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Oestradiol Increases Phosphorylation of a Dopamine- and Cyclic AMP-Regulated Phosphoprotein (DARPP-32) in Female Rat Brain

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Abstract

Recent studies suggest that oestrogen and progestin receptors may be activated by the neurotransmitter dopamine, as well as by their respective ligands. Because intracerebroventricular infusion of D_1, but not D_2, dopaminergic receptor agonists increases oestrous behaviour in oestradiol-primed rats, we wanted to determine if treatment with oestradiol alters the activity of D_1 receptor-associated processes in steroid receptor-containing areas in female rat brain. One D_1 receptor-associated phosphoprotein that may be influenced by oestradiol is a dopamine- and cyclic *A*MP-regulated phosphoprotein, $M_r = 32\,000$ (DARPP-32). Because DARPP-32 is phosphorylated in response to dopamine acting via a cAMP-dependent protein kinase, it provides a useful marker to examine where in the brain a particular stimulus might be altering the activity of D_1 receptor-containing neurones. To determine if oestradiol alters the phosphorylation of DARPP-32, we stained immunocytochemically brain sections of female rats treated with behaviourally relevant doses of oestradiol or oil vehicle with an antibody that detects only the threonine 34-phosphorylated form of DARPP-32. Behaviourally effective doses of oestradiol increase the phosphorylation of DARPP-32 within the medial preoptic nucleus, bed nucleus of the stria terminalis, paraventricular nucleus of the hypothalamus and the ventromedial nucleus of the hypothalamus, 48 h after treatment. These data suggest that oestradiol increases the activity of D_1 dopamine receptor-associated processes in oestrogen receptor-containing areas of female rat forebrain.

Ovarian steroid hormones affect both behaviour and physiology in female rats in some cases by binding to intracellular neuronal receptors (1, 2). Upon binding, steroid hormones cause a conformational change in their receptors that may allow the activated steroid-receptor complex to bind to DNA where it can interact with various combinations of coregulatory proteins to influence genomic transcription (3, 4). The outcome of steroid hormone action includes changes in second messenger systems (5), neurotransmitter/peptide receptor levels (6, 7), neurotransmitter release (8) and oestrous behaviour in rodents (9, 10).

Recent studies suggest that steroid receptors may also be activated *in vitro* (11) and *in vivo* (12) in the absence of ligand by the neurotransmitter dopamine. Indeed, intracerebroventricular infusion of D_1, but not D_2, dopaminergic receptor agonists increases oestrous behaviour in oestradiol-primed rats, and treatment with progesterone antagonists or antisense oligonucleotides directed at the progestin receptor mRNA blocks this facilitation (12). Because treatment with dopamine D_1 agonists (12, 13) increases oestrous behaviour in oestradiol-primed rats, we wanted to determine if oestradiol alters the activity of D_1 dopamine receptor-associated processes in ovarian steroid receptor-containing areas of rat brain. One way to determine if steroid hormones alter the activity of D_1 dopamine receptors is to examine the phosphorylation state of particular proteins that are associated with D_1 dopamine receptors following treatment with steroid hormones. One D_1 dopamine receptor-associated phosphoprotein that may be influenced by steroid hormone treatment is a dopamine- and cyclic AMP-regulated phosphoprotein, $M_r = 32\,000$ (DARPP-32) (14, 15).

DARPP-32 is phosphorylated on threonine^{34} in response to dopamine acting via a cAMP-dependent protein kinase (PKA). Its phosphorylation is increased by D_1, but not D_2,

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dopamine receptors in rat brain (16–18). Stimulation of D_1 dopamine receptors, which activates adenylyl cyclase, increases cAMP levels and thereby activates PKA. One outcome of PKA activation in D_1 dopamine receptor-containing neurones is the phosphorylation of DARPP-32 (19, 20). DARPP-32, when phosphorylated by PKA at threonine[34], inhibits protein phosphatase-1 (21) resulting in an increase in phosphorylation of other phosphoproteins. An interesting feature of DARPP-32 is that it is present in virtually all D_1 receptor-containing neurones; however, not all DARPP-32 containing neurones located in the caudate-putamen also contain D_1 dopamine receptors (22). Because DARPP-32 is phosphorylated in response to dopamine acting via PKA, and more than 50% of DARPP-32 containing neurones also contain D_1 dopamine receptor-immunoreactivity (22), phospho-threonine[34]-DARPP-32 (pDARPP-32) immunoreactivity may provide a useful marker to examine where in the brain a particular stimulus alters the activity of D_1 dopamine receptor-containing neurones. Recently, it was shown with Western blot analysis that oestradiol treatment increases the phosphorylation of DARPP-32 on threonine[34] in mouse brain (23). In the present study, we used an immunocytochemical technique, which allows for more anatomical resolution, in order to determine if and in which neuroanatomical areas, oestradiol treatment alters the phosphorylation of DARPP-32 in female rat brain. Therefore, we immunocytochemically stained brain sections from rats treated with a behaviourally relevant dose of oestradiol or vehicle with an antibody that detects the threonine[34] phosphorylated form of DARPP-32 (24).

Materials and methods

Analysis of pDARPP-32 immunoreactivity

Animals

Female Sprague-Dawley rats (200–250 g) obtained from Charles River Breeding Laboratories, Inc (Wilmington, MA, USA) were group-housed for 1 week in a 14 : 10 light : dark cycle. All rats were then ovariectomized under methohexital sodium anaesthesia (52 mg/kg body weight, Brevital, Eli Lilly and Co., Indianapolis, IN, USA) prior to experiment.

Hormone treatment

One week following surgery, rats were injected with either 5 µg of oestradiol benzoate (dissolved in 0.1 ml sesame oil; n = 5) or oil vehicle (n = 6). The rats were perfused 48 h after injection.

Perfusion

Rats were anaesthetized with sodium pentobarbital (89 mg/kg) and chloral hydrate (425 mg/kg) dissolved in saline. The heart was then exposed, and the left ventricle was injected with 5000 units of sodium heparin dissolved in 1 ml of saline (0.15 M). The right atrium and the left ventricle were incised prior to insertion of a cannula, through the incision of the left ventricle, into the aorta. Seventy-five ml of saline preceded the flow of 250 ml of fixative (2% acrolein in 0.1 M sodium phosphate buffer; pH 7.2) through the cannula. Perfusion pressure was maintained at 100 mmHg with a flow rate of 25 ml/min for 10 min. After the brains were removed and blocked, they were placed into 0.1 M sodium phosphate buffer (pH 7.2) containing 20% sucrose overnight at 4°C. Forty µm sections from the preoptic area to the midbrain region were cut on a freezing microtome and placed into cryoprotectant (25) at −20°C until processed for immunocytochemical detection of the pDARPP-32.

pDARPP-32 immunocytochemistry

For each animal, every fourth section was removed from cryoprotectant and rinsed three times for 5 min each in Tris-buffered saline (TBS; pH 7.6). Sections were then pretreated in 1% sodium borohydride for 10 min to remove residual aldehydes. Following pretreatment and three additional rinses in TBS for 5 min each, sections were placed into 1% H_2O_2, 20% normal goat serum, and 1% bovine serum albumin for 20 min to reduce nonspecific staining and endogenous peroxidase activity. Sections were then incubated with pDARPP-32 antibody (mAB-23; 1 : 10 000 dilution), which was generated against a 10-amino acid peptide encompassing the sequence of rat DARPP-32 phosphorylated on threonine[34] that is selective for the phosphorylated form of DARPP-32 (24), diluted in modified TBS (4°C) containing 0.1% gelatin, 0.02% sodium azide, 0.5% Triton X-100 and 1% normal goat serum for 3 days at 4°C. Following three rinses with the above modified TBS buffer for 5 min each, the tissue sections were then incubated in a secondary antiserum (7.5 µg/ml of biotinylated goat antirabbit IgG; Vector Laboratories, Burlingame, CA, USA) diluted in the modified TBS for 90 min at room temperature. Sections were then rinsed twice in the modified TBS and once in TBS for five minutes each prior to incubation in the DH:biotinylated horseradish peroxidase H complex (1 : 100 in TBS; Vectastain Elite Kit; Vector Laboratories) for 90 min. Following three additional rinses in TBS of 5 min each, sections were treated with 0.05% diaminobenzidine and 0.05% H_2O_2 in TBS for 5 min. Immediately after diaminobenzidine treatment, sections were rinsed three times for 5 min each in TBS and mounted onto glass slides with Permount (Fisher Scientific, Pittsburgh, PA, USA). Omission of pDARPP-32 antibody from the procedure resulted in no immunostaining by the secondary antibody.

Computer-aided image analysis

A Leitz Dialux 20 microscope (Ernst Leitz Wetzler GMBH, Germany) was fitted with a MTI CCD72 camera (DAGE MTI, Michigan City, IN, USA) connected to a Macintosh Quadra 700 (Apple Computer, Cupertino, CA, USA). The software used for image analysis was the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Prior to tissue examination, the microscope was adjusted for Kohler illumination using 10×10 magnification. The minimum and maximum particle size to be analysed was set to 10 and 200 pixels, respectively. A microscope slide with a black dot was placed under the microscope and viewed on the monitor. The camera gain and black levels were adjusted so that black was at the maximum grey level of 255 pixel density and a clear portion of the slide was within single pixel digits. This allowed the grey level range of pDARPP-32 immunoreactivity to be from 1 to 255 pixel density. Once the camera gain and black levels were adjusted, they remained calibrated for all sections to be analysed through a given area. Average pixel density for each section was determined by averaging the mean pixel density and standard deviation for the area to be analysed. The density threshold option was then set to a maximum of five standard deviations above the mean pixel density and to a minimum of one pixel density, and then a new background mean and standard deviation was determined with this setting, as now foreground immunostaining no longer factored into the average background pixel density. Finally, the density threshold option was set to a minimum of five standard deviations above the new average background pixel density and a maximum of 255 pixel density. This procedure effectively determined the mean pixel density of the background area excluding the contributions of foreground immunoreactivity. One carefully matched section for each area per animal was then analysed bilaterally making adjustments for background variations only, using the above procedure with the experimenter blind to treatment groups.

Neuroanatomical areas analysed

Representative sections of neuroanatomical areas involved in reproduction, which also contain high levels of ovarian steroid receptors, were matched using the rat brain atlas of Paxinos and Watson (Fig. 1A–D). The areas examined were the posterodorsal part of the medial amygdaloid nucleus (MePD), medial preoptic nucleus (MPO), medial division of the bed nucleus of the stria terminalis (BSTM), paraventricular hypothalamic nucleus (Pa) and a rostral and caudal ventromedial nucleus of the hypothalamus (Fig. 1). In the rostral section of the ventrolateral ventromedial nucleus of the hypothalamus (rVMHVL), the progestin receptor-immunoreactive cells are not confined to this nucleus; rather the progestin receptor-immunoreactive cells range from the nissl-defined rVMHVL and extend dorsally toward the fornix. Thus, we defined this area as the ovarian steroid hormone (i.e. oestrogen and progestin) receptor-containing area associated with the rVMHVL that extends dorsally (rVMHVL-ORA) (26). In the caudal section, only the caudal ventrolateral aspect of ventromedial hypothalamus (cVMHVL) was analysed, as ovarian steroid receptors are confined to this region.

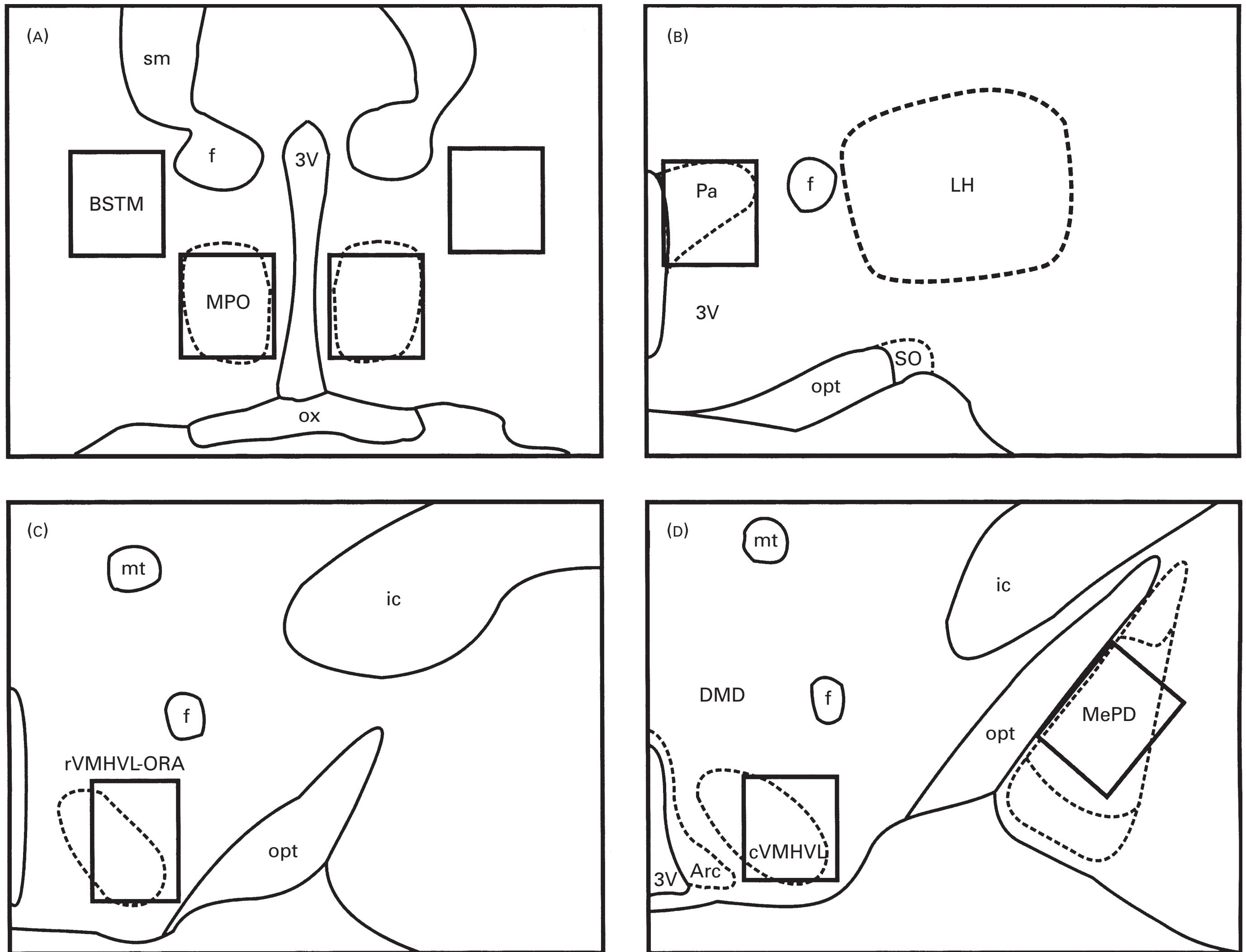

Fig. 1. Schematic drawings of areas in which pDARPP-32 immunoreactive cells were counted from the (a) medial preoptic nucleus (MPO); medial division of the bed nucleus of the stria terminalis (BSTM). (b) Paraventricular hypothalamic nucleus (Pa). (c) Ovarian steroid receptor-containing area associated with the rostral ventrolateral ventromedial nucleus of the hypothalamus (rVMHVL-ORA). (d) Caudal ventrolateral ventromedial nucleus of the hypothalamus (cVMHVL); posterodorsal part of the medial amygdaloid nucleus (MePD). f, fornix; 3V, third ventricle; SM, stria medullaris of the thalamus; ox, optic chiasm; SO, supraoptic nucleus; LH, lateral hypothalamic area; DMD, diffuse part of the dorsomedial hypothalamic nucleus; Arc, arcuate nucleus; mt, mammillothalamic tract; ic, internal capsule; opt, optic tract.

DARPP-32 and pDARPP-32 Western analysis

Animals

One week following ovariectomy, adult female rats were injected with either 5 µg of oestradiol benzoate (dissolved in 0.1 ml sesame oil, n = 3) or oil vehicle (0.1 ml sesame oil, n = 3). Approximately 48 h later, the rats were decapitated, and cortical and hypothalamic-preoptic tissue were dissected out at 0–4°C as described previously (27) and placed on dry ice.

Western immunoblotting protocol

The frozen tissue was homogenized by sonication in boiling 1% SDS, then boiled for an additional 10 min at 100°C. The protein concentration of each sample was determined as described previously (28). Four hundred µg of total protein of each sample were diluted with a SDS-polyacrylamide (PAGE) sample buffer containing 50 mM Tris/HCl, pH = 6.7, 10% glycerol, 2% SDS, 10% 2-mercaptoethanol (final concentration), and 0.01% bromophenol blue. Proteins were separated by SDS-PAGE on 12% polyacrylamide gels (Fisher Scientific) (29), then transferred to nitrocellulose membranes (0.2 m pore size) (30).

Briefly, nitrocellulose membranes were immunoblotted using the monoclonal antibody directed against the phosphorylated form of DARPP-32 (mAB-23). Membranes were blocked for 30–60 min in a solution (Blotto) containing phosphate buffered saline (PBS; 124 mM NaCl, 4 mM KCl, 10 mM Na_2HPO_4 and KH_2PO_4, pH = 7.2), 5% nonfat dry milk and 0.2% Tween-20. Membranes were then transferred to Blotto containing mAB-23 (1 : 500 dilution), incubated for 2 h then rinsed three times (10 min each) with PBS containing 0.2% Tween-20. The final rinse solution was replaced with Blotto containing goat antimouse HRP-linked IgG (1 : 8000 dilution; Pierce Chemical Co., Rockford, IL, USA), and the membranes were incubated for an additional 30 min. They were then rinsed several times (10 min per rinse) with PBS containing 0.2% Tween-20, and antibody binding was detected using enhanced chemiluminescent detection followed by exposure to X-ray film. pDARPP-32 immunoreactivity was quantified using a BioRad Video densitometer and 1D Analyst software (BioRad Laboratories, Richmond, CA, USA).

The membranes were then stripped of primary and secondary antibody by incubation at 50°C for 60 min in a solution containing 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH = 6.7. After rinsing the membranes (two times for 10 min in PBS containing 0.2% Tween) and

blocking for 30 min in Blotto, the membranes were immunoblotted to determine total DARPP-32 levels. For this purpose the membranes were incubated (1 : 8000 dilution) with a monoclonal antibody which detects DARPP-32, but which does not differentiate between phosphorylated and unphosphorylated forms of the protein. This antibody (C246a) has been previously characterized (31). The membranes were rinsed (two times for 10 min each in PBS containing 0.2% Tween), then incubated in Blotto containing rabbit antimouse secondary antibody (1 : 1000 dilution; Calbiochem, La Jolla, CA, USA) for 30 min. After rinsing (two times for 10 min each) incubation was continued in ^{125}I protein A (0.1 μCi/μl specific activity; 1 : 1000 dilution; New England Nuclear) in Blotto for 2 h. The membranes were rinsed extensively in PBS containing 0.2% Tween (five to six times for 10 min each), dried, and exposed for PhosphorImager analysis. The data are presented as arbitrary density units ±SEM.

Statistical analysis

Bilateral measurements of each area were pooled and analysed using SigmaStat Statistical Analysis System 1.01 (Jandel Scientific, Corta Madera, CA, USA). Immunocytochemical results were analysed using a *t*-test at $P<0.05$. Western data from two different experiments ($n=3$ and $n=4$ per group in each experiment) were considered statistically significant with a *t*-test at $P<0.05$.

Results

pDARPP-32 immunoreactivity

The number of pDARPP-32 immunoreactive cells was increased dramatically by oestradiol treatment in the medial preoptic area (143%), bed nucleus of the stria terminalis (164%), caudal ventromedial hypothalamus (134%) and the paraventricular hypothalamic nucleus (111%) when contrasted to oil-injected controls (Fig. 2; $P<0.05$; Fig. 3). However, treatment with oestradiol was without statistically significant effect on pDARPP-32 immunoreactivity within the rostral ventromedial hypothalamic area and the posterodorsal part of the medial amygdaloid nucleus ($P>0.05$).

DARPP-32 and pDARPP-32 immunoblots

Since the pDARPP-32 antibody detects several high molecular weight cross-reactive protein bands in addition to pDARPP-32, it was important to confirm that increased pDARPP-32 immunoreactivity was associated with an actual increase in DARPP-32 phosphorylation. This was done by performing Western blot analysis of hypothalamus from oestradiol-treated and oil-treated control rats to measure levels of DARPP-32 and pDARPP-32 in these rats.

A protein band with apparent M_r of approximately 34 000 was revealed by the monoclonal antibody (mAB-23) which detects the phosphorylated form of DARPP-32 (24). There was a two-fold increase in phosphorylated DARPP-32 in hypothalamic tissue of oestradiol-treated female rats compared to vehicle-treated controls. However, oestradiol treatment did not affect the phosphorylation of DARPP-32 in the cerebral cortex (Fig. 4). Analysis of bands identified with the monoclonal antibody, C246a, which does not differentiate between phosphorylated and unphosphorylated forms of the protein (31), suggests that there were no significant changes in total levels of DARPP-32 in hypothalamic or cortical tissue after oestradiol treatment compared to vehicle-injected controls.

Discussion

Treatment with behaviourally effective doses of oestradiol increased the immunostaining for the threonine^{34}-phosphorylated form of DARPP-32 within the medial preoptic area, bed nucleus of the stria terminalis, caudal ventromedial hypothalamus and the paraventricular hypothalamic nucleus of female rats contrasted to vehicle-injected controls (Fig. 2).

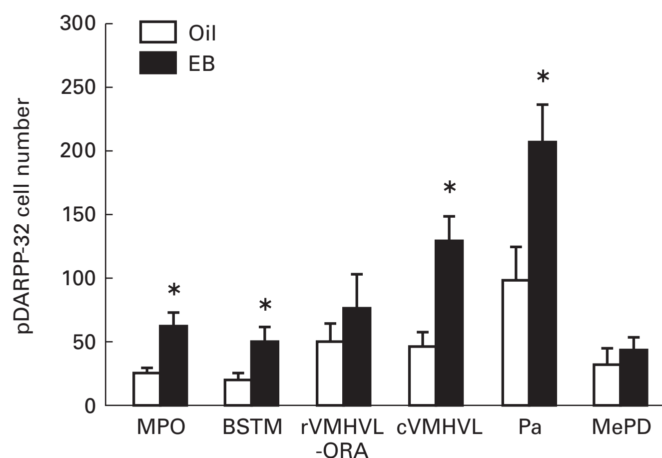

Fig. 2. Mean number (±SEM) of pDARPP-32 immunoreactive cells counted bilaterally for each area from vehicle (Oil) or oestradiol benzoate-treated (EB) treated rats. *$P<0.05$.

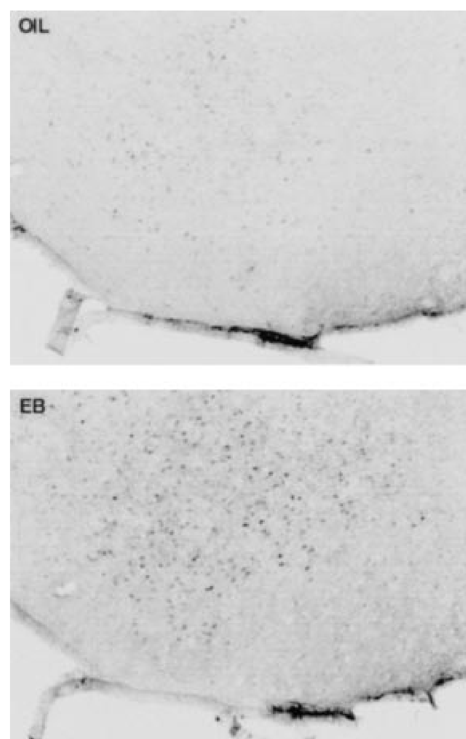

Fig. 3. Photomicrographs of pDARPP-32 immunoreactive cells within the caudal ventrolateral ventromedial nucleus of the hypothalamus from vehicle (Oil) or oestradiol benzoate-treated (EB) treated rats.

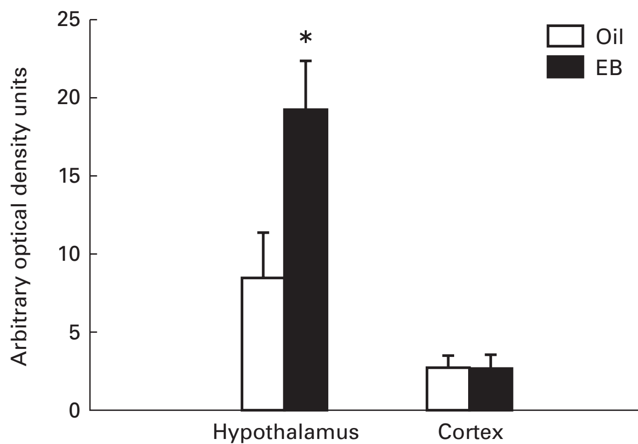

Fig. 4. The pDARPP-32 Western blot analysis of pDARPP-32 immunoreactivity in hypothalamic tissue presented as mean number of arbitrary density units ±SEM from vehicle (Oil) and oestradiol benzoate-treated (EB) treated rats. $^{*}P < 0.05$.

However, treatment with oestradiol had no statistically significant effect on pDARPP-32 immunoreactivity within the rostral ventromedial hypothalamic area or the posterodorsal part of the medial amygdaloid nucleus.

We found that areas expressing high levels of oestradiol-induced progestin receptors, such as the medial preoptic area, bed nucleus of the stria terminalis and caudal ventromedial hypothalamus, exhibited increased phosphorylation of DARPP-32 following oestradiol treatment. The posterodorsal part of the medial amygdaloid nucleus, an area that expresses only high levels of oestrogen receptors, did not exhibit an increase in DARPP-32 phosphorylation following oestradiol treatment. Therefore, oestradiol-induced phosphorylation of DARPP-32 correlates more with the distribution of progestin receptors rather than the distribution of oestrogen receptors. The two exceptions are the paraventricular nucleus and the rostral portion of the ventromedial hypothalamus. Oestradiol treatment increased the phosphorylation of DARPP-32 within the paraventricular nucleus; however, this area does not express progestin receptors nor is it critically involved in steroid-mediated sexual behaviour (32). Although oestradiol treatment increased the phosphorylation of DARPP-32 within the caudal portion of the ventromedial hypothalamus, it did not increase the phosphorylation of DARPP-32 within the rostral portion of the ventromedial hypothalamus. This differential sensitivity is consistent with other data showing differences in dopamine-related outcomes within the caudal versus rostral ventromedial hypothalamus, suggesting a level of specificity between these two related regions. For example, we have previously reported that vaginal-cervical stimulation increases the phosphorylation of DARPP-32 within the caudal but not the rostral portion of the ventromedial hypothalamus (33); however, both areas exhibit increased Fos protein expression following vaginal-cervical stimulation, suggesting that both areas do respond to vaginal-cervical stimulation (26). In addition, injection of the dopamine D1 receptor agonist, SKF-38393, increases the expression of Fos protein within the caudal but not rostral portion of the ventromedial hypothalamus in oestradiol-primed female rats (34). These studies suggest that the caudal portion of the ventromedial hypothalamus is more sensitive to changes in dopamine-related pathways involved in sexual behaviour contrasted to the rostral portion of the ventromedial hypothalamus.

The specificity of the monoclonal antibody (mAB-23) generated against a 10-amino acid peptide encompassing the sequence of rat DARPP-32 phosphorylated on threonine[34] was confirmed in immunoblot assays for pDARPP-32. Consistent with the immunocytochemical results, the immunoblots for pDARPP-32 indicated a two-fold increase in phosphorylated DARPP-32 within the hypothalamus of oestradiol-treated female rats contrasted to vehicle treated controls (Fig. 4). However, oestradiol did not increase the phosphorylation of DARPP-32 in cerebral cortex. To determine if there were changes in total DARPP-32 levels, nitrocellulose membranes were incubated with a monoclonal antibody (C246a) which does not differentiate between phosphorylated and unphosphorylated forms of the protein (31). There were no significant changes in the levels of DARPP-32 in either hypothalamic or cortical tissue after oestradiol treatment compared to vehicle controls.

There are several possible explanations for altered phosphorylation of DARPP-32 by oestradiol in female rat brain. One possible way that oestradiol could influence the phosphorylation of DARPP-32 in rat brain is by altering the number of available D_1 dopamine receptors. Previous studies suggest that treatment with oestradiol influences both the density and distribution of D_1 dopamine receptors in male (35, 36) and female rat brain (37). During the oestrous cycle, striatal D_1 dopamine receptor density is maximal on diestrous II when oestradiol levels are increasing (37). In a recent study, oestradiol treatment increased the expression of D_{1B} dopamine receptors in neonatal rat hypothalamic cell cultures (38). More importantly, the D_{1B} dopamine receptors that are increased by oestradiol appear to be constitutively active and generate cAMP in a ligand-independent manner, which could result in the phosphorylation of DARPP-32. Therefore, increases in the phosphorylation of DARPP-32 by oestradiol may be due to increased expression of D_1 dopamine receptors. Another possibility is that oestradiol action alters the linkage of D_1 dopamine receptors to signal transduction pathways that affect the phosphorylation of DARPP-32. Changes in coupling of noradrenergic receptors to cAMP production have been reported to occur following treatment with steroid hormones (39). Alternatively, oestradiol could increase the phosphorylation of DARPP-32 by altering dopamine release (40–42). Oestradiol treatment of extracted hypothalamic tissue increases dopamine release within 2 h (40, 41). Furthermore, treatment with behaviourally effective doses of oestradiol increases dopamine content in the medial basal hypothalamus of female rats within 48–96 h (42). Therefore, oestradiol may increase phosphorylation of DARPP-32 by increasing the expression of D_1 dopamine receptors, the release of dopamine, the coupling of dopamine receptors to G-proteins, or some combination of these factors. It will be interesting to determine if DARPP-32 is phosphorylated in response to oestradiol treatment in neurones that contain oestrogen receptors or in neurones that receive afferent input from oestrogen receptor-containing neurones.

Although dopamine is a likely candidate by which oestradiol can influence the phosphorylation of DARPP-32 in female rat brain, it is possible that factors other than dopamine may influence the phosphorylation of DARPP-32 in the hypothalamus. For example, DARPP-32 is phosphorylated in the epithelial cells of the choroid plexus following treatment with forskolin, isoproterenol, vasoactive intestinal peptide, atrial natriuretic peptide, or 5-HT, but not following treatment with dopamine (24). Moreover, it was recently reported that activation of adenosine A_{2a} receptors leads to the phosphorylation of DARPP-32 in striatal neurones (43); however, there are no adenosine A_2 receptors found within the hypothalamus (44, 45).

Another possibility for DARPP-32 phosphorylation is via a nitric oxide/cGMP mediated pathway (46). Treatment with either the nitric oxide donor, sodium nitroprusside, or 8-bromo-cGMP can increase the phosphorylation of DARPP-32 (46). Furthermore, oestradiol treatment can increase nitric oxide synthase within the ventromedial hypothalamus (47), and nitric oxide synthase is coexpressed with oestrogen (48) and progestin (49) receptors. Both cGMP (50) and nitric oxide (51) increase female sexual behaviour in oestradiol-primed female rats, and the increase in sexual behaviour by cGMP appears to activate progestin receptors in a ligand-independent manner (50). Therefore, it is possible that oestradiol can influence sexual behaviour and the phosphorylation of DARPP-32 in female rats by altering the activity of nitric oxide/cGMP pathways within steroid receptor-containing regions.

Dopamine D_1 receptor binding (52), D_1 receptor immunoreactivity (53, 54) and D_1 mRNA (55, 56) have been localized within the amygdala, preoptic area, and ventromedial hypothalamus, all of which also contain pDARPP-32 immunoreactivity. While this does not confirm that dopamine is responsible for the phosphorylation of DARPP-32 within these areas following steroid hormone treatment, it is consistent with this possibility. Although the amygdala contains both D_1 dopamine receptors and DARPP-32, oestradiol treatment does not significantly increase pDARPP-32 immunoreactivity at 1 h within this area. It is possible that oestradiol may increase pDARPP-32 immunoreactivity at a different time point than the one examined. The lack of an increase in pDARPP-32 immunoreactivity in the amygdala also demonstrates the heterogeneous response to oestradiol in rat brain. Indeed, oestradiol induced progestin receptor-immunoreactivity in fewer neurones within the amygdala than within the preoptic area and ventromedial hypothalamus.

It is not clear what the consequences might be for the increased phosphorylation of DARPP-32 by oestradiol in steroid receptor-containing areas. If the phosphorylation of DARPP-32 is in fact a marker for dopamine D_1 receptor activation, and D_1 receptor activation leads to progesterone-independent activation of progestin receptors, then perhaps oestradiol treatment leads to activation of progestin receptors in a ligand-independent manner to influence neuronal changes. Indeed, it was recently reported that oestradiol treatment increases the release of serum follicle-stimulating hormone (57) or luteinizing hormone (58) in female rats, and that these increases can be blocked with progestin antagonists. The authors suggest that the progestin antagonist, RU 486, may block progesterone-independent activation of progestin receptors, as the decreases in gonadotropin secretion are seen in the absence of ovarian progesterone. The release of luteinizing hormone-releasing hormone is also under the influence of nitric oxide. Oestradiol increases nitric oxide synthase, and nitric oxide stimulates cGMP, which has been shown to activate progestin receptors in a ligand-independent manner (59). Nitric oxide/cGMP pathways also increase the phosphorylation of DARPP-32 (46). Therefore, it is possible that oestradiol influences the release of luteinizing hormone via stimulation of nitric oxide/cGMP pathways, phosphorylation of DARPP-32, and activation of progestin receptors in a ligand-independent manner.

In contrast to the experiments demonstrating the dependence of oestradiol-induced changes in gonadotropin secretion on progestin receptors, sexual receptivity that is induced by oestradiol alone may not be dependent on progestin receptors (60). The fact that oestradiol-induced sexual behaviour is not inhibited by progesterone antagonists argues against the involvement of progestin receptors in oestradiol-induced sexual receptivity. In addition, mice that have a targeted disruption of the progestin receptor gene, and which do not express progesterone-facilitated sexual behaviour, do not differ from their wild type counterparts in their sexual behavioural response to high doses of oestradiol alone (61), suggesting again that progestin receptors are not essential for sexual behaviour induced by oestradiol alone. Interestingly, oestradiol-treated mice that have a targeted disruption of the gene encoding for DARPP-32 do not respond to progesterone with increased sexual behaviour (23). However, it is not known if these mice show disruptions in sexual behaviour induced by high doses of oestradiol.

In summary, our results indicate that oestradiol increases the phosphorylation of DARPP-32 in many areas of female rat brain suggesting that particular signal transduction pathways that are mainly associated with D_1 dopamine receptors can be altered by oestrogen. The results also suggest that particular behavioural and physiological events associated with the dopaminergic system can be affected by oestradiol. Finally, these data suggest that some of the effects of ovarian steroid hormones in the brain may be mediated by phosphorylation of DARPP-32.

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