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Research report

Neonatal oxytocin alters subsequent estrogen receptor alpha protein expression and estrogen sensitivity in the female rat

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ABSTRACT

In most species, the effects of oxytocin (OT) on female reproductive behavior are dependent upon estrogen, which increases both OT and OT receptor expression. It is also becoming apparent that OT neurotransmission can influence estrogen signaling, especially during development, as neonatal OT manipulations in prairie voles alter ERα expression and estrogen-dependent behaviors. We tested the hypothesis that OT developmentally programs ERα expression and estrogen sensitivity in female Sprague-Dawley rats, a species previously used to establish the estrogen-dependence of OT signaling in adulthood. OT treatment for the first postnatal week significantly increased ERα-immunoreactivity in the ventromedial nucleus of the hypothalamus (VMH), but not in the medial preoptic area (MPOA). Conversely, neonatal OT antagonist (OTA) treatment significantly reduced ERα-immunoreactivity in the MPOA, but not in the VMH. Both treatments increased OT-immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN) and reduced estrogen sensitivity, indicated by reduced sexual receptivity following chronic estradiol benzoate (EB) administration. Behavioral deficits in OTA-treated females were apparent during both paced and non-paced tests with 0.5 μg EB (but not 5.0 or 10.0 μg EB), whereas deficits in OT-treated females were only observed during the initial paced test with 0.5 and 5.0 μg EB (but not 10.0 μg EB). The current results demonstrate that OT can positively regulate ERα expression within the MPOA and VMH during development; however, endogenous OT selectively programs ERα expression within the MPOA. Thus, exogenous OT or OTA exposure during development may have long-term consequences on behavior through stable changes in ERα and OT expression.

1. Introduction

In mammals, oxytocin (OT) regulates all three fundamental classes of motivated behaviors, including ingestive, defensive and reproductive behavior [1]. During the neonatal period, the predominant actions of OT are on ingestive and defensive behaviors, such that OT signaling reduces both food intake and defensive responses, including the production of ultrasonic vocalizations, and facilitates stress-coping responses, such as grooming [2–7]. In adulthood, the effects of OT on ingestive and defensive behaviors are maintained and the regulation of reproductive behavior emerges after sexual maturation, in part due to the estrogen-dependent regulation of OT receptor (OTR) expression in the medial preoptic area (MPOA) and ventromedial nucleus of the hypothalamus (VMH) [8–10]. Within the MPOA and VMH, OT neurotransmission acutely facilitates female sexual behavior, whereas OTR antagonism inhibits sexual receptivity [11–13].

In addition to the acute effects of OT on motivated behaviors in the neonate and adult, OT signaling during the neonatal period may also contribute to the developmental programming of OT-dependent reproductive behaviors expressed in adulthood. In female rats, neonatal OT treatment delays vaginal opening and the onset of first estrus and increases maternal behavior, whereas neonatal OT antagonist (OTA) treatment had no effect on sexual maturation, but reduced maternal behavior [14,15].

The ability of neonatal OT manipulations to influence the expression of behaviors that are acutely regulated by OT in the adult are most likely mediated by either direct or indirect effects on the developmental patterning of the central OT-OTR system. It has previously been demonstrated that neonatal OT manipulations in prairie voles alter the subsequent expression of ERα [16,17], OT [18] and reproductive behavior [19,20]. Additionally, natural variations in the quality of maternal care received during the neonatal period can program adult reproductive behavior through changes in estrogen receptor (ER) α expression, estrogen sensitivity, and estrogen-induced OTR in the MPOA [21,22]. There-

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fore, we hypothesized that OT positively regulates ERα expression in the MPOA and VMH during the neonatal period and functions to program adult reproductive behavior through stable changes in ERα protein expression. Thus, we predicted that neonatal blockade of endogenous OT signaling by OTA would decrease, whereas exogenous OT would increase, subsequent ERα-immunoreactivity in the MPOA and VMH in adulthood. Additionally, we hypothesized that neonatal OT manipulations might also permanently reorganize OT expression in the paraventricular nucleus of the hypothalamus (PVN), the primary source of OT within the brain [1].

As ERα expression in the MPOA and VMH is critical for sexual receptivity in female rats, we also examined lordosis quotients in experimental subjects as a behavioral index of estrogen sensitivity and to characterize potential consequences of neonatal OT manipulations on an ERα-dependent behavior. While female reproductive behavior involves a complex suite of behaviors, including proceptivity and pacing, we focused solely on sexual receptivity, as this parameter is highly dependent upon ERα expression in the MPOA and VMH [23–26] and can be elicited by chronic estrogen treatment in ovariectomized female rats in the absence of exogenous progesterone, unlike most proceptive behaviors [27]. We predicted that neonatal OT treatment would increase, whereas OTA administration would decrease, subsequent estrogen sensitivity indicated respectively by leftward and rightward shifts in the estrogen dose–response curve for sexual receptivity.

2. Materials and methods

2.1. Experimental animals

Female Sprague-Dawley rats were obtained from our breeding colony that was originally established from rats purchased from Taconic Farms (Germantown, NY). On the day of birth (designated postnatal day 1, PD1) all animals were sexed and received a single toe-clip for subsequent identification and litters were culled to a maximum of ten individuals. Only animals from mixed sex litters were used in this study and all litters were culled to include at least 2 male littermates. Individual female neonates within litters were randomly assigned to receive single daily injections of oxytocin (OT, 1 mg/kg bodyweight), an OT antagonist (OTA, 0.1 mg/kg bodyweight) [$[d(CH_2)_5$, $Tyr(Me)^2$, $Orn^8]$-Vasotocin, Peninsula Laboratories, a division of Bachem, Belmont, CA, USA] or the isotonic saline (SAL) vehicle on PD1 through PD7. Doses were based on previous research demonstrating significant effects of similar neonatal treatments on the development of the hypothalamus and reproductive maturation [15,16,18]. A lower dose of OTA was used because the affinity of the antagonist for the OT receptor is approximately 10 times greater than the affinity of the natural ligand [28]. The OTA used in this study is selective for the OTR, but can also bind the vasopressin V1aR, although with much lower affinity [29,30]. Previous use of this compound at the current dose yielded results suggestive of selective blockade of the OTR over the V1aR [31,32]. All compounds were administered i.p. due to the disruptive nature of central injections. Additionally, the blood–brain barrier of neonates is permeable to peptides in the dose range used in the current study, which is supported by differential neural activation following i.p. administration of OT and OTA in neonatal prairie voles given at doses similar to those used here [33,34]. In order to minimize potential litter effects on brain neurochemistry and estrogen sensitivity, no treatment group was represented more than once per litter. At PD21, animals were weaned into single-sex sibling dyads or triads and then ovariectomized (OVX) at approximately PD45 and given two weeks to recover. In order to test for the effects of neonatal OT manipulations on estrogen sensitivity, OVX females were randomly assigned to receive one of three doses of estradiol benzoate (EB), 0.5 μg EB, 5.0 μg EB or 10.0 μg EB in 50.0 μl of sesame oil for seven days, or sesame oil vehicle only ($n = 7$ females per neonatal treatment for every level of EB or oil). Chronic treatment with EB has been shown to induce sexual receptivity without requiring progesterone [27]; therefore, the chronic EB paradigm allowed assessment of the effects of neonatal OT manipulations on estrogen sensitivity and was intended to minimize potential confounding influences of neonatal treatment effects on downstream estrogen-dependent progesterone signaling. All procedures were performed according to a protocol approved by the University of Illinois Animal Care and Use Committee.

2.2. Behavior testing

Sexual receptivity was examined twice in adulthood, 24 h after the third and seventh injections of oil or EB. During each testing session females participated in a paced mating test, in which the female has greater control over sexual contact with the male, followed by a non-paced mating test. Paced mating paradigms are more ethologically relevant but can be influenced by the motivation and social history of the animal [35,36]; therefore, non-paced tests were also conducted in order to get an additional measure of sexual receptivity. All tests began at least 1 h into the dark phase of the 12:12 h light–dark cycle and were completed within 4 h. Tests were videotaped under conditions of dim red illumination. Stimulus males were sexually experienced and displayed mounting behavior with estrogen- and progesterone-primed stimulus females prior to each test. Paced sex tests were conducted in a similar manner to Frye et al. [36]; briefly, stimulus males were placed into one side of the 2-chamber testing arena and allowed to habituate for at least 45 min prior to the start of the test (each chamber measured 40 cm $L \times 32$ cm $W \times 32$ cm H and were connected by a 4.5 cm diameter hole in the partition between the 2 chambers). Experimental females were then placed into the empty compartment and allowed to habituate for at least 5 min prior to uncovering the opening between the female- and male-occupied chambers. After the completion of the 30-min paced sex test, experimental females were returned to their home cages and allowed to rest for 30 min before being placed into a single-chambered testing arena for the non-paced sex test, which also lasted for 30 min (single chamber measured 40 cm $L \times 32$ cm $W \times 32$ cm H). Females were paired with different males for all tests in order to minimize potential effects of repeated exposure to the same individual. The sexual receptivity of each female was evaluated in paced and non-paced tests by calculating the lordosis quotient (LQ) from the first 10 mounts by the stimulus males (LQ = number of lordosis responses per 10 mounts) and only females receiving at least 10 mounts during the test were used for the behavioral analysis. Both paced and non-paced tests lasted for 30 min in order to ensure that all experimental females received at least 10 mounts during both types of behavioral tests and to create a standardized interval between the start of the first and second behavior tests. A single trained observer who was blind to experimental treatments manually scored all tests. As the objective of the study was to examine the effects of neonatal OT manipulations on the developmental programming of ERα and OT protein expression, brains were collected from experimental females two weeks after the last behavior test in order to evaluate differences in the levels of ERα- and OT-immunoreactivity in the absence of exogenous estrogen, which can induce variation in the number and intracellular localization of estrogen receptors. Additionally, differences in OT-immunoreactivity following the behavior tests might have reflected differences in central OT release, transport or estrogen-dependent synthesis. Brains were fixed via transcardiac perfusion with 4% formaldehyde and 2.5% acrolein (pH 7.4), equilibrated in 25% sucrose and sectioned at 30 μm on a freezing sliding microtome into four series and stored in cryoprotectant at −20 °C until processed for immunohistochemistry.

2.3. Immunohistochemistry

As we were interested in examining the effects of neonatal treatments on the levels of ERα and OT protein expression in adulthood, and not the effects of adult estrogen treatment itself, only a subset of the animals from the behavioral study were examined ($n = 8$ per neonatal treatment group). In order to minimize potential effects of prior adult steroid regime on ERα- and OT-immunoreactivity, the number of animals from each steroid condition was balanced within each neonatal treatment group. One quarter of the brain (consisting of every fourth section throughout the entire rostro-caudal extent of the brain) was stained for ERα-immunoreactivity and one quarter was stained for OT-immunoreactivity; all three neonatal treatment groups were equally represented in all staining runs for each antigen. Briefly, sections were rinsed in potassium phosphate-buffered saline (KPBS) to remove the cryoprotectant and incubated in 1% sodium borohydride to neutralize unreacted aldehydes from the fixation procedure. Endogenous peroxidases were inactivated by incubation in 0.014% phenylhydrazine and then sections were incubated in primary antibody (anti-ERα, C1355, Upstate, Lake Placid, NY, diluted 1:8000; the highly specific anti-OT sera was generously provided by Dr. Mariana Morris, diluted 1:100,000) in KPBS with 0.04% Triton X-100 (KPBST) for 1 h at room temperature followed by a 48-h incubation at 4 °C. Tissue was then rinsed in KPBS and incubated with a biotinylated goat α-rabbit IgG in KPBST for 1 h (Vector Laboratories, Burlingame, CA, diluted 1:600). Antigens were visualized with an avidin-biotinylated HRP (A/B) system (Vector Laboratories, Burlingame, CA) using 3,3′-diaminobenzidine (DAB) as a substrate for labeling OT and nickel-intensified DAB for labeling ERα. The specificity of C1355 has been previously demonstrated in rats tested/using preadsorption with the synthetic peptide used to produce the C1355 ERα antibody [37] and immunoblot with rat ERα transfected Cos-1 cells [38]. Stained sections were mounted on gelatin-subbed slides, air-dried overnight and dehydrated and cleared through a graded series of ethanol and xylene. Slides were cover-slipped with Entellan rapid mounting medium (Merck & Company, NJ, USA).

2.4. Image analysis

A single observer, blind to treatment groups, scored slides from each animal at 40× magnification. IPLab software was used to quantify the number of ERα-immunoreactive cells in discrete forebrain regions, whereas OT-immunoreactive neurons in the paraventricular nucleus (PVN) were hand-counted. Different techniques were used for counting the number of ERα- and OT-immunoreactive neurons due to the different staining properties of each antigen. The nuclear localization and relatively uniform size of ERα-immunoreactivity is conducive to automated counting of discrete cell nuclei and an approximation of the number of nuclei contained in