adhesion were not significantly reduced by the loss of PI3Kγ. Teflon-coated 12-well glass slides (Marienfeld) were coated with fibronectin (20 μg/ml Sigma) solution. Calcein-AM (Molecular Probes)–loaded PMNs (20 μl) were applied to the glass slides. After stimulation, nonadherent cells were removed by washing. Fluorescence of attached cells was measured in a BioTek FL600 fluorescence plate reader (excitation, 485 nm, 20-nm slit; emission, 530 nm, 25-nm slit).


17. Supplemental Web data are presented on Science Online at www.sciencemag.org/feature/data/1044275.sht.


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Requirement for DARPP-32 in Progesterone-Facilitated Sexual Receptivity in Female Rats and Mice

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DARPP-32, a dopamine- and adenosine 3’,5’-monophosphate (cAMP)–regulated phosphoprotein (32 kilodaltons in size), is an obligate intermediate in progesterone (P)–facilitated sexual receptivity in female rats and mice. The facilitative effect of P on sexual receptivity in female rats was blocked by antisense oligonucleotides to DARPP-32. Homozygous mice carrying a null mutation for the DARPP-32 gene exhibited minimal levels of P-facilitated sexual receptivity when compared to their wild-type littermates. P significantly increased hypothalamic cAMP levels and cAMP-dependent protein kinase activity. These increases were not inhibited by a D1 subclass dopamine receptor antagonist. P also enhanced phosphorylation of DARPP-32 on threonine 34 in the hypothalamus of mice. DARPP-32 activation is thus an obligatory step in progestin receptor regulation of sexual receptivity in rats and mice.

Progesterone (P) and dopamine (DA) facilitation of sexual receptivity in female rats requires intact, intracellular progestin receptors (PRs) (1). Wild-type female mice exhibit high levels of P- and DA-facilitated lordosis, whereas homozygous females carrying a null mutation for the PR gene show minimal reproductive behavior (2, 3). These observations substantiate a critical role for the PR as a transcriptional mediator for the signal transduction pathways initiated by P and DA.

DA, signaling through the D1 subclass of receptors in the neostriatum, induces increasing levels of cAMP and PKA in the striatum (4). Dopaminergic stimulation facilitates the expression of DARPP-32 and cAMP-regulated phosphoprotein-32 (DARPP-32) in the striatum (5). 

In a parallel experiment, intracerebroventricular (icv) administration of the selective D1 agonist SKF 38393 also facilitated a lordosis response in EB-primed rats. The response was reduced by antisense but not by sense oligonucleotides to DARPP-32 (6). In contrast, antisense oligonucleotides to DARPP-32 had no effect on serotonin-facilitated sexual receptivity in these animals (7). These results were confirmed with two separate sets of oligonucleotides to DARPP-32 mRNA and their matched sense oligonucleotide controls.

DA and P facilitation of sexual receptivity were also examined in mice carrying a null mutation for the gene encoding DARPP-32 (8). Wild-type and DARPP-32 knockout mouse show similar levels of hypothalamic PRs (9). Ovariectomized wild-type, heterozygous, and homozygous female mice were tested for a lordosis response in the presence of wild-type DARPP-32 males 30 min after P administration (3, 5). Icv P after EB priming resulted in high levels of lordosis in wild-type and heterozygous mice, whereas homozygous mice exhibited significantly lower levels of lordosis (Fig. 2A). The lordosis response of the wild-type mice to the treatments did not differ from those of the parental mouse strains C57BL/6 and 129SvEv, indicating that the behavioral alterations observed in knockout mice were not due to variations in genetic background.

Icv administration of SKF 38393 48 hours after EB priming also facilitated a reliable lordosis response in the parental strains and in wild-type and heterozygous female mice. Homozygous mutant mice, however, responded to the icv injection of SKF 38393 with minimal levels of lordosis (Fig. 2B). The lordosis response did not significantly differ between wild-type, heterozygous, and homozygous mice upon icv injection of cAMP phosphodiesterase–1 (PP-1), increases the state of phosphorylation of DARPP-32 and cAMP-dependent protein kinase activity. This is consis-

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hours later, the oligonucleotide treatment was repeated. (A) P (2 μg), the D, agonist SKF 38393 (SKF; 100 ng), or (B) serotonin (Ser; 100 ng) was administered by icv injection 48 hours after EB priming. EB and P were dissolved in sesame oil and the oligonucleotides and neurotransmitters in saline. The sexual receptivity of female rats was observed and scored as described (5, 6). Control groups of EB-primed and non-EB-primed animals received vehicle (Veh) only. The antisense and sense oligonucleotides used for the experiments illustrated correspond to the rat DARPP-32 mRNA sequence 5'-GCCAGCGC-CCGCCATGGAC-3' (C57BL/6 and 129SvEv: wild-type (129) and 129 SvEv (129)). Another set of oligonucleotides corresponding to the sequence in the DARPP-32 mRNA 5'-CCGCCATGGACCAGG-3' gave similar results. Statistical analysis (ANOVA followed by Dunn's test) indicated no significant effects on P-, SKF-, or Ser-facilitated lordosis activity in the presence of C57BL/6 male mice (3, 5). Control groups included EB-primed (EB) and non-EB-primed vehicle (Veh) and parental strains C57BL/6 (C57) and 129SvEv (129), which were similarly treated and tested. Statistical analysis (25) indicated significant differences (P < 0.01) in P- or SKF-facilitated lordosis in animals that received DARPP-32 antisense oligonucleotides as compared to EB+P or EB+SKF controls, respectively. Sense oligonucleotides had no significant effect (P > 0.05) on P-, SKF-, or Ser-facilitated lordosis (n = 6 animals in each group).

Wild-type mice exhibited high levels of lordosis, with lordosis quotients (LQs) similar to those of the parental strains C57BL/6 and 129SvEv. Homozygous mice exhibited no significant difference in P-facilitated lordosis response (Fig. 3A). Likewise, icv injection of SKF 38393 or serotonin produced no significant differences in the lordosis response between wild-type and homozygous EB-primed mice (Fig. 3B).

We examined the lordosis response in mice that were null mutants for the genes encoding DARPP-32 and I-1 (12). The DA- and P-facilitated lordosis response was significantly reduced in these double knockout mice (Fig. 3C). The serotonin-facilitated lordosis response was unaffected (Fig. 3C). These results indicate that DARPP-32 (and
not I-1) was required for the P- and DA-facilitated lordosis response in mice.

In the neostriatum, DA increases cAMP levels, PKA activity, and phosphorylation of DARPP-32 on Thr14. This result is in increased phosphorylation (through decreased dephosphorylation) of substrate proteins (1, 2). Thus, we examined the possibility that P-initiated pathways might increase intracellular cAMP levels and PKA activity, thereby regulating the state of phosphorylation of DARPP-32 in the hypothalamus (3). Icv administration of P to EB-primed rats resulted in a significant increase in hypothalamic cAMP levels as compared to those of vehicle controls (164%). This increase in cAMP was not inhibited by the D1 antagonist SCH 23390. In contrast, the SKF 38393-stimulated cAMP increase (159%) was inhibited by SCH 23390 (Fig. 4A). Concomitant with these findings, a significant increase in hypothalamic PKA activity was also observed upon icv administration of either P or SKF 38383 to EB-primed rats (EB+P, 236%; EB+SKF, 223%) (Fig. 4B). The SKF 38393-stimulated, but not the P-stimulated, increase in PKA activity was depressed by SCH 23390. Thus, the P-initiated pathway is distinct, and its effects on cAMP and PKA are not secondary to modulation of DA receptors by P. These results are in agreement with earlier reports that neither the density of D1 receptors nor the release and turnover of DA in the hypothalamus is altered by P administration to ovariectomized, EB-primed female rats (4).

Icv administration of Rp-cAMPS, a compound that blocks the cAMP signal transduction cascade by inhibiting PKA, inhibited P- (as well as DA-) facilitated sexual receptivity in EB-primed female rats (Fig. 4C). These observations are consistent with earlier reports demonstrating increased hypothalamic cAMP levels on the evening of proestrus, concomitant with the exhibition of sexual behavior (5) and the facilitatory effects of cAMP analogs and phosphodiesterase inhibitors on sexual behavior in female rats (6).

Finally, we examined the possibility that P regulates the state of phosphorylation of DARPP-32 in the hypothalamus of ovariectomized mice (7) (Fig. 4D). EB, P, and D1 agonist administered separately each increased DARPP-32 phosphorylation (1.7-, 1.7-, and 1.5-fold, respectively). Moreover, DARPP-32 phosphorylation was significantly enhanced by the combined action of EB with P (2.7-fold) and of EB with D1 agonist (2.3-fold). Taken together, these results suggest that P increases DARPP-32 phosphorylation by activation of PKA in the neurons of the hypothalamus, resulting in an enhanced lordosis response. Increased immunoreactive phospho–DARPP-32 cells in PR-containing areas of the rat hypothalamus have also been seen after vaginal-cervical stimulation, a somatosensory stimulation that increases expression of sexual behavior (18).

Progesterone and D1 agonists are both able to induce lordosis in EB-primed rats and mice. The ability of DA, like that of P, to induce lordosis can be prevented by either antisense oligonucleotides to the PR or by deletion of the PR gene (1, 3). In the present study, the P-facilitated lordosis response was reduced by antisense oligonucleotides directed against DARPP-32 in rats and in DARPP-32 mutant mice. Because phosphorylated DARPP-32 inhibits the activity of PP-1, resulting in increased phosphorylation of PP-1 substrates, it is likely that the phosphorylation of the PR or of associated coactivators is modulated by DARPP-32. This is consistent with our earlier findings that a PP-1 inhibitor, okadaic acid, stimulated PR- (and DA-) mediated gene transcription (19).

Our results suggest a signaling system induced in P-regulated sexual behavior in the brain. In our model, P initiates activation of the PR by stimulating two distinct pathways. P; perhaps through activation of the membrane-bound PR, stimulates the PKA pathway; this results in activation of DARPP-32 and decreased dephosphorylation of the PR and its associated coactivators. Simultaneously, P binds to the PR and allosterically activates it to promote interactions with nu-
The deleterious effects of ethanol on the developing human brain are poorly understood. Here it is reported that ethanol, acting by a dual mechanism [blockade of N-methyl-D-aspartate (NMDA) glutamate receptors and excessive activation of GABA<sub>A</sub> receptors], triggers widespread apoptotic neurodegeneration in the developing rat forebrain. Vulnerability coincides with the period of synaptogenesis, which in humans extends from the sixth month of gestation to several years after birth. During this period, transient ethanol exposure can delete millions of neurons from the developing brain. This can explain the reduced brain mass and neurobehavioral disturbances associated with human fetal alcohol syndrome.

The most disabling features of FAE/FAES are neurobehavioral disturbances ranging from hyperactivity and learning disabilities to depression and psychosis (2, 3). It is thought that the brain is particularly sensitive to the neurotoxic effects of ethanol during the period of synaptogenesis, also known as the brain growth spurt period, which occurs postnatally in rats but prematurationally (during the last trimester of gestation) in humans (4–6). Thus, ethanol treatment of neonatal rats causes reproductive effects relevant to FAE/FAES, including a generalized loss

Intraterine exposure of the human fetus to ethanol causes a neurotrophic syndrome (1) termed fetal alcohol effects (FAE) or fetal alcohol syndrome (FAS), depending on severity.

REFERENCES AND NOTES

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5. Ovariecotomized female rats were prescreened for sexual receptivity [6] by subcutaneous [sc] administration of estradiol (E2) followed by progesterone (P) 48 hours later. Stereotaxic surgery was performed on sexually receptive rats (6) and mice (3), which were then used in experiments to compare the effects of P, D1 agonist, and serotonin. Behavioral testing was performed during the dark phase of the reversed light/dark cycle as described (3, 6). The experimental observer was blind to the treatment conditions and mouse genotypes.


9. EB-induced hypothalamic cytoplasmic PRs in mice carrying the wild-type gene encoding DARPP-32 (+/+) and the null mutation (−/−) were assayed by one-point linear regression analysis as described previously (3). The following PR concentrations were adopted: 2 fmol/mg of protein as a standard range of phospho-DARPP-32 and total DARPP-32. Phospho-DARPP-32 and total DARPP-32 bands were obtained in the range of the linear range of signals for densitometry was obtained by comparison of phospho-DARPP-32 and total DARPP-32 (24). Phospho-DARPP-32 and total DARPP-32 bands were quantified by densitometry with the use of a PhosphorImager MP (Molecular Dynamics). The phospho-DARPP-32 signals in hypothalamic tissue extracts were represented as percent of vehicle controls. The tissues were stored at −80°C until processed. Fresh samples were processed and immunoblotted for phospho-DARPP-32 and total DARPP-32 (24). The microdissection of the hypothalamus was performed as described (23), and the tissues were stored at −80°C until processed. Fresh samples were processed and immunoblotted for phospho-DARPP-32 and total DARPP-32 (24). Phospho-DARPP-32 and total DARPP-32 bands were obtained for the standard range of phospho-DARPP-32 and total DARPP-32 (24). The linear range of signals for densitometry was obtained by exposing the chemiluminescent membranes to x-ray film for varying periods of time. The linearity of measurements was confirmed by calibrating the values obtained to a standard range of phospho-DARPP-32 and total DARPP-32 concentrations in striatal tissues under conditions of basal and D1 activation. The exposure conditions that yielded linear measurements for phospho-DARPP-32 were obtained by comparison of phospho-DARPP-32 signals in hypothalamic tissue extracts with those obtained from signals in striatal tissue measurements.


18. Statistical analysis was done either of the following two methods as appropriate. For each significant analysis of variance (ANOVA), post-hoc comparisons were made using Dunn’s method for comparison of all groups versus the control group or the Tukey-Kramer method for multiple comparisons. Instat (Graph Pad, San Diego, CA) was used for statistical analyses.

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