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adhesion were not significantly reduced by the loss of PI3K $\gamma$ . Teflon-coated 12-well glass slides (Marienfeld) were coated with fibronectin (20  $\mu$ g/ml; Sigma) solution. Calcein-AM (Molecular Probes)-loaded PMNs (20  $\mu$ l) were applied to the glass slides. After stimulation, nonadherent cells were removed by washing. Fluorescence of attached cells was measured in a Bio-Tek FL600 fluorescence plate reader (excitation, 485 nm, 20-nm slit; emission, 530 nm, 25-nm slit).

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G. Frascaroli, W. Luini, G. Montrucchio, M. Laffargue, and R. Calvez for their support and assistance. We also thank G. Tarone, P. Defilippi, L. Fumagalli, and G. Topley for critically reading the manuscript; B. Vanhaesebroeck, R. Wetzker, A. Klippel, and B. Hemmings for antibodies; C. Dahinden for C5a; and B. Moser for chemokines. Supported by the Progetto Finalizzato Biotecnologie, CNR (project biotechnology), Associazione Italiana per la Ricerca sul Cancro (AIRC), San Salvatore Foundation, Telethon grant E 635, and Swiss National Science Foundation grant 31-50506.97.

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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 D<sub>1</sub> 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 D<sub>1</sub> subclass of receptors in the neostriatum, induces increases

in the levels of adenosine 3',5'-monophosphate (cAMP) and activates cAMP-dependent protein kinase (PKA) (*4*). Dopamine- and cAMP-regulated phosphoprotein-32 (DARPP-32) is phosphorylated by PKA. In its phosphorylated state, this molecule, by inhibiting the activity of protein phosphatase-1 (PP-1), increases the state of phosphorylation of many substrate proteins, leading to the induction of physiological responses (*4*). To determine whether DARPP-32 might be involved in P and DA actions on the hypothalamus, we examined its role in the facilitation of sexual receptivity in female rats and mice (*5*).

Antisense oligonucleotides to the PR inhibit P-facilitated lordosis in female rats (*6, 7*). We used a similar strategy to examine the role of DARPP-32 in P- and DA-facilitated sexual receptivity. Ovariectomized, estradiol benzoate (EB)-primed, Sprague-Dawley female rats with stereotaxically implanted stainless steel cannulae in the third cerebral ventricle (*5*) exhibited high levels of P-facilitated lordosis in the presence of males (Fig.

1A). This P-facilitated lordosis response was significantly reduced in the animals that received antisense oligonucleotides to DARPP-32 but not in control animals receiving sense oligonucleotides to DARPP-32 (Fig. 1A).

In a parallel experiment, intracerebroventricular (icv) administration of the selective D<sub>1</sub> 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 (Fig. 1A). In contrast, antisense oligonucleotides to DARPP-32 had no effect on serotonin-facilitated sexual receptivity in these animals (Fig. 1B). 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 mice 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 serotonin (Fig. 2B), corroborating the DARPP-32 antisense experiments in rats indicating that DARPP-32 is not an integral part of the serotonin signaling pathway. This is consis-

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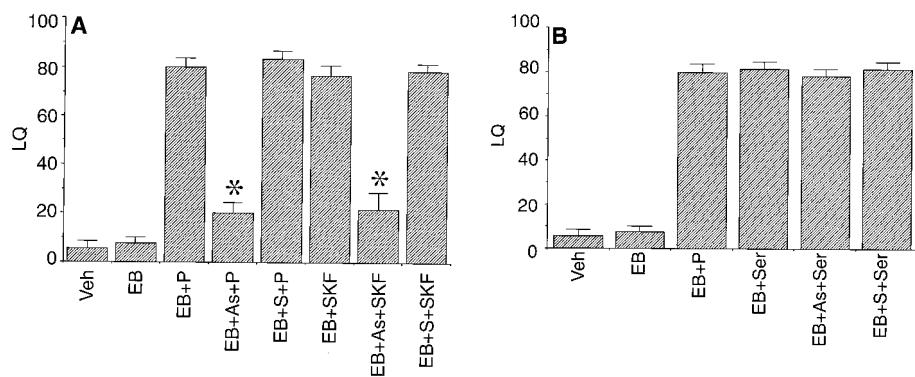
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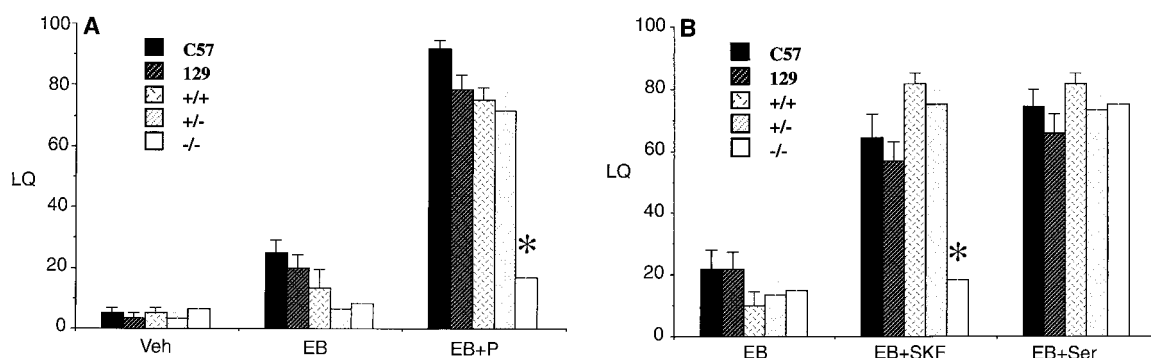
**Fig. 1.** Effect of DARPP-32 oligonucleotides on the sexual receptivity (LQ) of female rats. Ovariectomized and cannulated rats (5) were primed sc with EB (2  $\mu$ g) and were concurrently given 4 nmol of antisense (As) or sense (S) DARPP-32 oligonucleotides (2  $\mu$ l). Twenty-four hours later, the oligonucleotide treatment was repeated. (A) P (2  $\mu$ g), the D<sub>1</sub> 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'-CCGCCATGGACCCCAAGG-3'. Another set of oligonucleotides corresponding to the sequence in the DARPP-32 mRNA 5'-GCCAGCCGCGCCATGGAC-3' gave similar results. Statistical analysis (25) indicated significant (\**P* < 0.01) differences 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).

tent with our previous studies demonstrating that mice lacking the PR also respond to serotonin, but not to P or DA, by an increased lordosis response (3).

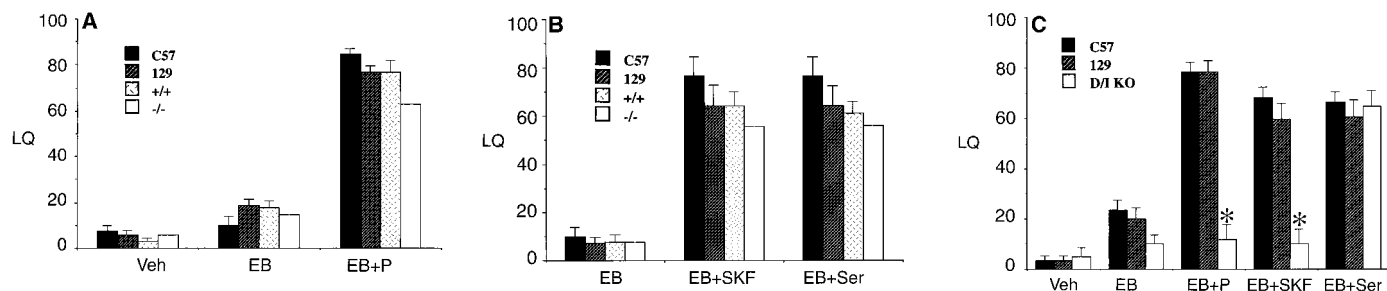
Inhibitor-1 (I-1) is a phosphoprotein that is closely related structurally, enzymologically, and functionally to DARPP-32 (10). Therefore, DA- and P-facilitated sexual receptivity was tested in mice carrying a null mutation for the gene encoding I-1 (11). Wild-type mice exhibited high levels of lordosis, 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

**Fig. 2.** Effects of P, SKF, or Ser on sexual receptivity (LQ) in DARPP-32 mutant mice. Ovariectomized and cannulated wild-type (+/+), heterozygous (+/-), and homozygous (-/-) mice (8) were primed sc with EB (0.5  $\mu$ g), followed 48 hours later by icv administration of (A) P (1  $\mu$ g), (B) SKF (50 ng), or Ser (50 ng) and were tested for lordosis response 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 129 SvEv (129), which were similarly treated and tested. Statistical analysis (25) indicated



significant differences (\**P* < 0.01) in SKF- or P-facilitated lordosis responses, but not in Ser-facilitated responses, of the homozygous mice as compared to those of wild-type and heterozygous mice (*n* = 6 animals in each group).



**Fig. 3.** Effects of (A) P, (B) SKF, or Ser on sexual receptivity (LQ) in I-1 mutant mice. Ovariectomized and cannulated wild-type (+/+) and homozygous (-/-) mice were EB-primed, then given icv injections of P, SKF, or Ser 48 hours after EB priming. Control groups and experimental procedures were analogous to those described in the legend of Fig. 2. ANOVA followed by Dunn's test indicated no significant effects of P, SKF, or Ser on lordosis responses between the wild-type and the homozygous mice (*P* > 0.05) (*n* = 8 to 11 animals in each group). (C)

Effect of P, SKF, or Ser on lordosis response in double knockouts for both DARPP-32 and I-1 (D/I KO). Experimental procedures were analogous to those described in the legend of Fig. 2. ANOVA followed by Tukey-Kramer multiple comparisons indicated statistically significant differences (\**P* < 0.001) in P- or SKF-facilitated lordosis responses, but not in Ser-facilitated responses of double knockout mice as compared to the C57BL/6 (C57) and 129 SvEv (129) mice (*n* = 6 animals in each group).

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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 Thr<sup>34</sup>. This results in increased phosphorylation (through decreased dephosphorylation) of substrate proteins (4). 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 (13). 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 D<sub>1</sub> 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

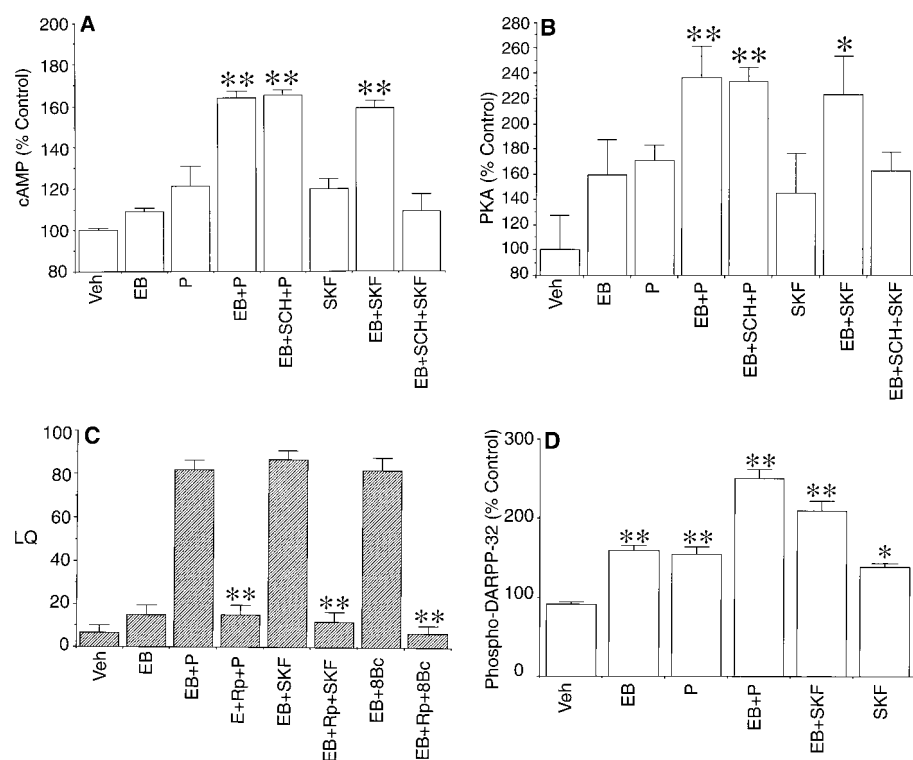
D<sub>1</sub> receptors nor the release and turnover of DA in the hypothalamus is altered by P administration to ovariectomized, EB-primed female rats (14).

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 (15) and the facilitatory effects of cAMP analogs and phosphodiesterase inhibitors on sexual behavior in female rats (16).

Finally, we examined the possibility that P regulates the state of phosphorylation of DARPP-32 in the hypothalamus of ovariectomized mice (17) (Fig. 4D). EB, P, and D<sub>1</sub> 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 D<sub>1</sub> 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 D<sub>1</sub> 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-



**Fig. 4.** Effect of SKF and P on (A) cAMP levels and (B) PKA activity in the hypothalamus. Icv vehicle (Veh), SKF (100 ng), or P (2  $\mu$ g) was administered to cannulated rats 48 hours after EB priming (2  $\mu$ g sc). Two groups of EB-primed rats received icv SCH 23390 (SCH; 100 ng) 30 min before SKF or P. The animals were killed 30 min after icv administration, and hypothalami were isolated and assayed for cAMP and PKA as described (13). cAMP levels and PKA activity are expressed as percentage of vehicle (Veh) control (means  $\pm$  SEM; 100%). Statistical analysis (25) indicated significant differences in P or SKF treatments as compared to treatment with vehicle alone (\*\* $P < 0.001$ ; \* $P < 0.01$ ). The experiments were repeated three times ( $n = 6$  animals for each group). (C) Effect of the cAMP antagonist Rp-cAMPS (Rp) on P-, SKF-, and 8-bromo-cAMP (8Bc)-facilitated lordosis. Icv Rp (100 ng per 1  $\mu$ l of saline) was administered 30 min before icv P (2  $\mu$ g), SKF (100 ng), or 8Bc (100 ng), and sexual receptivity was scored as described (5, 6). Dunn's test for multiple comparisons (25) indicated statistically significant differences (\*\* $P < 0.001$ ) in P-, SKF-, or 8Bc-facilitated lordosis in animals that received Rp as compared to EB+P, EB+SKF, or EB+8Bc controls, respectively. Rp alone had no significant effect on basal lordosis levels. Lordosis levels were similar to those seen in vehicle- and EB-treated animals ( $n = 6$  animals in each group). (D) Effect of SKF, EB, and P on DARPP-32 phosphorylation in the hypothalamus of C57BL/6J mice. Ovariectomized mice (0.5  $\mu$ g) received P (100  $\mu$ g sc) or SKF (5 mg per kg of body weight by intraperitoneal injection) 48 hours after EB priming. The animals were killed 4 hours (P) or 30 min (SKF) after injection; tissues were isolated and processed; and phospho-DARPP-32 levels were determined (17). Controls included EB- and non-EB-primed P, SKF, and vehicle treatments ( $n = 5$  animals for all groups). Phospho-DARPP-32 levels are expressed as percentage of control (means  $\pm$  SEM). Statistical analysis (25) indicated significant differences (\*\* $P < 0.001$ , \* $P < 0.05$ ) in the phospho-DARPP-32 levels of EB-, P-, SKF-, EB+P-, or EB+SKF-treated animals as compared to those of vehicle-treated animals.

clear coactivators in the conventional manner. The allosteric ligand activation and the phosphorylation work synergistically to promote receptor-mediated gene function. Finally, DA reinforces P action through a direct ligand-independent activation of the PR (I) as well as indirectly through activation of DARPP-32. It is implicit that cross-talk between these two pathways is important for integration of the multitude of signals that modulate reproductive behavior.

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5. Ovariectomized female rats were prescreened for sexual receptivity (6) by subcutaneous (sc) administration of EB (2 µg) followed by P (100 µg) 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, D<sub>1</sub> 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.
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9. EB-induced hypothalamic cytosol PRs in mice carrying the wild-type gene encoding DARPP-32 (+/+) and the null mutation (-/-) were assayed by one-point binding analysis as described previously (3). The following PR concentrations (in fmol/mg of protein) were obtained: vehicle (+/+) = 2.5 ± 1.2; vehicle (-/-) = 2.6 ± 0.87; EB (+/+) = 8.75 ± 1.5; EB (-/-) = 8.4 ± 1.8. Each value is the mean ± SEM of six independent determinations.
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12. Mice homozygous for the I-1 and DARPP-32 targeted mutations were generated by cross-breeding the two mutant lines. Resulting double heterozygotes were then crossed to yield mouse lines homozygous for each mutation and also the corresponding wild-type controls. Additional wild-type and double mutant mice were then generated by inbreeding wild types and double mutants.
13. Rats were decapitated and the brains removed. The hypothalamus was dissected from coronal sections submerged in oxygenated ice-cold artificial cerebrospinal fluid without Ca<sup>2+</sup> or Mg<sup>2+</sup>, and the tissues were processed for cAMP and PKA assays. Sample processing and cAMP assays were performed according to the procedures of Moore *et al.* (20). PKA assays using Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) as a substrate were carried out with 5 µg of protein as described (27). The amount of cAMP and PKA activity in each sample was normalized to the total amount of protein in the homogenate. Preincubation of protein extracts with the PKA-specific peptide inhibitor Walsh peptide demonstrated a concentration-dependent inhibition of substrate phosphorylation (a substrate:inhibitor ratio of 1:10 = 85% inhibition; 1:100,000 = 15%), confirming that the phosphorylation of the substrate peptide was specific to PKA.
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17. Mice were killed by exposure of the head to microwave irradiation for 900 ms, for which a Muromachi Microwave Applicator (Stoelting, Wood Dale, IL) set at 1.5 power was used. The brains were isolated from the crania, and 1-mm coronal sections bracketing the hypothalamic area were prepared with the aid of a mouse brain matrix (Activational Systems, Ann Arbor, MI), following the stereotaxic coordinates of Franklin and Paxinos (22). The microdissection of the hypothalamus was performed as described (23), and the tissues were stored at -80°C until processed. Frozen samples were processed and immunoblotted for 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:SF (Molecular Dynamics). The phospho-DARPP-32 values were normalized for the amount of total DARPP-32 present in the samples, and data were represented as percent of vehicle controls. 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 concentrations in striatal tissues under conditions of basal and D<sub>1</sub> 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.

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25. Statistical analysis was done by 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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# Ethanol-Induced Apoptotic Neurodegeneration and Fetal Alcohol Syndrome

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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.

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

The most disabling features of FAE/FAS 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 prenatally (during the last trimester of gestation) in humans (4-6). Thus, ethanol treatment of neonatal rats causes reproducible effects relevant to FAE/FAS, including a generalized loss

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