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26. Briefly, the cells were lysed in 1 ml of 10 m*M* tris, *p*H 7.4, 10 m*M* NaCl, 1.5 m*M* $MgCl_2$, 1% SDS, 0.5% β-mercaptoethanol, 10 m*M* vanadylribonucleotide complex, and 350 μg of proteinase K per milliliter, and heated to 42°C for 15 min. Each sample was then sonicated three times for 30 s each and then heated to 42°C for another 15 min. EDTA was added to give a final concentration of 10 m*M* and incubated another 5 min at 42°C, followed by addition of LiCl to give a concentration of 0.5*M*, and then the samples were cooled to room temperature. The extracted RNA was passed over oligo(dT) cellulose [H. Aviv and P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408 (1972)] to obtain poly(A)$^+$ RNA. RNA was estimated by an absorbance at 260 nm.
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32. The authors acknowledge D. Clark, J. Dickey, and S. Call for excellent technical assistance; C. Hart, J. Forstrom, and M. Murray for the generous gifts of monoclonal antibody to PDGF and yeast-derived PDGF-AA and PDGF-BB; R. Seifert for kindly providing $^{125}$I-labeled PDGF-AA and $^{125}$I-labeled PR7212 and for helpful discussions during the course of this work; M. Murray for providing PDGF B-chain cDNA; C.-H. Heldin for providing PDGF A-chain cDNA; D. Morris for providing β-actin probe; C. Carroll for drafting the figures; and M. Hillman for editorial assistance in preparation of this manuscript. This research was supported in part by the National Heart, Lung, and Blood Institute grant HL-18645.

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# Secretion of Activin by Interstitial Cells in the Testis

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**Activin, a dimer formed by the β subunits of inhibin, has an effect that is opposite to that of inhibin in a number of biological systems. Which cell types secrete activin in vivo is not known. TM3 cells, a Leydig-derived cell line, contained messenger RNAs that hybridized with human $\beta_A$ and $\beta_B$ complementary DNA probes and were similar in size to the porcine messenger RNA for the β subunits of inhibin. No hybridization to the inhibin α subunit was detectable in the TM3 cells. Conditioned medium from TM3 cells and from primary cultures of rat and porcine interstitial cells stimulated the release of follicle-stimulating hormone in a pituitary cell culture assay. It is likely that, in the testis, the Leydig cells secrete activin and the Sertoli cells produce inhibin, or a combination of both.**

INHIBIN IS A POLYPEPTIDE CAPABLE OF suppressing follicle-stimulating hormone (FSH) release from the pituitary without affecting the release of leutinizing hormone (LH) (*1*). The isolation, cloning, and sequencing of the inhibin cDNA showed that inhibin is a heterodimer composed of an α and one of two possible β chains ($\beta_A$ or $\beta_B$) (*2–5*). Inhibin activity has been isolated from both the ovary and the testis (*1, 6, 7*). During the purification of inhibin from porcine follicular fluid, proteins were found that have FSH-stimulating activity (*2*) and have been shown to be the dimers of the inhibin β ($\beta_A\beta_A$ or $\beta_A\beta_B$) subunits (*3*). It has been suggested that these molecules be called activin A and activin AB to signify that they have biological effects opposite to inhibin (*3, 8*). It is not known what cell type, or types, secrete activin, but it has generally been assumed that activin and inhibin are secreted by the same cell type in the ovary (granulosa) and in the testis (Sertoli) (*2*). The control of processing that might lead to the production of one or the other hormone expressed in the same cell is not understood.

A clonal cell line derived from mouse testicular Leydig (TM3) cells (*9*) was screened by RNA blot analysis for the presence of sequences hybridizing with human cDNA probes for the α, $\beta_A$, and $\beta_B$ subunits of inhibin. The TM3 cell line contained mRNA that hybridized with both the $\beta_A$ (Fig. 1A) and, in a lesser amount, the $\beta_B$ (*10*) probes. These two mRNAs were the same size as those from porcine ovary. No hybridization could be detected with the cDNA for the α subunit of inhibin under any conditions tried (Fig. 1B). These results suggested that TM3 cells express only the β mRNAs and may therefore be a source of activin. To determine whether these cells were capable of secreting a protein with the properties of activin, we tested conditioned medium from the TM3 cell line in a dispersed pituitary cell bioassay system (*8*). This medium elicited an increase in FSH, but not LH, release from the pituitary cell cultures compared to the cell-free control.

To confirm these results and rule out the possibility that the established cell line is expressing a gene not normally expressed in vivo, we prepared interstitial cell cultures from immature rat and pig testis that were enriched for Leydig cells. Sertoli cell–enriched cultures were prepared and assayed

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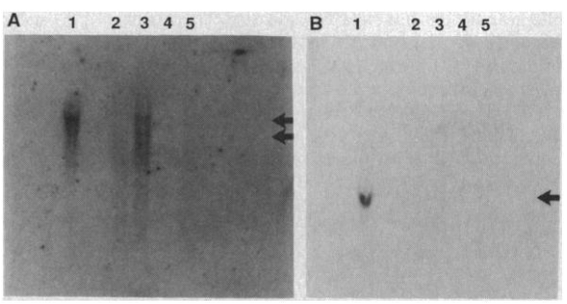


**Fig. 1.** RNA blot analysis of mRNA isolated from porcine follicles (lane 1), the TM3 Leydig cell line (lanes 2 and 3), and the TM4 cell line (a non-Leydig mouse testis line) (*9*) (lanes 4 and 5). Ten micrograms of total (lanes 1, 2, and 4) or polyadenylated (lanes 3 and 5) mRNA was loaded on each lane. The blots were hybridized with full-length cDNA probes for (**A**) the human inhibin $\beta_A$ subunit or (**B**) the α subunit. The cDNA probes were prepared and hybridization was carried out as previously described (*5*). The TM4 did not show hybridization to either cDNA. Arrows indicate the position of porcine $\beta_A$ mRNA [7.2 kb (top) and 4.5 kb (bottom)] in (A) and porcine α mRNA (1.3 kb) in (B).

for inhibin activity concurrently. Conditioned medium was collected from cultures grown under several different defined conditions, and the bioassay was performed.

Conditioned medium from rat Sertoli cell cultures caused a significant suppression of FSH release from rat pituitary cell cultures as measured by specific radioimmunoassay (RIA) (Fig. 2). This suppression was enhanced by pregnant mare's serum gonadotropin (PMSG), which has FSH-like and LH-like effects but will not interfere with the FSH or LH assays performed on the pituitary cell culture medium. This is in keeping with the previously reported stimulation of inhibin release by FSH (*1, 11*). Porcine follicular fluid elicited a similar suppression of FSH release, whereas the cell-free medium had no effect. A suppression of FSH release in pituitary cultures was also seen when conditioned medium from pig Sertoli cell–enriched cultures was assayed (Fig. 2). The isolated porcine Sertoli cells produced α, $\beta_A$, and $\beta_B$ mRNAs of the same size as those isolated from porcine ovary as determined by RNA blot analysis (*10*). It thus seems that in the pig, as in the rat, the Sertoli cell is the source of inhibin.

In contrast, the conditioned medium from the rat interstitial cell cultures caused a significant increase in FSH release from the pituitary cells (Fig. 2). This increase was also enhanced by PMSG, suggesting that activin release from Leydig cells may also be under gonadotropin (in this case, LH) control. There was no significant change in LH release in these cultures compared to the cell-free controls. Human activin A, produced by expressing the cDNA for human inhibin $\beta_A$, was used as a control and showed a similar pattern of increased FSH, but not LH, secretion by the pituitary cell cultures (Fig. 2, controls). Medium from porcine interstitial cell cultures also elicited an increase in FSH secretion.

To determine whether the activity produced by the interstitial cells was similar to authentic activin, we compared dose response curves for human recombinant (rh) activin A (rh-A), porcine follicular fluid (pff), and rat interstitial cell–conditioned medium (ICCM) in the pituitary bioassay (Fig. 3). Interstitial cell–conditioned medium has also been shown to contain material that cross-reacts with antisera specific for activin A and polyclonal antiserum to rh-inhibin, recognizing both α and β subunits, by immunoblot analysis and RIA (*10*).

Activin and transforming growth factor–β (TGF-β) have related sequences and structure (*2*). In addition, activin and TGF-β have been reported to have similar, but not identical, effects in a number of in vitro systems, including regulation of Leydig cell steroidogenesis and granulosa cell differentiation (*12, 13*). It was, therefore, of interest to determine whether the results reported above could be attributed to TGF-β produced by the TM3 and interstitial cell cultures rather than to activin. The mRNA hybridization shown in Fig. 1 is not TGF-β mRNA since it is the wrong size and the homology is not extensive enough to allow hybridization under the conditions used (*4, 14*). Purified recombinant human TGF-β was tested in the bioassay concurrently with the rh-activin and did not affect the FSH secretion from the pituitary cells. Finally,

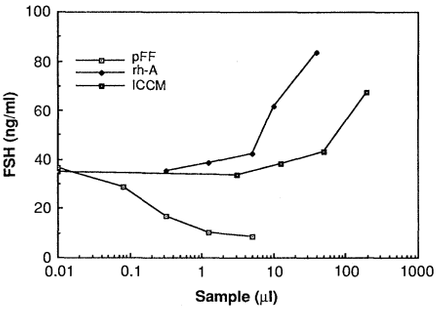


**Fig. 3.** Conditioned medium from rat Leydig cell cultures prepared as described in Fig. 2 (4F conditions) was assayed at several dilutions in parallel with rh-A and charcoal-stripped pFF standards. The follicular fluid caused a dose-dependent decrease in FSH secretion from the pituitary cell cultures, whereas the rh-A and the rat interstitial cell medium elicited a dose-dependent increase in FSH secretion. The activin standard contained 1 μg of rh-activin per milliliter.

the antibodies to activin that react with the interstitial cell–conditioned medium did not recognize human TGF-β, nor do antibodies to TGF-β give a similar immunoblot pattern. It thus seems unlikely that our results are due to TGF-β.

These data suggest that the source of activin in the testis is an interstitial cell. Because the clonal TM3 cell line expresses the genes for activin, the Leydig cell appears to be the interstitial cell that produces activin. Since the TM3 cells contain mRNA for both the $\beta_A$ and $\beta_B$ subunits, the observed activin bioactivity could be due to activin A, activin AB, activin B, or a combination of these molecules.

It is not possible at this time to determine whether Sertoli cells produce activin, by pre- or postsecretory association of the β subunits in addition to producing inhibin from the association of the α and β subunits. Activin bioactivity (as measured in the pituitary cell assay) in Sertoli cell–conditioned medium would most likely be masked by the inhibin activity. This is the case in follicular fluids, which contain both proteins and yet exhibit inhibin bioactivity in the pituitary cell bioassay (*2*).

The data presented above support the hypothesis that the FSH-stimulating activity found in the testis is produced by a cell in the interstitial tissue, probably the Leydig cell. By analogy, it is possible that the activin and inhibin found in follicular fluid are produced by different cell types in the ovary.

The hypothesis that inhibin and activin are secreted by different cells in the testis suggests novel possibilities for the endocrine regulation of the pituitary-gonadal axis and for paracrine and autocrine regulation in the testis by these two factors. Because of the evidence in support of extra-gonadal actions of these factors in erythroid differentiation

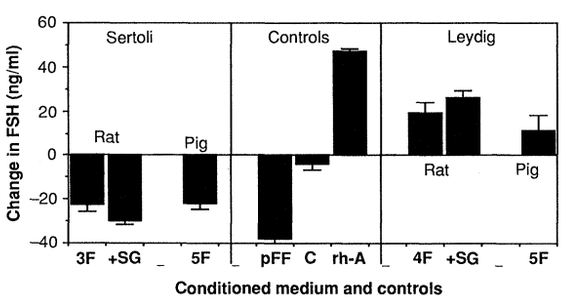


**Fig. 2.** Rat and pig Sertoli and Leydig cell cultures were prepared by the collagenase method as previously described (*18–20*). Rats were 17 days old at the time of culture, and porcine testis were obtained by castration at 2 to 3 weeks of age. Sertoli cell cultures were essentially free of Leydig cells. The rat Leydig cell cultures contained no detectable Sertoli cell contamination, whereas pig Leydig cultures were estimated to contain approximately 20% Sertoli cells. Cells were cultured in Ham's F12 nutrient mixture–Dulbecco's modified Eagle's medium (1:1) supplemented with insulin (10 μg/ml), human transferrin (10 μg/ml), and epidermal growth factor (10 ng/ml) (condition 3F) or 3F + α-tocopherol (condition 4F), and 0.1% fetal bovine serum (condition 5F) with or without PMSG (condition +SG) (2 μg/ml) as indicated. Cells were plated on day 0, the medium was changed on day 1 to the conditions indicated, collected on day 4, and stored at −20°C until assayed on dispersed pituitary cell cultures (*8*). Cultures were carried out in duplicate, and the medium was assayed in triplicate with 100 μl per assay. Controls shown for the bioassay are: medium only (condition C), medium containing the indicated hormones (condition 4F), charcoal-stripped porcine follicular fluid (condition pFF), or rh-A (20 ng per assay). Values shown are the mean ± SD. Data are expressed as the difference between the various conditions and the medium only control (control value for FSH was 44.6 ± 0.8 ng/ml). Controls were run with each assay and the Sertoli and Leydig cultures from each rat or pig preparation were assayed together. Similar results were seen in five separate rat preparations from animals ranging from 17 to 24 days of age, and in three separate pig primary culture preparations. LH values did not vary significantly from the control in any of the conditions shown.

(*15*) and their presence in the pituitary, adrenal, bone marrow, kidney, spinal cord, and brain as well as ovary, testis, and placenta (*16*), it seems likely that these factors also play an important role in a number of nonreproductive functions. The discovery of a protein homologous to this gene family in *Drosophila* (*17*) emphasizes the potential importance and widespread occurrence of these factors. Production of inhibin and activin by two associated cell types may then be one of a number of ways in which these factors regulate the growth and differentiated function of organs.


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# Role for Excitatory Amino Acids in Methamphetamine-Induced Nigrostriatal Dopaminergic Toxicity

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**The systemic administration of either methamphetamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to experimental animals produces degenerative changes in nigrostriatal dopaminergic neurons or their axon terminals. This study was conducted to determine if excitatory amino acids, which appear to be involved in various neurodegenerative disorders, might also contribute to the dopaminergic neurotoxicity produced in mice by either methamphetamine or MPTP. MK-801, phencyclidine, and ketamine, noncompetitive antagonists of one subtype of excitatory amino acid receptor, the *N*-methyl-D-aspartate receptor, provided substantial protection against neurotoxicity produced by methamphetamine but not that produced by MPTP. These findings indicate that excitatory amino acids play an important role in the nigrostriatal dopaminergic damage induced by methamphetamine.**

OVERACTIVITY OF EXCITATORY AMINO acid (EAA) neurotransmission may be associated with the pathophysiology of a number of neurodegenerative disorders, including epilepsy (*1*), olivopontocerebellar atrophy (*2*), stroke or hypoxia-induced brain damage (*3*), hypoglycemia-induced brain damage (*4*), spinal cord injury (*5*), and possibly even Huntington's disease (*6*) and Alzheimer's disease (*7*). The infusion of EAA agonists such as kainate, ibotenate, or *N*-methyl-D-aspartate (NMDA) directly into a particular brain region such as the cortex (*8*), hippocampus (*9*), or striatum (*6*) of experimental animals causes neurodegeneration within that brain region. Excitotoxin-induced cell death appears to be mediated by excessive stimulation of EAA receptors, of which there are at least three subtypes: NMDA, kainate, and quisqualate (*10*).

The excitotoxins cause a prolonged neuronal depolarization (*11*), an influx of $Ca^{2+}$ (*12*), and a depletion of adenosine triphosphate with a concomitant increase in purine catabolites (*13*). Each of these effects, alone or in combination, could contribute to cell death. Cultured cerebellar neurons can be protected from excitotoxin-induced death by compounds that reduce levels of superoxide or hydroxyl radicals (*14*). These latter findings are consistent with the premise that excitotoxin-induced neuronal degeneration may be mediated by oxidative stress associated with the production of superoxide and hydroxyl radicals.

Parkinson's disease (PD) is a neurodegenerative disorder of unknown etiology that is characterized by a loss of nigrostriatal dopaminergic neurons. One theory of neurodegeneration in PD is that oxidation products derived from dopamine (DA), such as hydrogen peroxide, superoxide radicals, and hydroxyl radicals, are neurotoxic (*15*). There are several experimental models of PD, one of which is the amphetamine- or methamphetamine (METH)–treated rodent or nonhuman primate. The amphetamines cause damage to nigrostriatal dopaminergic neurons as evidenced by marked decrements in the neostriatal content of DA and its metabolites, the number of high-affinity DA uptake sites, and the activity of tyrosine hydroxylase (TH), as well as histochemical indications of nerve terminal degeneration within the neostriatum (*16*). The amphetamines cause a release of newly synthesized

**Table 1.** Effect of (+)MK-801 on METH-induced decreases in TH activity and DA content in the mouse neostriatum. Mice received four intraperitoneal injections of METH at the doses indicated at 2-hour intervals. Other groups were injected intraperitoneally with (+)MK-801 at the doses indicated 15 min before and 3 hours after the first injection of METH. Results are the means ± SD of three to five mice per group killed 3 days after treatment.

| (+)MK-801 (mg/kg) | METH (mg/kg) | DA (μg/g) | TH activity (nmol/g per hour) |
|---|---|---|---|
| | | 14.9 ± 1.8 | 465 ± 54 |
| | 1.25 | 13.3 ± 0.4 | 367 ± 16 |
| | 2.5 | 5.3 ± 4.2* | 197 ± 111* |
| | 5 | 3.3 ± 0.8* | 154 ± 44* |
| | 10 | 1.1 ± 0.6* | 64 ± 26* |
| 0.5 | 5 | 10.5 ± 3.4*† | 240 ± 82* |
| 1.0 | 5 | 14.3 ± 1.0† | 369 ± 80† |
| 2.5 | 5 | 12.6 ± 2.6† | 358 ± 77*† |
| 2.5 | 10 | 15.4 ± 0.8† | 420 ± 50† |

*Statistically different ($P < 0.05$) from naive group (analysis of variance with Duncan's multiple range test). †Statistically different ($P < 0.05$) from METH-only group.

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