greene, University of Chicago, Chicago, IL) was used at 1:5000; antibody to green fluorescent protein (AbCam) was used at 1:25,000 to confirm expression of AAV.ALP and AAV.ER α in dialysis experiments). Sections were washed and then incubated in the secondary antibody (1:600), 2% NGS, and 0.2% Triton X-100 for 1 h. After several washes the sections were transferred to the Vectastain ABC reagent (Vectastain Elite ABC kits, Vector Labs) solution for 1 h. After washing, sites of antibody-antigen binding were visualized with the chromogen diaminobenzidine. Sections were mounted, dried, dehydrated and coverslipped.

Western blots

Immunoprecipitation and Western blot analysis for ER α were performed using striatal tissue from female rats, at 3 weeks post-OVX, according to previously published methods (Singh et al., 1999; Toran-Allerand et al., 2002). Females were OVX to remove the endogenous source of estradiol which can induce variation in the number and intracellular location of ER. Western blots were derived from at least four independent experiments, a representative of which is shown in Figure 5.

The specificity of the signal is determined by the apparent molecular weight of the protein detected, along with direct comparison with the appropriate protein lysate. Negative controls to test for the specificity of the interactions are performed by immunoprecipitation of the pre-cleared protein lysates with mouse IgG, instead of specific antibodies.

Statistical analyses

Behavioral data were analyzed by two-way ANOVA with pairwise comparisons by the Bonferroni correction. Microdialysis data was analyzed by two-way ANOVA with repeated measures and planned paired *t* tests.

Results

Rotational behavior

Animals that received the AAV.ER α in the dominant striatum made more net rotations (turns contralateral to the striatum with the AAV.ER α minus turns ipsiversive), both during the first 15 min (Fig. 1) and throughout the hour after receiving EB (data not shown), than did the control group ($F_{(1,21)} = 14.48, p < 0.001$). These rats also turned more after the EB injection than after vehicle or during the habituation period ($p < 0.01$) (Fig. 1). The control animals did not exhibit a significant asymmetry in terms of net rotations. Thus, the introduction of the AAV.ER α in the striatum enhanced the slight endogenous asymmetry in the striatum, and transgene expression resulted in turning after treatment with EB.

When animals were tested with 0.85 mg/kg AMPH (Fig. 2) the AAV.ER α group again exhibited significantly more turning contralateral to the striatum with the AAV.ER α than did the AAV.AL P group (Group effect: $F_{(1,20)} = 7.097, p < 0.15$; Time effect $F_{(2,2)} = 8.462, p < 0.001$; Group \times Time: $F_{(2,40)} = 3.363, p < 0.05$). As can be seen in Figure 2, there was only modest behavioral activation on the first day of testing in both groups, yet the AAV.ER α group exhibited greater rotational behavior than the control group ($p < 0.015$). On the second and third days of testing, the AAV.ER α group also exhibited significantly more turning than did the AAV.AL P group ($p < 0.002$). There was also significant sensitization of rotational behavior in the AAV.ER α group with animals turning more on day 3 than they did on day 1 ($p < 0.008$) or day 2 ($p < 0.03$).

The AAV.AL P group did not exhibit behavioral sensitization of rotational behavior contralateral to the side of the transgene implant. They did, however, show activation induced by AMPH (Table 1), and both groups exhibited an increase in the total number of rotations (contralateral + ipsilateral) over the 3 d of AMPH testing indicating sensitization of overall activity (Table 1). There were no differences between the groups in total rotations on any day.

Table 2. The effects of viral vector expression, hormonal treatment, and exposure to K⁺ on striatum dialysate concentrations of GABA

Group	Basal GABA in $\mu\text{M} \pm \text{SEM}$	GABA post-EB injection in $\mu\text{M} \pm \text{SEM}$	GABA postrecovery from K ⁺ stimulation in $\mu\text{M} \pm \text{SEM}$
AAV.AL P + OIL	0.11 \pm 0.01	0.109 \pm 0.008	0.088 \pm 0.009
AAV.AL P + EB	0.14 \pm 0.03	0.12 \pm 0.02	0.11 \pm 0.02
AAV.ER α + OIL	0.10 \pm 0.01	0.09 \pm 0.01	0.08 \pm 0.01
AAV.ER α + EB	0.10 \pm 0.02	0.07 \pm 0.01	0.07 \pm 0.02

Electropherograms were collected, and the mean dialysate concentration was obtained before (basal) and after (postinjection) an injection of 5 μg EB or OIL vehicle to OVX female rats. There was no main effect of treatment ($F_{(1,539)} = 4.34, p = 0.061$) on basal GABA concentration and no interaction between treatment and time of sample collection ($F_{(49,539)} = 0.68, p = 0.949$). Following recovery from the K⁺ stimulation, the GABA dialysate concentration was also determined. Basal extracellular GABA concentrations in dialysate were compared with GABA dialysate concentrations post-EB injection and postrecovery in the same rats. Two-way ANOVA with repeated measures showed that there was no main effect of treatment ($F_{(1,550)} = 4.13, p = 0.067$) on poststimulation GABA dialysate concentration and no interaction between treatment and time of sample collection ($F_{(50,550)} = 0.75, p = 0.894$).

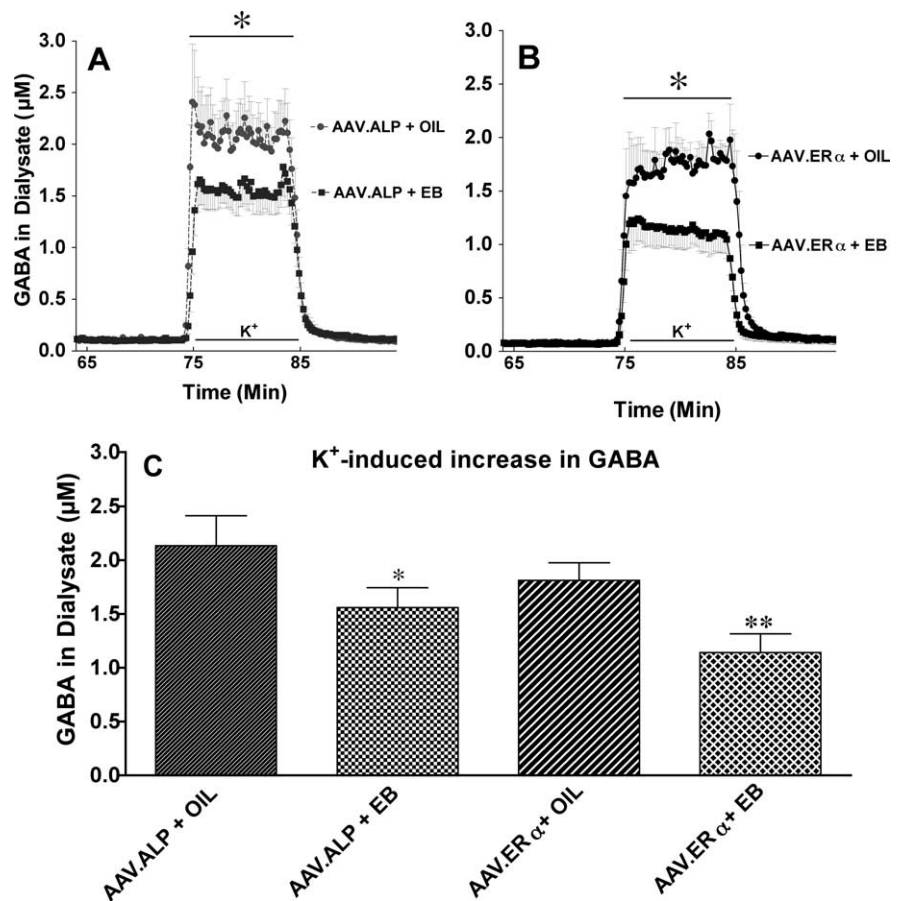


Figure 3. Effect of AAV.AL P or AAV.ER α and EB or OIL pretreatment on K⁺-evoked GABA in dialysate from striatum. **A**, The time course of the effect of AAV.AL P and EB or OIL pretreatment on K⁺-evoked GABA in dialysate from striatum. **B**, The time course of the effect of AAV.ER α and EB or OIL pretreatment on K⁺-evoked GABA in dialysate from striatum. For **A** and **B**, the bar depicts the period during which K⁺ was dialyzed into striatum through the dialysis probe. *There was a main effect of treatment ($F_{(3,29)} = 4.28, p < 0.013$), a treatment \times time interaction ($F_{(29,426)} = 2.94, p < 0.0001$); and an effect of time of sample collection ($F_{(29,142)} = 126.37, p < 0.0001$). **C**, Effect of AAV.AL P or AAV.ER α and EB or OIL pretreatment on the mean \pm SEM increase in K⁺-evoked GABA in dialysate from striatum. *AAV.AL P + EB less than AAV.AL P + OIL ($p < 0.043$). **AAV.ER α + EB less than AAV.ER α + OIL ($p < 0.002$), and AAV.AL P + EB ($p < 0.02$). There was no significant difference between the oil treated groups.

Microdialysis

During the microdialysis experiments the basal extracellular concentration of GABA did not differ among the four groups ($F_{(1,11)} = 1.353, p = 0.2694$) (Table 2). Furthermore, the basal extracellular GABA concentration was not significantly affected by treatment with EB or vehicle. Post-K⁺ stimulation baseline values were also not statistically different (Table 2).

Local perfusion of 75 mM K⁺ through the dialysis probe for 10

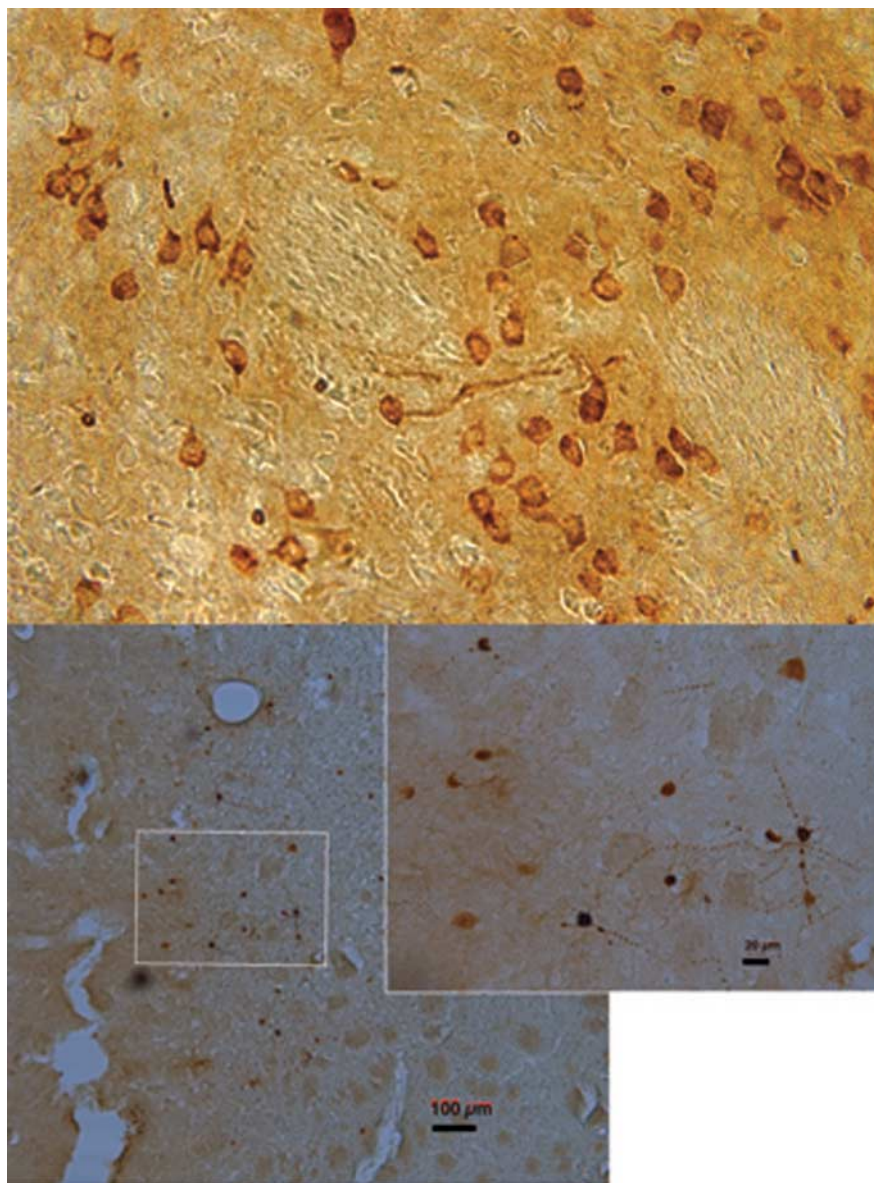


Figure 4. Expression of ER α (top) and GFP (bottom) in striatum after adeno-associated viral vector injection. Top, A representative image from an animal in the behavioral study. Bottom image is from an animal in the dialysis study; inset depicts cells with expression of GFP adjacent to the track. Magnification 40 \times ; scale bars: bottom right, 100 μ m; inset, 20 μ m.

min produced a transient increase of GABA in the extracellular fluid in all four groups of animals. Treatment with EB significantly attenuated the K⁺-induced increase in extracellular GABA concentration in dialysate (Fig. 3*A,B*). When results were analyzed by two-way ANOVA with repeated measures, there was a main effect of treatment ($F_{(3,29)} = 4.28, p < 0.013$), a treatment \times time interaction ($F_{(29,426)} = 2.94, p < 0.0001$); and an effect of time of sample collection ($F_{(29,142)} = 126.37, p < 0.0001$).

t tests were planned a priori to compare differences between the group pairings. Importantly, the AAV.ER α + EB group had significantly lower stimulated GABA release compared with the AAV.ALP + EB ($t = 2.335, p < 0.02$) indicating that AAV.ER α enhanced the response to EB (Fig. 3). As previously shown, EB attenuated the K⁺-induced increase in GABA in both the AAV.ALP and AAV.ER α groups (AAV.ALP + EB vs AAV.ALP + OIL, $t = 2.036, p < 0.043$; AAV.ER α + EB vs AAV.ER α + OIL

group, $t = 3.191, p < 0.002$). There were no significant differences between the AAV.ER α + OIL and the AAV.ALP + OIL groups.

Immunocytochemistry

The expression of the AAV.ER α transgene in striatal neurons as indicated by immunocytochemistry for ER α was confined primarily to the dorsolateral striatum exclusively on the side of the injection. Expression was quite variable in terms of the number of cells expressing the transduced genes, but expression was observed in all animals receiving the AAV.ER α . Expression of ER α was localized to the membrane/cytosol region of neurons, with most cells showing little nuclear staining (Fig. 4, top) and expression extending beyond the cell body into the surrounding processes. Animals that received AAV.ALP exhibited expression of GFP in the striatum (data not shown). For the microdialysis experiments, only animals with GFP expression around the dialysis probe (Fig. 4, bottom) were included.

Western blots

Western blots were performed as a positive control to measure endogenous ER α expression in the striatum. The blot indicated a low-level expression of ER α in the membrane fraction from striatum of OVX rats (Fig. 5). ER α expression was higher in the cytosol/membrane fraction than the nuclear extract taken from homogenized striatal tissue.

Discussion

The expression of recombinant ER α in the dorsolateral striatum resulted in enhanced turning contralateral to the side of the AAV.ER α expression compared with animals with AAV.ALP. Furthermore, animals with the AAV.ER α exhibited a greater estradiol-dependent attenuation of the K⁺-induced increase in GABA relative

to controls. Coupling these behavioral and neurochemical results to the Western blot results showing expression of endogenous ER α in striatum, these data suggest that endogenous ER α in striatum may enhance the striatal DA response to estradiol, and this effect may be mediated by an attenuation of GABA release resulting in a release of inhibition on DA terminals.

Striatal GABAergic neurons are known to have recurrent collaterals that have synapses in close proximity to DA terminals (Sizemore et al., 2004). GABA release onto GABA_B receptors located on DA presynaptic terminals tonically inhibits DA release in the striatum (Smolders et al., 1995; Charara et al., 2000). This inhibition of DA release mediated via GABA_B receptors on DA terminals is thought to be due to inactivation of voltage-dependent Ca²⁺ channels on the presynaptic terminal (Cardozo and Bean, 1995). Combining these anatomical results along with electrophysiology (Mermelstein et al., 1996) and microdialysis

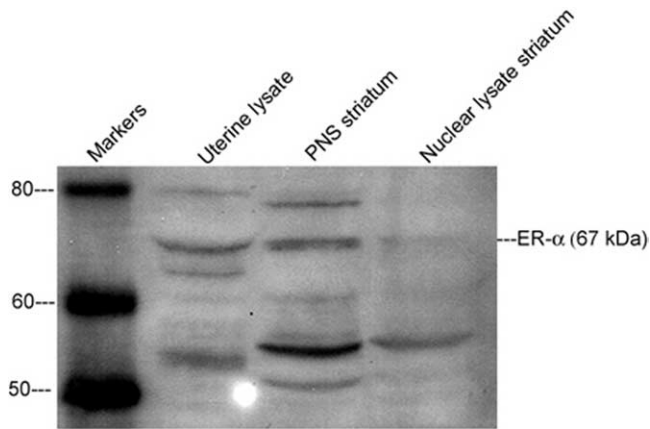


Figure 5. Western blot using an antibody to ER- α (Millipore, C-1355) with 100 μ g protein from striatal tissue obtained from female rats 3 weeks post-OVX showing the presence of ER- α in the membrane fraction (labeled PNS) from striatum

(Hu et al., 2006) data showing the inhibitory effects of estradiol on GABAergic neurons suggests a linkage between the DA-enhancing effects of estradiol and the reduction in GABA release. The twofold increase in attenuation of K^+ -evoked GABA seen with ER α overexpression suggests that this receptor plays a role in linking these two neurotransmitter systems in the striatum. The decrease in GABA release is hypothesized to result in a release of inhibition, which enhances DA release when DA neurons are activated (for discussion, see Becker and Hu, 2008; Becker and Taylor, 2008). Thus, when behaviorally active rats receive estradiol, we postulate that there is an enhanced release of DA on the side of the striatum with the AAV.ER α , relative to the other striatum, and animals turn in circles away from the more active side.

Behavioral sensitization to AMPH occurs due to long-term changes in DA neurons. The activation of DA systems is necessary to induce sensitization with AMPH, and behavioral sensitization is prevented by cotreatment with DA antagonists, given either systemically or directly into the ventral tegmental area (Kuczenski and Leith, 1981; Vezina and Stewart, 1989; Weiss et al., 1989). The evidence implicating D1 receptors is quite strong, although the involvement of D2 receptors is more controversial (White and Wolf, 1991; Stewart and Badiani, 1993). When AAV.ER α injected animals treated with estradiol receive AMPH, they exhibit sensitization of rotational behavior in the direction away from the striatum with the transgene. Both groups showed enhanced total rotations on the second test day although they did not receive additional estradiol treatment before AMPH. This is likely due to the combined effects of the neural changes associated with sensitization and the residual serum estradiol which was still elevated at the time of the second AMPH test. Interestingly, only the AAV.ER α animals showed a greater rotational behavior in the direction away from the side of the transgene expression. We hypothesize that the sensitization relative to the side of the transgene in the animals with the AAV.ER α is caused by enhanced postsynaptic changes due to either greater DA release on the side of the transgene expression, or the effects of ER α transgene expression in the postsynaptic cell, or both processes. The control animals did not exhibit sensitization of rotational behavior contralateral to the control transgene implant, but they did exhibit an increase in total rotations. Thus, both groups showed behavioral sensitization, but in the AAV.ER α group the transgene apparently enhanced activity in the striatum on the side containing the transgene resulting in an asymmetry in

the striatal response to AMPH. Since rotational behavior is related to the magnitude of the asymmetry in striatal DA (Robinson and Becker, 1986), this is indirect evidence of enhanced DA activity.

Studies to identify where estradiol acts in the brain of adult rats have previously reported that the striatum is not an area that concentrates estradiol in the nucleus or that expresses ER α mRNA (Pfaff and Keiner, 1973; Shughrue et al., 1997). However, ER α mRNA is present in the striatum during development at days 10–12 in the female rat, and there is specific binding to ER at this time (Toran-Allerand et al., 1992). In animals expressing AAV.ER α , shown in Figure 4, it is intriguing that there is little nuclear localization of the ER α . This suggests that the protein is treated differently in the striatum than in other brain regions (e.g., in the ventromedial hypothalamus) where expression is seen primarily in the nucleus (Musatov et al., 2007). Immunocytochemistry for ER α protein in the striatum of control rats finds low levels of protein immunoreactivity associated with striatal neuronal membranes of females (preliminary results from the Becker laboratory), but with the high background seen in striatum, images are not conclusive in animals not expressing ER α . Our results from Western blots indicate that there is a low level of endogenous ER α expression in striatum.

It is now apparent that all steroid hormones can have both long-term and rapid effects on target tissues (Hammes, 2003). The intracellular receptors mediating the long-term effect of steroid hormones through initiation of transcription have been known for many years (Greene et al., 1986). Alternate forms of estradiol receptors have also been identified that include ER β (Kuiper et al., 1996) and ER-X (Toran-Allerand et al., 2002; Toran-Allerand, 2004, 2005). Furthermore, there is now evidence for functional interaction between the membrane and nuclear receptors (Levin, 2005; Pedram et al., 2006).

The recent finding that there are membrane progesterin receptors in fish, with homologous genes found in humans and mice raises the possibility that there may be other families of receptors involved in the rapid signaling effects of other steroid hormones as well (Zhu et al., 2003a,b). These novel progesterin receptors code proteins that appear to have seven transmembrane domains characteristic of G-protein-coupled receptors. Further research will be required to determine if there are estrogen receptors homologous to these progesterin receptors.

Results reported here are consistent with a role for ER α in mediating the rapid behavioral and neurochemical effects of estradiol in the striatum. It is possible that other ERs may participate in the endogenous effects of estradiol in the striatum. The results reported here demonstrate that over-expressed ER α enhances the behavioral and neurochemical effects of estradiol that are usually seen. These results support the idea that ER α is involved in the effect of estradiol on striatal-mediated behaviors.

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