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MIGRATION OF LHRH-IMMUNOREACTIVE NEURONS FROM THE OLFACTORY PLACODE RATIONALIZES OLFACTO–HORMONAL RELATIONSHIPS

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Summary—Nerve cells that express luteinizing hormone-releasing hormone (LHRH), essential for reproductive functions, originate in the epithelium of the medial olfactory placode. While the peripheral origin of this physiologically important brain peptide is surprising, associations between olfactory and reproductive systems are well documented in behavioral studies of pheromones and in clinical studies of disorders including hypogonadotropic hypogonadism with anosmia or olfactory–genital dysplasia. Mechanisms underlying this migration include a close association with neural cell adhesion molecules (NCAM), but are likely also to involve other physical and chemical factors.

Luteinizing hormone-releasing hormone (LHRH, or gonadotropin hormone-releasing hormone, GnRH) is essential for reproductive functions. It regulates the release of both luteinizing hormone and follicle-stimulating hormone from gonadotropes of the anterior pituitary gland [1, 2] which in turn stimulate the secretion of steroid hormones by the testes and ovaries. In addition, it has been shown to facilitate reproductive behavior [3, 4]. A decapeptide, LHRH is found in neurosecretory neurons in the brain and nose of all vertebrates, including humans. In the brains of adult animals, these LHRH-containing neurons have been localized by immunocytochemical procedures and found to form a rostral-to-caudal continuum from the ventromedial forebrain and olfactory tubercle, through the medial septal and preoptic areas, to the hypothalamus and median eminence of the tuber cinereum [5]. The axons of these cells, for the most part, project to the median eminence where they terminate on the capillaries of the primary portal plexus, and thus gain access to the pituitary gonadotropes. In the nose, LHRH-immunoreactivity has been localized in axons and in a population of ganglion cells of the terminalis nerve. Throughout the peripheral, intracranial and central course of this nerve, the LHRH-immunoreactive cells and fibers are found in close association with blood vessels.

LHRH-IMMUNOREACTIVITY IN THE ADULT VERTEBRATE TERMINALIS NERVE

The terminalis nerve is a cranial nerve (also known as the nervus terminalis or zeroeth cranial nerve) that develops, along with the vomeronasal nerve and organ, from the epithelium of the medial olfactory pit. The central processes of the terminalis and vomeronasal nerves, ascend on the nasal septum, course through the cribriform plate of the ethmoid bone medial to the olfactory nerves and enter the ventromedial surface of the forebrain, just caudal to the olfactory bulb. While the vomeronasal nerve ends in the accessory olfactory bulb, the terminalis nerve ends in three or more fine rootlets that fan out into the medial septal and preoptic areas of the brain [6, 7]. The terminalis nerve can be distinguished from the vomeronasal nerve by the presence of ganglia along its course, the largest of which is the "ganglion terminale", usually found at the point where the terminalis nerve rootlets enter the brain.

A population of LHRH-immunoreactive neurons has been localized in the ganglia of the terminalis nerve, in a variety of mammals, including guinea pigs [8, 9], rats [10, 11], hamsters [12], mice [13], opossums [14], monkeys [15]

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and dolphins [16, 17]. In adult animals, the LHRH cells are outnumbered by non-LHRH-immunoreactive cells in the ganglia in a ratio of about 10:1 (personal observation).

ASSOCIATION BETWEEN THE DEVELOPMENT OF LHRH NEURONS AND THE TERMINALIS NERVE

Investigators, studying the development of LHRH neurons in embryonic material that included both the brain and the nasal regions observed, by immunocytochemical methods, that the earliest-appearing LHRH-immunoreactive neurons were found in the nose, in association with branches or in ganglia of the terminalis nerve. These observations were made in guinea pigs [8, 9], rats [11], hamsters [12], monkeys [15, 18] and in non-mammalian vertebrates, including platyfish [19], teleost fishes [20] and amphibians [21–23]. In these species, LHRH-immunoreactive cells were rarely seen in the epithelium of medial olfactory pit, or vomeronasal organ. The greater number of LHRH cells were seen in the nasal mesenchyme, just out of the epithelium of the olfactory pit or coursing with fibers of the vomeronasal and terminalis nerves through the cribriform plate of the ethmoid bone into the forebrain. It was interesting to speculate that these LHRH-immunoreactive neurons, like the terminalis and vomeronasal nerves, arose from the medial olfactory placode, but the possibility that these cells originated in the forebrain could not be ruled out. Pearson [24], in his study of the development of the terminalis nerve in humans concluded that the major ganglion of the terminalis nerve, the "ganglion terminale", was formed by migrations of cells from both the medial olfactory placode and from the forebrain.

ORIGIN AND MIGRATION OF LHRH NEURONS FROM THE EPITHELIUM OF THE MEDIAL OLFACTORY PIT

Immunocytochemical studies of the development of the LHRH neurons in mice led to the discovery that these neurons originate, at about 11 days of embryonic life (the first day of pregnancy counted as "day 1"), in the epithelium of the medial olfactory pit, just after its formation, and migrate into the brain along branches of the terminalis and vomeronasal nerves [25–28]. The time course over which these developments take place appears to be highly ordered, and the age of an embryonic mouse could be determined (+/− day) by noting the location of the LHRH-immunoreactive neurons along the migration route [25, 26]. The LHRH-immunoreactive cells begin to migrate out of the epithelium of the olfactory pit by about day 11.5, with axons of the terminalis and vomeronasal nerves, and by days 12 and 13, thick cords of LHRH cells are seen migrating along these nerves in the nasal mesenchyme. By day 14 of embryonic life, the LHRH cells enter the forebrain, following one of two courses: (1) the greater number of cells traverse the ganglion terminale and follow the central roots of the terminalis nerve into the anlagen of the septal and preoptic areas and hypothalamus or (2) a smaller number follow the vomeronasal nerve to the accessory olfactory bulb. The migration of LHRH-immunoreactive cells into the brain is generally over by 16 days of gestation, although a few cells may still be seen in the epithelium of the medial olfactory pit, and along the migration route. At the time of birth, the olfactory bulb is well formed and the ganglion terminale has come to resemble that seen in adult animals. Within the ganglion terminale, the LHRH-immunoreactive cells are now greatly outnumbered by non-LHRH-immunoreactive cells, essentially the reverse of the ratio seen at 14 days of embryonic life, when the ganglion terminale was a major thoroughfare for the entry of LHRH cells into the forebrain. Throughout adulthood and into old age, in mice (personal observation), LHRH-immunoreactive cells are seen with branches of the terminalis nerve, and in its ganglia, the apparent remainder of the original migration route.

Is the LHRH gene really expressed in those cells which appear to originate in the epithelium of the medial olfactory pit? *In situ* hybridization studies of the epithelium of the olfactory pit and the adjacent nasal mesenchyme of 10, 11, 12 and 13-day-old embryonic mice with probes specific for LHRH mRNA [28–30] showed gene expression for LHRH in cells in the epithelium of the olfactory pit and in the nasal mesenchyme. Wray *et al.* [28] reported cell expressing LHRH mRNA in the epithelium of the olfactory pit on embryonic day 11.5, and on the developing nasal septum on day 12.5. Similar findings were reported by Zheng [29, 30], in 12 and 12-day-old embryonic mice. He also noted a tendency of LHRH-expressing cells to clump together.

GENERALITY OF THE PHENOMENON OF LHRH CELL ORIGIN AND MIGRATION ACROSS SPECIES

Different species of vertebrates have been examined to determine if the origin and migration of LHRH neurons from the epithelium of the olfactory pit is a general phenomenon in vertebrates, and to test the hypothesis that the olfactory pit is the only source of the LHRH neurons. In guinea pigs [8, 9], rats [11]. and opossums [14], LHRH-immunoreactive cells were seen early in gestation on the nasal septum with branches of the terminalis nerve, and few LHRH cells were actually detected in the epithelium of the olfactory pit. In these animals, it may be that the LHRH neurons originate in the olfactory placode, but do not actually begin to synthesize an immunocytochemically detectable form of LHRH until they have migrated out onto the nasal septum. In support of this possibility, a recent study in fetal rats by Daikoku-Ishido *et al.* [31] localized precursor molecules of LHRH in the epithelium of the newly-formed medial olfactory pit at 13.5 days of gestation with antiserum to rat gonadotropic hormone-releasing hormone associated peptide 28–56 (rGAP) but not with antiserum to LHRH.

Recent immunocytochemical and *in situ* hybridization studies in rhesus macaques [18] and immunocytochemical studies in embryonic chickens [32] show convincing evidence that LHRH neurons originate in the epithelium of the medial olfactory pit and migrate into the brain along branches of the terminalis nerve, in a pattern of development similar to that seen in mice.

An immunocytochemical study (described below), carried out in this laboratory, of a 19-week-old fetus that had Kallmann syndrome (hypogonadotropic hypogonadism with anosmia), provides evidence that the generality of this phenomenon of LHRH cell origin and migration may be extended to humans.

CHARACTERISTICS OF LHRH NEURONS

Our studies of the time of origin of LHRH neurons, using tritiated thymidine and antiserum to LHRH [33] showed LHRH-immunoreactive cells with heavily-labeled nuclei only in those mice whose mothers had been injected with the radioactive precursor on day 10 of pregnancy (the first day of pregnancy counted as day 1). This finding of a very narrow timeframe for the origin of these cells corroborates that of Wray *et al.* [28].

Zheng *et al.* [34, 35] examined the ultrastructure and immunocytochemistry of the migrating neurons from the epithelium of the olfactory pit and nasal mesenchyme and found two populations of cells with similar morphology among them, only one of which contained LHRH-immunoreactivity. Before and during migration, LHRH-immunoreactivity was not detected in Golgi bodies or in neurosecretory vesicles, and we speculated that the migrating LHRH neurons do not have a secretory function before they reach their target organ. This observation is supported by evidence presented by Daikoku-Ishido *et al.* [31] in a study in rats, in which intraventricular transplants of nasal placode and specific brain tissues suggested that LHRH neurons acquired secretory activity in the presence of the medial basal hypothalamus.

A distinctive characteristic of the LHRH-immunoreactive neurons is the tendancy of these cells to cluster, and to form thick cords as they migrate across the nasal mesenchyme into the forebrain [25, 26, 36]. In adult rats and mice, a clump of LHRH cells is sometimes seen in the ventral forebrain or in the medial septum, giving the appearance of a group of migrating cells that failed to disperse after entering the brain. Such a group stands out from other LHRH-immunoreactive cells, which are characteristically not found in clusters in the brain. Jorgenson (personal communication) cultured cells from the olfactory epithelium and the adjacent nasal mesenchyme of 12 and 13-day-old embryonic mice, carried out immunocytochemistry on them and observed that the LHRH cells grew best only when they were closely packed together. Studies are underway, in this laboratory, to examine the possibility that a trophic substance may be secreted between these cells that is essential for their maintainence and/or their migration. Farbman and Squinto [37] studying the development of olfactory receptor cell axons, described migrating cells in the lamina propria of the nose as showing epithelioid characteristics, with little space between them, and desmosomal junctions between adjacent cells.

SPECIFICITY AMONG NEUROENDOCRINE PEPTIDES

Examination of Bouin's-fixed, paraffin-embedded tissue sections through the brain and

nasal regions of embryonic mice, with antisera to thyrotropin releasing hormone, corticotropin releasing hormone, oxytocin, vasopressin, neuropeptide Y and somatostatin,, showed no immunoreactive cells or fibers [29, 30]. It appears that the peripheral origin and the migration of LHRH neurons into the central nervous system, is unique among neuroendocrine cells.

MECHANISMS OF LHRH CELL MIGRATION: RELATION TO NEURAL CELL ADHESION MOLECULE

Cell adhesion molecules are large cell surface glycoproteins with homologies to immunoglobulins [38], and tend to be homophilic (as an example, NCAM sticks to NCAM). The chemical makeup of the migration route was examined in 10 to 18-day-old embryonic and fetal mice by immunocytochemical procedures using antisera to cytotactin, CTB proteoglycan, fibronectin, laminin, and neural cell adhesion molecule (NCAM). In order to follow the development and organization of the migration route and to determine if any of these possible components were co-localized with LHRH, antiserum to LHRH double-label immunocytochemical procedures were used in this study [39, 40]. Of the components tested, only NCAM-immunoreactivity was found to be present along the migration route.

At about day 10 of embryonic life, the olfactory placode, either side of the midline, invaginated to form the simple olfactory pit, NCAM-immunoreactive cells migrated out of the epithelium and formed an aggregate between the olfactory pit and the developing forebrain. Shortly after, NCAM-immunoreactive axons, which appeared to be the central processes of the olfactory nerves, emerged from the epithelium into the nasal mesenchyme, and then into the NCAM-immunoreactive cellular aggregate, which by day 11 had grown into contact with the ventromedial forebrain. At about this same time, a secondary recess has formed in the medial wall of the olfactory pit (the anlage of the vomeronasal organ) and two events followed closely: (1) axons of the vomeronasal and terminalis nerves, also showing NCAM-immunoreactivity began to migrate out of the epithelium and into the NCAM-immunoreactive cellular aggregate and (2) LHRH-immunoreactive neurons appeared in the epithelium of the olfactory pit. A few hours later, by day 11.5, the LHRH neurons began their migration out of the epithelium of the medial olfactory pit, coursing along the NCAM-immunoreactive fascicles of the terminalis and vomeronasal nerves into the forebrain. Certain features concerning the "pioneer" NCAM-immunoreactive cells and the migrating LHRH neurons were consistent throughout all the litters of embryonic mice examined in this study: (1) LHRH-immunoreactive cells were detected in the epithelium of the olfactory pit only after formation of the secondary recess in its medial wall, the anlage of the vomeronasal organ, (2) the migrating LHRH neurons, in the nasal mesenchyme, were never seen independant of the NCAM-immunoreactive fascicles of the vomeronasal and terminalis nerves, (3) the NCAM-immunoreactive fibers of the olfactory, vomeronasal and terminalis nerves did not grow directly into contact with the developing forebrain, but rather into the aggregate of NCAM-immunoreactive "pioneer" cells, which, in turn, came to merge with the rostral tip of the forebrain, and (4) no NCAM-immunoreactive neurons were observed in the forebrain before the "bridge" of NCAM-immunoreactive cells and fibers was in place between the olfactory pit and the forebrain(Fig. 1).

By about day 12, in transverse sections through the head of the embryonic mouse, two parts of the NCAM-immunoreactive cellular aggregate could be distinguished: a lateral part that received the axons of the olfactory nerves from the dorsal and lateral parts of the olfactory pit, and a medial part that received the ingrowing axons of the vomeronasal and terminalis nerves, and the migrating LHRH-immunoreactive neurons, from the medial part of the olfactory pit. Finally, the lateral part of the cellular aggregate will form the olfactory nerve layer of the olfactory bulb, and the medial part, which serves as the entrance for LHRH cells into the brain, will form the ganglion terminale of the terminalis nerve.

Since NCAM appeared to be a major component of the route along which LHRH neurons migrate into the brain, we examined the effects of antiserum to NCAM (A-NCAM) on the migration of these cells into the brain [41]. A 1 μl injection of A-NCAM (10 mg/ml) into the area of the olfactory pit of 10-day-old embryonic mice was sufficient to retard migration of LHRH-immunoreactive neurons out of the epithelium of the olfactory pit. Control animals which received a 1 μl injection of IgG

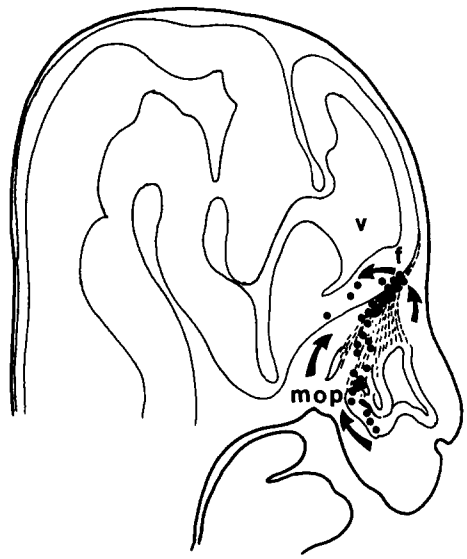

Fig. 1. Microprojection drawing of a 14-day-old fetal mouse head in the sagittal plane, showing diagramatically the NCAM scaffold and migration route of LHRH-immunoreactive neurons from the nose to the forebrain. LHRH-immunoreactive neurons are indicated by black dots and NCAM-immunoreactive fibers are indicated by dashed lines. Medial olfactory pit (mop); forebrain (f); and ventricle (v). NCAM-immunoreactive fibers from the dorsal and lateral parts of the olfactory pit are central projections of the olfactory nerves and those from the medial parts are central projections of the vomeronasal and terminalis nerves. LHRH-immunoreactive cells originate from the same part of the olfactory pit that gives origin to the vomeronasal and terminalis nerves and migrate into the brain along the NCAM-immunoreactive scaffolding provided by these developing nerves.

(10 mg/ml) into the area of the olfactory pit, or mechanical damage to the olfactory pit, showed at 12 days of embryonic life a normal distribution of LHRH-immunoreactive neurons among the NCAM-immunoreactive fascicles on the migration route. These results provide support for our thesis [26] that LHRH cells migrate from the olfactory placode to the brain.

ASSOCIATIONS BETWEEN DEFECTS OF THE OLFACTORY AND REPRODUCTIVE SYSTEMS

We were intrigued by the fact that the cells that produce an essential reproductive hormone, LHRH, originate in an olfactory placode-derived structure. Over the years, investigators have noted that particular defects of the reproductive system are frequently accompanied by defects of the olfactory system. Among the first to make an association between these defects was the German anatomist Heschl, who, in 1861, noted at an autopsy of an adult male, "...testes the size of beans without evidence of seminal canals...." and the absence of both olfactory bulbs [42]. At about the same time, a Spanish pathologist, Maestre de San Juan, reported similar findings at autopsy [43], and the condition of hypogonadotropic hypogonadism with anosmia came to be known as the "Sindrome de Maestre de San Juan." In 1944, the American medical geneticist, Kallmann, carried out an extensive study of the familial occurance of gonadal failure accompanied by anosmia (11 cases collected from three families). He and his collegues, Schoenfeld and Barrera [44], were the first to postulate a genetic basis for hypogonadotropic hypogonadism with anosmia, which is now most often referred to as "Kallmann Syndrome." This rather rare genetic disorder is more commonly found in males, but occurs in females as well [45]. The anosmia is usually traced to uni- or bilateral agenesis of the olfactory bulbs, and the hypogonadism is attributed to an absence of LHRH in the brain.

Additional evidence for an association between defects of the olfactory and reproductive systems comes from the work of de Morsier [46] who made an important correlation between a failure of gonadal development and a lateral cerebral dysplasia that results in an arrest of development of the anterior part of the brain. Termed "olfactogenital dysplasia" by de Morsier, this congenital defect affects cranial structures just lateral to midline, resulting in the absence of one or both olfactory bulbs, and it is often accompanied by hypogonadism. Histological examination of the hypothalamus of each of three individuals with this disorder showed atrophy of the anterior hypothalamus and of the median telencephalic structures situated in front of it, including the olfactory, the septal and the preoptic areas [47].

IMMUNOCYTOCHEMICAL EXAMINATION OF THE BRAIN AND NASAL REGIONS OF A HUMAN FETUS WITH KALLMANN SYNDROME

Shortly after presenting evidence of LHRH cell origin and migration in mice, we were given the opportunity to examine, by immunocytochemical procedures, the brain and nasal regions of a 19-week-old male human fetus that had Kallmann syndrome, and to compare the distribution of LHRH-immunoreactive cells in this fetus with that found in three normal fetuses of the same age and sex [48]. In the normal fetuses, we found a distribution of LHRH-immunoreactive cells and fibers in the brain and

nose that was in agreement with that described by other investigators [49, 50]. In the Kallmann fetus, no LHRH-immunoreactive cells or fibers were seen in any part of the brain. In contrast, in the nose of this fetus, on either side just lateral to midline, clumps and clusters of LHRH-immunoreactive cells and thick fascicles of LHRH-immunoreactive fibers were seen in a distribution fitting that of the terminalis nerve, an important part of the migration route. The olfactory bulbs were absent, and neither the olfactory, the vomeronasal, nor the terminalis nerves were in contact with the brain. We believe that the absence of LHRH in the brain of the Kallmann fetus was due to a failure of the LHRH neurons to migrate into the brain, and that these data give further evidence for the generality of an olfactory placode-derived origin and migration of LHRH neurons.

MIGRATION OF LHRH NEURONS RATIONALIZES OLFACTO—HORMONAL RELATIONSHIPS

The well-documented effects of the olfactory system on reproduction include the classic studies of Bruce [51] who demonstrated that pregnancy may be blocked in recently-mated female mice by the introduction of a strange male, and that this effect is abolished by prior removal of the olfactory bulbs of the female, and those of Whitten [52] who observed that female mice, housed together, will synchronize their estrous cycles, an ability that is lost when the olfactory bulbs are completely ablated. A similar phenomenon has been shown to take place in humans, in which the menstrual cycles, of a group of women living together in close quarters, become synchronized [53]. More recent studies in mice, rats, and guinea pigs [54] show that removal of the vomeronasal organ is as effective as removal of the olfactory bulbs, in blocking these effects.

While the importance of the olfactory system in normal social and reproductive behavior has been demonstrated experimentally [55, 56], little is known about the primary sensory receptor or of the neuroanatomical pathways which conduct the sensory signal to those areas of the brain (the medial hypothalamus, the preoptic area, and the amygdala) which are believed to correlate mating behavior and the neuroendocrine aspects of reproduction [57, 58].

The olfactory nerves carry airborne volatile signals from the olfactory epithelium to the main olfactory bulb and thence into the central nervous system. The vomeronasal nerves transport both volatile and non-volatile signals, introduced into the vomeronasal organ by licking or nuzzling, to the accessory olfactory bulb and thence to the amygdala, the bed nucleus of the stria terminalis, the preoptic area and the hypothalamus. The function of the terminalis nerve, still a subject of conjecture, might serve to signal physical changes in the nasal septal mucosa, and through the ganglia and central roots of this nerve, relay this information to the septal and preoptic areas and the hypothalamus. Throughout its course, the LHRH-immunoreactive axons of the terminalis nerve are found in close proximity to blood vessels of the nose and the forebrain, and to the cerebrospinal fluid within the subarachnoid space.

Does an olfactory placode origin of LHRH neurons lead the way to unified insights about the control of reproductive physiology by the brain? A tentative answer rests on the proximity, during development, of the hypophyseal and olfactory placodes. According to the studies of Verwoerd and van Oostrom [59] the first mesenchyme developing in the head area is actually of mesodermal origin. These new mesenchymal cells appear beneath the ectoderm and look like ectodermal cells, and are as a result called "ectodermal mesenchyme." At first the ectodermal mesenchyme only lies beneath the thinned ectoderm, but later it will be split into separate cell clusters, which then can be seen to migrate to specific parts of the head primordium. If these cells contribute to the formation of the placodes (described as thickenings of the ectoderm on the lateral sides of the head), they could be mesodermal cells contributing to the olfactory placode as well as to the hypophyseal placode. One could imagine such stem cells contributing both to populations of LHRH cells and of gonadotropes in the olfactory and hypophyseal placodes, respectively. The controlling cell, expressing LHRH, would be subject to chemosensory influence. We realize that this would deviate from the standard theories of development of the hypophyseal and olfactory placodes, which consider both structures to be derivatives of ectoderm.

Another interesting possibility is that LHRH neurons actually derive from the neural plate (see Groth, cited in Verwoerd and van Oostrom [59]). The medial parts of the olfactory placode (which give rise to the vomeronasal and terminalis nerves as well as the LHRH neurons) immediately adjoin the basal side of the neural

plate early in development, at about the 20–21 somite stage in embryonic mice. Similarly, the hypophyseal placode, at this stage of development, is still situated immediately adjacent to the neural ectoderm. This arrangement of placodal tissues, lends itself to speculation that the LHRH neurons and the pituitary gonadotropes may be "cut from the same cloth." Finally, LHRH neurons may be derived from the neural crest. In any case, the true origin of the LHRH-expressing cells in the epithelium of the olfactory pit will shed light on the manner in which these cells contribute to olfacto–hormonal relationships.

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