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Effects of sex and reproductive experience on the number of orexin A-immunoreactive cells in the prairie vole brain

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ABSTRACT

Large populations of cells synthesizing the neuropeptide orexin (OX) exist in the caudal hypothalamus of all species examined and are implicated in physiological and behavioral processes including arousal, stress, anxiety and depression, reproduction, and goal-directed behaviors. Hypothalamic OX expression is sexually dimorphic in different directions in laboratory rats (F > M) and mice (M > F), suggesting different roles in male and female physiology and behavior that are species-specific. We here examined if the number of hypothalamic cells immunoreactive for orexin A (OXA) differs between male and female prairie voles (*Microtus ochrogaster*), a socially monogamous species that pairbonds after mating and in which both sexes care for offspring, and if reproductive experience influences their number of OXA-immunoreactive (OXA-ir) cells. It was found that the total number of OXA-ir cells did not differ between the sexes, but females had more OXA-ir cells than males in anterior levels of the caudal hypothalamus, while males had more OXA-ir cells posteriorly. Sexually experienced females sacrificed 12 days after the birth of their first litter, or one day after birth of a second litter, had more OXA-ir cells in anterior levels but not posterior levels of the caudal hypothalamus compared to females housed with a brother (incest avoidance prevents sibling mating). Male prairie voles showed no effect of reproductive experience but showed an unexpected effect of cohabitation duration regardless of mating. The sex difference in the distribution of OXA-ir cells, and their increased number in anterior levels of the caudal hypothalamus of reproductively experienced female prairie voles, may reflect a sex-specific mechanism involved in pairbonding, parenting, or lactation in this species.

1. Introduction

Very large populations of cells synthesizing the neuropeptide orexin (OX), also known as hypocretin, are found in the lateral and perifornical/dorsomedial regions of the caudal hypothalamus of all mammals examined. These include laboratory rats [46,52,63], mice [69], grass rats (*Arvicanthis niloticus*) [15,49], Syrian hamsters (*Mesocricetus auratus*) [44], degus (*Octodon degus*) [47], domesticated cats [69], African green monkeys (*Cercopithecus aethiops*) [16] and humans [20]. OX exists in two isoforms (A and B) that are produced by the prepro-OX polypeptide and these isoforms have differential affinity for the OX receptors, with OXA preferentially binding the OX_1 receptor but OXA and OXB having similar affinity for the OX_2 receptor [66]. Through their widespread inputs and outputs [46,52,66], hypothalamic OX cells integrate a broad range of internal and external signals underlying arousal, stress, emotions and mood, reward processing, and the performance of goal-directed behaviors [2,58,66]. Studies of mice with null mutations of the OX receptor genes demonstrate that OX's role in arousal is mediated by the OX2 receptor while its other effects (*e.g.*, reward processing and goal-directed behaviors) are more likely controlled by the OXA-preferring OX1 receptor [43].

The goal-directed behaviors influenced by OX include the social interactions necessary for reproduction. For example, infusion of OXA into the mPOA (a site well-studied for its role in copulatory behaviors [26,30,54]) facilitates sexual motivation and performance in male rats [27]. Conversely, antagonism of OX1 receptors by systemic injection of SB-334867 increases the latency for male rats to intromit and decreases their frequency of ejaculation [45; but see 4]. Furthermore, expression of the immediate-early gene *c-fos* increases in hypothalamic OXergic cells when male rats are exposed to conspecific female sensory cues, sexually interact with a female, or are placed in a chamber associated with sexual reward [17,18,45]. Given these findings it is surprising that destruction of hypothalamic OXergic neurons does not affect

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sexual performance or motivation in experienced male rats, but it does facilitate the onset of a sexual interaction in inexperienced males, which has been attributed to the disrupted OX signaling reducing their novelty-induced anxiety [17]. In postpartum laboratory mice intracerebroventricular infusion of OXA dose-dependently decreases maternal attacks on an unfamiliar male mouse and disrupts nursing behavior [14]. Consistent with the latter result, inhibiting endogenous OX signaling with a peripheral injection of SB-334867 tends to increase some aspects of nursing [14].

Sex and endocrine state are two factors affecting OX synthesis and, therefore, its influence on reproductive and other behaviors. Hypothalamic prepro-orexin mRNA has been seen to be >50% higher and OXA content almost twice as high in virgin female rats compared to males [34,64], but the direction of this sex difference may be species specific because female mice have 15–20% fewer OXA-ir cells in the hypothalamus than do male mice [8]. Gonadal and other hormones circulating during adulthood contribute to the magnitude of this sex difference. In male rats the number of hypothalamic prepro-OX-ir cells decreases after castration in conjunction with the decrease in copulation, but both can be maintained by exogenous estradiol, presumably naturally a result of the aromatization of endogenous testosterone [45]. The more numerous studies on how gonadal hormones affect OXA-ir cells in females are equivocal. Hypothalamic OXA content has been seen to be particularly low during late proestrus or in response to exogenous estradiol, perhaps reflecting increased OXA release and depletion of intracellular stores [57], but others have found prepro-OX mRNA or OXA levels to be at their peak during late proestrus [53,60]. Still others have found that OX synthesis does not differ across the estrus cycle [70]. Pregnancy and lactation also have unclear effects on OX in female laboratory rats, being associated with higher, lower, and no change in expression [7,9,23,36,61,70].

All of the studies described above examined polygamous laboratory rodents, but it would be interesting to study the relationships among the hypothalamic OX system and social interactions, sex, and reproductive experience in a monogamous rodent such as the prairie vole (*Microtus ochrogaster*). Male and female prairie voles share more aspects of their behavioral repertoires when compared to most polygamous rodents, with both sexes of prairie vole forming lifelong pairbonds after mating and later contributing parental care to their offspring [11,76]. Furthermore, reproductive experience and pairbonding alter anxiety-related behavior [32,37,39] and energy balance [10] in male prairie voles in ways that are more typically found in postpartum female mammals [see 40, 74]. Prairie voles also have smaller sex differences compared to polygamous rodents in some (but certainly not all) aspects of their hypothalamic anatomy and neurochemistry [38,59]. Finally, OX interacts with the oxytocin and vasopressin neuropeptide systems [1,3,67] that have been so well studied for their contributions to prairie vole behavior and physiology [11,75].

Considering these points above, it is reasonable to suggest that OX could be a component of the neural systems necessary for maintaining the rewarding pair bond, parental caregiving, emotional state, or energy balance in prairie voles. If so, one might expect upregulated OXA in the hypothalamus of both sexes of pair bonded and parental prairie voles. If OXA is instead more involved in sex-specific processes in this species, any influence of reproductive experience and parental state might be expected to differ between male and female prairie voles. To begin evaluating which of these possibilities is more likely, we first determined if the number of OXA-ir cells in the prairie vole hypothalamus is sexually dimorphic. We then determined if reproductive experience and its suite of behavioral and physiological changes produce a similar or different influence on the number of OXA-ir cells in male and female prairie voles.

2. Materials and methods

2.1. Subjects

Subjects were male and female prairie voles (*M. ochrogaster*) from our breeding colony that descended from wild voles captured in Urbana, Illinois and described previously [13]. Animals were maintained on a 14:10-h light:dark cycle, an ambient temperature of $21 \pm 1\,^{\circ}C$, and housed in clear plastic cages ($48\,cm \times 28\,cm \times 16\,cm$) containing wood chips, wood shavings, and a substantial covering of hay. They were provided water and a food mixture containing cracked corn, whole oats, sunflower seeds, and rabbit chow (Tekland rodent diet No. 2031) in a ratio of 1:1:2:2 *ad libitum*. Subjects were weaned from their parents at 20 days of age and housed in mixed-sex sibling groups, where they remained until being used in the experiment when they reached 70–95 days of age. Prairie voles are induced ovulators and incest avoidance prevents them from mating with siblings, even if the siblings are separated for at least up to a week before a reunion [24]; this mixed-sex housing after weaning permitted us to use familiar opposite-sex sibling pairs as the reproductively inexperienced control groups (see below). All procedures were in accordance with the Institutional Animal Care and Use Committees at Michigan State University.

2.2. Housing and reproductive experience conditions

Subjects ($N = 25$) were taken from their home cages and socially isolated in a clean cage for four days. They were then placed in a soiled cage containing a similarly separated unfamiliar prairie vole of the opposite sex to elicit female estrus and mating [11] or they were placed in a soiled cage containing a similarly separated familiar opposite-sex sibling. For the sexually experienced subjects, cages were inspected daily starting 2–3 days before the expected day of parturition for the presence of pups. The sexually experienced females and males were sacrificed 12 days after the birth of their first litters ($n = 9$ females, 5 males) or one day after a female give birth to a second litter ($n = 6$ females, 5 males). Thus, the first group of dams were both lactating and pregnant and the second group of dams were lactating, recently parturient, and in a postpartum estrus. In the group sacrificed one day after birth of the second litter, the older litter of pups remained in the home cage until the time parents were sacrificed. The reproductively inexperienced females and males were yoked to mated animals and sacrificed between 35 and 40 days after rehousing with a sibling (shorter cohabitation: $n = 5$ females, 8 males) or 42–50 days after rehousing with a sibling (longer cohabitation: $n = 6$ females, 6 males). At sacrifice, it was confirmed that the uterine horns of the sexually experienced females killed 12 days after the birth of their first litter contained fetuses, and that the uterine horns of sexually inexperienced females housed with their brothers contained no fetuses and that no pups were living in those home cages. Although we confirmed that these sibling pairs did not have fetuses or pups at the time of sacrifice, so were reproductively inexperienced, we cannot be absolutely certain they were also sexually inexperienced because it is conceivable that mating between siblings could occur without producing fetuses or live offspring.

2.3. Perfusion, tissue collection and immunocytochemistry

Subjects were overdosed with an anesthetic containing ketamine (62.5 mg/kg), xylazine (7.5 mg/kg), and acepromazine (0.8 mg/kg) and perfused through the heart with 100 mL of 0.9% saline followed by 100 mL of 4% paraformaldehyde in sodium phosphate buffer (NaPB; pH = 7.6). Brains were removed, post-fixed overnight in 4% paraformaldehyde in NaPB, and submerged in a