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A role for mesenchyme-derived tachykinins in tooth and mammary gland morphogenesis

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SUMMARY

Tachykinin peptides such as substance P (SP) function as neurotransmitters and neuromodulators in the mammalian central and peripheral nervous systems. Here, we provide evidence that they may also play an important role in the morphogenesis of some nonneural organs where epithelial-mesenchymal interactions are involved. We show the following. (1) mRNA encoding tachykinin precursor proteins is expressed transiently in condensing mesenchyme during the development of mouse tooth germ, mammary gland, limb bud, external auditory meatus and genital tubercle. (2) In developing tooth germ and mammary gland; mRNA encoding the neutral endopeptidase (NEP) that degrades secreted tachykinins is spatially and temporally co-expressed with tachykinin precursor mRNA. (3) SP and the mRNA encoding SP receptors are also expressed in the developing tooth germ. (4) Tooth development in explant cultures is blocked both by tachykinin-precursor-specific antisense oligonucleotide and by an SP receptor antagonist: in both cases the block is relieved by exogenous SP. Together, these findings suggest a surprising new role for tachykinins in tooth and mammary gland morphogenesis, and possibly also in limb, ear and external genitalia morphogenesis.

Key words: tachykinin, morphogenesis, tooth, mammary, mesenchyme-epithelium interaction, mouse

INTRODUCTION

Interactions between mesenchyme and overlying epithelial are crucially important for the development of many vertebrate organs, including mammary glands, teeth, skin appendages, ears and limbs. The signalling between the two tissues is complex and reciprocal and changes as development proceeds.

Mammary gland development, for example, is initiated by the underlying mesenchyme, which induces the neutral epidermis to become mammary gland epithelium; the invaginating epithelium then interacts with the surrounding condensing mesenchyme (Kratochwil, 1969; Sakakura et al., 1976; Sakakura, 1991). Tooth development, by contrast, is initiated by the oral epithelium, which induces the jaw mesenchyme to aggregate at specific sites; reciprocal interactions then cause the epithelial cells to differentiate into enamel-secreting ameloblasts and the mesenchymal cells to differentiate into dentine-secreting odontoblasts (Mina and Kollar, 1987; Ruch, 1984, 1987; Thesleff and Humerinta, 1981). For the most part, the molecular mechanisms involved in these epithelial-mesenchymal interactions are poorly understood, although a number of growth factors (Partanen et al., 1985; Lyons et al., 1990; Jones et al., 1991; Kronmiller et al., 1991; Vaahtokari et al., 1991; Mitsiadis et al., 1992, 1993; Vainio et al., 1993; Chai et al., 1994) and extracellular matrix components (Vainio et al., 1989, 1991) have been implicated. Here we report the surprising finding that tachykinins, produced by mesenchymal cells, are apparently among the signaling molecules involved.

These are at least five distinct mammalian tachykinin peptides. Four of these, substance P (SP), neurokinin A (NKA) and its two extended derivatives neuropeptide K and neuropeptide γ, are proteolytically processed from a precursor protein encoded by a single gene, preprotachykinin (PPT)-A. There are three variants of the precursor protein, each encoded by a splicing variant of PPT-A mRNA (Krause et al., 1987, 1989).

Tachykinins are widely distributed in the central and peripheral nervous system, where they function as neurotransmitters and neuromodulators (reviewed in Krause et al., 1989; Maggio, 1988). The best characterized of these neuropeptides, the undecapeptide SP, has excitatory effects on both peripheral and central neurons. It also has a variety of effects on nonneuronal cells, including the stimulation of smooth muscle contraction, the stimulation of exocrine and endocrine secretion, the enhancement of vascular permeability, and the regulation of immune and inflammatory responses (Krause et al., 1989; Maggio, 1988; Payan, 1989). Tachykinin responses are mediated by at least three high affinity receptors, designated NK-1, NK-2 and NK-3, that are encoded by separate genes and differ in their relative affinities for the different tachykinin peptides (Hershey and Krause, 1990; Sasai and Nakanishi, 1989; Shigemoto et al., 1990; Yocota et al., 1989).

Secreted tachykinins are thought to be cleared from the extracellular space by the cell-surface-bound neutral endopeptidase 24.11 (NEP, also known as CD10 or enkephalinase). The same endopeptidase also degrades a number of other secreted peptides, including enkephalins, bombesin-like peptides, angiotensins and the chemotactic peptide fMet-Leu-Phe (Erdos and Skidgel, 1989; McKelvy and Blumberg, 1986).

There is indirect evidence that some of the peptides initially defined by their neural activity – and therefore called neuropeptides – may play additional roles in the development of nonneural tissues. Some opioid peptides, for example are expressed in mesodermal lineages during organogenesis (Keshet et al., 1989), some neuropeptides such as SP, bombesin and vasoactive intestinal peptide (VIP) act as growth factors for nonneural cells (Nilsson et al., 1985; Payan, 1985; Represa et al., 1988; Rozengurt and Sinnett-Smith, 1983; Tanaka et al., 1988; Gressens et al., 1993), and NEP is transiently expressed during the development of certain organs (Dutriez et al., 1992) and seems to be involved in lung development (Sunday et al., 1992; King et al., 1993).

Prompted by these findings, we initiated a study to examine whether tachykinins play a role in organogenesis. We find that PPT-A and NEP mRNAs are expressed transiently in developing mammary glands, teeth, genital tubercle, ear and limb bud, mainly in the condensed mesenchyme surrounding the invaginating epithelium. In addition, we show that NEP mRNA is temporally and spatially co-expressed with PPT-A mRNA in developing teeth and mammary glands and that SP and mRNA encoding SP receptors are expressed in developing tooth germs. Finally, we demonstrate that agents that interfere with tachykinin signalling inhibit tooth morphogenesis in explant cultures, suggesting that tachykinins play a critical role in tooth development, and possibly in the other organs where they are transiently expressed.

MATERIALS AND METHODS

Embryos

Hybrid mice (C57/Bl×Balb/C) were time-mated and the day of locating the vaginal plug was designated as embryonic day 0 (E0). Embryos were removed at days E10-E17, fixed overnight in 4% paraformaldehyde in phosphate buffer saline and processed for in situ hybridization.

Organ culture

The first mandibular arch was removed from E10 embryos, (30-34 somite pairs, Theiler's stage 16; Theiler, 1972). Arches were cultured in BGjb medium(GIBCO), supplemented with vitamin C (50 mg/ml) and 10% fetal calf serum, as described by Kronmiller et al. (1991). After 10 days the tissue was fixed and processed as described above.

In situ hybridization

In situ hybridization was performed as previously described (Hogan et al. 1986). In brief, 10 μm-thick frozen sections were collected on poly(L)lysine-coated glass slides, refixed in and dehydrated in graded ethanol solutions 30%, 60%, 80%, 95% and 100%, each for 2 minutes. Before hybridization, sections were pretreated successively with 0.2 M HCl, pronase (0.125 mg/ml), 4% paraformaldehyde in PBS, and 0.25% acetic anhydride in 0.1 M triethanolamine buffer pH 8. Hybridization was carried out at 50°C overnight in a solution containing 50% (vol/vol) formamide in 0.3 M NaCl and ^{35}S-labeled RNA probe (2×10^8 cts/minute/ml). Washing was performed under stringent conditions that included an incubation at 50°C for >14 hours in 50% formamide in 0.3 M NaCl and a 30 minute incubation at 37°C in RNaseA (20 mg/ml). Autoradiography was performed using Kodak NTB-2 nuclear track emulsion with a 5-9 day exposure. Control hybridizations with riboprobes in the 'sense' orientation were carried out for each probe.

Hybridization probes

The following cDNA clones were used as templates for cRNA synthesis. (1) A cDNA containing the entire coding region of mouse PPT-A cDNA was cloned in our laboratory from a mouse brain cDNA library (whole brain cDNA in a λgt10 vector) and was identified by sequencing as the spliced variant γ-PPT. The cDNA shares 98% homology in its coding region with the respective rat cDNA. (2) A 0.9 kb long cDNA composed of the coding region and the 3′-noncoding region of mouse NEP was cloned in our laboratory from the same mouse brain cDNA library and shown to be identical to the previously described NEP cDNA sequence (Chen et al., 1992). (3) cDNAs encoding the rat tachykinin receptors NK-1 and NK-2 (Sasai and Nakanishi, 1989; Yocota et al., 1989) were kindly provided by Dr S. Nakanishi of Kyoto University.

cDNAs were cloned into the polylinker of a Bluescript SK vector (Stratagene) and were linearized by digestion with the appropriate restriction endonuclease to allow the synthesis of an ^{35}S-labeled complementary RNA in either the antisense or sense orientation, using T3 or T7 RNA polymerase. RNA probes were fragmented by a mild alkaline treatment prior to use for in situ hybridization. For blot-hybridization analysis, the same cDNA fragments were labeled with ^{32}P by randomly primed DNA synthesis.

Reverse Transcription-PCR analysis (RT-PCR)

The region of the first mandibular molar tooth was dissected from E13-E16 embryos. Tissue was homogenized and RNA extracted in RNasol solution (Stratagene), using 200 μl/10 mg tissue, followed by extraction in chloroform, precipitation in isopropropanol and washing in ethanol.

Oligo(dT)-primed cDNA was synthesized from 10 μg of total RNA, using a cDNA synthesis kit (Amersham) and their recommended procedure for first-strand cDNA synthesis. cDNAs were amplified with the indicated primers in 30 rounds of PCR amplification. Each cycle included a 60 second incubation at 94°C, a 2 minute incubation at 54°C, and a 90 second incubation at 72°C. PCR products were electrophoresed through a 1.5% agarose gel and analysed by blot-hybridization. The following oligonucleotides were used for PCR amplification: (1) 5′-CTTCTTTCGTAGTTCTGCAT-3′ and 5′-TGCCAACGATGATCTAAATT-3′, specific for PPT-A cDNA; (2) 5′-ATGGATAACGTCCTTCCTATG-3′ and 5′-ATAATTGGTCACTGTCCTCAT-3′, for NK-1 cDNA; (3) 5′-GAAGCGCGCGGTACCCAGAC-3′ and 5′-GCCAGGTAGACCTGCTGGAT-3′, for NK-2 cDNA.

SP radioimmunoassay

Mandibles from E14, E15 and E17 mice were dissected, and individual molars were excised and cleaned of surrounding tissues. Pools of 15-20 molars from each age were homogenized in 2 M acetic acid, containing 1 mg/ml PMSF (Sigma) and 0.3 M β-mercaptoethanol. Homogenates were centrifuged twice at 7000 ***g*** for 10 minutes and supernatants were lyophilized and stored at −70°C until use.

SP concentrations in molar extracts were determined by radio immunoassay (RIA), using an SP detection kit (Incstar Corp., Stillwater, Minnesota), following the procedure recommended by the manufacturer. SP concentrations were determined relative to SP standards and the amount of SP per molar tooth was calculated.

Inhibition of molar development by PPT-A antisense oligonucleotides and NK-1 receptor antagonist

E14 molar explants were grown in culture on top of 2 mm-thick, 1% agarose disks in 35 mm culture dishes (Nunc). Disks were immersed in 0.5 ml serum-free BGjb medium, supplemented with transferrin (50 μg/ml), Vitamin C (50 μg/ml) and antibiotics. Cultures were grown at 37°C for 6 days in 5% CO_2, with a medium change every other day.

For antisense inhibition experiments, oligonucleotides were added to the culture medium at a final concentration of 10 μM. Sulfor-modified, 18-mer oligonucleotides were synthesized by Oligos Etc. Inc.(Wilsonville, OR). The oligonucleotides [5′-ATGAAAATC-CTCGTGGCC-3′] and [5′-GGCCACGAGGATTTTCAT-3′] were used as sense and antisense oligonucleotides, respectively. These sequences correspond to the translational initiation codon and downstream sequences of mouse PPT-A cDNA.

CP-96345 [(2S, 3S)-*cis*-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octane-3-amine], a potent nonpeptide anatagonist of the NK1 SP receptor (Snider et al., 1991), was a generous gift from Pfizer Inc. (Groton, CT). CP-96345 (dissolved in distilled water) was added to the organ culture medium to a final concentration of 8 μM.

Rescue of antisense-inhibited development and CP-96345-inhibited development was carried out by adding a mixture of SP (10 μM, Sigma) and the specific NEP inhibitor phosphoramidon (10 μM, Sigma) together with the inhibiting reagent.

RESULTS

To identify tachykinin-producing cells in the mouse embryo, we cloned a mouse PPT-A cDNA and used it to make a PPT-A-specific RNA probe, which we then used for in situ hybridization studies. In northern blot analyses of adult brain mRNA, the probe detected all three of the alternatively spliced PPT mRNA species that encode the four PPT-A-derived Tachykinin peptides (not shown).

We carried out a comprehensive in situ hybridization analysis of mouse embryos from day 12 to 17 postcoitum. Complex patterns of expression of PPT-A mRNA were detected throughout this developmental period in the central and peripheral nervous systems. Expression of tachykinins in the nervous system, however, has been addressed by previous studies (e.g. Brene, 1990) and is beyond the scope of this study. (Nonetheless, central nervous system was included in most sections analyzed to serve as an internal positive control for signals detected elsewhere). We focused instead on the local production of tachykinins in nonneural tissues, especially in tissues engaged in active organogenesis. While the present study is mostly concerned with the role of tachykinins in tooth and mammary gland development, PPT-A mRNA was also detected in other developing organs, including the mesenchyme of the external auditory meatus, limb buds and genital tubercle (Fig. 1). It was also detected in the forming lip furrow (see Fig. 3F, arrowhead). It was not detected, however, in several other organs where epithelial-mesenchymal interactions are implicated, such as the developing kidney, lung and gut (see Fig. 3F).

Expression of PPT-A mRNA in the developing mammary bud

The mammary gland anlage is first evident as a thickening of the abdominal epithelium along paired longitudinal lines known as the mammary streaks. Beginning at E12, epidermal cells at determined sites along the mammary streak invaginate into the underlying mesenchyme and become associated with condensed mesenchyme to form five pairs of mammary buds.

At E12, the mesenchyme underlying the invaginating epithelium already expressed PPT-A mRNA and was clearly distinguishable from the flanking, non-expressing mesenchyme (Fig. 2A). By E13, the mesenchyme surrounding the bulb-

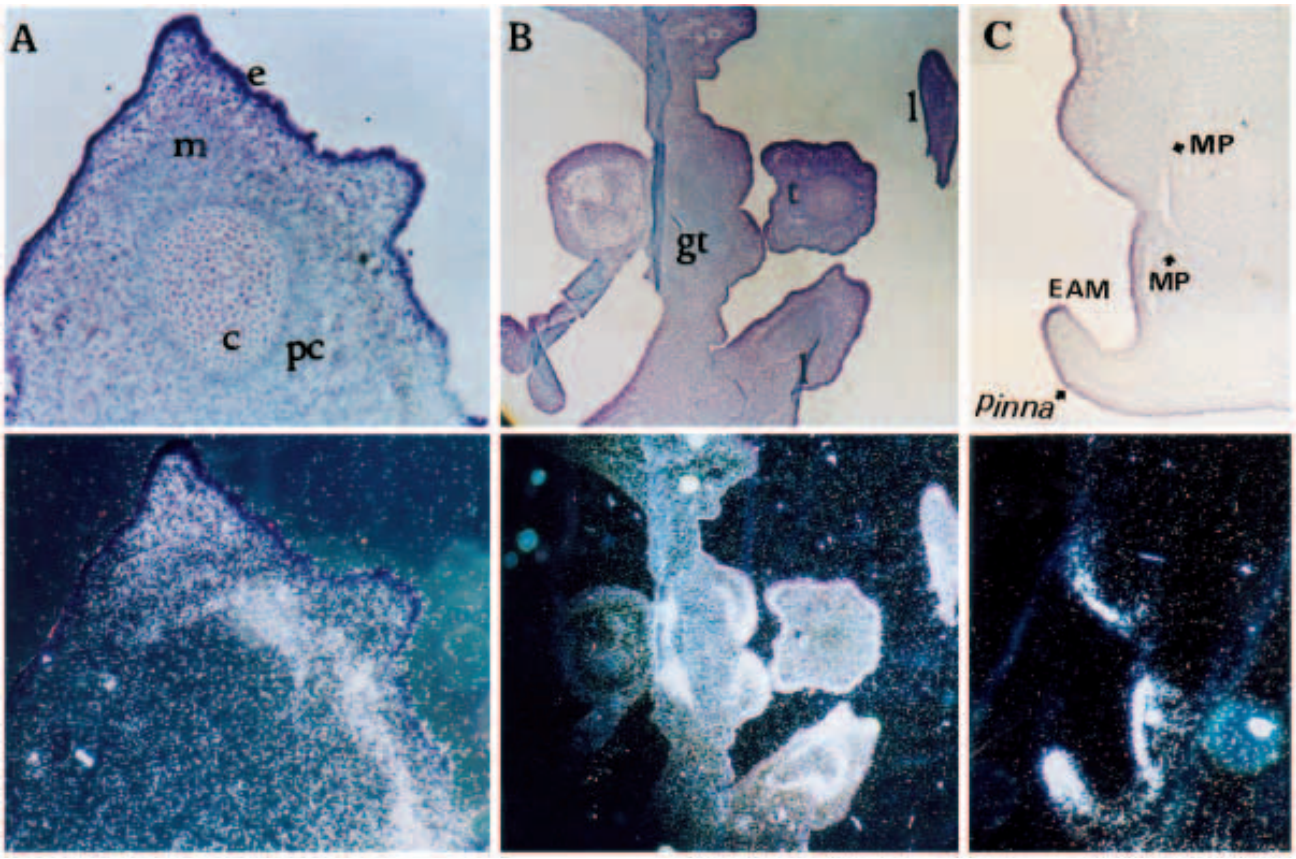

Fig. 1. Expression of PPT-A and NEP mRNAs in limb, genital tubercle and external ear development by in situ hybridization. Bright-field and corresponding dark-field images of transverse sections of E15 (A,B) and E14 (C) mouse embryos showing the forelimb bud in A, the genital tubercle, hind limb and tail in B, and the developing external ear in C. Hybridization was with a PPT-A-specific probe. Note that the hybridization signal is mainly in the condensed mesenchyme in these organs. m, mesenchyme; e, epithelium; c, cartilage; pc, perichondrium; gt, genital tubercle; t, tail; l, hindlimb; EAM, external auditory meatus; MP, meatal plate.

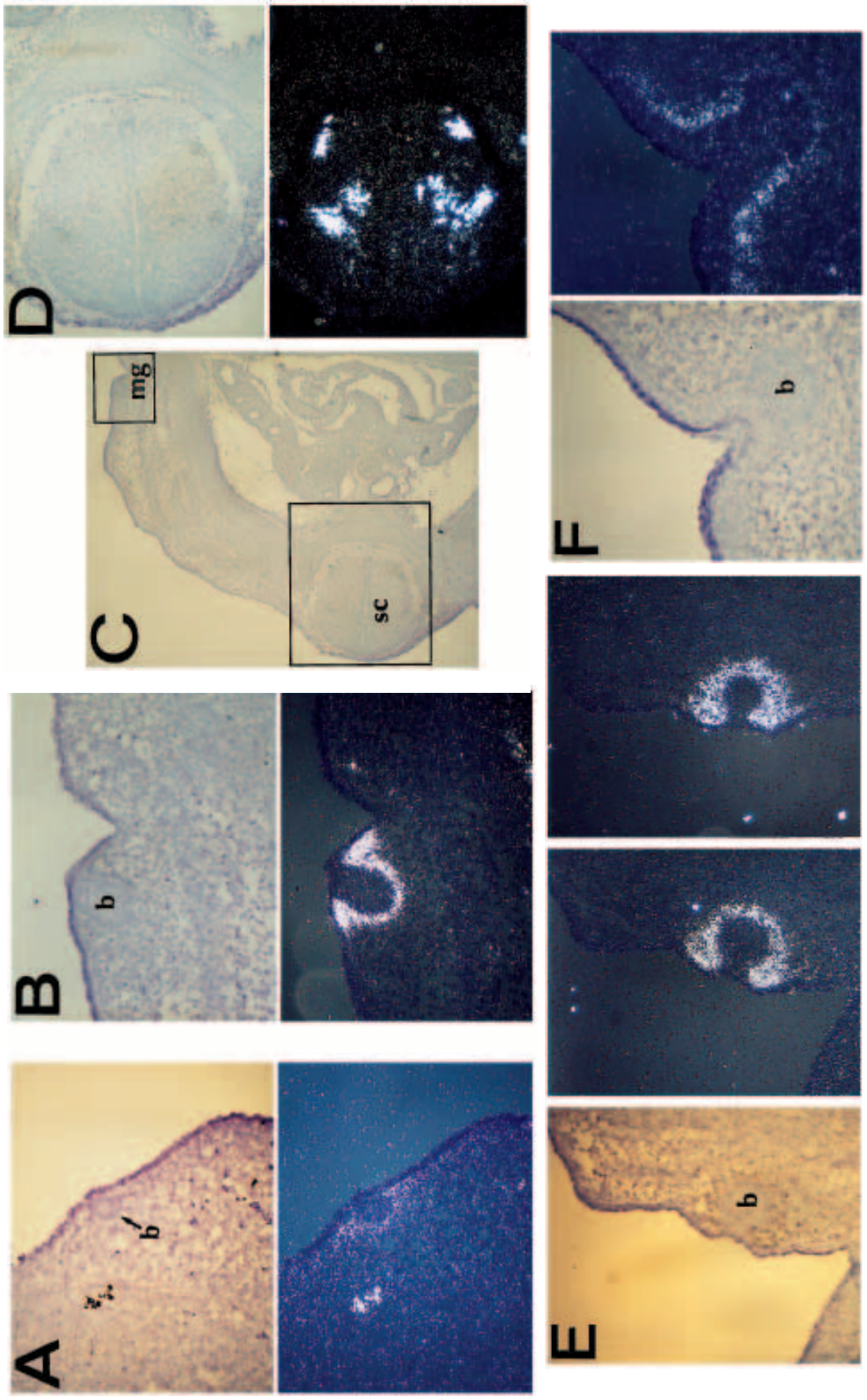

Fig. 2. Expression of PPT-A and NEP mRNAs in mammary gland development by in situ hybridization. Bright-field and corresponding dark-field images of transverse sections of E12 (A), E13 (B-D), E14 (E), and E15 (F) mouse embryos showing the mammary bud (b) and in D, spinal cord. Hybridization was with a PPT-A-specific probe, except for the far right-hand dark-field image in E, which shows an adjacent section hybridized with an NEP-specific probe. The boxed areas of the developing mammary gland (mg) and spinal cord (sc) in C are shown enlarged in B and D, respectively. Except for C, the hybridization signal is concentrated in the condensed mesenchyme of the mammary bud.

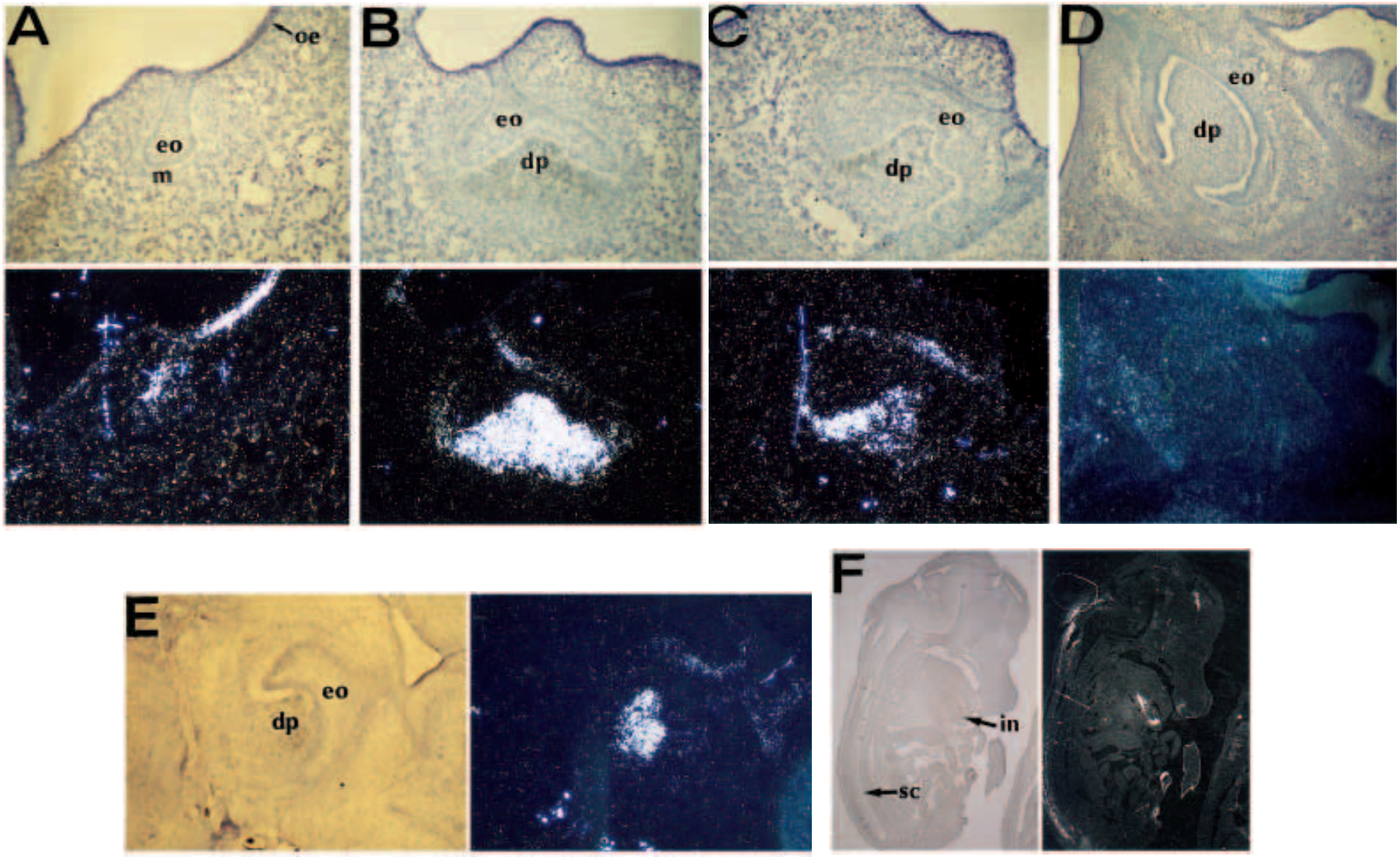

Fig. 3. Expression of PPT-A and NEP mRNAs in tooth germ development by in situ hybridization. Bright-field and corresponding dark-field images of transverse sections of E15 (A,B,C,E) and E17 (D) mouse embryos showing progressive stages of molar development – bud stage (A), cup stage (B,E), late cup stage (C) and bell stage (D). Hybridization was with a PPT-A-specific probe (A-D,F) or a NEP-specific probe (E). In F, the pattern of PPT-A mRNA expression in the spinal cord (sc) and incisor tooth germ (in) is shown in a parasagittal section of an E15 embryo. m, mesenchyme; dp, dental papilla; eo, enamel organ.

shaped mammary epithelium had further condensed and was now visible as a halo of fibroblasts, morphologically distinguishable from the adjacent loose mesenchyme; the intensity of the PPT-A mRNA signal was increased at this time compared to E12 (Fig. 2B). Abundant PPT-A mRNA expression remained confined to the condensed mesenchyme throughout the E14 (Fig. 2E), which is when the sexual phenotype of the mammary gland is determined. On E15, when the mammary bud was starting to elongate, expression of PPT-A mRNA decreased, particularly at the elongated end of the mammary bud (Fig. 2F).

Expression of PPT-A mRNA in developing tooth germs

Morphologically, tooth formation commences with a thickening in the oral epithelium, which then invaginates into the jaw mesenchyme to form a dental lamina. The lamina enlarges at a restricted number of sites, each marking the position of a future tooth. Each epithelial swelling (the future enamel organ) then becomes associated with condensed mesenchyme to form an individual tooth germ. Further development of the tooth germ is associated with cytodifferentiation and proceeds through well-characterized stages of bud, cup and bell-shaped structures, to form the adult tooth, with its epithelium-derived enamel and mesenchyme-derived dentin.

Fig. 3 shows the distribution of PPT-A mRNA during sequential stages of molar development. While PPT-A mRNA was detectable in the oral epithelium, the most intense signals were detected in the condensed mesenchyme. Although in the bud stage PPT-A mRNA could not be detected in the mesenchyme underneath the epithelial bud (Fig. 3A), in the cap stage, where the epithelium had expanded laterally to form the enamel organ, PPT-A mRNA was readily detected in the condensing mesenchyme (Fig. 3B), which is the forerunner of the dental papilla of the molar (and incisor) tooth germs (Fig. 3F). Mesenchymal expression of PPT-A mRNA diminished in the later cap stage, where cusps had formed (Fig. 3C), and could no longer be detected at the bell stage (Fig. 3D).

To identify the molecular species of PPT-A mRNA expressed in molars, we amplified cDNAs prepared from molar mRNA, using PPT-A-specific oligonucleotides derived from external exons shared by all of the alternatively spliced PPT-A mRNA species. As shown in Fig. 4, the most abundant mRNA species expressed in molars was γ-PPT mRNA (yielding a 270 bp fragment in this experiment), which encodes the tachykinin peptides SP, SK, and NPγ.

Expression of SP in developing tooth germs

To determine whether the SP peptide was produced in developing molars, we isolated first mandibular molars at ages E14, E15 and E17, and determined SP levels in extracts obtained from pools of 15-20 molars, using an SP-specific radioimmunoassay (see Materials and Methods). As shown in Table 1, SP was detectable at each of these ages.

Expression of tachykinin receptors in tooth germs

To determine whether mRNAs encoding tachykinin receptors were expressed in developing tooth germs, molars were carefully dissected from the mandibular process with as little as possible adjacent tissue and total RNA was extracted. RT-PCR analysis was carried out using NK-1- and NK-2-specific oligonucleotides as primers, as NK-1 and NK-2 are the two major surface receptors that mediate responses to PPT-A-encoded tachykinins. As shown in Fig. 4, NK-1 mRNA was detected in molar primordia from E13 to E16. In contrast, NK-2 mRNA was not detectable in the same RNA preparations, although it was readily detectable in RNA extracted from adrenal glands, which are known to express NK-2 receptors (Tsuchida et al., 1990). Unfortunately, we were not able to identify the cells expressing NK-1 mRNA by in situ hybridization analysis or by in situ binding of radiolabelled SP, presumably because the level of expression was too low to be detected in these assays.

Expression of NEP mRNA in developing tooth germs and mammary buds

To determine whether a specific peptidase capable of degrading tachykinin peptides is expressed at the same time and locations as the tachykinins, we carried out an in situ hybridization analysis with an NEP-specific RNA probe. Sections serial to those employed for identification of PPT-A-producing cells were used in these experiments. NEP mRNA was temporally and spatially co-expressed with PPT-A mRNA during both mammary bud and tooth germ development. NEP was co-expressed with PPT-A in the condensed mesenchyme adjacent

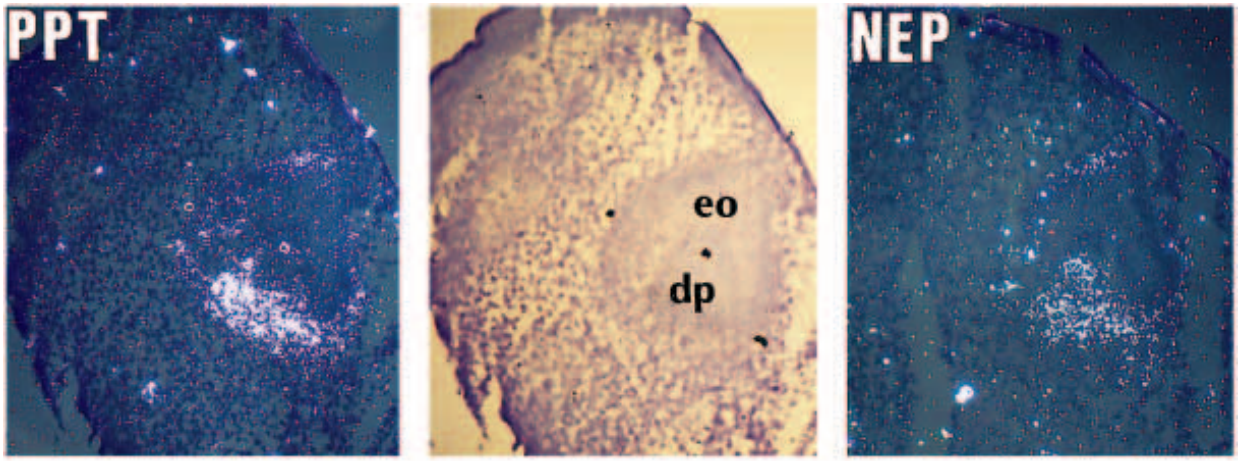

Fig. 4. Expression of PPT-A and NEP mRNAs during in vitro development of molars in an explant of a mandibular process. The culture was prepared from the first mandibular arch of an E10 embryos and was cultured for 9 days. Bright-field (middle) and dark-field images of adjacent sections, hybridized with a PPT-A-specific probe (left) and a NEP-specific probe (right) are shown. eo, enamel organ; dp, dental papilla.

Table 1. SP concentrations in extracts of developing molars by RIA

Age	SP (pg/molar)
E14	11
E15	13
E17	11

Extracts from pools of 15-20 molars at each age were assayed by RIA and the amount of SP per molar was calculated.

to the mammary bud epithelium (Fig. 2E) and in the condensed mesenchyme associated with the enamel organ epithelium (Fig. 3E). PPT-A and NEP mRNAs were also co-expressed in the mesenchyme of the developing external auditory meatus, the forming lip furrow, the genital tubercle and the limb buds, as well as in multiple sites in the spinal cord (not shown).

In vitro development of molars

To determine whether PPT-A and NEP mRNA expression in the developing tooth requires a nerve supply, we cultured explants of mandibular processes from E10 embryos (30-34 somites, Theiler's stage 16, a time prior to innervation; Lumsden and Buchanan, 1986). After 9 days in culture, both incisor and molar tooth germs had developed. Fig. 5 shows a molar anlage that developed to a late cup stage in vitro. In tooth germs that developed in culture, PPT-A and NEP mRNA were mostly expressed in the condensed mesenchyme underneath the enamel organ (Fig. 5), just as in vivo (Fig. 3C). Thus, the maintenance of PPT-A and NEP mRNA expression (and possibly also its initial induction) in the dental mesenchyme is apparently independent of innervation.

Inhibition of tooth development by antisense PPT-A oligonucleotides

To determine whether tachykinins are required for normal tooth development in vitro, we cultured explants of E14 molar anlagen at the early cup stage for 6 days, in either the presence or the absence of a PPT-specific antisense oligonucleotide (see Methods). The optimal concentration of oligonucleotide was determined by testing a range of concentrations between 5 and 10 μM. As shown in Fig. 6, both untreated explants and explants treated with the matching sense oligonucleotide progressed in culture to a more advanced stage of development, in which ameloblasts appeared and cusps formed. In contrast, explants treated with the antisense oligonucleotide failed to progress. When exogenous SP (together with the NEP inhibitor phosphoramidon) was added together with the antisense oligonucleotide, it neutralized the inhibitory effect of the antisense oligonucleotide (Fig. 6), attesting to the specificity of the antisense treatment and suggesting that SP itself may be involved in normal tooth development.

Inhibition of tooth development by an antagonist of the NK-1 receptor

As an alternative to the use of antisense oligonucleotides, we used the NK-1 receptor antagonist CP-96345 to inhibit tachykinin signalling in vitro. CP-96345 is a potent, nonpeptide, competitive antagonist of the NK-1 receptor (Snider et al., 1991), which has proven to be a valuable tool for studying the functions of SP (e.g. Prabhakar et al., 1993). Molar anlagen were isolated from E14 embryos and cultured as explants for 6 days, in either the presence or absence of CP-96345. The

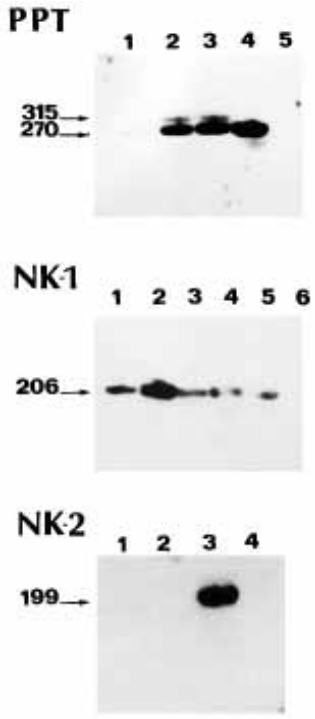

Fig. 5. Identification of PPT-A, NK-1, and NK-2 mRNAs in isolated first molars by RT-PCR. **Top figure:** amplification with PPT-A-specific primers. 1, E13; 2, E15; 3, E16; 4, brain mRNA; 5, no template. The 315 bp and 270 bp fragments amplified correspond to β-PPT and γ-PPT mRNA species, respectively. **Middle figure:** Amplification with NK-1-specific primers. 1, E13; 2, E14; 3, E15; 4, E16; 5, brain mRNA; 6, no template. **Bottom figure:** Amplification with NK-2-specific primers. 1, E14; 2, E15; 3, adrenal gland mRNA; 4, no template.

optimal concentration of antagonist was determined by testing a range of concentrations between 4 and 40 μM. As shown in Fig. 7, the antagonist (8 μM) greatly retarded tooth development and this effect was completely neutralized when exogenous SP (together with phosphoramidon) was added along with the antagonist.

The results of the inhibition experiments are summarised in Table 2.

DISCUSSION

Our findings indicate that mRNAs encoding three crucial components for tachykinin signalling, tachykinin precursor proteins, NK-1 tachykinin receptor and NEP, are all expressed during the development of mouse mammary glands and tooth germs. We also show that the tachykinin SP is present in the developing tooth germ. The PPT-A and NEP mRNAs are mainly expressed in the condensed mesenchyme associated with the epithelia of the mammary gland and enamel organ, and they are temporally restricted to the early stages of development, suggesting that tachykinins may play a role in an early step common to the development of both organs. To our knowledge, this is the first demonstration of the co-localization of mRNAs encoding NEP and a NEP substrate in the same group of cells in the context of a developmental process.

Morphogenesis in both mammary gland and tooth development depends on sequential and reciprocal epithelial-mesenchy-

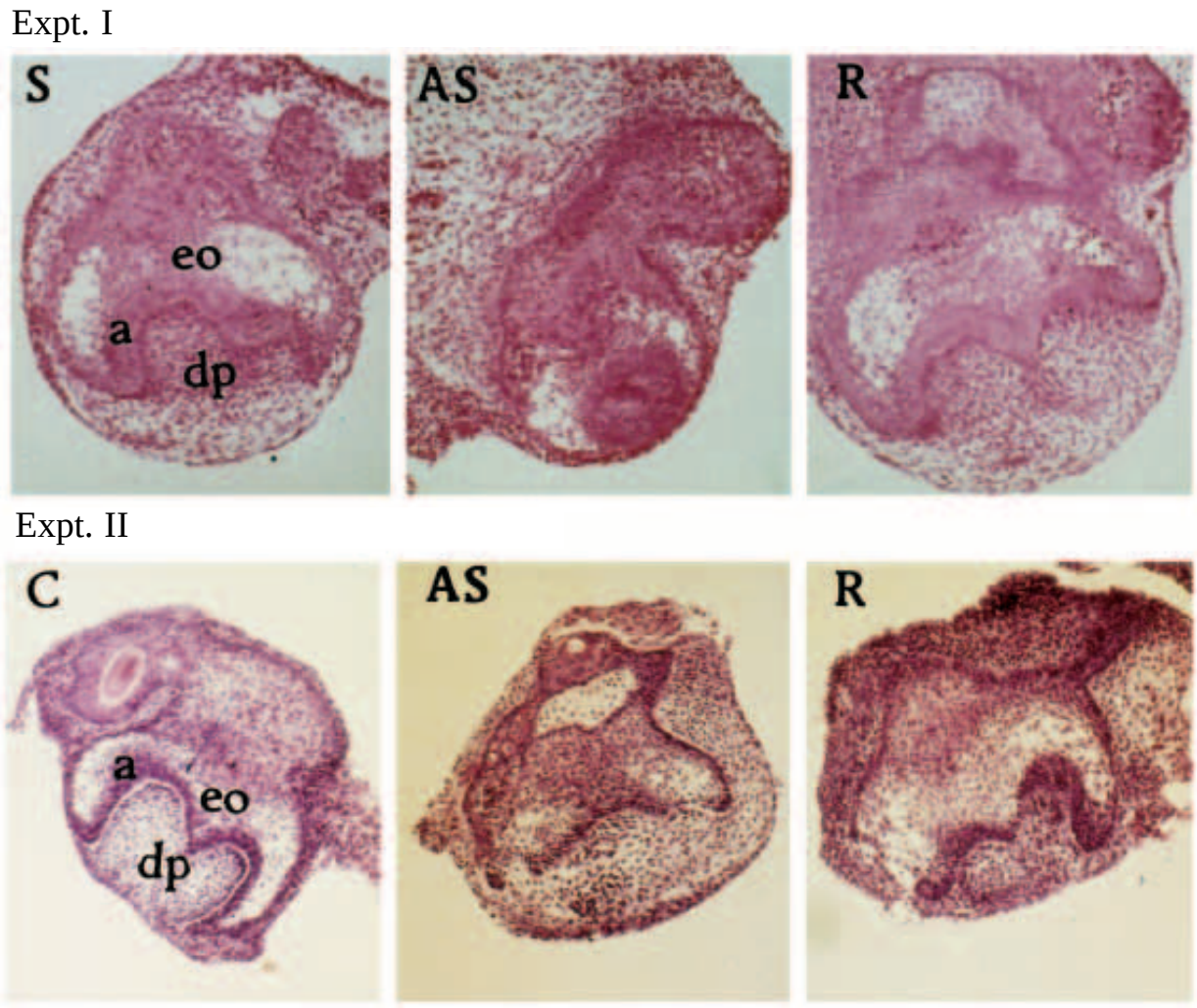

Fig. 6. Effect of PPT-A antisense oligonucleotide on molar development in culture. Cultures were treated with antisense (AS) or sense (S) oligonucleotides or without oligonucleotides with SP and phosphoramidon(C), as described in Materials and Methods. In rescue experiments (R) SP and phosphoramidon were added together with antisense oligonucleotides. E14 explants were cultured for 6 days, embedded in paraffin, sectioned and stained with hematoxylin and eosin. To visualize all structures, explants were sectioned from end-to-end and all of the sections were stained and examined. dp, dental papilla; eo, enamel organ; a, ameloblasts.

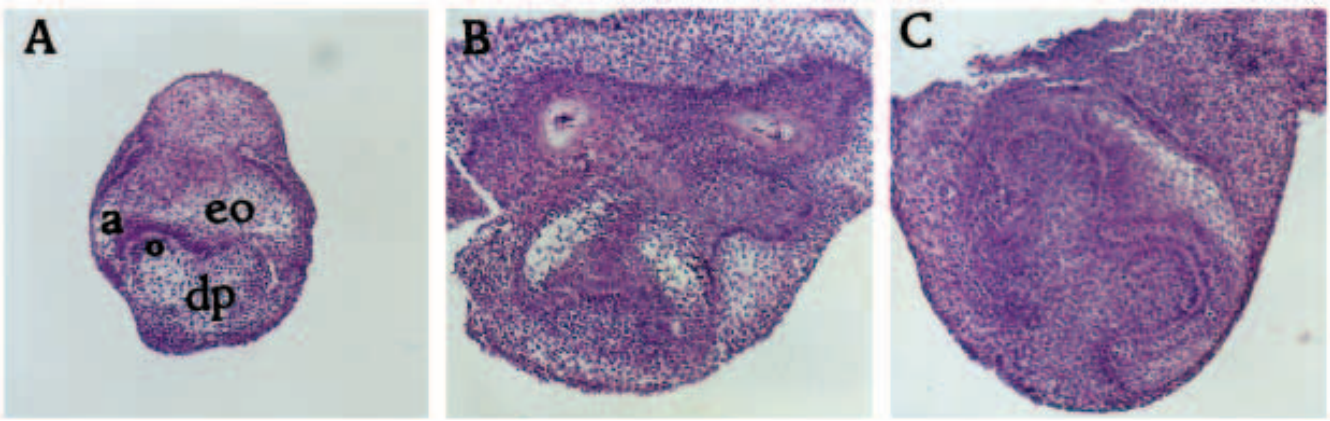

Fig. 7. Effect of the NK-1 receptor antagonist CP-96345 on molar development in culture. E14 explants were cultured for 6 days in the absence of antagonist (A), presence of antagonist (B), or presence of antagonist, SP and phosphoramidon (C), as described in Materials and Methods. They were embedded in paraffin, sectioned and stained with hematoxylin and eosin to visualize specific structures, as described in Fig. 6. dp, dental papilla; eo, enamel organ; a, ameloblasts.

Table 2. Summary of inhibition experiments with PPT-A antisense oligonucleotides and NK-1 receptor antagonist

Treatment	Proportion of cultures where molars developed to bell stage
PPT-A sense oligos	6/10
PPT-A antisense oligos	0/8*
PPT-A antisense oligos+SP and phosphoramidon	5/9
NK-1 receptor antagonist	0/11*
NK-1 receptor antagonist+SP and phosphoramidon	12/13
SP+phosphoamidon	9/13

Explants of E14 molars (early cup stage) were cultured for 6 days and the proportion of cultures that developed to the bell stage was determined.

Oligonucleotides (oligos), SP and phosphoramidon were used at 10 μM and the NK-1 receptor antagonist (CP-96345) was used at 8 μM.

*Significantly different ($p<0.004$) from controls (treatment with sense oligonucleotides or with added SP and phosphoramidon), when analyzed by χ^2 test, using Yates and Bonferroni corrections (Glantz, 1987).

mal interactions. In both processes the locations of the organs are determined in two steps. In the first, the lines along which the future teeth and mammary glands will form are laid down as linear thickenings in the epithelium. In the second, the site at which each individual tooth or mammary gland will form along this lines is determined by interactions between the epithelium and the underlying mesenchyme (Mina and Kollar, 1987; Ruch, 1984; Sakakura, 1991; Thesleff and Humerinta, 1981). The embryonic origins of the interacting mesenchyme in the two types of organ, however, are different: in tooth development the mesenchyme is of neural crest origin (Kollar and Lumsden, 1979; Lumsden, 1987, 1988), while in mammary gland it is not.

Although the transient spatial and temporal co-expression of the PPT-A and NEP mRNAs in the developing mammary gland and tooth is suggestive that tachykinins play a role in the development of these organs, to establish such a role one needs to show that interference with tachykinin signalling interrupts normal mammary gland and tooth development. We show this in explants of tooth anlagen in two ways. We show that interfering with either the translation of PPT mRNA (using antisense oligonucleotide) or the interaction of SP with its receptor (using a specific NK-1 receptor antagonist), starting at the early cup stage of development when PPT mRNA expression is maximal, impairs normal morphogenesis of the enamel organ. In these experiments, normal development is rescued by the addition of exogenous SP, attesting to the specificity of the inhibitors and suggesting that, among the different peptides that may be processed from the precursor PPT protein, SP is likely to play a role in tooth development. The findings that developing molars express NK-1 receptors and that an NK-1-specific antagonist blocks molar tooth development are consistent with this suggestion, as the ligand preferences of NK-1 are SP>NPγ>SK=NPK, whereas those of NK-2 are NPK>NPγ>SK>SP (Krause et al., 1989). Similar rescue experiments using other peptides may determine whether additional PPT-encoded peptides are also involved in tooth development.

Although our findings suggest that the condensed mesenchymal cells produce tachykinins, they do not indicate which cells respond to these peptides. While the RT-PCR studies indicate that mRNA encoding NK-1 receptors are present in developing tooth germs, we were not able to identify the cells expressing the NK-1 receptors, either by in situ hybridization analysis or by in situ binding of radiolabelled SP. Since NEP mRNA expression co-localizes with PPT-A mRNA expression in the condensed mesenchyme and, in all previously studied systems, NEP co-localized with cells bearing the relevant peptide receptors, it is possible that NK-1 receptors are also expressed on the condensed mesenchyme, which would suggest an autocrine function for the tachykinins in tooth development.

Our experiments also do not indicate what role tachykinins normally play in tooth or mammary gland development. Although it has been shown that both substance P and neurokinin A (substance K) are mitogens for some nonneural cell types (Nilsson et al., 1985; Payan, 1985; Tanaka et al, 1988), a mitogenic role is unlikely in mammary gland development, as PPT-A mRNA expression peaks at a time when cell proliferation is mostly arrested (Sakakura, 1991).

Irrespective of the role of tachykinins in organogenesis of tooth germs or mammary glands, PPT-A and NEP mRNAs can serve as new molecular markers for these processes, to distinguish between the mesenchymal cells that interact with adjacent epithelium during early morphogenesis and the flanking mesenchymal cells. The mRNAs start to be expressed in the condensed mesenchyme of tooth anlagen at the same time as a number of other known early markers of these cells, including syndecan (Vainio et al., 1991), tenascin (Chiquet-Ehrismann et al., 1986; Thesleff et al., 1987), Hox-7 (Msx-1) (Mackenzie et al., 1991) and LEF-1 (van Genderen et al., 1994). In the developing mammary gland, PPT-A mRNA starts to be expressed at the same time (E12) as androgen receptors (Durnberger and Kratochwill, 1980) but earlier than the second commonly used marker of mammary mesenchyme, tenascin, which is first detectable at E14 (Chiquet-Ehrismann et al., 1986).

Our findings that PPT-A mRNA is transiently expressed in the condensing mesenchyme of the developing limb bud, external auditory meatus and genital tubercle suggest that tachykinins may play roles in the development of these organs, as well as in the development of teeth and mammary glands. Moreover, our results suggesting new developmental roles for the tachykinins raise the possibility that other neuropeptides may also be involved in local cell-cell signalling in the development of various organs, including the development of the nervous system.

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REFERENCES

Brene, S., Lindefors, N., Friedman, W. J. and Persson, H. (1990). Preprotachykinin A mRNA expression in the rat brain during development. *Dev. Br. Res.* **57,** 151-162.

Chai, Y., Mah, A., Crohin, C., Groff, S., Bringas, Jr. P., Lee, T., Santos, V. and Slavkin, H. C. (1994). Specific transforming growth factor-β subtypes regulate embryonic mouse Meckel's cartilage and tooth development. *Dev. Biol.* **162,** 85-103.

Chen, C-Y, Salles, G., Seldin, M. F., Kister, A. E., Reinherz, E. L. and Shipp, M. A. (1992). Murine common acute lymphoblastic leukemia antigen (CD10 neutral endopeptidase 24.11). *J. Immunol.* **148,** 2817-2825.

Chiquet-Ehrismann, R., Mackie, E. J., Pearson, C. A. and Sakakura, T. (1986). Tenascin: An extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell* **47,** 131-139.

Durnberger, H. and Kratochwil, K. (1980). Specificity of tissue interaction and origin of mesenchymal cells in the androgen response of the embryonic mammary gland. *Cell* **19,** 465-471.

Dutriez, I., Sales, N., Fournie-Zaluski, M. C. and Roques, B. P. (1992). Pre- and post-natal ontogeny of neutral endopeptidase 24-11 (enkephalinase) studied by in vitro autoradiography in the rat. *Experientia* **84,** 290-300.

Erdos, E. G.and Skidgel, R. A. (1989). Neutral endopeptidase 24.11 (enkephalinase) and related regulators of peptide hormones. *FASEB J.* **3,** 145-151.

Glantz, S. A. (1987). How to analyze rates and proportions. In *Primer of Biostatistics.* (ed. B. Kaufman, B. White and J. White), pp. 122-132. New York: McGraw-Hill Inc..

Gressens, P., Hill, J. M., Gozes, I., Fridkin, M. and Brenneman, D. E. (1993). Growth factor function of vasoactive intestinal peptide in whole cultured embryos. *Nature* **362,** 155-158.

Hershey, A. D. and Krause, J. E. (1990). Molecular caracterization of a functinal cDNA encoding the rat substance P receptor. *Science* **247,** 958-962.

Hogan, B., Constantini, F. and Lacy, E. (1986). Techniques for visualizing genes and gene products. In *Manipulating the Mouse Embryo: A Laboratory Manual*. pp. 228-242. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Jones, C. M., Lyons, K. M. and Hogan, B. L. M. (1991). Involvement of bone morphogenetic protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in the mouse. *Development* **111,** 531-542.

Keshet, E., Polakiewicz, R. D., Itin, A., Ornoy, A. and Rosen, H. (1989). Proenkephalin A is expressed in mesodermal lineages during organogenesis. *EMBO J.* **8,** 2917-2923.

King, K. A., Hua, J., Torday, J. S., Drazen, J. M., Graham, S. A., Shipp, M. A. and Sunday, M. E. (1993). CD10/neutral endopeptidase 24.11 regulates fetal lung growth and maturation in utero by potentiating endogenous bombesin-like peptides. *J. Clin. Invest.* **91,** 1969-1973.

Kollar, E. J. and Lumsden, A. G. S. (1979). Tooth morphogenesis: the role of the innervation during induction and pattern formation. *J. Biol. Buccale* **7,** 49-60.

Kratochwil, K. (1969). Organ specificity in mesenchymal induction demonstrated in the embryonic development of the mammary gland of the mouse. *Dev. Biol.* **20,** 46-71.

Krause, J. E., Chirgwin, J. M., Carter, M. S., Xu, Z. S. and Hershey, A. D. (1987). Three rat preprotachykinin mRNAs encode the neuropeptides substance P and neurokinin A. *Proc. Natl. Acad. Sci. USA* **84,** 881-885.

Krause, J. E., MacDonald, M. R. and Takeda, Y. (1989). The polyprotein nature of substance P Precursors. *BioEssays* **10,** 62-69.

Kronmiller, J. E., Upholt, W. B. and Kollar, E. J. (1991). EGF antisense oligonucleotides block murine odontogenesis *in vitro*. *Dev. Biol.* **147,** 485-488.

Lumsden, A. G. S. and Buchanan, J. A. G. (1986). An experimental study of timing and topography of early tooth development in the mouse embryo with an analysis of the role of innervation. *Archs Oral Biol.* **31,** 301-311.

Lumsden, A. G. S. (1987). Contribution of the cranial neural crest to tooth development in mammals. In *Developmental and Evolutionary Aspects of the Neural Crest* (ed. P. F. A. Maderson), pp.261-300. New York: John Wiley.

Lumsden, A. G. S. (1988). Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ. *Development* **103 Supplement,** 155-169.

Lyons, K. M., Pelton, R. W. and Hogan, B. L. M. (1990). Organogenesis and pattern fomation in the mouse: RNA distribution patterns suggest a role for bone morphogenetic protein-2A (BMP-2A). *Development* **109,** 833-844.

Mackenzie, A., Leeming, G. L., Jowett, A. K., Ferguson, M. W. J. and Sharpe, P. T. (1991). The homeobox gene Hox 7.1 has specific regional and temporal expression patterns during early murine craniofacial embryogenesis, especially tooth development in vivo and in vitro. *Development* **111,** 269-285.

Maggio, J. E. (1988). Tachykinins. *Ann. Rev. Neurosci.* **11,** 13-28.

McKelvy, J. F. and Blumberg, S. (1986). Inactivation and metabolism of neuropeptides. *Ann. Rev. Neurosci.* **9,** 415-434.

Mina, M. and Kollar, E. J. (1987). The induction of odontogenesis in non-dental mesenchyme combined with early murine mandibular arch epithelium. *Archs Oral Biol.* **32,** 123-127.

Mitsiadis, T. A., Dicou, E., Joffre, A. and Magloire, H. (1992). Immunohistochemical localization of nerve growth factor (NGF) and NGF receptor (NGF-R) in the developing first molar tooth of the rat. *Differentiation* **49,** 47-61.

Mitsiadis, T. A., Couble, P., Dicou, E., Rudkin, B. B. and Magloire, H. (1993). Patterns of nerve growth factor (NGF), proNGF, and p75 NGF receptor expression in the rat incisor: comparison with expression in the molar. *Differentiation* **54,** 161-175.

Nilsson, J., von Euler, A. M. and Dalsgaard, C-J. (1985). Stimulation of connective tissue cell growth by substance P and substance K. *Nature* **315,** 61-63.

Partanen, A.-M., Ekblom, P. and Thesleff, I. (1985). Epidermal growth factor inhibits morphogenesis and cell differentiation in cultured mouse emryonic teeth. *Dev. Biol.* **111,** 84-94.

Payan, D. G. (1985). Receptor-mediated mitogenic effects of substance P on cultured smooth muscle cells. *Biochem. Biophys. Res. Comm.* **130,** 104-109.

Payan, D. G. (1989). Neuropeptides and inflamation: The role of Substance P. *Ann. Rev. Med.* **40,** 341-352.

Prabhakar, N. R., Cao, H., Low III, J. A. and Snider, R. M. (1993). Selective inhibition of the carotid body sensory response to hypoxia by the substance P receptor antagonist CP-96345. *Proc. Natl. Acad. Sci. USA* **90,** 10041-10045.

Represa, J. J., Miner, C., Barbosa, E. and Giraldez, F. (1988). Bombesin and other growth factors activate cell proliferation in chick embryo otic vesicles in culture. *Development* **102,** 87-96.

Rozengurt, E. and Sinnett-Smith, J. (1983). Bombesin stimulation of DNA synthesis and cell division in cultures of Swiss 3T3 cells. *Proc. Natl. Acad. Sci. USA* **80,** 2936-2940.

Ruch, J. V. (1984). Tooth morphogenesis and differentiation. In *Dentin and Dentinogenesis* (ed. A. Linde), pp. 47-79. Boca Raton: CRC Press.

Ruch, J. V. (1987). Determinisms of odontogenesis. *Cell. Biol. Rev. RBC.* **14,** 1-112.

Sakakura, T., Nishizuka, Y. and Dawe, C. J. (1976). Mesenchyme-dependent morphogenesis and epithelium-specific cytodifferentiation in mouse mammary gland. *Science* **194,** 1439-1441.

Sakakura, T. (1991). New aspects of stroma-parenchyma relations in mammary gland differentiation. *Int. Rev. Cytol.* **125,** 165-201.

Sasai, Y. and Nakanishi, S. (1989). Molecular caracterization of rat substance K receptor and its mRNAs. *Biochem. Biophys. Res. Commun.* **165,** 695-702.

Shigemoto, R., Yocota, Y., Tsuchida, K. and Nakanishi, S. (1990). Cloning and expression of a rat neuromedin K receptor cDNA. *J. Biol. Chem.* **265,** 623-628.

Snider, R. M., Constantine, J. W., Lowe III, J. A., Longo, K. P., Lebel. W. S., Woody, H. A., Drozda, S. E., Desai, M. C., vinick, F. J., spencer, R. W. and Hess, H-J. (1991). A potent nonpeptide antagonist of the substance P (NK1) receptor. *Science* **251,** 435-437.

Sunday, M. E., Hua, J., Torday, J. S., Reyes, B. and Shipp, M. A. (1992). CD10/neutral endopeptidase 24.11 in developing human fetal lung. Patterns of expression and modulation of peptide-mediated proliferation. *J. Clin. Invest.* **90,** 2517-2525.

Tanaka, T., Danno, K., Ikai, K. and Imamura, S. (1988). Effects of substance P and substance K on the growth of cultured keratinocytes. *J. Invest. Dermatol.* **90,** 399-401.

Theiler, K. (1972). *The House Mouse*. pp. 60-65. Berlin-Heidelberg: Springer-Verlag.

Thesleff, I., Mackie, E., Vainio, S. and Chiquet-Ehrismann, R. (1987). Changes in the distribution of tenascin during tooth development. *Development* **101,** 289-296.

Thesleff, I. and Humerinta, K. (1981). Tissue interactions in tooth development. *Differentiation* **18,** 75-88.

Tsuchida, K., Shigemoto, R., Yocota, Y. and Nakanishi, S. (1990). Tissue distribution and quantitation of the mRNAs for three rat tachykinin receptors. *Eur. J. Biochem.* **123,** 751-757.

Vaahtokari, A.,Vainio, S. and Thesleff, I. (1991). Associations between transforming growth fwctor β1 RNA expression and epithelial-mesenchymal interactions during tooth morphogenesis. *Development* **113,** 985-994.

Vainio, S., Jalkanen, M. and Thesleff, I. (1989). Syndecan and tenascin expression is induced by epithelial-mesenchymal interactions in embryonic tooth mesenchyme. *J. Cell Biol.* **108,** 1945-1954.

Vainio, S., Jalkanen, M., Vaahtokari, A., Sahlberg, C., Mali, M., Bernfield, M. and Thesleff, I. (1991). Expression of syndecan gene is induced early, is transient, and correlates with changes in mesenchymal cell proliferation during tooth organogenesis. *Dev. Biol.* **147,** 322-333.

Vainio, S., Karavanova, I., Jowett, A. and Thesleff, I. (1993). Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues dueing early tooth development. *Cell* **75,** 45-58.

van Genderen, C., Okamura, R. M., Farinas, I., Quo, R.-G., Parslow, T. G., Bruhn, L. and Grosschedl, R. (1994). Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice. *Genes Dev.* **8,** 2691-2703.

Yocota, Y., Sasai, Y., Tanaka, K., Fujiwara, T., Tsuchida, K., Shigemoto, R., Kakizuka, A., Ohkubo, H. and Nakanishi, S. (1989). Molecular characterization of a functional cDNA for rat substance P receptor. *J. Biol. Chem.* **264,** 17649-17652.

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