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FACTORS INVOLVED IN THE MIGRATION OF NEUROENDOCRINE HYPOTHALAMIC NEURONS

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INTRODUCTION

The hypothalamus integrates physiological processes critical for survival and reproduction. The development of these control mechanisms is under the strict control of several cues and their developmental defect might represent a cause of clinical defects. Pathways essential for the induction of the ventral midline of the hypothalamus or for the differentiation of specific hypothalamic lineages have the potentiality to cause endocrine disorders.

The hypothalamus is generated predominantly from the third ventricular neuroepithelium in a "lateral early to medial late" pattern; the earliest-generated neurons occupy the lateral zone, and the latest-generated ones lie in the periventricular zone. This may not be the result of migration *per se*, as the third ventricle appears to be receding medially in response to ongoing cell proliferation (1). Although neuroendocrine neurons are mainly located in the periventricular nuclei, the latest generated, they show a relatively early birthdate, therefore the peak generation of parvicellular neurons precedes the generation of the nuclei they will ultimately occupy. This phenomenon suggests that these neurons could follow a delayed migrational strategy, despite limited extension.

Among the neuroendocrine neurons, the most impressive neuronal migration is undertaken by the neurons that produce the hypothalamic neurohormone luteinising hormone-releasing hormone (LHRH) also known as gonadotropin-releasing hormone (GnRH). Actually, the observation that, GnRH-secreting neurons do not originate in the brain is certainly intriguing. These neurons are born in the medial wall of the olfactory placode and, during embryonic life, migrate along the vomeronasal and the terminal nerves to gain access to the forebrain and to reach their final destination along the rostral-to-caudal continuum, extending from the medial septo/preoptic region of the forebrain to the posterior hypothalamus (2, 3). Once they have reached the hypothalamus, their axons project to the median eminence, where they release

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GnRH into the pituitary portal vessels to induce the release of gonadotropins from the anterior pituitary into the general circulation. These steps are key events for the development of a series of normal reproductive functions. In fact, an impaired migration of GnRH neurons was found to be the pathogenic factor of the hypogonadotropic hypogonadism (HH) occurring in patients affected by the X-linked Kallmann's syndrome (4).

The mechanisms involved in the development of the migration route, as well as the molecular signals which control the movement of GnRH neurons along it, are not yet well understood; however, the study of the migration of GnRH neurones has recently received a new impulse since the description of the human disease and of the animal models in which a putative defect of this process has been postulated. In the last few years a series of factors able to affect the migration of GnRH neurones have been identified using several experimental models (5). On the basis of their time of expression and of their regional distribution, it has been suggested that these factors may act at different levels of the progression of the migratory process. In addition, some of these factors were found to affect indirectly the migration of GnRH neurons, i.e. by altering the development of the GnRH neurons themselves, or the components of the migratory route.

USE OF IMMORTALISED NEURONS TO STUDY THE MIGRATION OF GnRH NEURONS

In order to study the factors that are directly involved in the mechanisms controlling the migration of GnRH neurons, it is necessary to work in well-defined experimental conditions in which one can dissect out the specific actions of the several factors investigated. Unfortunately, the study of the GnRH-secreting neurons in animal models is generally hindered by their peculiar development and anatomical distribution. Several subpopulations of GnRH neurons have been described; in addition, they migrate during a developmental narrow window of time (from embryonic day 11 to 18, in rodents) and these neurons are very limited in number and scattered into the septo/hypothalamic region in adulthood. The study of GnRH neurons has been facilitated by the development of cell lines of immortalised pure mouse GnRH-secreting neurons. Two different cell lines, the GT1 cells (which include GT1-1, -3 and -7 subclones) (6) and the GN cells (with GN10, GN11 and NLT subclones) (7) have been obtained by genetically targeted tumorigenesis of GnRH neurons in mice. Biochemical and immunological studies have shown that both cell lines express neuronal markers, and retain the biological features of GnRH-secreting neurons. Moreover, a series of similarities between these immortalised neurons and normal GnRH neurons "in vivo", indicate that immortalised GnRH neurons can be considered an adequate model to study the biology of GnRH-secreting neurons (8). It is interesting to underline that the GT1 and GN cell lines are representative, respectively, of well differentiated post-migratory and of immature migratory GnRH neurons (9, 10). Under this perspective, we were the first to demonstrate, by different

techniques and experimental paradigms (microchemotaxis assay, collagen gel invasion tests, etc.), that GN11 cells retain a strong chemomigratory response *in vitro* (9, 11, 12) suggesting their utilisation to study the multiple factors involved in the control of the migration of GnRH neurons in well controlled culture conditions (8).

FACTORS AFFECTING THE MIGRATION OF GnRH NEURONS: STUDIES *IN VIVO* AND *IN VITRO*

As already reported, we found that GN11 cells show a very low spontaneous motility in the absence of chemotactic stimuli; however, foetal bovine serum (FBS) was highly efficient in stimulating in a concentration-dependent manner the chemotaxis of these cells; we found that the chemotaxis of GN11 cells is activated by FBS-derived factors falling in the molecular weight range of 30-60 KDa. Considering that cells in all vertebrate organisms are in contact with serum, such a sensitive response to FBS indicates the presence of specific factors, other than the classical growth and survival factors, that may affect the spontaneous motility of GnRH migrating neurons, providing the tropic support to such a phenomenon.

However, it has been recognised that during migration GnRH neurons have to overcome different critical steps such as: (I) the initial acquisition of cell motility and migration out of the olfactory placode, (II) the survival and the progression of migration into the nasal mesenchyme along the olfactory nerve, (III) the passage through the nasal-forebrain junction and the migration into the forebrain, (IV) the interruption of the migration at different levels of the septo/preoptic region of the forebrain and posterior hypothalamus, and (V) the terminal differentiation with axonal elongation and synaptogenesis (8).

Here we will summarise the data collected in our laboratory on the characterisation of some factors possibly involved in the progress of migration of the GnRH neurons along these steps (Fig. 1).

Recently, it has been found that the null mutant mice for *Ebf2* (Early B-cell factor 2), are hypogonadic and show a defect of migration of GnRH neurons (13). *Ebf2* is a component of the HLH (helix-loop-helix) family of transcription factors implicated in various aspects of neural development and neuronal functions. The study of the null embryos indicates that Ebf2 is required neither for the appearance of GnRH-neurons, nor for the development of their migratory pathway; in these animals, GnRH neurons exit the nasal mesenchyme later than in wild-type mice, and show signs of degeneration. As the *Ebf2* gene is expressed in GnRH neurons, it has been proposed that the observed migration defect is cell-autonomous. To the authors' knowledge this is the first report of a single genetic defect that directly affects GnRH-neuron migration, with no gross alterations of their migration route. In preliminary experiments we found that GN11 cell do express *Ebf2* and that its inactivation by transfection with a vector coding for an *Ebf2* dominant negative induces the formation of cellular inclusions and apoptotic cell death; its effect on the migratory activity of GN11 cells is still under investigation.

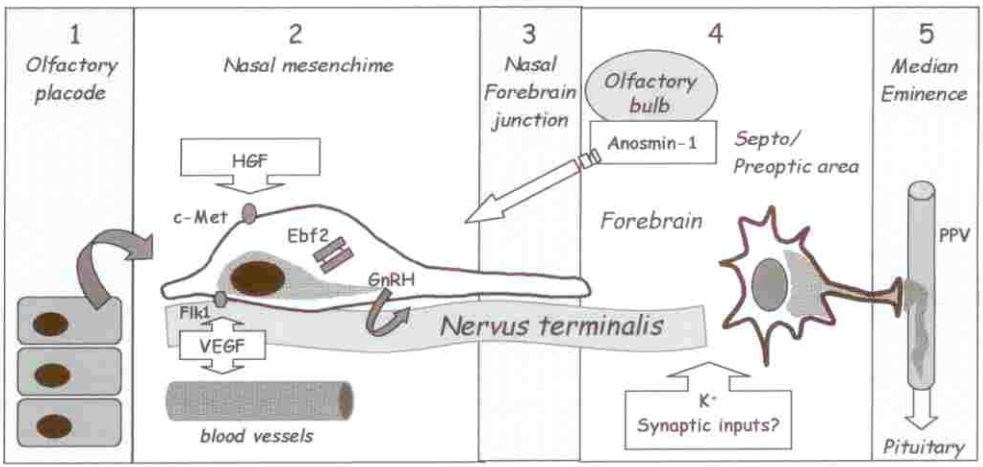

Fig. 1. - *Critical steps of the migration of GnRH neurons from the olfactory placode to the septo/preoptic area.*
The figure also reports a summary of the factors so far identified that affect the migration of GnRH neurons at selected phases of the migratory process. Abbreviations: HGF (Hepatocyte Growth Factor) and c-Met (HGF receptor) (12); VEGF (vascular endothelial growth factor); Ebf2 (Eb-cell factor2) (13); Anosmin-1 (KAL1 gene product); PPV (pituitary portal vessels); black dots (GnRH).

A series of tissue-derived factors and of classical growth factors have been found to exert chemotropic effects on neuronal cells. Among them, hepatocyte growth factor/scatter factor (HGF/SF) plays a major role acting via its receptor c-Met (14). Of interest HGF/SF and c-Met are present in olfactory regions such as the olfactory bulb, the olfactory epithelium, and the olfactory nerve layer as early as in the E11.5 phase, which is a stage corresponding to the beginning of the GnRH migratory process. HGF/SF expression in the target tissue of this neuronal migration suggests that molecular signalling between the forebrain and the olfactory placodes might influence the development of the olfactory nerve pathway. Actually, we have found that HGF/SF may play a role in the migration of GnRH neurons. In particular, it has been found that in the mouse, a population of migrating GnRH neurons expresses c-Met receptors (12); GN11 cells also show transcripts and immunoreactivity for c-Met and the application of HGF/SF induces a specific and intense migration of GN11 cells in chemotaxis assays (12). Based on the pre-existing literature and the present results, it is possible to postulate that HGF/SF plays an instructive/permissive role for the migration of GnRH cells.

Intense vasculogenesis has been observed in the nasal mesenchyme during brain development; moreover, the migration of GnRH neurons from the olfactory placode is concomitant to the migration of N-CAM immunoreactive cells, that act like migration pioneers and aggregate around the developing blood vessels forming a structure named "cellulovascular strand" (15). It has been proposed that vasculogenesis may provide a signal to direct the development of the migration route and the migration of GnRH neurons. Considering these findings, we have then investigated the possible role of vascular endothelial growth factor (VEGF), an angiogenic

growth factor, as a candidate for the control of GnRH neuronal migration in the nasal region. Actually, VEGF shows the characteristics of pleiotropic molecules; it is also expressed in neurons, where it may interact with their specific receptors (Flt-1, Flk-1/KDR) and with other receptors already known to be involved in the development of the neuronal networks (neuropilin).

Data from the authors' laboratory indicate that GN11 cells exposed to a gradient of VEGF show a significant chemomigratory activity via an interaction with the Flk-1 receptors, suggesting that angiogenic factors produced by the nasal mesenchyme during the development of the olfactory system, might also provide some guidance cues for the migration of GnRH neurons. Finally, other factors (e.g., IGF-I, endothelin, fibrinogen) have been found to stimulate GN11 cell chemomigration (unpublished observations), even though their mode of action has to be further characterised.

A possible correlation between migration of neurons and their electrical steady-state properties has been described (16) suggesting that depolarisation could inhibit migratory activity. Patch-clamp recording experiments have revealed that, while GT1-7 cells show a picture of mature neurons (9), GN11 cells do not show any action potential or current even under exposure to depolarising stimuli (9). The only current detected in these cells is a Ba^{++} and Cs^{++}-sensitive K^+ inward-rectifier type of conductance. These results are supported by the identification of an electrically silent population of GnRH neurons in developing mice unable to generate action potentials (17). We have then performed microchemotaxis assays during depolarising conditions. In particular, during the depolarisation induced by high $[K^+]_o$, or by blocking the K^+-inward rectifier current induced by the presence of Cs^{++} ions, the chemomigratory response to FBS appeared to be strongly reduced suggesting that depolarisation might also be one of the stop signals for migration of GnRH neurons. It could be hypothesised that depolarisation of GnRH neurons during the late phase of migration might be induced by the presence of different concentrations of K^+ in extracellular fluids, which may be regulated by astrocytes, along the migratory pathway, or by the progressive activation of the multiple neuronal inputs, that will define the function of these neurons in adulthood.

Finally, with the GN11 cell model we have investigated the role of anosmin-1, the product of the KAL1 gene. This is responsible for the X-linked form of Kallmann's syndrome (KS) (18) a genetic disease characterised by infertility and inability to smell. It has been demonstrated that targeting of olfactory axons to the olfactory bulb and the migration of GnRH neurons are impaired in KS (4).

Anosmin-1 is a secreted protein (18), that shares significant homologies with adhesion factors molecules (i.e. NCAM, L1, TAG-1, F3/contactin), expressed at the level of the olfactory bulb and along the migratory route of olfactory axons and GnRH neurons.

On the basis of its structural characteristics and site of expression, it has been proposed that KAL protein might play a role in the control of migration and targeting of the axons of the olfactory neurons; however, no direct evidence is so far available on a possible direct effect of KAL on the migration of GnRH neurons. In a very recent study (19), we show for the first time a direct action of anosmin-1 on the

migratory activity of GnRH neurons. Specifically, we exposed immortalised migrating GnRH neurons (GN11 cells) to conditioned media (CM) of COS or CHO cells transiently transfected with KAL1 gene in microchemotaxis and collagen gel assays. We found that anosmin-1-enriched media produced a cell-specific chemotactic response of GN11 cells. None of the CM enriched with three forms of anosmin-1 carrying different missense mutations (N267K, E514K, F517L) found in patients affected by X-linked KS, affected the chemomigration of GN11 cells (Fig. 2A). Anosmin-1 binds to the GN11 cell surface (Fig. 2B) by interacting with the heparan-sulphate proteoglycans and the chemotactic effect of anosmin-1-enriched CM can be specifically blocked by heparin or by heparinase pretreatment. These results strongly suggest an involvement of anosmin-1 in the control of the migratory behavior of GnRH neurons, and provide novel information on the pathogenesis of KS.

In conclusion, the study of the migration of GnRH neurons is of true neurobiological interest, and it presents potential therapeutic implications for reproductive diseases (as the Kallmann's syndrome) that even if not lethal, may be highly invalidant not only due to infertility but also due to a series of variable CNS defects. Moreover, the study of the molecular cues that affect the migration of GnRH neurons may provide novel insights on common biochemical events controlling neuronal development and migration.

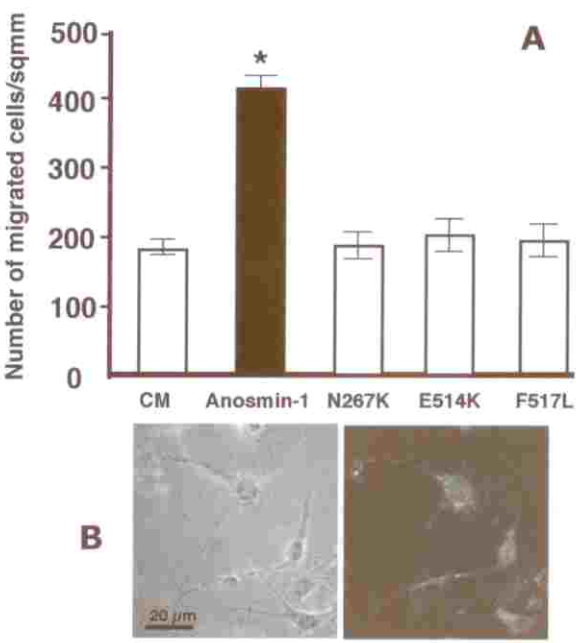

Fig. 2. - A) Chemotactic effect of anosmin-1 on GN11 immortalised GnRH neurons. Microchemotaxis experiments were performed in Boyden's chamber using control conditioned media (CM), from untransfected COS-7 cells, anosmin-1 enriched CM (anosmin-1) or the conditioned media of COS-7 cells transfected with the three anosmin-1 mutants N267K, E514K, F517L. The results are expressed as number of cells that migrated *per* square mm of the porous membrane in 3 hrs. Means ± SD obtained from three independent experiments. * Significant ($P < 0.05$) *vs.* CM.
B) Immunodetection of anosmin-1 on GN11 neurons. GN11 cells (left panel; phase contrast) were co-cultured with anosmin-1-expressing COS cells and labelled with monoclonal 9E10 anti-myc antibody (right panel, immunofluorescence) in unpermeabilised conditions. The pictures show that anosmin-1 is associated to the surface of GN11 cells.

SUMMARY

Neuroendocrine control of physiological functions needs a complex developmental organisation of the hypothalamic parvicellular neurons, which synthesise and release hypophysiotropic hormones.

Among the hypothalamic neuroendocrine cells, Gonadotropin-releasing hormone (GnRH) neurons represent a unique class; they are generated in the olfactory placode and, during embryonic life, migrate to the septo/hypothalamic region along terminal and vomeronasal nerves. At this level GnRH neurons undergo terminal differentiation and start to release GnRH to modulate the secretion of pituitary gonadotropins. All these steps are under the strict control of several developmental cues and their defect might represent a cause of clinical disorders. A number of factors have been proposed to be involved in the migration of GnRH neurons, but their role is still unclear. By using gene knockout techniques it has been found that mice carrying a targeted deletion of *Ebf2* gene, a component of Olf/Ebf bHLH transcription factors, show a defective migration of GnRH neurons, providing the first evidence of a mouse model of such defect. Since the investigation of GnRH neurons is hindered by their peculiar anatomical distribution, other studies has been forwarded by the availability of immortalized GnRH-expressing neurons (GN11 cells) that retain a strong chemomigratory response "in vitro". Among the factors analysed, we found that hepatocyte growth factor/scatter factor (HGF/SF) and vascular endothelial growth factor (VEGF) induce specific chemotaxis of GN11 neurons, suggesting that migratory signals can arise from nasal mesenchyme and from the concomitant vasculogenesis. Finally, anosmin-1 (the product of the gene responsible of the X-linked form of Kallmann's disease) was found to induce a significant chemotactic response of GN11 cells, confirming a permissive/instructive role of KAL1 gene product in the migratory behaviour of GnRH neurons. In conclusion, the migration of the GnRH neurons appears to be a complex process, which involves the interplay of multiple molecular cues. These studies may provide new insights on the etiopathogenesis of the large proportion of reproductive dysfunctions that affect humans and could provide novel insights on common biochemical events controlling neuronal development and migration.

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