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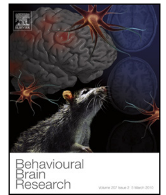

Research report

Activation of the olfactory system in response to male odors in female prepubertal mice

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HIGHLIGHTS

- We identified parts of the olfactory pathway responding to male odors in prepubertal female mice.
- Brain nuclei differentially respond to intact male odors as early as post-natal day 24.
- The accessory olfactory pathway seems to be involved in the processing of male odors.

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ABSTRACT

Exposure to male odors during the prepubertal period accelerates puberty, a phenomenon known as the Vandenbergh effect. This experiment identifies the parts of the olfactory pathway that respond to male odors in prepubertal female mice. Female mice were kept in a room free of adult male odors from birth until odor exposure. At post-natal day 21, 24 or 28, (ages representing time points early, intermediate, and late in the prepubertal period) mice were exposed to clean bedding, soiled bedding from castrated males, or soiled bedding from intact males. Each group was exposed to odor in separate rooms to prevent cross contamination. Ninety minutes after odor exposure, mice were sacrificed, the brains removed and prepared for c-Fos immunohistochemistry. The numbers of neurons expressing c-Fos were counted in a defined area of the following nuclei: AOB mitral layer, AOB granular layer, MOB, MEPV, MEPD, Aco, BNST, MPOA, and VMH. There was a significant effect of age on c-Fos-expression in the MEPV, MEPD, Aco, MPOA, BNST and piriform cortex. There was a significant effect of odor on c-Fos-expression in the MEPV, MEPD, Aco, MPOA, and VMH, showing that these areas are differentially sensitive to intact male odors vs. clean bedding and that these brain areas may be responsible for communicating odor information that drives puberty acceleration.

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1. Introduction

For more than 50 years it has been known that the entry into puberty can be accelerated by the presence of adult male conspecifics, a phenomenon called the Vandenbergh effect [1–3]. Exposure to soiled male bedding during the prepubertal period stimulates LH release in female mice [4], and the presence of male urine alone is sufficient to accelerate puberty even when exposed for as little as 2–3 h per day starting at post-natal day 21 [5–8] Urine from female mice or castrated male mice does not have the same puberty-accelerating affects, but castrated males treated with testosterone for as little as 3 days once again produce urine that is capable of eliciting puberty acceleration, suggesting that the source of these odors is androgen dependent [9,10]. Furthermore, urine from dominant males that typically have higher circulating androgens, is more effective at accelerating puberty onset compared to urine from subordinate males [8]. Several compounds have been identified in male mouse urine as candidate chemosignals responsible for puberty accelerating activity, including major urinary proteins (MUPs), which are large non-volatile compounds, as well as several smaller volatile compounds that may or may not associate with MUPs [11–14].

Although there has been much research identifying the signals present in male urine that accelerate puberty in female mice, there remains a need to further study the central mechanism by which exposure to male chemosignals accelerate puberty in female mice.

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The detection of these chemosignals takes place in the accessory and main olfactory systems, which, respectively, detect the non-volatile and volatile components of odors, and may work together to communicate odor information [15,16] to the reproductive system. Indeed, a direct pathway exists between the medial amygdala, which receives direct input from the accessory olfactory bulb (AOB), and the ventromedial hypothalamus (VMH) and bed nucleus of the stria terminalis (BNST), both nuclei associated with the control of reproduction [17]. Adult female mice exposed to odors from dominant males show more c-Fos-expression in the posteroventral part of the medial amygdala (MEPV), the bed nucleus of the stria terminalis (BNST), and the medial preoptic area (MPOA) [18], further implicating the accessory olfactory system as the primary system for the detection of opposite sex chemosignals.

While the central nuclei responsible for the detection of male chemosignals have been identified, the mechanism by which this chemosensory information is integrated with the reproductive system is unknown, but it may involve the neuropeptide kisspeptin. There is considerable evidence that the kisspeptin system plays an important role in the onset of puberty in female mice (reviewed by [19–21]). In female rats, treatment with kisspeptin accelerates puberty [22] while treatment with kisspeptin antagonists delays the start of puberty, characterized by day of vaginal opening [23]. In humans, inactivating mutations in either kisspeptin or the kisspeptin receptor, GPR54, can result in hypogonadotropic hypogonadism and failure to enter puberty [24,25]. Other support for the importance of kisspeptin in the control of puberty in female mice is that the sexually dimorphic population of kisspeptin neurons in the rostral periventricular area of the 3rd ventricle (RP3V), which consists of the anteroventral paraventricular nucleus (AvPv) and the rostral and caudal parts of the paraventricular nucleus (rPeN and cPeN, respectively), does not develop until around puberty onset, between p25 and p30 [26–28]. It is possible that chemosensory information is integrated with other information (such as energy availability) and relayed to the reproductive via kisspeptin neurons. Thus, this experiment will determine if a single exposure to male odors during the prepubertal period is sufficient to induce c-Fos-expression in kisspeptin neurons in the anterior hypothalamus.

Most research on the areas activated by exposure to male odors has been done on adult female mice, so it is necessary to investigate whether the same brain nuclei that are stimulated in response to a potential mate during adulthood show the same response during the prepubertal period. The current experiment tests the hypothesis that the brain areas implicated in both olfaction and reproduction are differentially sensitive to odors from intact and castrated males upon initial exposure during the prepubertal period. To this end, we exposed female mice to soiled bedding from intact or castrated males at time points early, intermediate, and late in the prepubertal period. If this hypothesis is supported, it is expected that brain areas known to communicate between the olfactory and reproductive systems would show increased c-Fos-expression after exposure to bedding soiled by intact males, but not bedding soiled by castrated males or clean bedding.

2. Materials and methods

2.1. Animals

All animals were housed at the central animal facility at the French National Institute for Agricultural Research (INRA), in Nouzilly, France. The mice were housed and all experimental protocols were executed in rooms kept at constant temperature 21–22 °C and with a 12:12 light dark cycle, food (pellets, Safe, Augy, France) and water was available ad libitum. All the procedures were conducted in accordance with the European directive 2010/63/EU on the protection of animals used for scientific purposes and approved by an Ethical Committee for Animal Experimentation (CEEA VdL, Tours, France, no. 2012-10-2).

Male and Female mice of the CD1 (Swiss) strain were purchased from R. Janvier Breeding Center (Le Genest, Saint-Isle, France) and maintained in the breeding colony at INRA. For breeding, female mice housed two per cage were given access to a male mating partner overnight and pregnancy was confirmed with the presence of a vaginal plug. The breeding males were removed from the cages and all pregnant females were housed in a separate room free of odors from adult males to avoid odor exposure during early life of the pups. The pups were born and litters reduced to five males and five females at postnatal day 2. At post-natal day 20 (p20) the pups were weaned and the males were removed from the room and placed with the main colony, while females remained in the room and were housed singly in polycarbonate cages ($23 \times 16 \times 14$ cm) containing 75 g of clean bedding (mix of Sterilabo® ad Copolabo®, Sisca, Alfortville, France).

2.2. Collection of soiled bedding

In a separate room, gonadally intact and castrated adult, male mice were individually housed in cages containing 75 g of clean bedding. After 5 days, the soiled bedding was collected. Soiled bedding from all intact males was combined to provide the intact male bedding stimulus, and soiled bedding from all castrated males was combined to provide the castrated male bedding stimulus. The castrated males used to provide soiled bedding were adult males whose testes were completely removed via a midline incision closed with absorbable sutures under isoflurane anesthesia at least 3 weeks before bedding collection began.

2.3. Odor exposure

Groups of female mice were exposed to odors from clean bedding, castrated male bedding, or intact male bedding on post-natal day 21, post-natal day 24, or post-natal day 28 (p21, p24, p28, respectively). The groups in this experiment were as follows: p21 clean bedding ($n = 8$), p21 castrated male bedding ($n = 8$), p21 intact male bedding ($n = 8$), p24 clean bedding ($n = 9$), p24 castrated male bedding ($n = 9$), p24 intact male bedding ($n = 10$); p28 clean bedding ($n = 8$), p28 castrated male bedding ($n = 10$) p28 intact male bedding ($n = 11$). These ages were selected to represent times early, intermediate, and late in the prepubertal period. Our lab has monitored VO in mice exposed to male odors from p22 through p38 and found no VO at p22, 25% of animals showing VO at p24, and almost 80% showing VO at p28 [29]. VO was thus only recorded in p24 and p28 mice to determine the reproductive state of the animals. In addition, the number of kisspeptin cells in the RP3V was counted at all ages to confirm the development of the reproductive axis, since the kisspeptin system has been shown to directly influence GnRH secretion and to be closely linked to the control of puberty onset (reviewed by [30]).

During odor exposure, 25 g of bedding was removed from the home cage and was replaced by 25 g of clean bedding, soiled bedding from castrated males, or soiled bedding from intact males. Each odor exposure took place in separate rooms to reduce any possibility of cross-contamination of odors. After 90 min of odor exposure, the mice received a lethal dose of anesthetic (Ketamine/Domitor) before intracardiac perfusion with sodium nitrite, followed by 4% paraformaldehyde. The brains were removed, post-fixed in 4% paraformaldehyde for 1 h at room temperature, and then placed in sucrose at 4 °C until they sank. The brains were then frozen in dry ice, and were cut into 30 μm sections on a freezing microtome (CM3505S, Lieca microsystems). Six sets of 30 μm coronal sections were cut from the rostral preoptic

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