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Serotonin Regulates Mammary Gland Development via an Autocrine-Paracrine Loop

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Summary

Mammary gland development is controlled by a dynamic interplay between endocrine hormones and locally produced factors. Biogenic monoamines (serotonin, dopamine, norepinephrine, and others) are an important class of bioregulatory molecules that have not been shown to participate in mammary development. Here we show that mammary glands stimulated by prolactin (PRL) express genes essential for serotonin biosynthesis (tryptophan hydroxylase [TPH] and aromatic amine decarboxylase). TPH mRNA was elevated during pregnancy and lactation, and serotonin was detected in the mammary epithelium and in milk. TPH was induced by PRL in mammosphere cultures and by milk stasis in nursing dams, suggesting that the gene is controlled by milk filling in the alveoli. Serotonin suppressed β-casein gene expression and caused shrinkage of mammary alveoli. Conversely, *TPH1* gene disruption or antiserotonergic drugs resulted in enhanced secretory features and alveolar dilation. Thus, autocrine-paracrine serotonin signaling is an important regulator of mammary homeostasis and early involution.

Introduction

To discover potentially novel aspects of mammary gland physiology and development, we set out to identify genes for which expression is altered by prolactin (PRL). We focused our attention on discovering PRL-regulated genes that do not encode milk proteins or other conventional milk-associated gene products (Groner and Gouilleux, 1995; Hennighausen et al., 1997). The gene expression profiling studies we report here led us to the unexpected observation that the mammary gland expresses the serotonin-biosynthetic enzyme tryptophan hydroxylase (TPH). This discovery provoked a series of physiological studies leading us to the conclusion that the mammary glands possess an autocrine-paracrine serotonergic system that communicates important local homeostatic information.

Serotonin (5-hydroxytryptamine, 5-HT) is a central neurotransmitter that is widely implicated in the regulation of mood and cognition, and is a peripheral signaling molecule that affects hemostasis, immune function, intestinal physiology, and other systems (Hardman and Limbird, 2001). There are multiple types of 5-HT receptors that are distributed widely among endocrine, cardiovascular, immune, and gastrointestinal tissues. Receptors for 5-HT fall into one of four distinct families (5-HTR_1, 5-HTR_2, 5-HTR_3, 5-HTR_{4-7}), which are characterized by different signal transduction mechanisms and physiological roles.

TPH is the rate-limiting enzyme for 5-HT synthesis (Fitzpatrick, 1999) and is encoded by both the classical TPH gene (*TPH1*) and a newly discovered neuronal gene (*TPH2*), which is preferentially expressed in the brain (Walther et al., 2003). The discovery of a dual genetic basis for central and peripheral 5-HT biosynthesis, which is conserved in mammals, birds, and fish, suggests that both neural and nonneural functions of 5-HT have essential and distinct physiological roles.

Mammary gland development and lactation are regulated by a dynamic interplay of endocrine and locally produced (autocrine-paracrine) factors. These signals conspire to facilitate both the dramatic changes necessary for development and lactogenesis (homeorrhesis) and the adjustments required to maintain the steady-state quality and quantity of milk during lactation (homeostasis). Prolactin (PRL) is the major endocrine inducer of mammopoiesis and milk secretion (Horseman, 1999; Vomachka et al., 2000). Nursing stimulates a reflex secretion of PRL, which induces a variety of milk protein genes and secretory functions. A well-characterized PRL signaling pathway operates by activation of a nonreceptor tyrosine kinase (Jak2) and a latent transcription factor (Stat5) (Groner and Gouilleux, 1995). A separate, Stat5-independent signaling pathway involves arachidonic acid mobilization, and this pathway appears to be more important for stimulating milk secretion (Lkhider et al., 2001). Thus, lactation is driven by an endocrine signaling system that is strongly influenced by nursing activity and affects expression of milk genes and secretory activity through multiple signal transducers.

An autocrine-paracrine homeostatic feedback opposes the endocrine stimulation of mammary development and milk secretion. The feedback system has been characterized by numerous physiological studies in rodents and ruminants (Daniel and Smith, 1999). As milk fills the glands, secretion is rapidly downregulated, protein gene expression is suppressed, and involution is initiated (Peaker et al., 1998). In spite of the careful physiological characterization of the local feedback regulation in the mammary gland, the specific biochemical

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components of this regulatory system have not been identified.

Results

A Gene Encoding TPH Is Induced in the Mammary Glands

Suppression subtractive hybridization cloning (Diatchenko et al., 1996) was used to identify genes that were differentially expressed in mammary gland tissues from PRL-deficient (*PRL*$^{-/-}$) mice (Horseman et al., 1997) compared with mice made hyperprolactinemic by pituitary grafting (18 days) and mice that were treated by implanting progesterone-containing tablets (25 mg, 18 days). Pituitary grafting was used because it causes sustained elevation of PRL without elevation of other pituitary hormones (Adler, 1986). The level of PRL after pituitary grafting was in the range of 50–150 $\mu g \cdot ml^{-1}$ (unpublished data), which results in substantial stimulation of mammary gland development (similar to the latter half of pregnancy). Of the several genes identified as being PRL dependent, eight of an initial 25 clones that we sequenced encoded cDNA fragments for tryptophan hydroxylase (EC1.14.16.4, TPH, Genbank accession number: AF273257), which catalyzes the conversion of tryptophan to 5-hydroxytryptophan (5-HTP).

Several tests were done to confirm the induction of TPH by PRL in the mouse mammary gland. Differential hybridization to arrayed mouse cDNAs (Clontech Mouse Atlas version 1.2, Palo Alto, CA) revealed an induction of TPH mRNA in PRL-stimulated breast, and the signal intensity indicated that the transcript was relatively abundant in PRL-stimulated mammary gland (being among the upper 10% of 1200 genes, data not shown). Virtual Northern blotting (Southern hybridization to full-length cDNA) was used to identify the mammary TPH transcripts (Figure 1A). TPH transcripts of 1.8 and 3.8 kilobases, which is in agreement with other nonneuronal tissues (Stoll and Goldman, 1991), were detected in the mammary gland extracts after pituitary grafting but were undetectable in glands of control or progesterone-treated mice. In the mouse mammary gland, in situ hybridization revealed that the *TPH* gene was expressed in the epithelial cells of pituitary-grafted but not control virgin mice (Figures 1B and 1C). The gene encoding TPH was expressed in normal human breast and brain tissues (total RNA, Stratagene, Inc., La Jolla, CA) and in both nontransformed human breast epithelial cells (MCF10A) and T47D human breast cancer cells (Figure 1D).

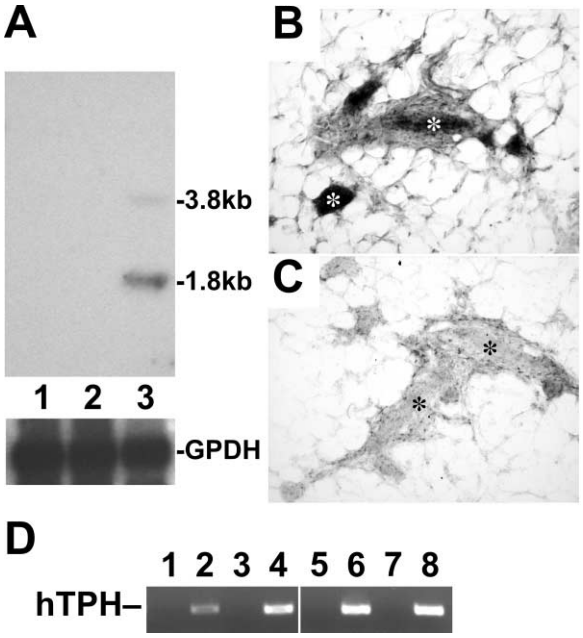


Figure 1. TPH mRNA Is Expressed in Mouse and Human Mammary Glands

(A) Mammary gland mRNA was reverse transcribed and Southern blotted (virtual Northern blot) using alkaline phosphatase-labeled mouse TPH cDNA probe. The TPH signal was induced following 18 days treatment with pituitary grafting (lane 3) when compared with progesterone-treated (lane 2) or sham-operated control (lane 1) mice. The molecular sizes of the bands (kilobases, kb) are labeled in the right margin. Glyceraldehyde-3-phosphate dehydrogenase (GPDH) is shown as an internal positive control.

(B and C) In situ hybridization analysis of TPH in a pituitary graft-stimulated (B) and unstimulated (C) mammary gland. Antisense probe-specific signal (*) was observed in epithelial cells. Digoxygenin-labeled antisense cRNA probe corresponded to the 5′ end of TPH mRNA (~200 bases). The corresponding sense-strand probe was used as a negative control (data not shown).

(D) Human TPH mRNA was identified by RT-PCR in extracts of normal human breast (lanes 1 and 2) and brain (lanes 3 and 4) and in human breast-derived epithelial cells, MCF-10A (lanes 5 and 6), and T47D (lanes 7 and 8). Controls (lanes 1, 3, 5, and 7) were from RNA samples that were handled identically but without reverse transcription. Fragments with the expected size (confirmed by sequencing) were amplified from cDNA samples (lanes 2, 4, 6, and 8).

Serotonergic Enzyme Gene Expression in Mouse Mammary Glands

TPH converts L-tryptophan to 5-HTP, which is metabolized to 5-HT by aromatic amine decarboxylase (AADC, EC4.1.1.28). In the pineal gland, 5-HT is converted to melatonin through serotonin *N*-acetyltransferase (SNAT, EC2.3.1.5). Representative results of assays for expression of the indoleaminergic enzymes are shown in Figure 2. TPH1 expression was highly induced by PRL, AADC mRNA was modestly, albeit consistently, elevated in PRL-stimulated mammary extracts, and SNAT was not detected in the mammary glands (Figure 2A). The lack of SNAT expression is consistent with results from a previously published report showing no melatonin synthesis in rat mammary glands (Chen et al., 1999). Mammary TPH cDNA was amplified with primers specific for the peripheral TPH1 isoform but not with TPH2-specific primers (Figure 2B). The pattern of enzyme gene expression implied that the mammary gland can synthesize and secrete 5-HT. To test this, we performed two types of experiments: immunohistochemistry for 5-HT and measurements of 5-HT in milk.

Serotonin in the Mammary Epithelium and Milk

Immunoreactive 5-HT was localized specifically in the epithelium of mammary glands from pituitary-grafted female mice (Figures 2C and 2D). We also noted a low level of 5-HT staining in the adipose stroma. It was difficult to determine whether the stromal staining was specific because of its low intensity and dispersion, but we

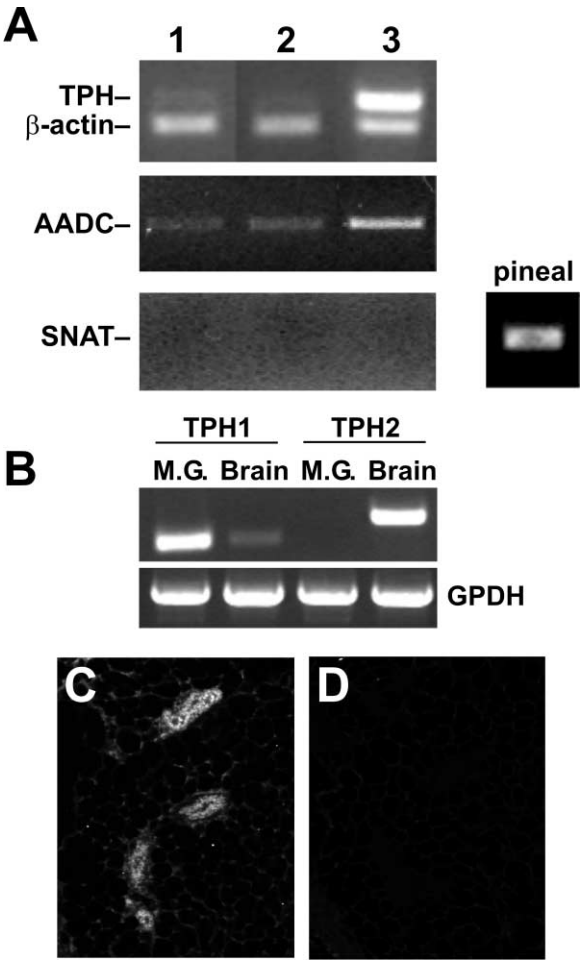


Figure 2. The Serotonin Biosynthetic System in the Mouse Mammary Gland

(A) Expression of mRNA for indoleamine-synthesizing enzymes was examined by RT-PCR (see Supplemental Table S1 for primer sequences at http://www.developmentalcell.com/cgi/content/full/6/2/193/DC1), and mRNAs for the two enzymes necessary for 5-HT synthesis (TPH and AADC) were seen to be expressed in the mammary gland. In contrast, serotonin N-acetyl transferase mRNA (SNAT), which is necessary for melatonin synthesis, was not detectable in mammary gland but was detected in the positive control tissue (pineal). Lane 1, control; lane 2, progesterone treated; lane 3, pituitary grafted. Primers specific for the classical (TPH1) and neuronal (TPH2) isoforms of TPH were used to determine which gene was expressed in the mammary gland (B). TPH1-specific but not TPH2-specific primers yielded an amplified product in mammary gland tissue, whereas TPH2 was preferentially expressed in the brain. (C) and (D) show immunostaining patterns for 5-HT in the mammary gland. Paraffin sections of mouse mammary tissue taken on day 18 after pituitary grafting were immunostained with monoclonal anti-5-HT (C) or control IgG (D). Accumulation of 5-HT (lucent signal) was detected in the mammary gland epithelium with a low level of staining in the adipose stroma.

have observed 5-HT receptor gene expression in the stroma (unpublished data), which suggests that some of the apparent stromal staining could be accounted for by receptor bound 5-HT. The epithelial cells were uniformly positive, and the background in the epithelium was very low.

Preliminary experiments showed that 5-HT was present in milk expressed from the nipples of five mice (data not shown), but it was difficult for us to collect sufficient and consistent volumes of fresh milk from individual mice, so we chose to study milk 5-HT by dialysis of flowing duct milk in situ. To this end, main ducts of the #4 glands of mice that were 4–9 days postpartum were cannulated with dialysis probes, and 5-HT was measured in the dialysates by HPLC/EC (Raymond et al., 1978; Shankaran and Gudelsky, 1999). The milk within mammary gland ducts contained substantial quantities of both 5-HT and its primary metabolic product, 5-HIAA. The values we report here are from samples collected in the second 30 min after oxytocin injection, and repeated sampling of the dialysates showed that 5-HT was secreted into the milk for at least 1.5 hr. The concentrations of 5-HT and 5-HIAA in milk were 16.0 $\pm$ 4.5 ng•ml^{-1} and 11.5 $\pm$ 1.5 ng•ml^{-1}, respectively (n = 6 mice). While it is conceivable that 5-HT in the milk or epithelium could be derived from a source other than the mammary gland, the most parsimonious inference is that the primary source of this 5-HT was the mammary epithelium. These experiments established that the mammary gland expresses the peripheral isoform of TPH and AADC and that 5-HT is present in the mammary epithelium and milk. Because TPH is the rate-limiting step in 5-HT synthesis and appeared to be tightly regulated in mammary glands, we undertook a series of experiments to study TPH gene regulation.

TPH Gene Regulation In Vivo by Reproductive Development, Hormones, and Milk Stasis

TPH and AADC mRNA levels were measured by real-time RT-PCR in normal wild-type mice at various stages of mammary gland development (Figure 3A). The highest levels of TPH mRNA were observed in pregnant mice. Low levels of TPH and AADC mRNA were observed in nulliparous mice and during involution. On day 10 of lactation, TPH mRNA was intermediate between that in nulliparous and pregnant animals. RT-PCR with primers specific for TPH1 and TPH2 demonstrated that the mammary gland expresses exclusively TPH1 at all developmental stages (unpublished data). The higher levels of TPH1 during pregnancy and lactation correspond with the elevation of lactogen (PRL or placental lactogens) during these phases of development.

To study TPH regulation by intrinsic mammary gland mechanisms, individual teats were sealed so that we could compare TPH expression in suckled and unsuckled glands that were exposed to the same stimulatory hormonal milieu (Li et al., 1997). Figures 3B and 3C show results from experiments in which TPH and milk protein mRNA levels were compared between #3 abdominal mammary glands that were left open on one side (suckled) while the contralateral gland was sealed (unsuckled). TPH mRNA was expressed in all the glands whether they were open or sealed. However, the level of TPH expression was consistently higher in the sealed glands compared with their contralateral open controls. In contrast, milk protein gene expression was downregulated by teat sealing, and the housekeeping gene standard (glyceraldehyde phosphate dehydrogenase, GPDH) was unaffected, as shown in representative electrophoretic

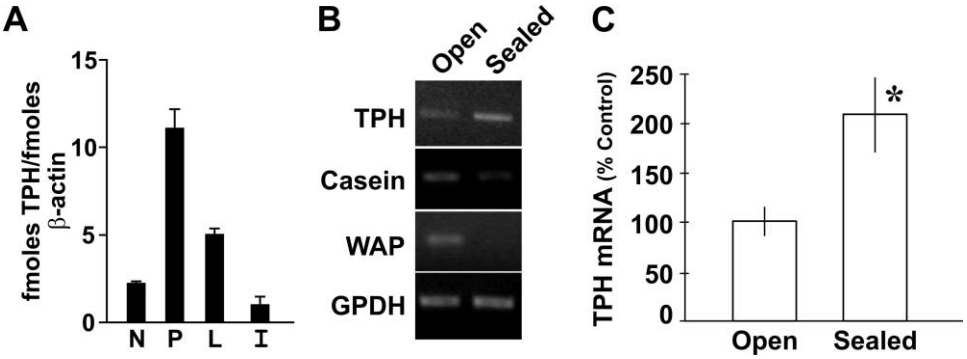


Figure 3. Mammary TPH Regulation during Development and Lactation

(A) Levels of TPH mRNA were measured by real-time RT-PCR in mammary gland extracts from nulliparous (N), pregnant (P, day 15 postcoitus), lactating (L, day 10 postpartum), and involuting (I, day 3 postweaning).

(B) Levels of expression for TPH, β-casein, whey acidic protein (WAP), and GPDH were measured in extracts of #3 glands with open or sealed teats 24 hr after sealing. Representative cDNA bands from RT-PCR assays showed that TPH was increased, whereas casein and WAP were decreased, and GPDH was unaffected by teat sealing.

(C) For quantification, average levels of TPH expression were determined by densitometry in extracts from six mice in which one gland was open and the other sealed. Values were normalized to GPDH and expressed as percent of the average level of the control (unsealed) glands (mean ± SEM). Open and sealed glands were compared by paired t test and were significantly different (*$p < 0.02$, $n = 6$).

patterns (Figure 3B). To make statistical comparisons of TPH expression, gel images were scanned and the fluorescence intensities (relative to GPDH internal standard) were averaged (Figure 3C). This test showed that there was a statistically significant difference in TPH mRNA comparing the open and sealed glands (two-tailed, paired t test, $p < 0.02$, $n = 6$).

TPH Gene Regulation in Three-Dimensional Mammary Epithelial Cell Cultures

We attempted to study TPH regulation using conventional cell culture experiments with an established mouse mammary epithelial cell line (HC11) or enriched primary mouse mammary epithelial cells (PMEC) cultured on plastic dishes, but these techniques produced inconsistent results in which TPH was expressed only at very low levels. To achieve a closer correspondence between an in vitro model and the in vivo situation, we studied TPH regulation in mammosphere cultures. Mammospheres develop when PMEC are suspended in a semisolid medium such as reconstituted basement membrane (Matrigel, BD Biosciences, Palo Alto, CA). Under these conditions, PMEC form spherical, cystic structures that are polarized so that the basolateral surfaces contact extracellular matrix proteins, and the apical surfaces line a closed central lumen. These complex structures, termed mammospheres, secrete milk proteins, fluids, and ions vectorially into the lumen and respond to lactogenic hormones (Blatchford et al., 1995). Mammosphere TPH mRNA increased robustly for at least 96 hr following additions of PRL to the medium (Figure 4A). This time course was slower than is typical for genes that are regulated directly by PRL such as the milk proteins, which indicated that a complex indirect process might be involved in TPH induction by PRL. The dose response for PRL (Figure 4B) was typical of many PRL effects in mammary cells and other cell types. Induction of TPH mRNA in mammospheres was blocked by either actinomycin D, which blocks transcription, or cycloheximide, which blocks translation (data not shown). Estradiol did not induce TPH expression above the PRL-stimulated level in the mammosphere cultures (Figure 4C). Progesterone caused a dose-dependent inhibition of PRL-stimulated TPH expression (Figure 4D).

Serotonergic Agents Affect Lobuloalveolar Development in Mammary Explants

Unlike peptide hormones and growth factors, for which there are very few drugs that have specific effects on synthesis, release, or receptor activation, there are numerous well-characterized agents that affect monoamine (e.g., serotonin) signal transduction at specific levels (Hardman and Limbird, 2001; Azmitia, 2001). The effects of PRL and serotonergic agents were studied in explants from midpregnant mouse mammary glands. Figure 5 shows representative hematoxylin-eosin stained sections through explants treated with PRL and serotonergic agents. For comparisons with normal development, the reader is referred to the mammary histology database (http://mammary.nih.gov). In the absence of PRL treatment, the control explants (Figure 5A) were regressed to simple, largely tubular structures that had a few small alveolar buds, compact cytoplasms, and few, if any, secretory vesicles or lipid globules. Explants treated with PRL (Figure 5B) took on a highly stimulated biosynthetic and secretory appearance. The epithelium formed lobuloalveolar clusters with dilated and filled lumens. Individual cells had large nuclei and expanded cytoplasms that were filled with basophilic material, secretory vesicles, and lipid globules. The epithelium was reminiscent of that in actively lactating mammary glands, although there was more adipose stroma in the PRL-stimulated explants than is typical of lactating glands. When 5-HT was added along with PRL (Figure 5C), the explants took on the appearance of involuting glands. The alveoli were collapsed, and the cells had small clear cytoplasms. Many cells had dense, pychnotic, and fragmented nuclei. Thus, even in the presence

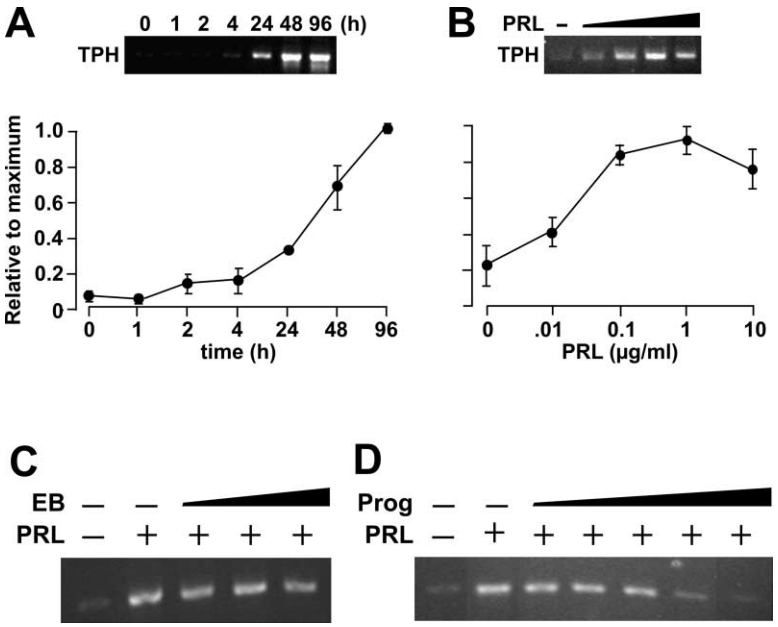


Figure 4. Profiles of TPH mRNA Induction in Primary Mouse Mammary Epithelial Cells Cultured as Three-Dimensional Organoids

(A) TPH levels during 4 days of hormone treatment (1 $\mu g \cdot ml^{-1}$ oPRL) are shown as representative electrophoretic bands (above) and as mean (± SEM) relative to the maximum value ("1") in the graph (n = 4 experiments).

(B) A dose-response relationship for oPRL concentrations ($\mu g \cdot ml^{-1}$) relative to the maximum induction is shown as representative electrophoretic bands (above) and as means (± SEM) for four experiments (below). Cells were cultured with the respective PRL concentrations for 48 hr.

(C) Representative RT-PCR results from two experiments on the effects of estradiol (EB, estradiol benzoate, 0.01, 0.1, 1.0 nM) in PRL-treated cultures after 48 hr incubation with hormones. Estradiol did not alter TPH mRNA in vitro.

(D) Representative results from two experiments showing that progesterone (Prog) inhibited PRL-stimulation of TPH mRNA in a dose-dependent manner (0.1, 1, 10 nM, 0.1, 1 μM). Cultures were treated for 48 hr with PRL and progesterone or were untreated (leftmost lane).

of PRL levels that otherwise would be stimulatory, 5-HT suppressed the secretory and biosynthetic activity of the epithelium in mammary explants. The adipose stroma also appeared to be affected by 5-HT, with smaller lipid globules and more fibrous stroma. Treatment with p-chlorophenylalanine (PCPA), which inhibits TPA activity, resulted in a very distinctive morphological change characterized by highly chromophilic epithelia and dense secretory material inside acinar clusters (Figure 5D). In explants treated with methysergide (a 5-HT receptor antagonist) and PRL, the secretory features within the epithelial cells and in the lumen were similar to PRL but greatly exaggerated (Figure 5E).

Lipid globules were counted to provide an objective way of summarizing the morphological results (Figure 5F). These data showed that 5-HT significantly ($p < 0.001$) inhibited the effect of PRL. PCPA treatment resulted in levels similar to PRL alone, but the globules were smaller on average with PCPA, so the counts may not include small globules. Methysergide significantly enhanced ($p < 0.001$) the PRL effect on lipid globules.

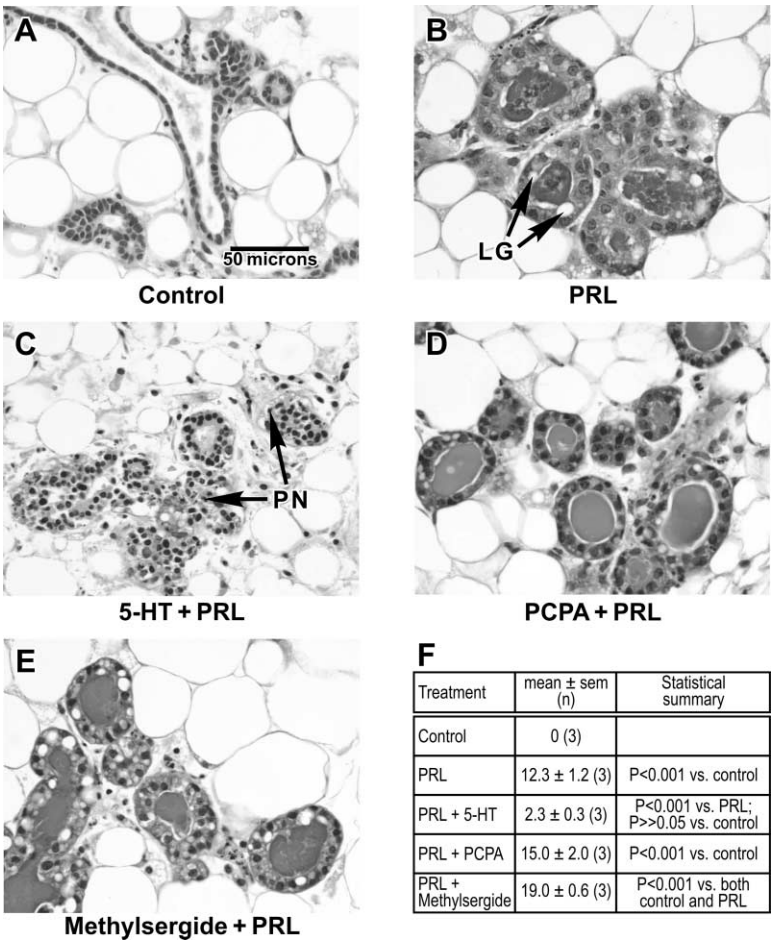
Milk Protein Gene Regulation by Serotonergic Agents

The effect of 5-HT on PRL-induced β-casein induction was measured in monolayer cultures of primary mammary epithelial cells (PMEC) (Figure 6A). Treatment of PMEC with PRL for 24 hr induced a high level of β-casein gene expression. The induction of β-casein was strongly attenuated by 5-HT in a dose-dependent manner. Neither PRL nor 5-HT had any effect on GPDH expression. The highest dose of 5-HT caused extinction of β-casein expression, and the fact that housekeeping gene expression was unaffected indicated that the action of 5-HT could not be explained by nonspecific or toxic effects.

Having observed that 5-HT inhibited β-casein gene expression, we hypothesized that blocking 5-HT receptors would enhance PRL-induced milk gene expression in mammospheres. Milk protein mRNA levels were assayed in extracts of mammosphere cultures treated with combinations of PRL and methysergide, an antagonist of multiple 5-HT receptor subtypes. Methysergide increased the expression levels of multiple milk protein mRNAs (Figure 6B), and the effects were highly robust in replicate experiments. The induction by methysergide was consistently greatest for GlyCAM-1 (more than 10-fold) and similar for β-casein, WAP, and α-lactalbumin (2- to 3-fold). GPDH and the tight junction protein occludin were relatively unaffected by methysergide as were other genes such as Na^+-K^+-ATPase and galactosyltransferase (data not shown).

Serotonergic Agents Affect Mammosphere Development

The foregoing experiments showed that 5-HT signaling affected mammary morphology using intact tissue explants and that it affected gene expression in isolated epithelial cultures. To test whether morphological development was affected by 5-HT at the level of isolated epithelial cells, mammoshere cultures were used.Under control conditions (1% serum-containing medium), mammospheres were small cystic structures comprising a darker boundary layer surrounding a lighter interior luminal space (Figures 7A and 7B). Qualitatively, those treated with 5-HT (Figures 7C and 7D) had a distinctly different appearance with irregular diameters and resembling solid ball-like structures or cysts with a small lumen containing cell debris. PCPA treatment resulted in dilated mammospheres with opaque, thin boundaries and strongly refractile luminal contents under phase contrast (Figures 7E and 7F). Mammospheres treated with methysergide were similar in appearance to the controls, with a light lumen and darker boundary layer



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Treatment	mean ± sem (n)	Statistical summary
Control	0 (3)	
PRL	12.3 ± 1.2 (3)	P<0.001 vs. control
PRL + 5-HT	2.3 ± 0.3 (3)	P<0.001 vs. PRL; P>>0.05 vs. control
PRL + PCPA	15.0 ± 2.0 (3)	P<0.001 vs. control
PRL + Methylsergide	19.0 ± 0.6 (3)	P<0.001 vs. both control and PRL

Figure 5. Serotonergic Effects on Mammary Gland Explant Cultures

Organ cultures of mammary glands from day 15 of pregnancy were cultured on floating lens paper rafts (see Experimental Procedures for details). Explants were treated with combinations of PRL (1 μg/ml) and either 5-HT, PCPA, or methysergide (2×10^{-4} M for each), as labeled beneath each panel, for 10 days with medium changed every second day. The explants were sectioned and stained with hematoxylin and eosin and photographed with 60× objective magnification. (A) shows control tissue, cultured in the presence of serum-free complete medium, containing insulin, aldosterone, and hydrocortisone (Ginsburg and Vonderhaar, 2000). (B) shows a typical PRL-stimulated alveolar cluster. Lipid globules are labeled (LG), and the lumen contains amorphous secretory material. (C) shows the effects of the combination of PRL and 5-HT, and examples of pychnotic nuclei (presumptive apoptotic figures) are labeled (PN). Note the paucity of secretory material and lipid globules and the collapsed and disorganized appearance of the alveolar clusters. (D) shows a PCPA and PRL-treated explant with a cluster of acinar epithelial structures characterized by very densely staining cytoplasms and dense secretory material. (E) is from an explant that was treated by incubation with PRL and methysergide which shows large lipid globules and exaggerated secretory features. (F) shows tabulated counts for lipid globules in each treatment group.

(Figures 7G and 7H), but the colonies treated with methysergide appeared to be more dilated than the control mammospheres.

We used an objective and quantitative morphometric method to determine the response of mammospheres to methysergide treatment because the effect of methysergide was less obvious than that of PCPA. The 5-HT-treated colonies had irregular crenated boundaries, so morphometry was not done on these. Mammosphere size was measured by employing the Macintosh computer-based public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Approximately 300 epithelial colonies in three replicate experiments were measured for each group. The thresholds were adjusted to outline automatically the mammospheres, and they were sorted into frequency distributions based on colony sizes (in arbitrary units). The size distribution of colonies was plotted by histogram (Figure 7I), and these distributions were tested for significance by a nonparametric t test, which is summarized as a comparison of the median colony areas (Figure 7J). The distribution of sizes was markedly shifted by drug treatment such that control colonies predominated among the smaller size classes (below 8 units), whereas methysergide-treated colonies were more frequent in all of the larger size classes (12 units and above). The difference in mammosphere size distribution was highly significant ($p < 0.005$; Mann-Whitney U test; Figure 7J).

TPH1 Gene Disruption Affects Mammary Gland Development

Having established by physiological and pharmacological studies that TPH1 is expressed in the mammary gland and that 5-HT signaling is inhibitory for mammary development and gene expression, we made use of mice with a disruption of the *TPH1* gene (Walther et al., 2003) to develop a genetic model in which to study mammary TPH function. Based on our initial observation that TPH was highly induced by pituitary grafting (high PRL) but not by progesterone treatment, we asked whether mammary development in pituitary-grafted mice would be affected by *TPH1* deficiency. The pharmacological studies led us to predict that the lobuloalveolar system would be more expansive and/or dilated in the $TPH1^{-/-}$ background. This prediction was confirmed by analysis of stained histological sections of mammary glands from $TPH1^{+/+}$ and $TPH1^{-/-}$ mice (Figure 8A). Of particular note, in both untreated and pituitary grafted $TPH1^{-/-}$ females the ducts and alveolar sacs had larger luminal diameters than the $TPH1^{+/+}$ glands. The stimulated $TPH1^{-/-}$ glands were filled with secretory material (Figure 8Aiv). Although these data were consistent with the in vitro studies showing direct actions of 5-HT in the

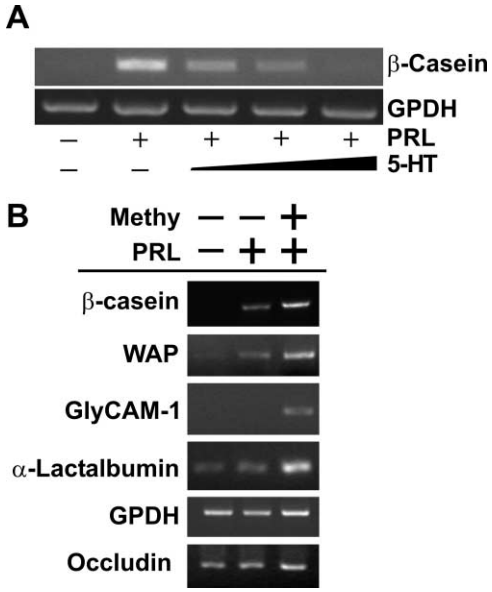

Figure 6. Serotonergic Agents Alter Mammary Gland Gene Expression

(A) Primary mammary epithelial cells (PMEC) in monolayers were cultured on rat tail collagen-coated plates in a 1% fetal bovine serum-containing medium including insulin and EGF. After preincubation for 7 days, the cells were treated with PRL either alone or in combination with increasing doses of 5-HT for 48 hr (2, 20, 200 μM). Expression levels of β-casein and GPDH were assayed in RNA extracts by RT-PCR.

(B) PMEC in three-dimensional mammosphere cultures were treated with PRL (1 μg•ml^{-1}) either alone or in combination with methysergide (methy, 100 μg•ml^{-1}), as shown above each lane. Expression levels of β-casein, WAP, GlyCAM-1. α-lactalbumin, GPDH, and occludin were assayed in RNA extracts by RT-PCR. The data shown for each panel are representative of three independent experiments.

mammary glands, it was possible that the hyperstimulated phenotype of pituitary grafted *TPH1*$^{-/-}$ mice could result from effects on other tissues. To test this, we analyzed mammary gland development in mice in which selected teats were sealed to provoke stasis-induced involution (Figure 8B). The actively lactating glands with open teats (Figures 8Bi and 8Biii) had complex lobuloalveoli surrounded by densely staining secretory epithelium. Typical of active lactation, the open glands had very little adipose stroma. After 3 days in milk stasis, the sealed glands of the normal (*TPH1*$^{+/+}$) females showed signs of early involution, including collapse of the lobuloalveoli into clusters of regressing cells and extensive reestablishment of the adipose stroma (Figure 8Bii). In contrast, glands in the *TPH1*$^{-/-}$ females were resistant to stasis-induced involution (Figure 8Biv). The degree of epithelial regression was variable but always less than that in the *TPH1*$^{+/+}$ controls, and there was no detectable reestablishment of the adipose stroma after 3 days of stasis. These results in the *TPH1* gene deletion model were consistent with the physiological and pharmacological studies, establishing that the mammary gland 5-HT biosynthetic system is an important component in the local control of development and secretory function.

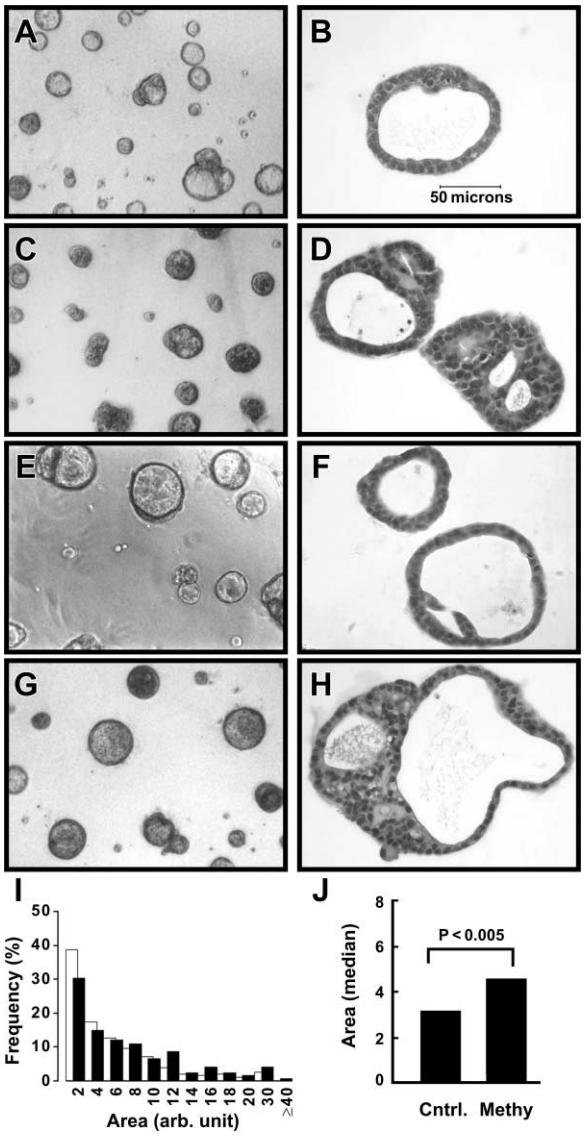

Figure 7. Effects of Serotonergic Agents on Cultured Mammospheres

Mammospheres cultured from normal virgin (*TPH*$^{+/+}$) mice were suspended in Matrigel and cultured in 1% fetal bovine serum-containing medium with addition of drugs, as described in Experimental Procedures. Qualitative comparisons of typical mammospheres are shown for cultures treated with control (A and B), 5-HT (C and D), PCPA (E and F), or methysergide (G and H). (A), (C), (E), and (G) are phase contrast images of mammosphere cultures in situ, and (B), (D), (F), and (H) are brightfield images of hematoxylin-eosin stained sections from paraffin-embedded mammospheres cultured identically. (I) Frequency distributions of sizes of colonies for control- (open bars) and methysergide-treated (closed bars) cultures. (J) Median areas (in arbitrary units) of mammospheres sorted by size ($n = 350$, two replicate experiments) of control- and methysergide-treated cultures ($p < 0.005$, Mann-Whitney U test).

Discussion

Mammary Expression of TPH and Regulation of 5-HT Synthesis

The mammary gland is known to synthesize a variety of peptide growth factors, cytokines, and neuropeptides (Daniel and Smith, 1999; Goldman and Frawley, 1996), but

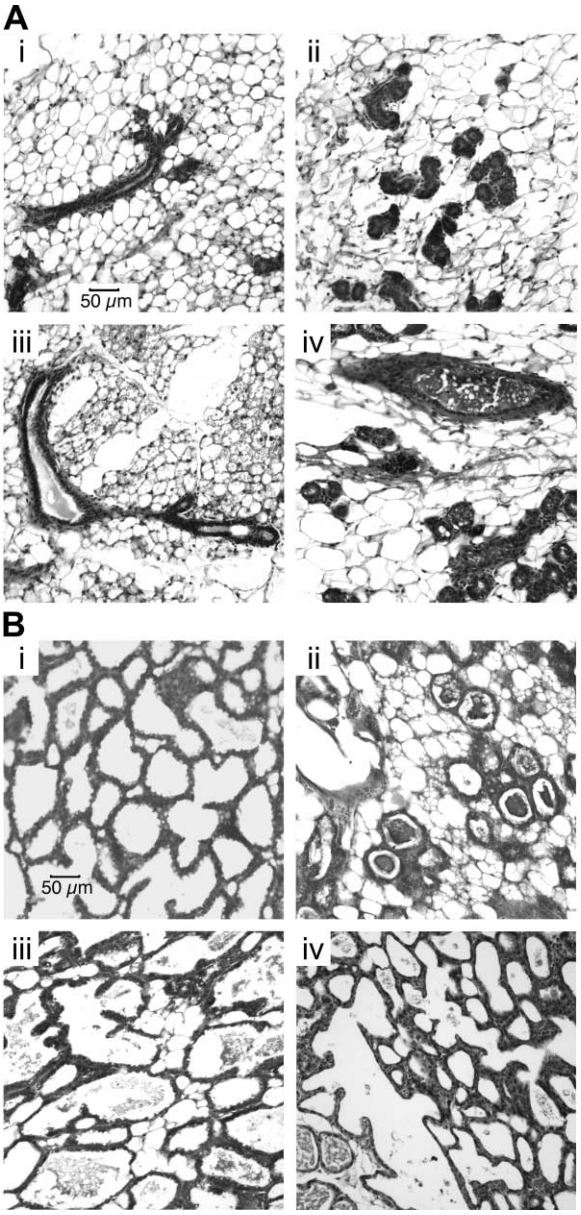

Figure 8. Mammary Gland Phenotypes in *TPH1*$^{-/-}$ Mice

(A) Hyperstimulation of *TPH1*$^{-/-}$ mammary glands by PRL. Normal *TPH*$^{+/+}$ (Ai and Aii) and *TPH1*$^{-/-}$ (Aiii and Aiv) mice were either untreated (Ai and Aiii) or PRL-stimulated by pituitary grafting (18 days; Aii and Aiv). In *TPH*$^{+/+}$ mice, PRL stimulated the growth of clusters of alveolar epithelium (Aii). *TPH*$^{-/-}$ mice had open ducts with obvious secretory material in the unstimulated controls (Aiii) and responded to PRL stimulation with exaggerated growth of alveolar clusters and large amounts of dense secretory material in the lumens of alveoli and ducts (Aiv).

(B) *TPH1* gene disruption prevents normal stasis-induced involution. Matched contralateral #4 glands from *TPH*$^{+/+}$ (Bi and Bii) and *TPH*$^{-/-}$ (Biii and Biv) mice were left open to suckling (Bi and Biii) or sealed (Bii and Biv) on day 10 of lactation. After 3 days of unilateral milk stasis, the glands were removed and prepared by histological sectioning and staining. Involution-related changes in the normal mice included collapse of the secretory lobuloalveoli into clusters of regressing cells and extensive filling of the surrounding stroma with adipose fat (Bii). In contrast, *TPH1*$^{-/-}$ glands retained an extensive lobuloalveolar morphology without the normal adipose filling of the stroma (Biv).

prior to our studies there was no evidence of biogenic monoamine synthesis in mammary tissues. Knowing the broad functional potential and ancient phylogenetic history of biogenic monoamine signaling, we suspected that there might be important biological implications of monoamine synthesis in the mammary glands. Consequently, we performed a variety of experiments regarding mammary 5-HT regulation and function.

The mammary glands are highly specialized skin glands with an uncertain evolutionary history. Although little data exist in mammalian skin, 5-HT is synthesized in large amounts in skin glands of fish and amphibians (Zaccone et al., 1994; Seki et al., 1995), suggesting that monoamine synthesis may have been a primitive characteristic in mammary gland evolution. Serotonin synthesis has been documented in the skin of hamsters (Slominski et al., 2002), but neither cells of origin nor functions of integumentary 5-HT were studied in the hamster. An unexpected duality in the 5-HT biosynthetic system was recently demonstrated by the discovery of a gene expressing a neuronal TPH isoform (TPH2), in addition to the classical *TPH1* gene, which is preferentially expressed in peripheral tissues (Walther et al., 2003). We observed that the peripheral *TPH1* gene, but not *TPH2*, was expressed in the mammary glands and that it encoded two mRNA sizes (1.8 and 3.8 kb), which may be accounted for by alternative mRNA processing of the primary transcript.

TPH1 mRNA was elevated in mammary glands during pregnancy and lactation compared with nulliparous virgin and involuting glands. In lactating mice, the level of TPH mRNA was doubled following 1 day of milk stasis (teat sealing), which disrupts the normal homeostatic balance between the extrinsic secretory drive (primarily PRL) and local feedback. Identifying the specific mechanisms by which TPH is induced by filling of the mammary glands will be of interest.

Functions of Mammary TPH and 5-HT

Treatment of mammary gland explants or mammospheres with 5-HT caused involution-related morphological changes. Conversely, inactivation of the 5-HT pathway by gene disruption, a TPH blocker, or a 5-HT receptor antagonist resulted in exaggeration of the developmental changes associated with secretion. Because these effects of serotonergic agents were similar in both explants and mammospheres, they appear to be mediated by 5-HT receptors on the epithelial cells.

Treatment of mammary epithelial cells with 5-HT suppressed PRL-induced β-casein gene expression in a dose-dependent manner, which suggested that 5-HT in the immediate microenvironment of the epithelial cells could function as an autocrine-paracrine regulator. In closed mammospheres, blocking 5-HT receptors permitted higher levels of expression for several milk protein genes, including β-casein, WAP, α-lactalbumin, and GlyCAM1. Based on this pharmacological evidence, we concluded that expression of specific mammary gland genes is restrained by activation of 5-HT receptors in the mammary epithelium. These initial studies have been limited to a few genes and pharmacological agents, but there are many other mammary functions and a large

variety of available serotonergic drugs. Detailed and extensive pharmacological studies with a variety of serotonergic agonists and antagonists will be needed to sort out the roles of 5-HT receptor signaling for specific functions in the mammary glands.

The recent establishment of *TPH1* gene-disrupted mice provided a valuable genetic model in which to test the roles of mammary 5-HT synthesis. Confirming all of our physiological studies, *TPH1*$^{-/-}$ mice were hyperresponsive to PRL (pituitary grafting) and were resistant to milk-stasis-induced involution. This animal model can serve as the basis for a variety of future studies on the role of mammary-derived 5-HT.

The first phase of involution of the lobuloalveolar cells may occur while PRL levels are high. After PRL levels fall, the involution process enters a second phase characterized by wholesale remodeling of the glands. Several of the local factors that mediate involution have been identified. Our results suggest that the first phase of involution may be directly associated with 5-HT signaling. Three other signaling molecules known to participate in the first phase of involution are transforming growth factor-β (TGF-β), p53, and Stat3 (Chapman et al., 1999; Nguyen and Pollard, 2000). Future experiments that link the pathways associated with these signaling molecules to 5-HT may provide valuable insights into the mechanisms of mammary development.

In conclusion, our findings show that the mammary gland contains a complex serotonergic regulatory system that includes biosynthetic and functional components. This autocrine-paracrine system appears to play an important role in the processes of development and homeostatic control of lactation and involution.

Experimental Procedures

Animals

Prolactin-deficient mice (Horseman et al., 1997), made congenic on C57Bl/6J by eight backcross generations, were treated by either grafting an anterior pituitary gland under the kidney capsule; implanting a 25 mg, 21 day release progesterone tablet (Innovation Research of America, Sarasota, FL, USA) subcutaneously for 18 days; or were sham operated. Progesterone-treated tissues were included in these experiments as a control so that we could distinguish genes that responded to PRL from those that were indirectly regulated by PRL via its stimulatory effect on the ovary (Brisken et al., 1999; Vomachka et al., 2000). Lactating female mice and nulliparous virgin control mice were purchased from Harlan Laboratories (Indianapolis, IN) and were studied between days 4 and 9 postpartum. Mice were maintained on an L, D: 14, 10 daily light, darkness cycle with food and water available ad libitum. Ovariectomy was done via bilateral incisions in the flanks. Surgeries were performed under general anesthesia and aseptic conditions. *TPH*$^{-/-}$ and corresponding controls (*TPH*$^{+/+}$) were mixed (129sv, C57Bl/6) genetic background. Mice were purchased for some studies from Jackson Laboratory (Bar Harbor, ME) or Taconic (Germantown, NY). All experiments were conducted under protocols that were approved by the Institutional Animal Care and Use Committee.

Differential Gene Expression

The cDNAs specifically expressed in the PRL-stimulated breast were cloned by suppression subtractive hybridization (Diatchenko et al., 1996) using reagents and protocols from CLONTECH Laboratories, Inc. (Palo Alto, CA). Virtual Northern hybridization (Southern hybridization to full-length cDNA) was performed following the manufacturer's protocol (CLONTECH Laboratories, Inc.). Briefly, full-length cDNA was prepared using the SMART primer method and was amplified for 14 cycles using TaKaRa Ex-Taq (PanVera Corp., Madison, WI); 100 ng of full-length cDNA was applied to each well of a 1.2% agarose gel, and Southern hybridization was done following manufacturer protocols for Hybond N$^+$ membrane and Alkphos direct kit (APBiotech, Uppsala, Sweden). The tissue content of mouse TPH mRNA was measured by real-time quantitative RT-PCR (Kang et al., 2000) using the Bio-Rad iCycler Thermal Cycler using SYBR-Green detection with protocols recommended by the manufacturer. Primers are described in Supplemental Table S1 (see http://www.developmentalcell.com/cgi/content/full/6/2/193/DC1).

In Situ Hybridization and Immunohistochemistry

Localization of TPH mRNA in mammary gland tissue sections was performed as described in Hou et al. (2000). Ten micrometers (μ) paraformaldehyde-fixed frozen sections were hybridized with digoxygenin-labeled (DIG RNA Labeling Kit, Roche Molecular Biochemicals, Indianapolis, IN) sense and antisense RNA probe corresponding to 5′ end of TPH mRNA (~200 bases). Immunohistochemical detection of 5-HT was performed on 5 μ paraffin-embedded sections using a monoclonal anti-5-HT (H209, Dako Corp., Carpinteria, CA), which was visualized by a Cy3-conjugated streptavidin-biotin secondary antibody system (Histomouse SP, Zymed, Corp., South San Francisco, CA).

Assay of 5-HT and 5-HIAA in Milk Dialysates

Microdialysis probes (2–4 mm length) were prepared and perfused as described previously in Yamamoto and Pehek (1990), and the dialysate concentrations of 5-HT and 5-hydroxyindole acetic acid (5-HIAA) were measured by high-pressure liquid chromatography with electrochemical detection (HPLC/EC) (Shankaran and Gudelsky, 1999). For collection of milk dialysate, the fourth inguinal mammary gland was surgically exposed, and the main milk duct was pierced with a 26 gauge needle to allow for insertion of the dialysis probe. Oxytocin (0.2 IU intraperitoneal) was injected prior to probe insertion to stimulate milk letdown. The dialysates were collected for 30 min intervals after discarding the first 30 min equilibration samples. Levels of 5-HT and 5-HIAA were stable for at least 1 hr, and the reported values are for the first 30 min sample. Recovery efficiencies were determined to be approximately 1% from milk for 5-HT and 5-hydroxyindole acetic acid (5-HIAA).

Cell and Organ Explant Cultures

Reagents and media for cell and organ cultures were purchased from Life Technologies, Inc. (Gaithersburg, MD), unless otherwise noted. PMEC were prepared from virgin mice (Swiss Black) by enzymatic digestion and differential trypsinization, modifying the method of Imagawa et al. (2000). In brief, excised mammary glands were minced with a razor blade, transferred into 50 ml conical tubes, and digested in M199 medium containing bovine serum albumin (2.5 mg•ml^{-1}, fraction V, Sigma) and collagenase type III (0.1%, Worthington Biochemical Corporation, Freehold, NJ), penicillin-streptomycin (1× pen strep, 50 units•ml^{-1}-50 μg•ml^{-1}, respectively), and amphotericin B (2 μg•ml^{-1}, Sigma Chemical Co., St. Louis, MO) for 3 hr at 37°C. Organoids were allowed to settle, washed twice in phosphate buffered saline, and incubated at 37°C under 5% CO_2 in DMEM/F12 (1:1) containing insulin (5 μg/ml), epidermal growth factor (10 μg•ml^{-1}), 1% fetal bovine serum (Harlan Bioproducts, Indianapolis, IN), and 1× pen strep (complete medium) for 5–7 days to let them attach to rat tail collagen-coated dishes (BD Biosciences, Palo Alto, CA). Fibroblasts were removed by four rounds of partial trypsinization. Resulting concentrated epithelial cells (2×10^5 cells per well) were plated on conventional plastic tissue culture dishes or on basement membrane (Matrigel, 1 ml per well, BD Biosciences, Palo Alto, CA) reconstituted in a 6-well plate. They were preincubated for 7 days in complete medium, which was changed each day. After preincubation, the cells were treated with ovine PRL (1 μg•ml^{-1}) for 2 days. Following treatments, the cells were dissociated with dispase (BD Biosciences, 150 units/well) and collected by centrifugation.

MCF10A cells are cultured on plastic dishes in medium containing DMEM/F12 with 5% horse serum, 10 μg•ml^{-1} insulin, 0.5 μg•ml^{-1} hydrocortisone, 1× pen strep, 0.25 μg•ml^{-1} amphotericin B, 2 mM L-glutamine, and 20 μg•ml^{-1} EGF. T47D cells were cultured on plastic dishes in DMEM with 10% fetal bovine serum, insulin (5

$\mu g \cdot ml^{-1}$), 1× pen-strep, 0.25 $\mu g \cdot ml^{-1}$ amphotericin B, and 2 mM L-glutamine.

Mammary gland explants were cultured according to the methods outlined in Ginsburg and Vonderhaar (2000). Briefly, mammary glands from pregnant C57Bl/6J mice (15 days postcoitus) were cultured in Waymouth's MB 752/1 medium (InVitrogen, Grand Island, NY); supplemented with L-glutamine (2 mM), penicillin (100 $U \cdot ml^{-1}$), streptomycin (100 $\mu g \cdot ml^{-1}$), gentamycin (50 $\mu g \cdot ml^{-1}$), 20 mM HEPES, 5 $\mu g \cdot ml^{-1}$ insulin, 100 $ng \cdot ml^{-1}$ aldosterone, 100 $ng \cdot ml^{-1}$ hydocortisone, and fatty acid free bovine serum albumin (1 $mg \cdot ml^{-1}$); and were floated at the medium interface on siliconized lens paper rafts. The cultures were maintained in a humidified atmosphere of 50% O_2, 5% CO_2, and 45% N_2 at 37°C. Agents were added fresh every second day, and the cultures were carried out for 10 days in total.

Drugs (methysergide, 5-HT, PCPA) were used over a dose range of 2×10^{-4} M – 2×10^{-7} M and did not have any negative effects on cell viability or expression of housekeeping genes relative to untreated cells. These doses of drugs were in the same range as previous studies of nonneuronal tissues in vitro (Tamura et al., 1996; Tuladhar et al., 2000). Where a single dose is shown, the results represent the maximum dosage.

Histology

For histological examination, tissues or mammospheres were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 7 μ (tissue) or 4 μ (mammospheres), and stained with conventional hematoxylin-eosin reagents (Pathology Department, University of Cincinnati). Mammary gland wholemounts were as described in Horseman et al. (1997). Lipid globules were quantified by establishing a threshold size (2 μ) and counting globules that were larger than the threshold within multiple high power fields using images captured by a digital camera. Averages were calculated for three separate samples in each group, which were compared by ANOVA with Dunnett's multiple comparison test.

Statistics and Experimental Replication

Each experiment in cultured cells and tissues was replicated two to four times as noted, and representative results are shown in the figures. Differences between means were tested by ANOVA for multiple groups. For comparison of two means, Student's t test (two tailed) or an equivalent nonparametric test (Mann-Whitney U test) was used. Significance was accepted for $p < 0.05$.

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