The role of the nucleus retroambiguus in the neural control of respiration, vocalization and mating behavior
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ABSTRACT

Recent studies have revealed brainstem-spinal pathways involved in the generation of receptive behavior in hamster and cat, and the enormous influence of estrogen on these pathways. The present study gives an overview of the location of estrogen receptor-alpha-immunoreactive neurons (ER-α-IR) in the brainstem of the female hamster. In the mesencephalon ER-α-IR cells were found in the arcuate and peripeduncular nuclei as well as throughout the rostrocaudal extent of the periaqueductal gray (PAG), and the laterally adjoining tegmentum. In the caudal brainstem, groups of ER-α-IR cells were present in the ventrolateral parabrachial nucleus, the solitary nucleus, and in contrast to the cat, in the nucleus retroambiguus. No ER-α-IR cells were found in any other part of the brainstem. The functional implications of these findings are discussed.

INTRODUCTION

In rat (Pfaff et al., 1994) and cat (Leedy and Hart, 1985) the ventromedial hypothalamus and the periaqueductal gray (PAG; Krieger et al., 1979) are one of the most important cell groups involved in mating behavior. In order to activate mating behavior, the PAG excites motoneurons in the lumbosacral cord via its projections to the nucleus retroambiguus (NRA) (Vanderhorst and Holstege, 1995; Holstege et al., 1997; Gerrits and Holstege, 1999). In the cat estrogen induces outgrowth of NRA to these motoneuronal cell groups (Vanderhorst and Holstege, 1997b), although it is not known how estrogen produces this effect. Recently, Gerrits and Holstege (1999) have demonstrated that in the female hamster the NRA, among other projections, has distinct projections, to the iliopsoas muscle motoneurons, a muscle involved in the lordosis response. These projections are presumed to be excitatory. This finding suggests that in this species a similar PAG-NRA-motoneuronal projection exists as in the cat, and which might serve as the final common pathway for mating behavior. However, its motor performance during mating differs strongly from the cat, and its much shorter estrous cycle suggest that there are important differences between the motor control of mating in cat and hamster. One possibility is the different localization of estrogen receptors in these two species. In the present study the location of estrogen receptor-alpha-immunoreactive neurons (ER-α-IR) was determined in
all parts of the brainstem of the golden hamster. The results suggest that ER-α-IR cells play a different role in the production of mating behavior in the hamster than in the cat.

**MATERIALS AND METHODS**

All surgical procedures, pre- and postoperative care, and handling and housing of the hamsters were in accordance with the protocols approved by the Faculty of Medical Sciences of the University of Groningen. Seven female hamsters, weighing 100-110 g, were bilaterally ovariectomized, under chloralhydrate (400 mg/kg, i.p.) anesthesia. Ovariectomy results in a strong decrease of circulating estrogen, producing an increase of unoccupied estrogen receptors. Since the H222 antibody is directed against unoccupied estrogen alpha receptors, ovariectomy leads to the demonstration of a larger number of ER-α-IR cells (Axelson et al., 1992). After a survival time of 2 to 4 weeks, the hamsters were re-anesthetized with an overdose of Nembutal (0.8 ml of 6% pentobarbital sodium, i.p.). Subsequently, the hamsters were transcardially perfused with 400 ml of heparinized saline, followed by 400 ml fixative consisting of 4% paraformaldehyde, 0.5% glutaraldehyde in a 0.1M phosphate buffer (pH 7.4). The brains and brainstems were removed and postfixed for 10 hours and stored in 0.05M tris-buffered saline (TBS; 0.05M, 0.09% NaCl, pH 7.6) at 4°C. The brainstem was cut transversally into 60 µm sections on a vibratome filled with TBS at 4°C. Free floating sections were pretreated with 0.01 M NaIO₄ and 1% NaBH₄ to remove the residual aldehydes, with 1% Triton X-100 to increase antibody penetration, and with 1% H₂O₂ to block endogenous peroxidase activity. All these substances were diluted in TBS. The pretreated sections were then incubated in H222 antibody (1:1000 in 0.05 M Tris, 0.5 M NaCl, 0.5% Triton X-100 (TBHS+; gift of G. Greene), a rat monoclonal antibody to the ligand-binding domain of the estrogen receptor alpha (Greene et al., 1984), for five days at 4°C. Subsequently, the sections were incubated in the second antibody, biotinylated sheep anti-rat immunoglobulin (1;100 in TBHS+, Amersham, RPN-1002) for 60 min at room temperature, followed by avidin-biotin-peroxidase-complex (1:400 in TBS, ABC Elite kit, Vector, PK-6100). All incubations were performed on a rocking table, and between all steps the sections were rinsed with TBS for 90 min. Finally, the sections were visualized using diaminobenzidine-nickelammoniumsulphate (0.04% DAB, 0.2% nickelammoniumsulphate, 0.012% H₂O₂ in TBS). Control sections were processed as described above, but without the first antibody. No specific immunoreactivity was observed in these control sections. All sections were mounted on slides, dehydrated and coverslipped with DePex mounting medium. The location of ER-α-IR positive neurons in the brainstem in one out of two sections was plotted in a Zeiss Axioskop equipped with the Neurolucida system of MicroBrightField. Drawings of the sections of the brainstem were made at intervals of 240 µm using Adobe Illustrator. Photomicrographs were taken from representative sections and processed using Adobe Photoshop.
Fig. 1. Drawings of sections, rostral (A) to caudal (Z2), showing the distribution of the ER-α-IR neurons in the brainstem in case 107. The distance between the sections is 240 µm. In each section (A-Z2) one dot represents one ER-α-IR cell. Scale bar, 2 mm. Arc, arcuate nucleus; LC, locus coeruleus; MG, medial geniculate nucleus; NRA, nucleus retroambiguus; PAG, periaqueductal gray; PB, parabrachial nucleus; Sol, solitary complex; Sp5C, spinal trigeminal complex, caudal; 3, oculomotor nucleus; 4, trochlear nucleus.
RESULTS

At rostral mesencephalic levels a group of ER-α-IR cells was present in the arcuate nucleus and in the peripeduncular nucleus just ventral to the medial geniculate nucleus (Fig. 1A-B). Furthermore, ER-α-IR neurons were found along the midline in the ventral, and a smaller group in the lateral part of the rostral PAG (Fig. 1A), and in the laterally adjoining tegmentum. Caudally, the midline cell group gradually disappeared (Fig. 1E-G), but the number of ER-α-IR neurons in the lateral PAG increased (Figs. 1F-H; 2A). At the level of the trochlear and the caudal pole of the oculomotor nucleus, the ER-α-IR neurons formed a distinct cell group in the ventrolateral PAG (Fig. 2B). Further caudally, this group gradually increased in size and split into two groups, one in the lateral and one in the ventrolateral PAG (Fig. 1H-I). These two cell groups did not extend into the caudal pole of the PAG, at which level only few ER-α-IR cells were found (Fig. 1J). Of all the ER-α-IR cells in the PAG 80% was located in its caudal half. At pontine levels a distinct group of ER-IR neurons was found in the ventrolateral parabrachial nucleus (Fig. 1K-L; 2C), and another, smaller group in the ventrolateral part of the periventricular gray, just rostral to the locus coeruleus (Fig. 1K-L). In the medulla oblongata ER-α-IR cells were only found caudal to the obex. One group was present in the solitary nucleus and another in the region of the nucleus retroambiguus (NRA; Fig. 1W-Z1; 2D). For the location of the NRA neurons projecting to the lumbosacral cord

Fig. 2. Brightfield photomicrographs of labeled ER-α-IR nuclei in neurons in mesencephalon, pons and medulla oblongata in case 117. (A) rostral PAG; (B) caudal PAG; (C) parabrachial nucleus; (D) nucleus retroambiguus. Scale bar, 50 µm.
ER-α-IR neurons in the hamster brainstem

see Gerrits and Holstege (Gerrits and Holstege, 1999). There were also cells found in the reticular formation between NRA and solitary nucleus (Fig 1V-1Z1). Other parts of the brainstem were devoid of ER-α-IR cells.

DISCUSSION

Estrogen receptors have been studied mainly in hypothalamic and other limbic structures in the brain, but to only a very limited extent in the brainstem. This is somewhat surprising, because the brainstem plays a crucial role in the motor control of copulatory activities. Although there are no reports about ER-α-IR cells in the brainstem of the hamster, in this species Krieger et al. (Krieger et al., 1976) have reported estradiol concentrating cells in the ventromedial quadrant of the PAG. However, in contrast to the present study, they only found these cells at one, relatively caudal, level, and not outside the borders of the PAG. In the hamster, ER-α-IR neurons have not been demonstrated before in the four cell groups at pontine and medullary levels, i.e. the ventrolateral parabrachial nuclei, the ventrolateral periventricular gray, the solitary nucleus and the NRA. In the rat (Amandusson et al., 1995) and sheep (Scott et al., 1998) ER-α-IR cells have been reported in the solitary nucleus. From the drawings in these studies the impression was gained that the NRA also contained ER-α-IR cells in these species, although it was not specifically stated. In the hamster there were no ER-α-IR cells in the caudal nucleus of the spinal trigeminal nucleus, similar to sheep (Scott et al., 1998), but unlike cat (see chapter 7 this thesis) and rat (Amandusson et al., 1995). The most interesting finding in the present paper is that the NRA in the hamster contains ER-α-IR cells, which seems not to be the case in the cat (see chapter 7 this thesis). The ER-α-IR neurons in the NRA are the same cells that project to the ilopsoas motoneurons (Boers and Holstege, 1998). One might speculate that this difference between hamster and cat reflects the difference in how estrogen induces mating behavior in these two species, in the cat by growth of NRA terminals on motoneurons, in the hamster by its effect on the NRA neurons themselves.