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Oxytocin and milk removal are required for post-partum mammary-gland development

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ABSTRACT

The oxytocin (OT)-neurophysin preprohormone is synthesized in the paraventricular and supraoptic nuclei of the hypothalamus. OT is cleaved from its precursor, transported from the magnocellular neurons to the posterior pituitary and secreted during labour and upon the suckling stimulus of pups. OT induces the contraction of myoepithelial cells surrounding the mammary alveoli, which leads to the ejection of milk. Mice deficient in OT are unable to nurse their young. Administration of OT enabled OT-deficient dams to nurse. We now show that OT and milk removal are also required for post-partum alveolar proliferation and mammary-gland function. Alveolar density and mammary epithelial-cell differentiation at parturition was similar in wild-type and OT-deficient dams. However, within 12 h after parturition approx. 2% of the alveolar cells in wild-type dams incorporated DNA and proliferated, but virtually no proliferation was detected in OT-deficient dams. Continuous suckling of pups led to the expansion of lobulo-alveolar units in wild-type but not in OT-deficient dams. Despite suckling and the presence of systemic lactogenic hormones, mammary tissue in OT-deficient dams partially involuted. Our studies demonstrate that post-partum alveolar proliferation requires not only systemic lactogenic hormones, such as prolactin, but also the presence of OT in conjunction with continued milk removal.

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INTRODUCTION

Prolactin (PRL) is synthesized in the anterior lobe of the pituitary gland and is required for the proliferation and functional differentiation of mammary lobulo-alveolar structures [1]. Oxytocin (OT) is released from the posterior lobe upon a suckling stimulus by the young. It induces the contraction of myoepithelial cells surrounding the alveoli, which results in the ejection of milk [2-4]. OT is a nonapeptide whose sequence was identified by Du Vigneaud and co-workers [5]. It is synthesized as an OT-neurophysin

preprohormone in specialized magnocellular neurons of the paraventricular and supraoptic nuclei of the hypothalamus [6]. After cleavage of the signal peptide, OT is processed during transport within secretory granules in magnocellular neurons to the posterior pituitary gland, where it is secreted into the circulation [7]. Some OT synthesis has been found in the uterus, placenta, corpus luteum, testes and the amnion of various species [8].

The ancestral gene for the OT/vasopressin superfamily was present in the Archaemetazoa about 600 million years ago [9]. The conservation of this gene in vertebrates and invertebrates suggests that OT conveys important functions that are not restricted to its 'galactogogic effect' in mammals. It has an uterotonic effect and plays an important role during the initiation and maintenance of parturition [10-13]. Furthermore, OT has been postulated to be required for memory [14], mating behaviour [15], natri-and anti-diuresis [16,17], fertility [18-20], and maternal behaviour in rodents [21,22]. However, recent studies in mice in which both alleles of the OT gene had been inactivated demonstrated that OT is essential only for lactation and milk ejection [23,24].

The concept of the neuroendocrine milk ejection reflex was first described by Ely and Petersen [3]. Nipple stimulation leads to the release of OT from the posterior pituitary gland into the bloodstream, and subsequently to milk ejection as a result of the contraction of the mammary myoepithelium. Mice deficient in OT are unable to nurse the litter, but exogenous administration of OT restored myoepithelial contraction and dams were able to feed their young [23,24]. There are indications that OT also contributes to the development of the mammary gland. From in ***vitro*** and ***in vivo*** studies, Sapino and co-workers [25] suggested that OT directly induces myoepithelial cell growth and differentiation by enhancing the effect of lactogenic hormones. Further support for a proliferative role of OT comes from in ***vitro*** studies of neuronal tissues. For example, the rate of proliferation in rat cortical and hypothalamic astroglia cells increased after OT administration ***in vitro*** [26]. However, OT may also have an inhibitory effect in some human breast-cancer cell lines [27]. As a potential releasing factor for PRL, OT could also act indirectly on mammary-gland development. The OT receptor (OT-R) is expressed in the lactotrophs of the pituitary gland [28], and OT might control PRL release [29-31]. As a lactogenic hormone, PRL is a principal mediator of mammopoiesis [1]. Recent studies have shown that the disruption of the PRL signalling pathway leads to an impaired development of lobulo-alveolar structures [32].

Initial analysis of OT-deficient darns revealed sparse mammary alveolar development [23]. We have now tested the hypothesis that OT controls, directly or indirectly, development of the mammary gland. In particular, we examined lobulo-alveolar proliferation and mammary function in post-partum dams. Our findings demonstrate that the impaired milk release caused by the absence of OT is linked to the repression of the post-partum proliferation of lobulo-alveolar structures. In addition, rapid programmed cell death (PCD) is initiated in mammary tissue in post-partum OT-deficient dams, even in the presence of suckling and the continued release of lactogenic hormones. In contrast, OT is not required for the terminal differentiation of myoepithelial cells and has no measurable effect on the release of lactogenic hormones from the anterior pituitary. Mice deficient in OT therefore served as an appropriate model to distinguish between the effects of systemic and local factors in maintenance and PCD of the lobulo-alveolar compartment.

RESULTS

Milk ejection is impaired in OT-deficient mice

Mice carrying an inactivated OT gene have been generated by gene targeting in embryonic stem cells [23]. OT-deficient mice gave birth to normal-sized litters, but were unable to feed their young. The inability to raise a litter was the result of a failure of milk ejection and post-partum mammary-gland development. Intraperitoneal injection of OT into OT-deficient dams within 12 h after parturition partially restored lactation, and milk was found in the stomachs of the pups (Figure 1). However, since the injection of neither physiological nor supraphysiological levels of OT (as described by Nishimori and co-workers [24]) fully established lactation for more than 24 h, we considered the possiblity that mammary development was impaired in these mice.

OT is not required for mammary development during pregnancy

To a large extent lobulo-alveolar outgrowth in the mammary gland occurs during pregnancy. Whole-mount analyses established that within 12 h after parturition the alveolar density in OT-deficient and wild-type dams was comparable (Figures 2A and 2B).

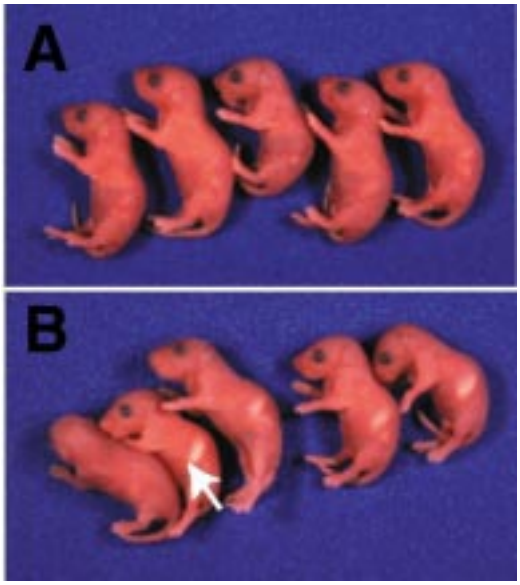

Figure 1 Pups (5 h old) from an OT-deficient mouse before (A) and shortly after (B) injection of OT. The arrow indicates milk in the stomach of a pup.

Histological sections confirmed that the alveoli were well developed and contained expanded lumina filled with milk (Figures 2C and 2D). Immunohistochemical analysis established that a major milk protein, the whey acidic protein (WAP), was synthesized and secreted into the alveolar lumina of OT-deficient dams (Figure 2C). Staining of WAP in the lumina of OT-deficient mice was more intense than in control mice (Figure 2D), demonstrating the accumulation of milk caused by the failure of milk ejection.

OT induces the contraction of myoepithelial cells surrounding the alveoli. These myoepithelial cells contain smooth-muscle contractile and cytoskeletal proteins. Serial injections of OT into OT-deficient dams restored lactation during the first 24 h but failed to induce continued lactation. To evaluate the morphology and differentiation status of the mammary myoepithelium, tissue sections from post-partum wild-type and OT-deficient dams were stained with antibodies specific against smooth-muscle actin (SM-actin). A layer of myoepithelial cells surrounding the luminal secretory epithelial cells was observed in both OT-deficient mice and wild-type littermates (Figures 2E and 2F). This demonstrates that the mammary myoepithelium in OT-deficient mice has a normal appearance on this level of resolution.

OT and milk release control post-partum mammary development

In addition to the extensive lobulo-alveolar growth during pregnancy, further proliferation of secretory mammary epithelium occurs after parturition. OT, local growth factors, the suckling stimulus and milk removal have been considered to contribute to post-partum development, but experimental evidence has been lacking. The OT-deficient mice allowed us to test the hypothesis that OT and milk removal are required for post-partum mammary alveolar proliferation. Cell proliferation in mammary tissue from post-partum OT-deficient and control mice was monitored by 5'-bromo-2'-deoxyuridine (BrdU) labelling. Whereas after parturition approx. 2% of the nuclei in wild-type mice were labelled by BrdU, less than 0.1% were labelled in the OT-deficient mice (Figures 2G, 2H and 3). In particular, smaller alveoli in the fringe region of the wild-type gland showed extensive cell proliferation within the first 3 days after parturition. No significant proliferation was observed in control mice at day 10 of lactation (results not shown), suggesting that within this period the full outgrowth was achieved. Our findings demonstrate that control but not OT-deficient mammary epithelial cells proliferate during the first few days after parturition.

The role of the suckling stimulus in the presence of systemic lactogenic hormones on post-partum mammary development was evaluated in control and OT-deficient dams 3 days after parturition (Figure 4). Since the OT-deficient mice cannot lactate, the suckling stimulus was maintained by replacing the litters every 12 h with litters from control mice. At parturition the density of alveolar outgrowth was similar between OT-deficient and control dams (Figures 2A and 2B). However, extensive glandular proliferation and development had occurred after 3 days of suckling in control but not in OT-deficient dams (Figures 4A and 4B). In contrast with the OT-deficient dams (Figure 4A), the fat pads of control mice were filled with secretory epithelium (Figure 4B), and the more expanded alveoli produced and secreted large amounts of milk to satisfy the needs of the growing offspring. No additional lobulo-alveolar development was observed in suckled OT-deficient dams, and the persisting alveoli failed to fully expand (Figure 4A). Nevertheless, mRNAs encoding milk proteins were detected in OT-deficient dams by Northern-blot analysis (results not shown), and mammary epithelial cells produced and secreted milk proteins into the lumen (Figure 4A).

Although the suckling stimulus and the presence of systemic lactogenic hormones did not induce further alveolar proliferation in post-partum OT-deficient dams, they were sufficient to reduce remodelling, and some lobulo-alveolar structures that secrete

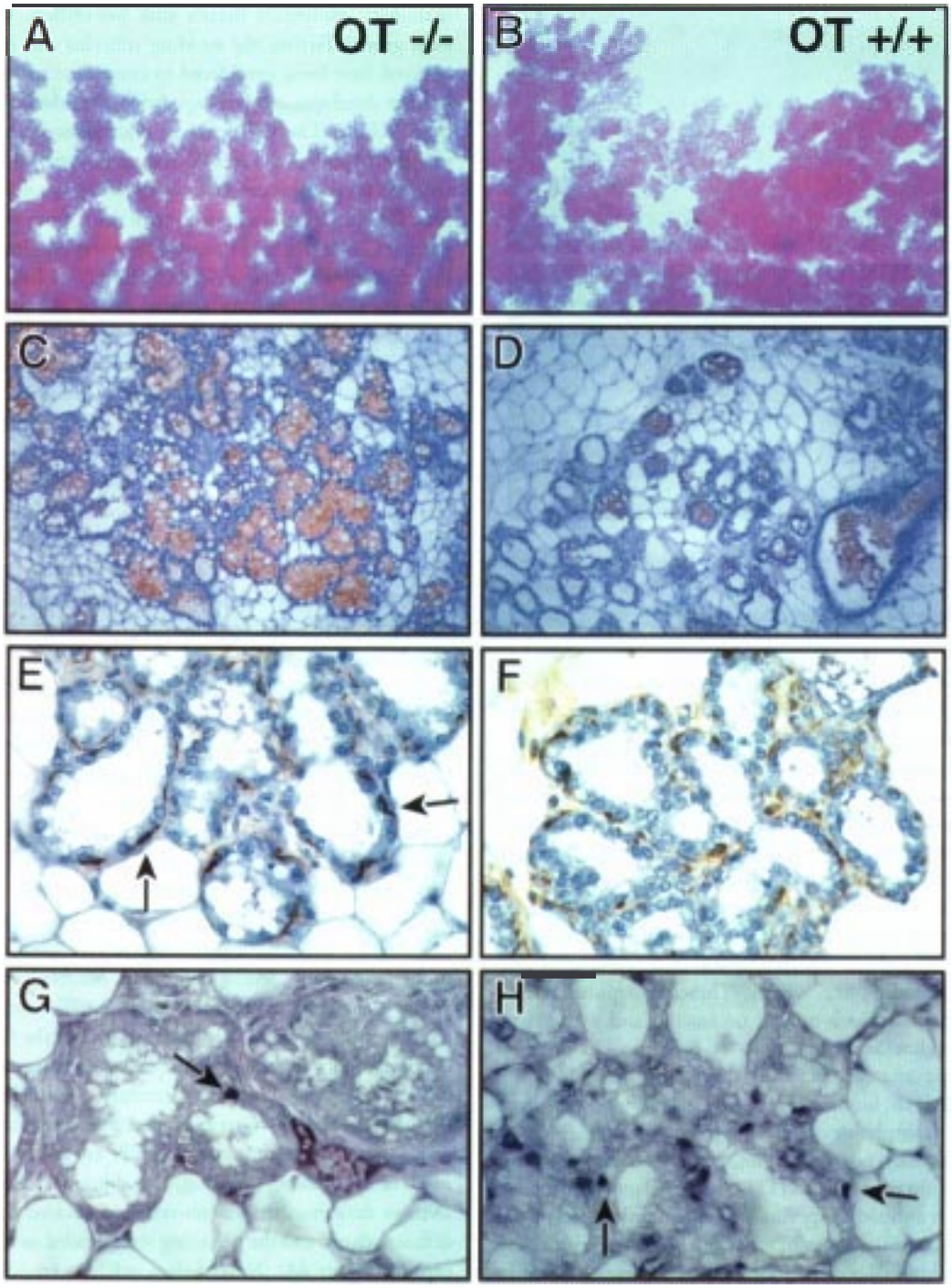

Figure 2 Whole mounts (A and B) and histological analysis (C-H) of mammary-gland tissues from mice deficient in OT (A, C, E and G) and their wild-type controls (B, D, F and H) a few hours after parturition. (A and B) Carmine Alum stained, magnification 50x; (C and D) immunohistochemical staining of WAP, 200x; (E and F) immunohistochemical staining of SM-actin in myoepithelial cells (arrows), 630X; (G and H) BrdU labelling of proliferating cells (arrows), 630X.

milk proteins were maintained (Figure 4A). The absence of a suckling stimulus for 3 days resulted in the rapid remodelling of secretory epithelium in both mutant and control dams (Figures 4C and 4D). After 10 days of lactation the mammary secretory epithelium of control dams is fully developed (Figures

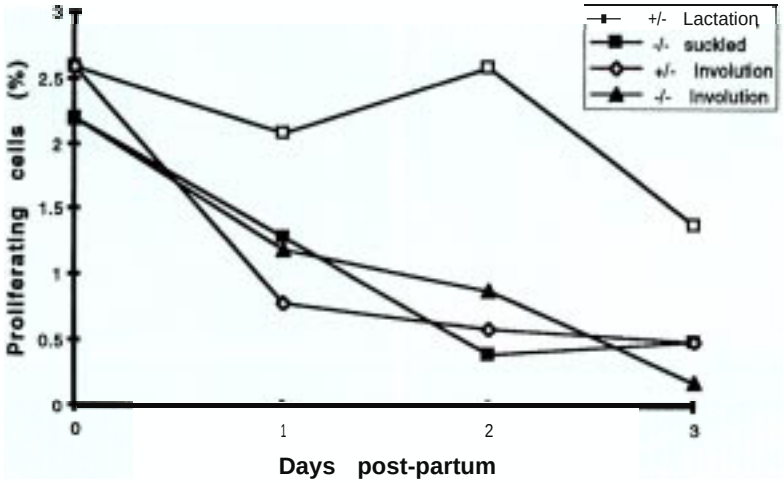

Figure 3 Proliferation in mammary tissue of OT-deficient and control dams. Mammary tissue sections were stained for BrdU-labelled cells, and 1000 cells from each genotype and each time point were analysed.

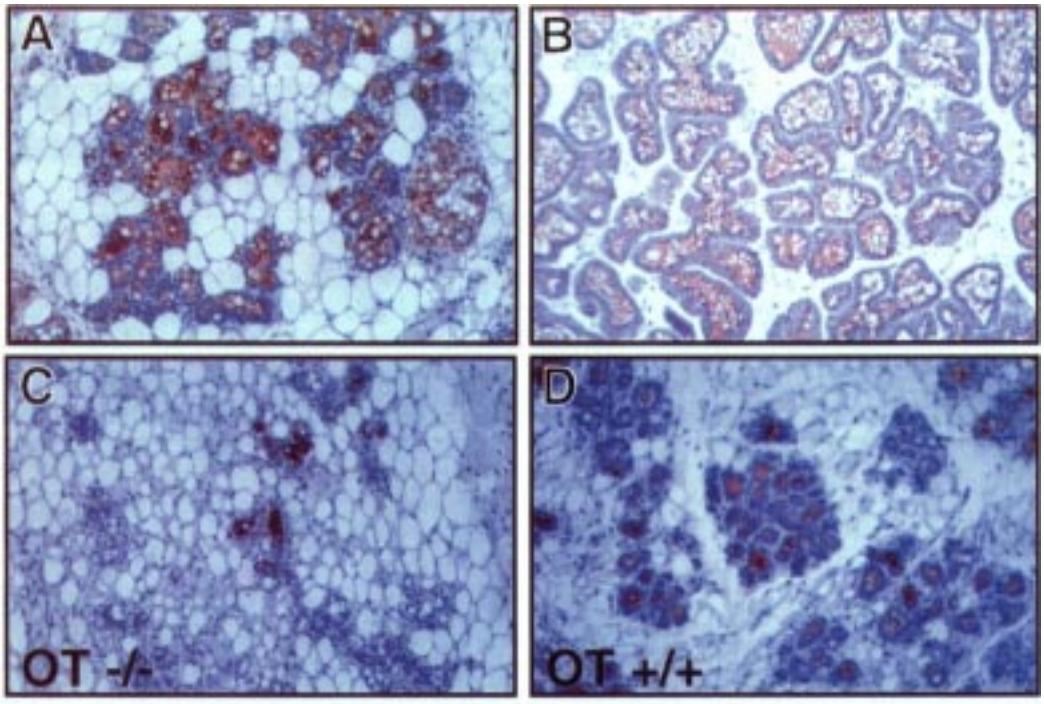

Figure 4 Immunohistochemical staining of WAP in mammary-gland tissue sections of mice deficient in OT (A and C) and their wild-type controls (B and D) 3 days after parturition; magnification 200X. (A and B) With continuing suckling stimulus, i.e. the litter remained with the mother; (C and D) without suckling stimulus, i.e. the litter was removed within 8 h after parturition.

5B and 5D). In contrast, the alveolar density in suckled OT-deficient dams was significantly reduced (Figures 5A and 5C). However, the remaining alveoli were filled with milk proteins (Figure 5C). In the absence of a suckling stimulus, mammary tissue from both control (Figures 5F and 5H) and OT-deficient (Figures 5E and 5G) dams had undergone extensive remodelling after 10 days.

Activity of the PRL signalling pathway

It has been hypothesized that PRL release is dependent on the presence of OT. To test rigorously this

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