A membrane-associated progesterone-binding protein, 25-Dx, is regulated by progesterone in brain regions involved in female reproductive behaviors

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The ventromedial hypothalamus (VMH) plays a central role in the regulation of the female reproductive behavior lordosis, a behavior dependent upon the sequential activation of receptors for the ovarian steroid hormones estradiol (E) and progesterone (P). These receptors function as transcription factors to alter the expression of target genes. To discover behaviorally relevant genes targeted by E and P in the VMH, we used the differential display PCR to identify messenger RNAs that are differentially expressed in the hypothalamus of ovariectomized (ovx) rats treated with E alone compared with ovariectomized rats treated with E and P. We show here that one interesting mRNA within the hypothalamus that is repressed by P after E priming encodes the protein 25-Dx, the rat homolog of the human membrane-associated P-binding protein Hpr6.6. Neurons in the brain containing the highest levels of 25-Dx are located in several nuclei of the basal forebrain, including the VMH. 25-Dx expression is also higher in the hypothalamus of female P receptor ''knockout'' mice than in their wild-type littermates. These findings suggest a mechanism in which the activation of nuclear P receptor represses expression of a membrane P receptor, 25-Dx, during lordosis facilitation.

Lordosis is a reproductive behavior, characterized by the rigid dorsiflexion of the spinal column and elevation of the hind quarters observed in mating female rodents. The lordosis posture allows intromission by the male and is due to elevated circulating levels of estradiol (E) and progesterone (P) from the ovaries, which occurs during proestrus, accompanied by gentle pressure on the vagina from the male (1). Lordosis is also observed in ovariectomized (ovx) females given exogenous hormone followed by gentle pressure on the perineum (1). This model system underscores the behavior's dependence on E, P, and tactile stimulation (2) and is widely used to examine the influence of E, P, and somatosensory effects on an easily identifiable reproductive behavior.

By tracing the somatosensory input, Pfaff *et al.* (2) have constructed a detailed map of the neural circuitry responsible for lordosis. One brain structure important for integrating the endocrine aspect with other neural events surrounding lordosis is the ventromedial hypothalamus (VMH) (2). The VMH contains an abundance of E and P receptors (2), ligand-activated transcription factors (3) that act within the nuclei of VMH neurons. Under a lordosis-producing hormone regimen, E, operating through the transcriptional activity of the activated estrogen receptor, has been shown to increase the transcription of oxytocin and the oxytocin receptor, gonadotropin-releasing hormone and the gonadotropin-releasing hormone receptor, preproenkephalin (PPE), the μ opioid receptor, and the progesterone receptor (PR) (4). The transcriptional targets of activated PR within this brain region have been more difficult to ascertain, but recent work has identified a few P-responsive genes in the brain that may reside within the lordosis pathway.

These include genes whose products play a general role in the cellular management of proteins and vesicles (e.g., Hsc73) (5) and others that may serve specific functions in intracellular trafficking in endocrine cells (e.g., SCAMP-25) (6). Here we have added to this list of P-responsive genes by performing the differential display technique on RNA isolated from the hypothalamus of rats treated with lordosis-producing amounts of E and P, and we report the discovery of an interesting P-repressed mRNA that encodes the protein 25-Dx (7), a putative membrane receptor for P.

Materials and Methods

Animals and Treatments. Intact and ovx adult female Sprague–Dawley rats were obtained from Charles River Breeding Laboratories and maintained on a 12-h light, 12-h dark schedule (lights on at 1000), with food and water supplied *ad libitum*. The ovx rats were allowed to rest for 10 days after surgery before any experimentation was performed. Hormone treatments of ovx rats were as follows: injections of estradiol benzoate (EB) (s.c.; 12.5 μg per rat) were followed 24 h later with injections of P (i.p., to guarantee a fast route; 500 μg per rat) (EB + P). Other groups of rats received either EB followed 24 h later with sesame oil vehicle (EB + vehicle) or vehicle only at both time points. Three hours after the final injection, all animals were CO_2 asphyxiated and killed. The estrous cycles of the intact rats were monitored by cytological examination of vaginal smears taken between 0900 and 1100. The animals were monitored for three consecutive 4-day cycles to ensure consistent normal reproductive physiology and killed on the afternoon (1400) of diestrus day 1. The brain from each animal was embedded in freezing media (TissueTek; Miles) for *in situ* hybridization or it was dissected and the hypothalamus was frozen in liquid nitrogen for RNA isolation.

PR ''Knockout'' (PRKO) Mice. Breeding pairs of mice were obtained from Lydon *et al.* (8), and a colony was established and housed with a 12-h light, 12-h dark schedule, with food and water supplied *ad libitum*. Genotyping was performed on tail DNA according to the method of Lydon *et al.* (8). All mice used in this

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study were adult (>12 weeks). To maintain consistent circulating hormone levels, the female mice were ovariectomized under metafane anesthesia, allowed to rest for 10 days before any experimentation was performed, and injected with hormone according to the regimen described above, except that 10 μg of EB and 500 μg of P per mouse were used. At the appropriate times, mice were killed by cervical dislocation, and the brain from each animal was obtained for RNA isolation.

RNA Isolation and Analysis. To obtain RNA for differential display PCR (DD-PCR) and Northern blots, the VMH was dissected from a 2-mm coronal section taken from the rostral edge of the optic chiasm. Anterior and lateral tissue was removed by cutting a trapezoid, around the third ventricle, inscribed by the fornix and the optic tracts. Total RNA from all tissues described above was then isolated by the guanidinium isothiocyanate method (9). To eliminate potential DNA contamination, RNA was treated with RNase-free DNase I (GenHunter, Nashville, TN). To isolate poly(A)$^+$ RNA, the total RNA was further purified with an Oligotex mRNA Kit (Qiagen, Chatsworth, CA). Northern blots were prepared by size fractionating 10 μg on 1.5% agarose gels containing 6.5% (1.1 M) formaldehyde, transferred to GeneScreen*Plus* nylon membranes (NEN/DuPont), and hybridized to randomly primed ^{32}P-labeled (Boehringer Mannheim) probe specific for the full-length cDNA of the 25-Dx gene. The blots were hybridized according to the supplier's instructions and washed at 65°C with a final stringency of 0.5× SSC, 1% SDS. The hybridization signal was quantified by PhosphorImager (Molecular Dynamics) analysis. Before the RNA was transferred to nylon membranes, the ethidium bromide-stained gels were photographed over UV light with Polaroid 665 positive/negative film. To correct for variations in the loading of RNA, the 18S rRNA bands visible in the negative image of the gels were quantified by densitometry (National Institutes of Health IMAGE). The PhosphorImager values were then divided by the densitometry values to normalize the 25-Dx signal. The results are expressed in arbitrary PhosphorImager units and presented as mean values obtained from three or more animals.

DD-PCR. The differential display technique was performed essentially as described by Liang and Pardee (10). Briefly, total RNA (100 ng) isolated from the VMH of the hormone-treated and control animals described above was reverse transcribed in a 20-μl reaction, using an anchored oligo(dT) primer (10) and Superscript-RT (Life Technologies, Grand Island, NY). One microliter of the reverse transcription reaction was used in PCR with arbitrary 10-mers and anchored oligo(dT) primers in the presence of [α-^{33}P]dATP according to the methods of Liang and Pardee (10, 11). The radioactive products were size fractionated on denaturing 6% polyacrylamide gels and visualized by autoradiography. The resulting autoradiographs were examined to locate cDNA bands that satisfy two criteria: first they must exhibit a differential intensity between treatment groups, relative to the majority that displayed a uniform intensity between groups; and second they must be present in each individual within the group. Adhering to these criteria ensured that the cDNAs chosen for further analysis were altered by the treatment of the animals and were not due to individual variation. Satisfactory bands were then cut from the dried polyacrylamide gels, reamplified by PCR (10), and cloned into the plasmid vector pCR-Script SK(+) (Stratagene) according to the supplier's instructions. The resulting clones were sequenced by the Protein/DNA Technology Center at Rockefeller University and confirmed to be differentially expressed by the "reverse Northern" slot blotting procedure of Liu and Raghothama (12). For this procedure, the plasmid clones were slot blotted (9), in duplicate, to GeneScreen*Plus* nylon membranes. The duplicate membranes were then hybridized separately to two different

cDNA probes synthesized by reverse transcriptase (Superscript-RT; Life Technologies) from poly(A)$^+$ RNA isolated from the VMH of rats treated with EB and EB + P. After hybridization, the blots were washed at 65°C to a final stringency of 0.1× SSC, 1% SDS and visualized by autoradiography. The DD-PCR clones that displayed a differential signal intensity in this analysis were considered "true positives." Clone identities were determined by performing BLAST searches against the GenBank database.

***In Situ* Hybridization.** The *in situ* hybridization methodology was essentially as described by Mello *et al.* (ref. 13; see also ref. 14). The riboprobe for 25-Dx was synthesized from the DD-PCR band cloned into pCR-Script SK(+), and the sequence corresponds to Fig. 1*e*. The PPE riboprobe was described (14). Slides containing brain tissue were hybridized and washed at 60°C and then treated with RNase (5 μg/ml; Boehringer Mannheim), to eliminate nonspecific binding. The final wash was performed at room temperature at a stringency of 0.1× SSPE (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA). After the signal was determined by PhosphorImager analysis, the slides were placed in contact with BioMax MR (Kodak) film and exposed for 1–2 weeks, and then dipped in photographic emulsion (NBT-2; Kodak) and exposed for 4 weeks in light-protected boxes at 4°C. Anatomically matched coronal brain sections were quantified by counting the number of silver grains per cell (100–120 cells per rat), using National Institutes of Health IMAGE software and the MACRO program of Beykirch (http://www.zippy.nimh.nih.gov/nih-image/user-macros/grain_counting.txt).

Statistical Analysis. All results are presented as mean values ± SEM. Student's *t* tests were used to compare the mean differences between groups. Statistical significance was accepted at $P < 0.05$ (two-tailed).

25-Dx/Green Fluorescent Protein (GFP) Fusion Construction and Analysis. The rat 25-Dx cDNA sequence was analyzed by the PRIMER program (Whitehead Institute) to identify primers suitable for PCR from the sequence around the 5′ and 3′ ends of the 25-Dx coding region. The primers, 25DXGFP-F1 (5′-TAGAATTCTC-CAGAGATCATGGCTGC-3′) and 25DXGFP-R1 (5′-TAG-GATCCCATGTTGTAAAGACAGACTGG-3′), were synthesized by the Protein/DNA Technology Center at Rockefeller University. Reverse transcription–PCR was performed on total RNA isolated from rat liver, and the resulting 693-bp fragment was directionally cloned into pEGFP-N1 (CLONTECH) predigested with *Eco*RI and *Bam*HI. The resulting construct, pEGFP/25-Dx, was sequenced through the cloning junctions to verify proper fusion of the two reading frames. However, after preparation of this paper, it was learned that an error existed in the initial report of the 25-Dx sequence near the carboxyl terminus (E. Falkenstein and M. Wehling, personal communication). The corrected sequence of 25-Dx is reported in an updated GenBank entry (accession no. P70580). Despite the consequent error in our fusion construct, pEGFP/25-Dx was still informative in demonstrating the localization of 25-Dx to the cell membrane, as expected. Using the Qiagen Effectene Reagent, we transfected pEGFP-N1 (CLONTECH) and pEGFP/25-Dx into GT1–7 cells. The cells were cultured on glass coverslips and mounted on microscope slides according to instructions from CLONTECH. To locate the fusion protein, the transfected cells were visualized under UV light, using FITC optics. Photographs were obtained with the SPOT digital camera and annotated with Adobe PHOTOSHOP.

Results

25-Dx Gene Expression in the Female Rat Hypothalamus Is Repressed by P. Using the DD-PCR (10, 11) with 20 primer pairs, we have so far screened approximately 10% (≈3,000) of the unique

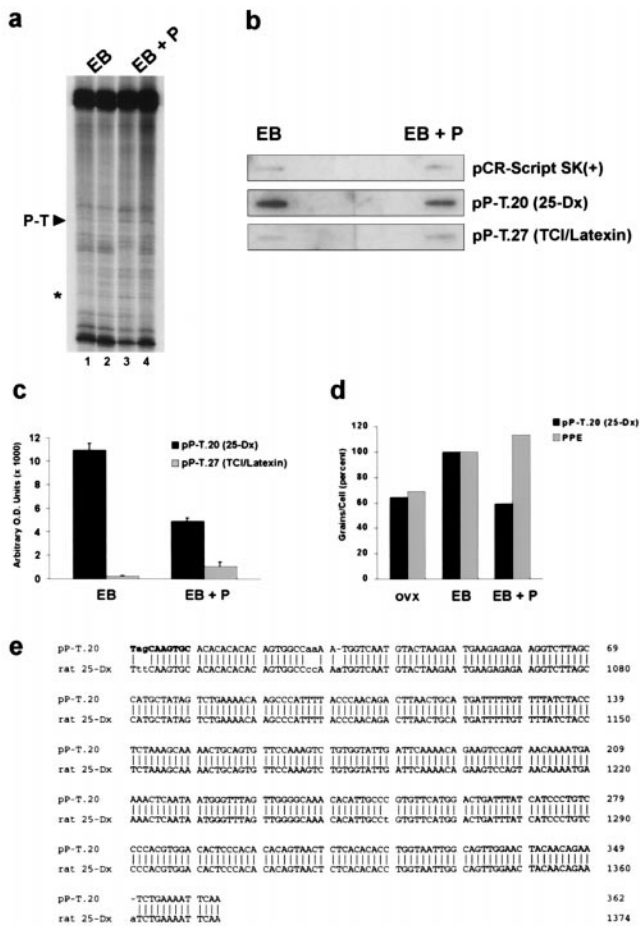<div style="text-align: right">NEUROBIOLOGY</div>

Fig. 1. Differential display identification of 25-Dx. (a) DD-PCR was performed on total hypothalamic RNA isolated from ovx female rats treated with EB (lanes 1 and 2) and EB + P (lanes 3 and 4) (see *Materials and Methods*). The band labeled P-T appears to be more intense in EB + P-treated animals than in animals treated with EB only. The asterisk (*) indicates another DD-PCR band obtained from this gel that was also differentially expressed (5). The cloning of band P-T into a plasmid vector resulted in two unique gene sequences. (b) Reverse Northern blot to elucidate the DD-PCR results. One of the transcripts (pP-T.27; tissue carboxypeptidase inhibitor/latexin) is up-regulated by P, in a pattern similar to that observed in the DD-PCR gel, whereas the other (pP-T.20; 25-Dx) is down-regulated by P. (c) Quantitative analysis of the results shown in b. Graphed are the means and standard errors from three separate slot blots hybridized with radiolabeled cDNA probes derived from two independent groups of hormonally treated animals. This precaution ensured replicatability of both the blotting and probe synthesis procedures. (d) 25-Dx expression is repressed by P after E priming. Riboprobe synthesized from pP-T.20 was hybridized *in situ* to the VMH of vehicle, vehicle + EB, and EB + P-treated ovx female rats. The results are expressed as the percentage of silver grains per cell normalized to animals treated with EB + vehicle (100%). (e) Alignment of the sequence of pP-T.20 with rat 25-Dx. The numbers correspond to the cDNA sequence according to Selmin *et al.* (7). Vertical lines indicate identity. The bold text indicates the sequence and location of the arbitrary primer, AP10, used in the differential display reaction.

mRNAs expressed in the female rat hypothalamus. Thus far, 32 cDNA bands displayed a differential intensity between ovx female rats treated with EB alone compared with ovx female rats treated with EB and P. Of these 32 cDNA bands, 28 were successfully cloned into the plasmid vector pCR-Script (Stratagene) and further evaluated by differential ''reverse Northern'' slot-blot analysis (12). Of these 28, 12 clones maintained a differential expression pattern. One of these 12, however, resulted in the isolation of two distinct cDNAs, pP-T.20 and

pP-T.27. Subsequent reverse Northern analysis revealed that the two cDNAs are differentially expressed (Fig. 1 *b* and *c*). The clones were sequenced, and a GenBank homology search was performed. One clone contained a sequence identical to tissue carboxypeptidase inhibitor (TCI)/latexin (15, 16), whereas the other contained a sequence identical to that of 25-Dx (7). TCI/latexin, the expression of which in the hypothalamus is substantially less than that of 25-Dx (Fig. 1*b* and data not shown), was increased by nearly 5-fold by P, whereas 25-Dx was reduced by 2.2-fold (Fig. 1*c*). Here we focused our attention on 25-Dx because previous biochemical data (17) indicate that it is a membrane-associated P-binding protein and thus more likely to be directly involved in facilitating steroid hormone responses in the brain than is TCI/latexin. The analysis of TCI/latexin will appear elsewhere.

To further evaluate the P-dependent expression of 25-Dx in the brain, we performed *in situ* hybridizations on ovx females treated with vehicle, EB + vehicle, and EB + P. Fig. 1*d* shows that the expression of 25-Dx in the VMH is enhanced by nearly 40% by EB; however, in animals treated with EB + P, 25-Dx expression is repressed to the level of animals not treated with hormone. This lower level of 25-Dx expression in EB + P-treated animals is consistent with the reverse Northern results (Fig. 1*c*) and further demonstrates that 25-Dx gene expression is repressed by P after EB treatment. Adjacent brain sections were also probed with PPE as a positive control, because this laboratory has shown that PPE expression is up-regulated in the VMH by EB (18) and remains elevated throughout proestrus (19). As expected, PPE mRNA levels are elevated by EB treatment and remain high in EB + P-treated animals (Fig. 1*d*).

25-Dx Is Expressed in Several Neuroendocrine Regions of the Brain. To identify additional sites within the female rat brain that express 25-Dx, we performed *in situ* hybridization studies on coronal sections from forebrain areas of bregma (-1.8 mm to the dorsal hypothalamus, bregma -3.3 mm) (20) of female rats in diestrus. Low expression was observed throughout the cortex and striatum (Fig. 2). However, the highest expression was observed in the zona incerta, amygdala, paraventricular nucleus, supraoptic nucleus, VMH, arcuate nucleus, and hippocampus (Fig. 2). No significant expression was observed in the cerebellum or other brain regions caudal to the third ventricle, including the ventral tegmental area (data from sagittal sections not shown). The lack of 25-Dx in the ventral tegmental area is of interest because the binding of P at the membrane in this region has been shown to facilitate lordosis (21). In addition, the failure to find 25-Dx mRNA in the cerebellum indicates that 25-Dx is probably not a component of membrane-associated P-binding complexes identified there (22).

25-Dx Gene Expression in the PRKO Mouse Hypothalamus Is Sexually Dimorphic. We began characterizing the 25-Dx gene in the context of nuclear PR by using Northern blots to examine 25-Dx expression in the hypothalamus of PRKO mice. Mice, like rats (7) and pigs (23), but in contrast to humans (24), have a single 1.9-kb transcript that corresponds in size to the cDNA isolated from rat liver. Humans have an additional 6-kb transcript not seen in these other species (24). In PRKO mice, a sexually dimorphic expression pattern was observed. In PRKO females, 25-Dx expression in the hypothalamus was significantly higher than in wild-type females, whereas expression in males did not vary with genotype (Fig. 3*b*). These results indicate that PR plays a gender-specific role in the regulation of 25-Dx expression in the mouse hypothalamus.

25-Dx Associates with the Cell Membrane of Neurons. The initial isolation of the porcine homolog of the 25-Dx protein involved chromatographic fractionation of liver membrane proteins
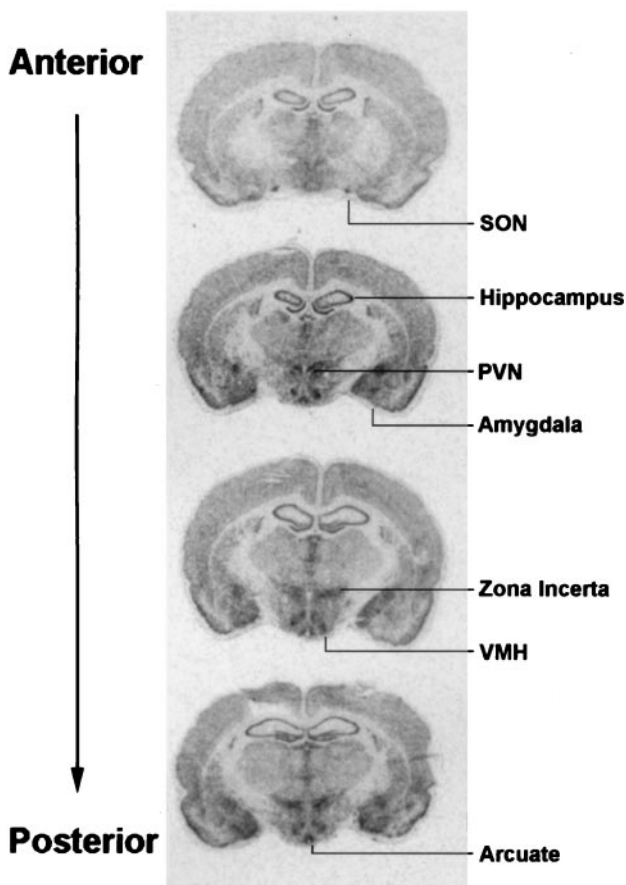
Fig. 2. 25-Dx is expressed in a variety of hypothalamic nuclei throughout the basal forebrain. *In situ* hybridization was performed on 10-μm coronal brain sections obtained from intact rats in diestrus. Representative sections are shown anterior to posterior (top to bottom). Dark areas indicate where 25-Dx expression is the highest. SON, supraoptic nucleus; PVN, paraventricular nucleus; VMH, ventromedial hypothalamus.

Fig. 3. The expression of 25-Dx is sexually dimorphic in the PRKO mouse hypothalamus. Northern blots were used to examine the expression of 25-Dx in the hypothalamus of male and female PRKO mice. (*a*) The expression of 25-Dx in the hypothalamus of female PRKO (ko) mice is higher than in wild-type (wt) littermates. The 25-Dx probe hybridizes to a single 1.9-kb transcript in the mouse hypothalamus (*Top*). The ethidium bromide-stained gels demonstrate the equal loading of RNA across lanes for the Northern blot (*Middle*) and the corresponding genotype of each animal, as determined by PCR (*Bottom*). (*b*) Quantitative analysis of Northern blots. Blots similar to the one shown in *a* were prepared for male (+/+, $n = 3$; +/−, $n = 12$; −/−, $n = 14$) and female (+/+, $n = 12$; +/−, $n = 13$; −/−, $n = 14$) PRKO mice and quantified by PhosphorImager analysis. *, $P < 0.05$ (two-tailed), **, $P < 0.001$ (two-tailed).

(17). Falkenstein *et al.* (25) further indicated subcellular localization to endoplasmic reticulum and Golgi structures of hepatocytes. To determine if a similar membrane-associated localization occurs in cells of neuronal origin, we fused the 25-Dx gene with the gene encoding the GFP and transfected the construct into GT1–7 cells in culture. GT1–7 cells are hypothalamic neurons immortalized by constitutive expression of the simian virus 40 large-T antigen under the control of the gonadotropin-releasing hormone promoter. The 25-Dx/GFP chimeric protein, which fluoresces green under UV light, is localized to the cell membrane (Fig. 4 *e* and *f*). This observation is in stark contrast to cells transfected with the GFP gene alone, in which fluorescence is seen throughout the cytoplasm (Fig. 4 *a–d*). Furthermore, the fluorescent areas of the membrane attributable to the 25-Dx/GFP fusion protein overlie the cell soma. A similar subcellular localization occurred in a transfected human neuroblastoma cell line, SH SY-5Y (data not shown).

Discussion

Steroid hormones, in general, interact with their receptors in the nuclei of steroid-responsive cells, and the activated receptors alter the expression of target genes (3). This genomic mechanism is temporally insufficient, however, to account for many of the rapid (<10 min) nongenomic effects of steroid hormones throughout the body. For example, P initiates the
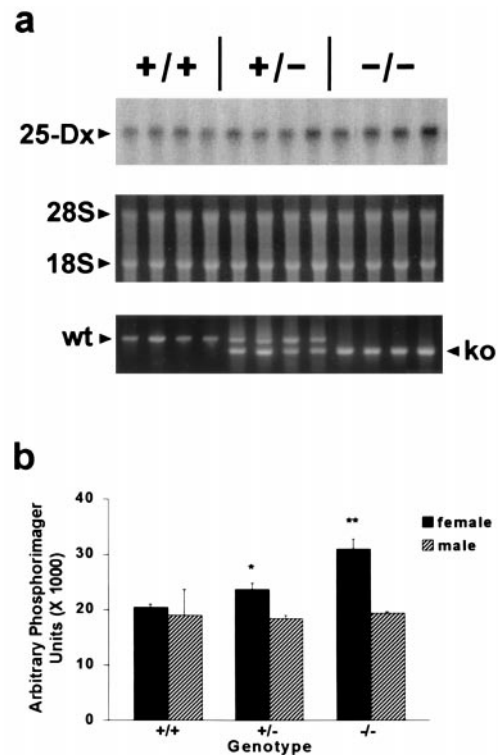
acrosomal reaction of sperm during conception (26), relaxes cardiovascular smooth muscle (27), and promotes the influx of calcium into hepatocytes (28). Progesterone also has analgesic properties (29) and rapid effects on female rodent reproductive behavior (30), both thought to be mediated by nongenomic membrane interactions (29, 30). Here we have shown that 25-Dx, a membrane-associated (Fig. 4) (25) P-binding protein (17), is expressed in several brain regions, including those known to facilitate female sexual behavior (Fig. 2) (2). Thus, 25-Dx appears to serve as a regulated membrane receptor for mediating nongenomic actions of P within neuroendocrine regions of the brain.

The 25-Dx gene encodes a 25-kDa protein localized to the plasma membrane (Fig. 4) (25) with a high affinity for P (17). It also has structural features similar to those of cytokine and peptide hormone receptors and is most closely related to the prolactin receptor (7). Given these receptor-like properties, one could view the P-mediated repression of 25-Dx (shown in Fig. 1 *c* and *d*) simply as evidence of feedback inhibition. For instance, when P binds 25-Dx at the cell surface, signal transduction cascades may terminate in the nucleus to attenuate transcription of the 25-Dx gene. This effect on transcription would effectively reduce the number of receptors at the cell surface and complete an autoregulatory feedback loop, typical of membrane receptors. However, our data from
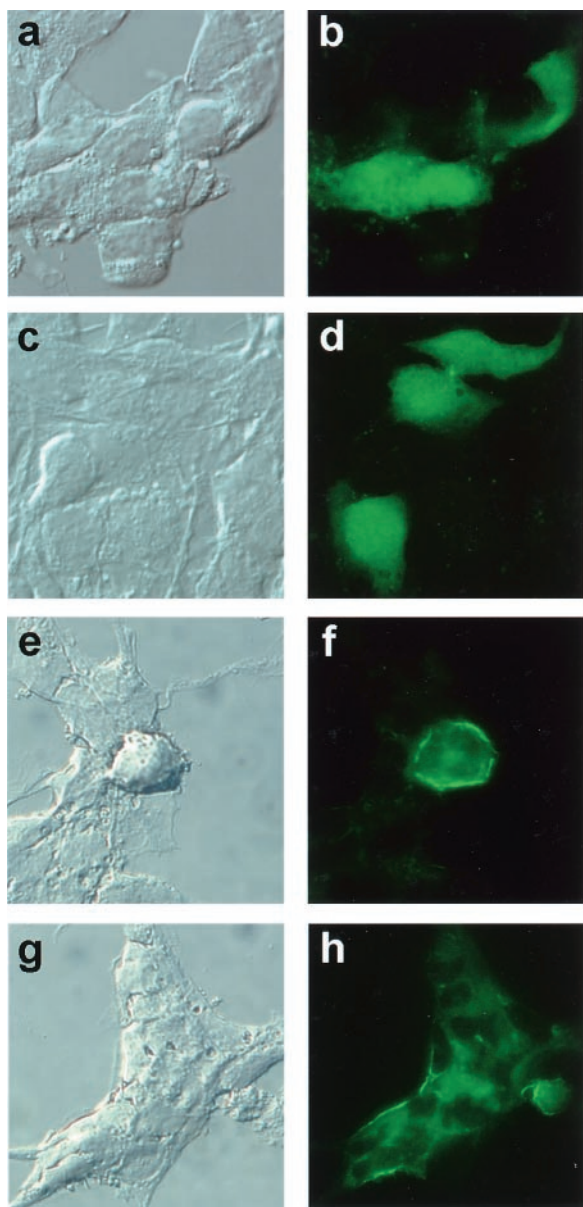
Fig. 4. 25-Dx is localized to the cell membrane of hypothalamic neurons. GT1–7 cells, grown on glass coverslips, were transfected with control GFP expression plasmid pEGFP-N1 (CLONTECH) (*a–d*) or the GFP/25-Dx fusion protein expression plasmid pEGFP/25-Dx (*e–h*). Forty-eight hours after transfection, the cells were fixed and mounted on microscope slides and photographed using bright-field microscopy enhanced with Nomarski optics (40× magnification) (*Left*). To the right of each bright-field image is the same visual field photographed under UV illumination, using a filter set designed for FITC fluorescence. The GFP protein fluoresces uniformly green throughout the cytoplasm (*b* and *d*), whereas the GFP/25-Dx fusion protein exhibits more intense green fluorescence at the cell border (*f* and *h*).

PRKO mice suggest that repression of the 25-Dx gene by P may also be mediated by the activated nuclear PR. The PR gene encodes two isoforms, A and B, which possess antagonistic activities; PR_B activates transcription, whereas PR_A represses transcription in certain cell types and of genes with certain promoter configurations (31). PRKO mice lack both the A and B isoforms of PR (8) and therefore also lack PR_B-mediated activation and PR_A-mediated repression. As shown here,

25-Dx expression is elevated in the hypothalamus of female (but not male) PRKO mice relative to their wild-type littermates (Fig. 3). Furthermore, 25-Dx mRNA is abundant in many areas of the female brain that also express nuclear PR (32) (Fig. 2). Together, these findings suggest that in females, 25-Dx expression is controlled by PR_A. Nevertheless, transcriptional analysis of the 25-Dx promoter, in the context of PR_A and PR_B, will be necessary to determine if repression of the 25-Dx gene by P, as we have shown here, is due to a negative feedback mechanism through signal transduction from the membrane or is mediated directly by PR_A.

In addition to P, 25-Dx can also bind a variety of steroid hormones with various relative affinities: P (100%), corticosterone (25%), testosterone (20%), cortisol (4%), and promegestone (R-5020) (2.5%) (17). Certain P metabolites, such as 3α-hydroxy-5α-pregnan-20-one and 5α-pregnan-3,21-diol-20-one, which have a low affinity for nuclear PR (33), can facilitate female sexual behavior, most likely because of their ability to bind the γ-aminobutyric acid type A ($GABA_A$) receptor (34). Our discovery of high levels of 25-Dx expression in several nuclei of the hypothalamus (Fig. 2) raises the possibility that these neurosteroids also interact with 25-Dx and thereby may be responsible for additional nongenomic P responses in the brain. Furthermore, the moderate affinity for 25-Dx by corticosterone and testosterone suggests that signaling through 25-Dx could also occur in response to these two steroids. In fact, Meyer *et al.* (17) have proposed that in individuals with modest amounts of P in circulation, such as males and postmenopausal women, 25-Dx could be occupied by corticosterone and testosterone to a greater extent than by P. Our finding that 25-Dx is expressed in the male PRKO hypothalamus, but does not appear to be altered by the genetic disruption of the PR gene (Fig. 3*b*), is a further interesting indication that 25-Dx is subject to sexually dimorphic regulation and perhaps dependent upon the types and amounts of steroid hormone in circulation. It remains to be determined which signal transduction molecules are activated when 25-Dx is bound by hormone and how these signals might influence the neuron's response, ultimately affecting behavioral endpoints such as lordosis.

Other behavioral considerations relevant to 25-Dx include those associated with dioxin exposure. The treatment of rats with the potent dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) enhances 25-Dx gene expression in the liver (7). Rats treated with TCCD also develop anorexia (35), and male rat pups exposed to the same compound early in life (*in utero* and during lactation) exhibit demasculinized and feminized male-typical sex behaviors as adults (36). The molecular targets of dioxins in the brain that cause these aberrant behaviors have not been identified, but our discovery of 25-Dx expression in hypothalamic nuclei known to regulate both feeding and reproductive behaviors is an intriguing indication that 25-Dx plays some role in their presentation. It will be of interest to see if 25-Dx is dioxin-inducible in the brain, as it is in the liver, and to determine the developmental stages in which it is expressed. Such studies would greatly enhance our understanding of how dioxins in the environment could adversely affect developmental pathways and disrupt vital neurological circuits that regulate feeding and reproduction.

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