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Deficiency in Mouse Oxytocin Prevents Milk Ejection, but not Fertility or Parturition

W. Scott Young, III, Emily Shepard, Janet Amico†, Lothar Hennighausen§, Kay-Uwe Wagner§, Mary E. LaMarca*, Cindy McKinney* and Edward I. Ginns*

Laboratory of Cell Biology and *Clinical Neuroscience Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, MD 20892–4068, USA.

§Laboratory of Biochemistry and Metabolism, National Institute of Diabetes, Digestive and Kidney Diseases

†Division of Endocrinology and Metabolism, University of Pittsburgh, School of Medicine and VA Medical Center, Pittsburgh, PA 15261, USA.

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Abstract

Oxytocin is a nonapeptide hormone that participates in the regulation of parturition and lactation. It has also been implicated in various behaviors, such as mating and maternal, and memory. To investigate whether or not oxytocin (OT) is essential for any of these functions, we eliminated, by homologous recombination, most of the first intron and the last two exons of the OT gene in mice. Those exons encode the neurophysin portion of the oxytocin preprohormone which is hypothesized to help in the packaging and transport of OT. The homozygous mutant mice have no detectable neurophysin or processed oxytocin in the paraventricular nucleus, supraoptic nucleus or posterior pituitary. Interestingly, homozygous mutant males and females are fertile and the homozygous mutant females are able to deliver their litters. However, the pups do not successfully suckle and die within 24 h without milk in their stomachs. OT injection into the dams restores the milk ejection in response to suckling. These results indicate an absolute requirement for oxytocin for successful milk ejection, but not for mating, parturition and milk production, in mice.

Oxytocin (OT) is a nonapeptide that was first identified in pituitary extracts through its effects on labor and milk secretion (1–3). Furthermore, the concept of neurosecretion arose from studies of OT and the other major neurohypophyseal hormone, the antidiuretic hormone vasopressin (VP) (4). The early determination of their amino acid sequences (5) permitted more detailed studies of their distributions and biological effects. Both OT and VP were shown to be made in the magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus (6, 7) and processed from their precursor forms along the axonal projection to the posterior pituitary (8). Subsequent cloning of the cDNAs encoding the bovine precursors for OT and VP (9, 10) opened the way for studies examining their levels of expression during various physiological and manipulated states (for review, see ref. (11)).

The original observations linking OT with lactation and parturition have been been confirmed in numerous studies. For example, opioids inhibit parturition in rats by reducing neurohypophysial secretion of OT (12). In addition, other functions for OT have been discovered or suggested. These include roles in mating and maternal behaviors (13–17), natriuresis (18) and antidiuresis (19), and memory (20). Nevertheless, OT has not been shown to be necessary for successful procreation. Attempts to examine whether OT is absolutely required in the various steps from mating through pregnancy to rearing have generally used OT antagonists or antibodies or selective brain lesions(21–24). The results of these studies have not been wholly consistent, have been confounded by the absence of other hormones, or reflect species differences. For example, OT antagonists prevent premature parturition in some patients, while other patients do not benefit from this form of therapy. Similarly, some rats escape inhibition by OT antagonists (25). In order to determine the functions for which OT is required, we used gene targeting to eliminate most of the first intron and the last two of three exons of the OT gene (26)(Fig. 1).

Results

We used methods previously described (27) to disrupt the OT gene. Embryonic stem cell clone #89, having a normal karyotype, was injected into C57BL/6 blastocysts that were subsequently reimplanted into foster mothers. Three chimeric male mice transmitted the altered OT allele in their germline. Heterozygous, and subsequent homozygous, mutant progeny were identified by PCR

Correspondence to: W. Scott Young, III, Building 36, Room 2D10, 36 Convent Drive, MSC 4068, Bethesda, MD 20892–4068, USA.

(Fig. 1) and, as expected, the mutation is transmitted in a Mendelian fashion.

No obvious gross anatomical or behavioral differences were noted between wild-type (WT), heterozygous (HE) or homozygous (HO) mutant pups. The size of the homozygous mutant mice is not significantly different from wild-type mice through at least two months (males: 23.1 ± 1.2g ($\pm$sdev) and 20.9 ± 0.7, df = 8; females: 19.3 ± 0.2 and 17.7 ± 0.6, df = 9). OT has natriuretic (18) and antidiuretic properties (19) and its receptor is present in the rat kidney (28, 29), so we measured two parameters that could reflect kidney dysfunction. There is no difference among males in plasma osmolalities (WT: 297 ± 2.4mOSM ($\pm$sem), HO: 297 ± 2.1, df = 16) or in their abilities to concentrate their urines after 24 hours of water deprivation (WT: 2945 ± 151 mOsm ($\pm$sem), HO: 3045 ± 360, df = 8).

To confirm that the production of OT was reduced, both hybridization histochemistry (30) and immunocytochemistry were performed. Fig. 2 shows that although abundant OT transcripts are detected in the PVN of the WT and HE mice using an exon A probe, greatly reduced levels are in the HO PVN. Quantitation reveals that the levels for the HE and HO PVNs are 53% ($P < 0.0001$, df = 13) and 1% ($P < 0.0001$, df = 15) of the WT level. Similar reductions are found in the SON. Immunohistochemistry using antibodies neurophysin or to a processed oxytocin intermediate, OT-GKR (31) showed abundant staining within neurons of the WT PVN and SON and within the posterior pituitary

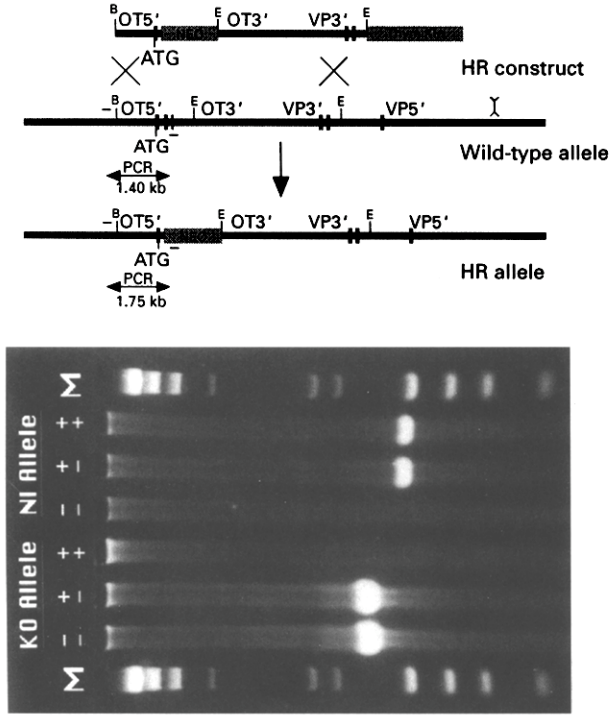

FIG. 1. The schematic shows the construct that was recombined into the wild-type mouse oxytocin allele, replacing its last 2 exons. Pairs of primers were designed for PCR that either detected the wild-type allele (1.4 kb) or the mutant allele (1.75 kb) as indicated. The gel shows the results of a typical PCR study of three mice (wild-type = +/+, heterozygote = +/−, and homozygous mutant = −/−) using the primer pairs separately for the wild-type or mutant (HR) allele. Abbreviations: HR, homologous recombination; NEO, neomycin resistance cassette; Thym Kin, thymidine kinase cassette; B, BamHI restriction site; E, EcoRI restriction site.

(Fig. 3A, C, E) and no staining above background in the mutant PVN, SON, or posterior pituitary (Fig. 3B, D, F). Although the OT-specific Pitt Ab-2 antibody (32) used below in the radioimmunoassays (RIA) had higher background staining, neurons of the PVN and SON were also clearly labeled in the WT mice, and not in the HO mice (data not shown). Liquid phase RIA of pituitary extracts from HO and WT mice was performed with a polyclonal antiserum specific for OT. The OT concentration in WT mice was 220 ± 20 (mean $\pm$ SEM) ng/pituitary whereas in HO mice, the OT level was < 1 ng/pituitary (800 ± 50 pg/pituitary). Plasma OT levels in the HO mice were undetectable (WT was 15–20 pg/ml).

The expression of other genes expressed in the PVN and SON was also examined by hybridization histochemistry. VP transcripts are reduced in the PVN by 26% ($P = 0.105$, df = 8) and by 30% in the SON ($P < 0.01$, df = 8) in the homozygote mutant mice. The level of expression in the HE is similar to the WT in the PVN but intermediate between the WT and HO in the SON. To determine if this reduction in VP expression is a nonspecific reduction due to disruption of the OT gene which is closely linked to the VP gene (26, 33), we also studied the expression of VP in the suprachiasmatic nucleus of the hypothalamus. There, VP expression is unaffected in the HE or HO. We also measured the expression of dynorphin which is co-expressed with VP (34). Dynorphin expression is elevated by 37% in the HO PVN neurons compared to the WT ($P < 0.01$, df = 10). Expression in the HE PVN is intermediate between that of the HO and WT PVN (although not significantly different from either). Neither the expression in the PVN of corticotropin-releasing factor (expressed in parvocellular VP neurons and in magnocellular OT neurons; ref. (35–38)) nor of enkephalin (expressed in parvocellular VP neurons and in magnocellular neurons; ref. (39, 40)) is altered (not shown).

WT, HE and HO mice were mated in different combinations to determine which, if any, behaviors and physiological functions essential for procreation were affected. We find that all three genotypes are fertile, even when homozygous males and females are mated. The homozygous mutant females carry normal-sized litters (9, 9, and 11 pups in three counted litters). The newborn pups fail to survive more than 24 h, dying without milk in their stomachs, despite apparently normal suckling. However, administration of 22.4 U of OT intraperitoneally, which does not change noticeably the mother's nursing behavior, rescues the pups who then have productive suckling within 30 min to 1 h (Fig. 4). The failure of the newborn pups to obtain milk is not just due to ineffective suckling, as 6 day old WT pups placed with the HO dams (that are within 24 h of parturition) are also unable to obtain milk, despite presumably more forceful suckling. In addition, whole mount examination of the mammary glands from HO dams showed milk accumulation prior to OT administration (data not shown).

Conclusions

Our results show that successful lactation in mice requires OT but appears unnecessary for parturition. OT has been considered an important, if not vital, hormone for parturition, given the rise in uterine sensitivity to OT of the myometrium that accompanies the rise in uterine OT receptor levels at birth (41–44) and the influence of OT agonists and antagonists on uterine contractions

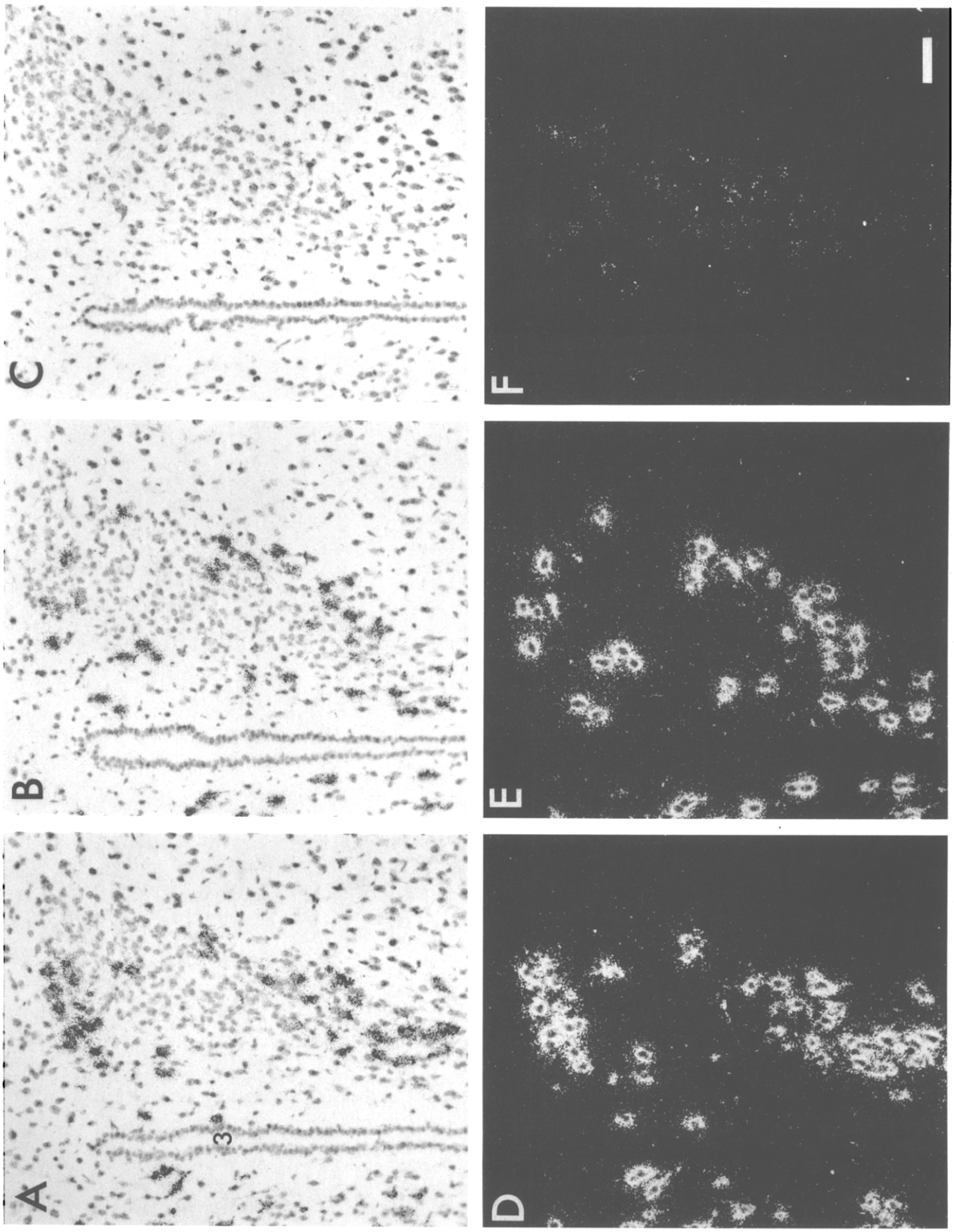

FIG. 2. Hybridization histochemistry with an OT probe was used on sections containing the PVN from wild-type (A, D), heterozygous (B, E) and homozygous mutant (C, F) mice and the sections were examined by brightfield (A–C) and darkfield (D–F) photomicroscopy. 3 is the third ventricle. Bar equals 50 μ.

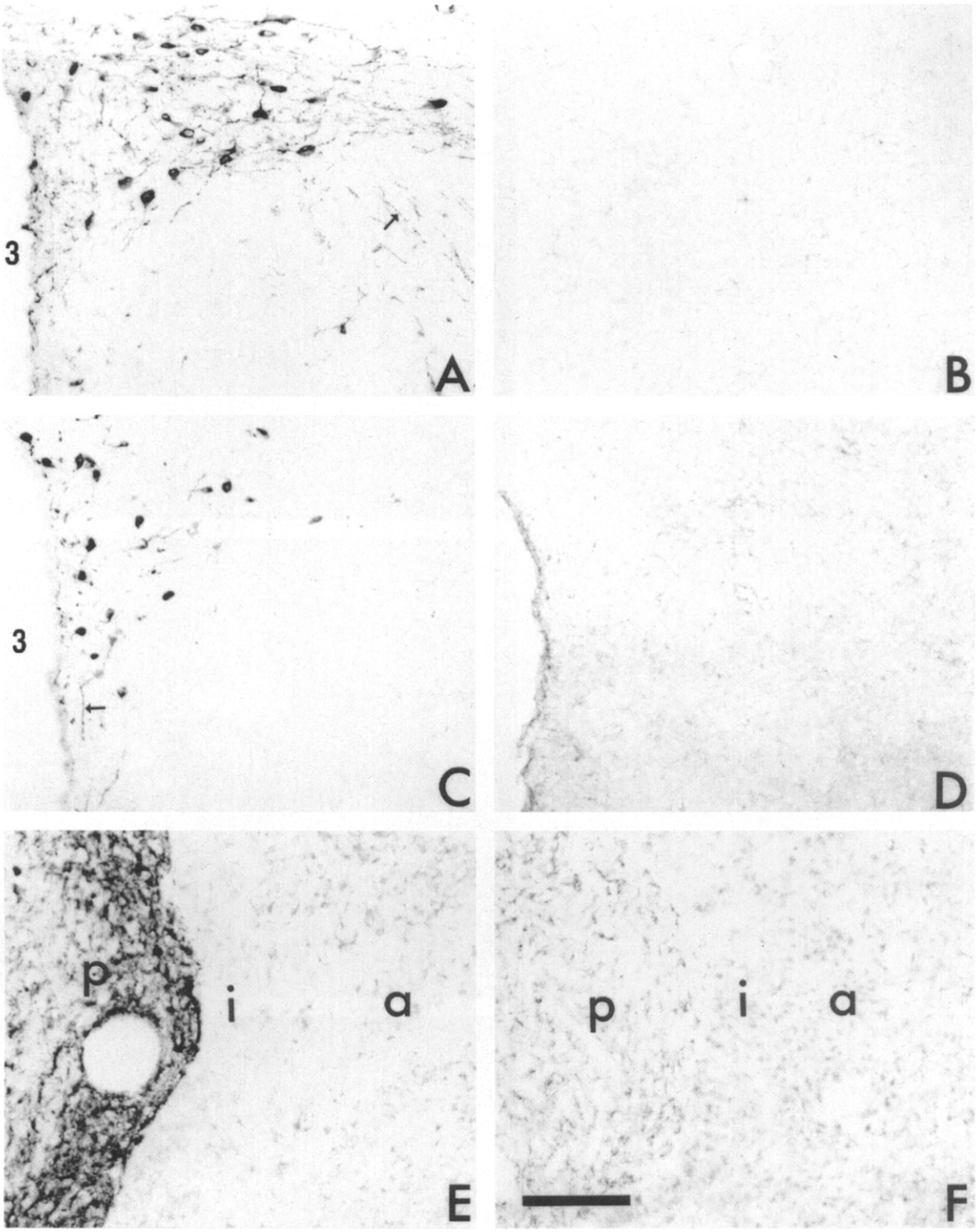

FIG. 3. Immunohistochemical detection of OT neurophysin using the mouse monoclonal antibody PS36 (A, B) or of OT-GKR using the rabbit antisera VA18 (C–F) in the PVN (A–D) or pituitary (E–F) of wild-type (A, C, E) and homozygous mutant (B, D, F) mice. There are numerous heavily stained neurons and axon fibers (arrows) present in the wild-type mice stained with either antibody. No staining was observed with either the neurophysin or the OT antibody in the mutant mouse. OT immunoreactivity was abundant in the posterior pituitary (p) of the WT mouse, but absent from that of the HO mouse. 3 is the third ventricle, a is anterior pituitary, i is intermediate lobe. Bar equals 1 mm.

(22–25). Inhibition of the release of rat neurohypophysial oxytocin by opioids also delays parturition (12). OT itself is also synthesized in the rat and human uterus, especially during parturition (45, 46). Of interest is that we have not been able to detect OT transcripts in the uteri from term or 1 day postpartum mice (unpublished observations). There are several potential explanations for the successful parturition in the absence of OT. One possibility is that circulating vasopressin is able to stimulate the OT receptor (47) sufficiently to allow parturition. Alternatively, other hormones, acting through other receptors, are able to

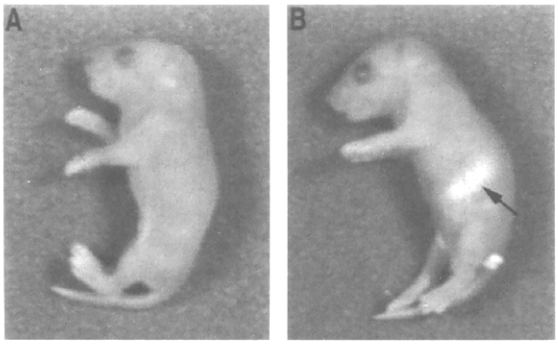

FIG. 4. Newborn pups (A) are unable to obtain milk from their homozygote dams and show no milk in their stomachs. However, 1 h after an intraperitoneal injection of oxytocin, the pups are able to obtain milk (arrow in B shows milk in stomach).

compensate for the lack of OT. These scenarios are under further study, as well as various parameters relating to parturition, such as whether there is any delay to the onset of parturition and the interval between pup deliveries. Targeted disruption of the OT receptor gene in mice should help elucidate to what extent there exists a functional relationship between vasopressin and the OT receptor.

Successful lactation is believed to result from the interplay of several hormones, including progesterone, prolactin, and OT (48), as well as nipple stimulation activating a neuroendocrine reflex (49). OT, released by suckling, is responsible for the myoepithelial contraction that then causes the milk ejection (50). Our results demonstrate unequivocally that OT is necessary for successful milk ejection, but not milk production.

In addition to its role in lactation, OT has been shown to participate in various maternal behaviors (13–17). During the initial 24 h of survival, the pups were found appropriately grouped together and cleaned. However, further studies are in progress to determine to what degree maternal (as well as other) behaviors are affected.

We do not understand why magnocellular VP expression is slightly depressed. It seems unlikely that this finding is a direct result of disrupting this particular locus, as the levels of VP in the suprachiasmatic nucleus are the same. In general, genes expressed in the magnocellular neurons of the PVN and SON vary in the same direction when the animal's physiology is perturbed (11). In our mutant mice, the expression of dynorphin and VP change in opposite directions. Although opioids are known to inhibit hormonal release from the pituitary (51–58), it is not clear why dynorphin expression should increase in the absence of OT.

Materials

Homologous recombination

We used methods previously described (27) except that FIAU (kindly donated by Lilly Research Laboratories, Indianapolis, IN, USA) was used instead of gancyclovir. A 129 SV mouse genomic library (kindly supplied by Drs H. Hellmich and A. Zimmer, NIMH) was screened and a clone that contained the OT gene and greater than 12 kb of flanking sequence was isolated. A 1.3 kbp BamHI/StuI fragment containing the OT promoter and first exon and a 4.6 kbp EcoRI fragment downstream of the coding region were inserted into the pPNT vector (59) 5′ and 3′, respectively, to the neomycin resistance cassette (Fig. 1). After selection in G418 and FIAU, 186 surviving clones were expanded, and isolated DNA was examined by PCR to identify 4 clones with the appropriate homologous recombination. Animal procedures were approved by the NIMH Animal Care and Use Committee.

Gene construction and analysis of genotype

To identify the genotypes of the mice, DNA from approximately 1cm of mouse tail was extracted and dissolved in 100 µl of 10 mM Tris-HCl, pH 7.5/1 mM EDTA. 10 µl PCR reactions containing 0.4 µl of DNA, 2.5 mM MgCl, 200 µM dNTPs, 0.1 µl Taq polymerase (Perkin-Elmer Cetus), and 0.1–0.25 µM primers were heated 5 min at 95 °C and then cycled 40 times at 95 °C for 1min and 72 °C for 3 min. Additional polymerase was added after 1 h. The primer pairs for the wild-type allele were TGGGC ACCCA CATAG GCCTA CCCAT GATCC and AGAGG GAGCC TAACA CTTCC CAAAG CCCTG GGTTC CCACG and for the mutant allele TGGGC ACCCA CATAG GCCTA CCCAT GATCC and ACCCC TTCCC AGCCT CTGAG CCCAG AAAGC GAAGG. The first primer in each pair is 5′ to the BamHI site at the 5′ end of the homologous recombination construct. The primer pairs for the wild-type and mutant alleles produced 1411 and 1753 bp fragments, respectively (Fig. 1).

Hybridization histochemistry

Hybridization histochemistry was performed as previously described (30) using male mice. The exposures were for 1–14 days against Fuji phosphorimaging plates which were used to quantitate the signals in a Fuji BAS2000 image analysis system (Stamford, CT, USA). Values were compared by ANOVA with Fisher's post-hoc test. For higher resolution, sections were subsequently dipped into Ilford K.5D nuclear emulsion and exposed for 1 mo.

Immunocytochemistry

Immunohistochemistry was performed on 12 µM thick fresh-frozen, 8% formaldehyde post-fixed or 4% paraformaldehyde/15% picric acid-perfused cryostat-sectioned tissues at room temperature. Slides with sections were warmed for 10 min and then incubated in 0.6% Triton X-100/3% normal goat serum for 1 h. Next, either the mouse monoclonal OT neurophysin antibody PS36 (60) at a 1:500 dilution or the rabbit polyclonal OT antisera VA18 (31) at dilutions of 1:5000–30,000 were added. The VA18 antisera is specific for the processed OT precursor, OT-GKR. The sections were incubated for another hour before continuing the incubation at 4 °C overnight. The sections were next washed in PBS and immersed into 1:1000–2000 dilution of anti-mouse or anti-rabbit IgG conjugated to biotin (Vector Labs) for 1 h. After several rinses in PBS, the sections were incubated in the ABC solution (Vector Labs) at 1:250–500 dilution for an additional hour. They were then transferred into 0.1 M Tris-HCl, pH 8, and developed using diaminobenzidine as a substrate. The sections were then coverslipped and viewed under a microscope.

Radioimmunoassay

For the assay of OT (61, 62), 250 µl of plasma (pooled from 3 mice for each sample) were mixed with 2 volumes of acetone and the supernatant was washed with 2 volumes of anhydrous ether. The acetone phase was dried and reconstituted to a total volume of 125 µl in 0.01 M potassium phosphate buffer containing 1.5 M NaCl, 0.25% BSA, and 0.1% Na azide, pH 7.4 (PBSA). For the assay, 50 µl of extracted plasma, 50 µl of oxytocin-specific antibody Pitt Ab-2 (32) (pH7.4) and 50 µl of iodinated OT were mixed and the volume adjusted to 500l with PBSA. The 125I-OT was added 1 day after the addition of the antibody and the incubation was continued for another 5 days. Separation of bound from free hormone was performed by precipitation with 25% polyethylene glycol after the addition of 50 µl bovine gamma-globulin (29 mg/ml) to the total assay volume. All samples were run in duplicate. The standard curve was prepared with synthetic OT (Bachem, Torrance, CA, USA). No displacement of iodinated tracer OT was found by assay of 100 mU arginine VP, 1ng lysine VP, or 1 ng of desamino-8D-arginine VP. The intra- and interassay coefficients of variation spanning the physiological concentrations of the assay are both <10%. The minimum detectable OT in extracted plasma is 1.0 pg/ml. OT immunoreactive peptide levels were measured in duplicate on acid extracts of individual pituitary glands. Extracts were diluted in assay buffer and multiple dilutions measured in the assay.

Studies of mammary function

The pups from the HO dams died without milk in their stomachs, so we examined the mammary glands to see if they failed to develop properly and thus were not able to produce milk, or whether milk was produced but milk ejection was not occurring. Individual mammary glands were biopsied and analyzed as previously described (63). Also, HO dams were injected intraperitoneally with 22.4 U of OT 12 h after parturition to see if the dams would then be lactate successfully.

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