

# The product of X-linked Kallmann's syndrome gene (*KAL1*) affects the migratory activity of gonadotropin-releasing hormone (GnRH)-producing neurons

Anna Cariboni<sup>1,†</sup>, Federica Pimpinelli<sup>1,†</sup>, Sophia Colamarino<sup>3,‡</sup>, Roberta Zaninetti<sup>1</sup>, Margherita Piccolella<sup>1</sup>, Cristiano Rumio<sup>2</sup>, Flavio Piva<sup>1</sup>, Elena I. Rugarli<sup>3</sup> and Roberto Maggi<sup>1,\*</sup>

<sup>1</sup>Laboratory of Developmental Neuroendocrinology, Department of Endocrinology, Centre of Excellence on Neurodegenerative Diseases and <sup>2</sup>Department of Human Morphology, University of Milano, Milano, Italy and <sup>3</sup>Telethon Institute for Genetic and Medicine (TIGEM), Naples, Italy

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**X-linked Kallmann's syndrome (KS) is a genetic disease characterized by anosmia and hypogonadism due to impairment in the development of olfactory axons and in the migration of gonadotropin-releasing hormone (GnRH)-producing neurons. Deletions or point mutations of a gene located at Xp22.3 (*KAL1*) are responsible for the disease. This gene encodes for a secreted heparin-binding protein (KAL or anosmin-1) which exhibits similarities with cell-adhesion molecules. In the present study, we show for the first time a direct action of anosmin-1 on the migratory activity of GnRH neurons. Specifically, we exposed immortalized migrating GnRH neurons (GN11 cells) to conditioned media (CM) of COS or CHO cells transiently transfected with human *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 on three forms of anosmin-1 carrying different missense mutations (N267K, E514K and F517L) found in patients affected by X-linked KS affected the chemomigration of GN11 cells. Anosmin binds to the GN11 cell surface by interacting with the heparan sulphate proteoglycans, and the chemotactic effect of anosmin-1-enriched CM can be specifically blocked by heparin or by heparitinase pretreatment. These results strongly suggest an involvement of anosmin-1 in the control of the migratory behaviour of GnRH neurons and provide novel information on the pathogenesis of KS.**

## INTRODUCTION

Idiopathic or isolated hypogonadotropic hypogonadisms (HH) are congenital disorders characterized by delayed puberty, infertility, low plasma levels of gonadotropins and, consequently, of gonadal steroids (1–4). Kallmann's syndrome (KS) is defined as the association of HH with an olfactory deficit (anosmia). Both olfactory and gonadotropin-releasing hormone (GnRH) neurons originate from the olfactory placode, which will later develop into the olfactory epithelium.

During normal embryo development, olfactory neurons project their axons to the olfactory bulb through the cribriform plate and the meningeal tissue, while GnRH neurons migrate along the pathway of the olfactory nerves (terminal and vomeronasal) to reach the septo-hypothalamic area (5,6). In the X-linked form of KS (X-KS), these processes are incomplete and both olfactory axons and GnRH neurons are clustered within the meninges above the cribriform plate (7). Intragenic deletions and point mutations of a gene located at Xp22.3 (*KAL1* gene) are responsible for X-KS (8–10).

\*To whom correspondence should be addressed at: Laboratory of Developmental Neuroendocrinology, Department of Endocrinology, Centre of Excellence on Neurodegenerative Diseases, University of Milano, Via G. Balzaretti, 9, 20133 Milano, Italy. Tel: +39 0250318233; Fax: +39 0250318204; Email: roberto.maggi@unimi.it

<sup>†</sup>The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

<sup>‡</sup>Present address: Laboratory of Genetics, The Salk Institute, La Jolla, CA, USA.

*KALI* is expressed in the developing olfactory bulb, the central target of olfactory axons, in the path through which GnRH-secreting neurons migrate, in other brain regions and in the developing kidney (11,12). *KALI* encodes a 680 amino acid secreted protein, named KAL protein or anosmin-1 (8,13), and shows significant homologies with molecules known to play specific roles in neural development. The N-terminal of anosmin-1 displays a 'four-disulphide-core domain' (WAP domain), a typical motif of serine protease inhibitors. This domain is followed by four fibronectin type III (FNIII) repeats, a common motif found in several extracellular matrix proteins as well as in cell-adhesion molecules (i.e. NCAM, L1, TAG-1 and F3/contactin), in protein kinases and in tyrosine-phosphatases (14–16). Anosmin-1 has been shown to bind to heparin, and its adhesive activity is dependent on cell surface heparan sulphate proteoglycans (HSPG) (17). *KALI* is evolutionally conserved in various species including *Caenorhabditis elegans* (GenBank accession no. AF342986) (18), *Drosophila* (GenBank accession no. AE003746), zebrafish (19) and birds (11,20). Unfortunately, studies on the putative functions of anosmin-1 have been limited by lack of a rodent model (e.g. knockout mice), since *KALI* has not yet been cloned so far in these species.

Several hypotheses have been offered to explain the physiological role(s) of anosmin-1 during brain development. Experiments performed in different species and in *in vitro* models revealed a role for anosmin-1 in the control of different cell functions, including cell adhesion and neurite/axonal elongation and fasciculation (17). More recent studies in *C. elegans* showed that *KALI* is expressed by a subset of neurons and affects neurite outgrowth by modulating branching (18). However, depending on the cellular context, heterologous *KALI* expression can also cause axon misrouting. The axon-branching and -misrouting activities were found to require different domains of anosmin-1 (21). Further *in vitro* and *ex vivo* studies have revealed that anosmin-1 greatly enhances axonal branching of dissociated rat olfactory bulb neurons, and that it acts as a chemoattractant for the axons of the rat piriform cortex neurons (22). An axon guidance defect has been postulated in X-KS, since some patients have been reported to display neurological symptoms, such as mirror movements, sensorineural deafness (23), eye-movement abnormalities, cerebellar ataxia and gaze-evoked horizontal nystagmus (24). On the basis of these information and its structural characteristics, it has been proposed that anosmin-1 might play a role in the control of migration and targeting of olfactory axons. Other defects of KS, which include unilateral renal aplasia and incomplete closure of the lip and of the palate, even if less common, also suggest alterations in cell migration (25,26). Nevertheless, no direct evidence on a possible effect of anosmin-1 on the migration of GnRH neurons is so far available. Recently, migrating GnRH-immunoreactive neurons have been observed in close association with anosmin-1-immunoreactive fibres in the musk shrew (27). Assuming that the protein is present at the surface of these fibres, this observation suggests a possible direct action of anosmin-1 on the migration of GnRH neurons in these species.

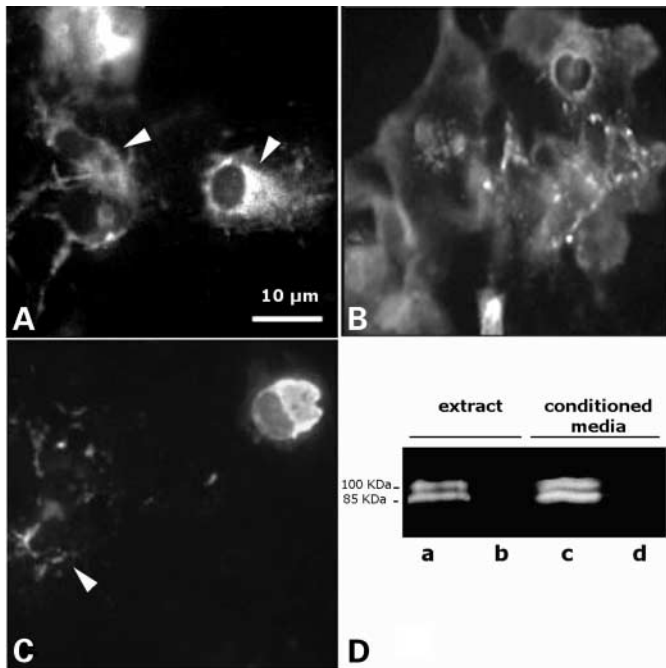
*In vivo* or *ex vivo* studies of GnRH neurons are hindered by their very small number and by their scattered distribution in

the septo-hypothalamic region (28). Experimental attempts to maintain a pure population of GnRH neurons in culture have not been successful (29,30). Two different cell lines, the GT1 cells (which include GT1-1, -3 and -7 subclones) (31) and the GN cells (with GN10, GN11 and NLT subclones) (32), 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, retain many characteristics of GnRH-secreting neurons (33–40) and represent good models to study the biology of GnRH neurons *in vitro*. It has been shown that GT1 cells are representative of differentiated postmigratory GnRH neurons; on the contrary, we were the first to demonstrate that GN11 cells retain an immature phenotype and a strong chemomigratory response *in vitro* (41–43). The present study was designed to verify the hypothesis that anosmin-1 might intervene in modulating the migration of GnRH neurons. The study includes the evaluation of the migratory activity of GN11 cells exposed to human anosmin-1 preparations using several methodological approaches. We present data indicating that anosmin-1 induces a cell-specific chemotactic response of GN11 cells and that none of three forms of anosmin-1, carrying different missense mutations (N267K, E514K and F517L), affects the chemomigration of GN11 cells. We also show that anosmin-1 exerts this effect by interacting with HSPG.

## RESULTS

### Anosmin-1 increases the chemomigration of GN11 cells

We selected GN11 cells as a model system to test a possible effect of anosmin-1 on migration of GnRH neurons, due to their immature phenotype and strong chemomigratory response *in vitro*. As a source of anosmin-1 for our experiments, we used conditioned media (CM) of COS-7 cells transfected with *KALI* expression vectors (see Materials and Methods) (44,45). Immunofluorescence analysis, using monoclonal anti-c-myc antibody (9E10), and direct fluorescence analysis were performed to detect the localization of the myc-tagged and the GFP-tagged anosmin-1, respectively, in COS-7 cells. As shown in Figure 1A, anosmin-1<sub>myc</sub> protein was highly expressed in COS-7 cells. In permeabilized COS-7 cells, anosmin-1<sub>myc</sub> staining appeared to be localized in the endoplasmic reticulum and in the Golgi region, a distribution typical of secreted proteins. However, in unpermeabilized cells anosmin-1<sub>myc</sub> immunoreactivity was restricted to the surface of the cells and adherent to the culture dish (Fig. 1B). A similar pattern was found while observing the distribution of GFP-tagged anosmin-1 by direct fluorescence of living cells (Fig. 1C). Western blot analysis of whole cell extracts and of CM revealed the presence of two major bands with apparent molecular weights of 100 and 85 kDa (Fig. 1D), which corresponds to two different glycosylated forms of anosmin-1 (44,45). GN11 cells, placed in the Boyden's chamber, were exposed for 3 h to a gradient of CM obtained from untransfected COS-7 cells (CM), and COS-7 cells transfected with the empty pMT21 vector (CM<sub>myc</sub>) or with pMT21-*KALI*<sub>myc</sub> (anosmin-1). Figure 2 shows the results of microchemotaxis experiments expressed as number

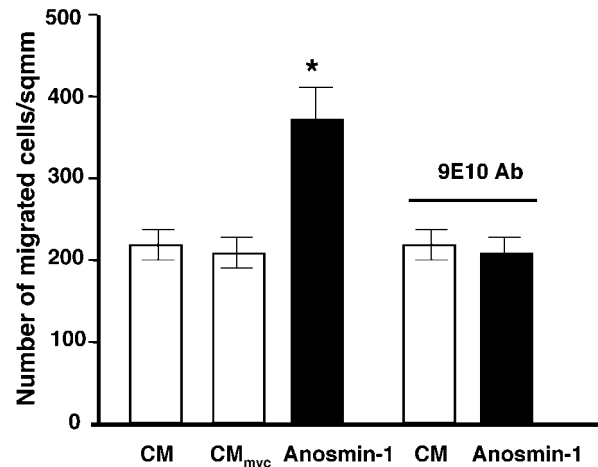


**Figure 1.** Cellular distribution and western blot analysis of anosmin-1 in COS-7 cells transfected with pMT21-*KALI*<sub>myc</sub> vector. Anosmin-1<sub>myc</sub> was detected by monoclonal 9E10 anti-myc antibody in permeabilized cells (A); the immunofluorescent staining appears to be localized in the endoplasmic reticulum and in the Golgi region as well as on the surface of untransfected cells (arrowhead). In unpermeabilized cells, anosmin-1 immunoreactivity was mainly restricted to the surface of the cells and of the culture dish (B). A similar distribution is also evident by direct fluorescence examination of COS-7 cells transfected with anosmin-1-GFP vector (C); arrowheads indicate anosmin-1-GFP signal on the cell surface. (D) Western blot analysis of anosmin-1<sub>myc</sub> protein produced by transfected COS-7 cells and present in their culture media conditioned for 16–18 h. Immunoblotting was performed using 9E10 anti-myc antibody; apparent molecular masses of the molecular weight markers are indicated on the left. The antibody reveals two major bands only in cell extracts and in CM of cells transfected with pMT21-*KALI*<sub>myc</sub> (lines a and c) but not in cells with pMT21<sub>myc</sub> (lines b and d). The apparent molecular masses of the two bands are of 100 and 85 kDa, corresponding to different glycosylated forms of anosmin-1.

of migrated cells per mm<sup>2</sup> of the porous membrane. Both CM and CM<sub>myc</sub> induced, with similar potency, a low chemotaxis of GN11 cells. When GN11 cells were exposed to anosmin-1-enriched media, they showed an even larger chemotactic response; this effect was significantly higher (45% increase) than those elicited either by CM or by CM<sub>myc</sub>. To confirm that this effect was due to the anosmin-1 secreted by transfected cells, we attempted to neutralize these effects by preincubation of the CM with the 9E10 anti-c-myc antibody. Figure 2 shows that immunoneutralization of the anosmin construct blocked the chemomigration of GN11 cells induced by anosmin-1, whereas the presence of the antibody did not affect the low response elicited by control CM on GN11 cells.

#### Missense point mutations induce a loss of the chemotactic effect of anosmin-1 on GN11 cells

To further confirm the role of anosmin-1 on GN11 migration, we investigated the effect of CM obtained from COS-7 cells

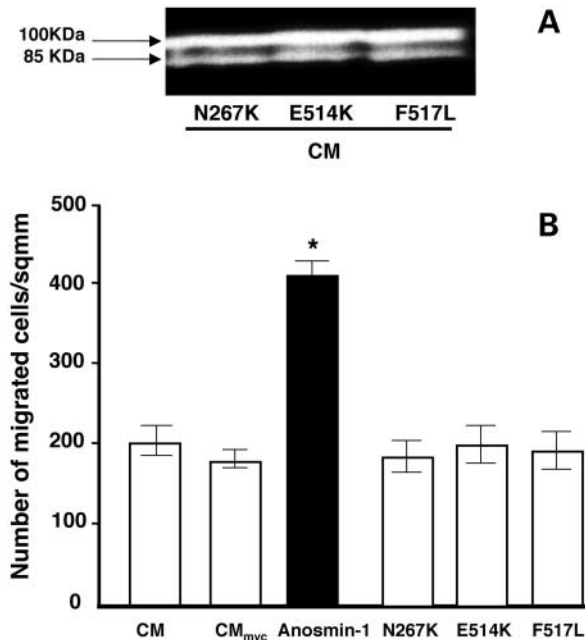


**Figure 2.** Chemotactic effect of anosmin-1-enriched media on GN11 immortalized GnRH neurons. Microchemotaxis experiments were performed in Boyden's chamber using CM from untransfected COS-7 cells (control CM), COS-7 cells transfected with the empty pMT21 vector (CM<sub>myc</sub>) or with pMT21-*KALI*<sub>myc</sub> (anosmin-1-enriched CM; anosmin-1) in the absence or in the presence of 9E10 anti-c-myc antibody to immunoneutralize anosmin-1. The results are expressed as number of migrated cells per mm<sup>2</sup> of the porous membrane in 3 h. Mean  $\pm$  SD obtained from more than 10 independent experiments. \*Significant ( $P < 0.05$ ) versus CM.

transfected with the expression vectors of three mutant forms of anosmin-1 found in patients affected by X-KS. In particular, microchemotaxis experiments were performed on GN11 cells exposed to CM enriched with different *KALI* mutants incorporating the N267K, E514K or F517L missense mutations (46). Western blot analysis of the CM enriched in anosmin-1 mutants with 9E10 antibody revealed the presence of two major immunoreactive bands, equivalent to those identified for the wild-type anosmin-1 (Fig. 3A). Immunofluorescence analysis also showed a cellular distribution similar to that observed for the wild-type anosmin-1 (data not shown). These results indicate that the mutated forms of *KALI* are correctly expressed and secreted by COS-7 cells. The results of the microchemotaxis experiments showed once again that the exposure of GN11 to CM enriched in anosmin-1 induced a strong migratory signal; on the contrary, exposure to the three mutant forms of anosmin-1 did not affect the chemomigration of GN11 cells; this response was of the same magnitude on that of GN11 cells exposed to control CM (Fig. 3B).

#### Anosmin-1 does not stimulate the chemomigration of GT1-7 and SK-N-SH cells

A possible migratory effect of anosmin-1 was also tested by microchemotaxis assay on other cell types, such as GT1-7 and SK-N-SH cell lines. [GT1-7 is another cell line of immortalized GnRH-secreting neurons, characterized by a more mature phenotype compared with GN11, whereas SK-N-SH is human neuroblastoma cell line showing moderate migration/invasive ability (47)]. These experiments showed that exposure to either control CM or anosmin-1 did not affect the motility of GT1-7 cells (Table 1). Neuroblastoma SK-N-SH



**Figure 3.** (A) Western blot analysis of CM obtained from COS-transfected with wild-type pMT21-*KALI*<sub>myc</sub> mutants leading to the N267K, E514K or F517L missense mutations. (B) Chemomigration of GN11 neurons exposed to control CM, anosmin-1-enriched CM (anosmin-1) or CM of COS-7 cells transfected with the three anosmin-1 mutants. The results are expressed as number of migrated cells in Boyden's chamber assay per mm<sup>2</sup> of the porous membrane in 3 h. Mean  $\pm$  SD obtained from three independent experiments. \*Significant ( $P < 0.05$ ) versus CM.

**Table 1.** Chemotactic responses of GT1-7 and SK-N-SH cells exposed to different chemoattractants

Chemoattractant	GT1-7	SK-N-SH
DMEM	52 $\pm$ 12	65 $\pm$ 12
10% FBS	54 $\pm$ 10	140 $\pm$ 25*
CM	53 $\pm$ 9	130 $\pm$ 13*
CM <sub>myc</sub>	56 $\pm$ 11	135 $\pm$ 14*
Anosmin-1	54 $\pm$ 12	137 $\pm$ 10*

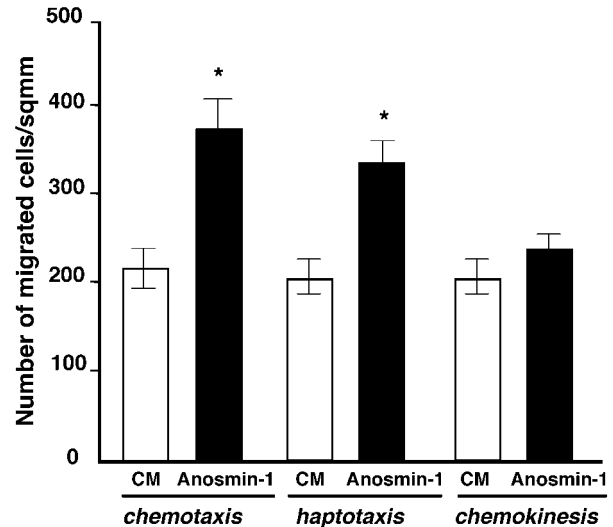
Values are mean  $\pm$  SD of the number of migrated cells per mm<sup>2</sup> of membrane.

\*Significant  $P < 0.05$  versus DMEM.

cells responded with a limited migratory activity to CM, but anosmin-1 did not induce any additional chemotactic response in these cells (Table 1). These data strongly support the specificity of the chemomigratory effect of anosmin-1 on GN11 cells.

### Anosmin-1 induces chemotaxis and haptotaxis rather than chemokinesis of GN11 cells

In order to test the nature of the effect of anosmin-1 on the motility of GN11 cells, we exposed these cells to anosmin-1 using different protocols designed to evaluate chemotaxis, chemokinesis and haptotaxis. To measure chemotaxis (i.e. the directed migration of cells toward a concentration gradient

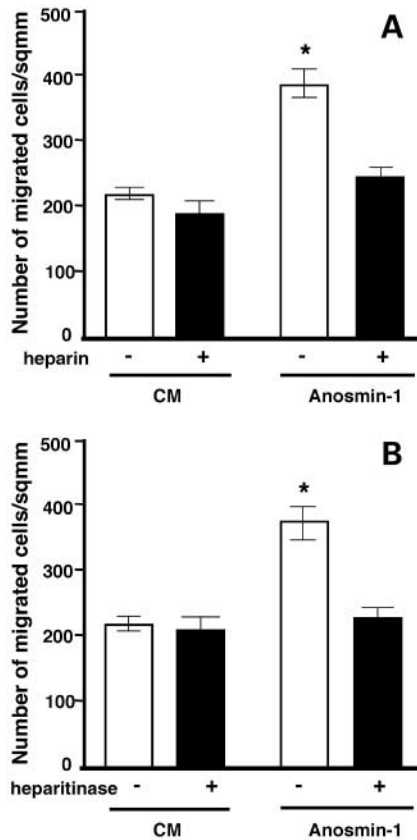


**Figure 4.** Anosmin-1 induces chemotaxis and haptotaxis rather than chemokinesis of GN11 cells. Microchemotaxis experiments were performed in Boyden's chamber using control CM and anosmin-1-enriched CM (anosmin-1) as chemoattractants; the results are expressed as number of migrated cells per mm<sup>2</sup> of the porous membrane in 3 h. Mean  $\pm$  SD obtained from more than 10 independent experiments. \*Significant ( $P < 0.05$ ) versus CM.

of chemotactic factors), the chemoattractants (e.g. control or anosmin-1-enriched CM) were placed only into the wells of the lower compartment of the chamber; haptotaxis (i.e. the directed movement up to a gradient of substratum-bound chemoattractant) was measured by coating the lower surface of the gelatine-treated porous membrane for 24 h with control CM or anosmin-1. Finally, the chemokinesis (i.e. stimulation of increased random cell motility) was assessed by placing the same concentration of chemoattractant in both the upper and lower wells of the Boyden's chamber, thereby eliminating the chemical gradient. These experiments indicated that anosmin-1 induces significant chemotaxis and haptotaxis of GN11 cells; on the contrary, no significant chemokinetic effects were observed when the cells were tested in the absence of a concentration gradient of anosmin-1 (Fig. 4).

### HSPG mediate the chemotactic effect of anosmin-1 on GN11 cells

Since heparin may compete with the binding of the *KALI*-encoded protein to HSPG, the response of GN11 cells to anosmin-1-enriched media was tested in the presence, or absence, of heparin. Figure 5A shows that the addition of heparin did not affect the chemomigration of GN11 cells induced by control CM; however, the increased migration induced by anosmin-1 was abolished by heparin treatment. In agreement with these results, a pretreatment of GN11 cells with the enzyme heparitinase blocked the chemomigration of GN11 induced by the presence of anosmin-1 in CM (Fig. 5B); the response of GN11 cells to the control CM was not affected by the enzyme pretreatment.



**Figure 5.** Role of HSPG in the chemotaxis of GN11 cells induced by anosmin-1. (A) For competition experiments, control and anosmin-1 CM (anosmin-1) were preincubated at 37°C with 30 µg/ml heparin for 30 min, prior to be used as a chemoattractant in Boyden's chamber assay. (B) Enzymatic pretreatment of the cells. GN11 cells were preincubated with heparitinase (H, 1 U/ml), at 37°C for 55 min, prior to be used in the microchemotaxis assays using control and anosmin-1-enriched CM as chemoattractants. The results are expressed as number of migrated cells per mm<sup>2</sup> of the porous membrane in 3 h. Mean ± SD obtained from three independent experiments. \*Significant ( $P < 0.05$ ) versus respective control CM.

### Anosmin-1 released from COS<sub>KAL</sub> cells binds to the surface of GN11 cells

Since we observed that anosmin-1 adheres to the surface of COS-7, we wanted to verify whether it could also bind to GN11 cells. To this purpose, the GN11 cells were co-cultured with anosmin-1-producing COS cells for 16 h. The immunostaining of the cultures, in permeabilized conditions, using both the 9E10 anti-c-myc antibody and the LR1 anti-GnRH antibody, revealed a clear c-myc-immunoreactive signal associated to anosmin-1-producing COS cells and showed that anosmin-1 signal co-localizes on GnRH-immunoreactive cells (Fig. 6A–C). In addition, we analysed co-cultures of anosmin-1-producing COS cells with GN11 cells stably expressing the green fluorescent protein (GN11-GFP); the cultures were then immunostained with 9E10 antibody in unpermeabilized conditions and processed for confocal microscopic examination. Double staining of the co-culture cells indicated that c-myc epitope immunoreactivity was present on the surface of GN11 cells (Fig. 6D–G). Incubations of

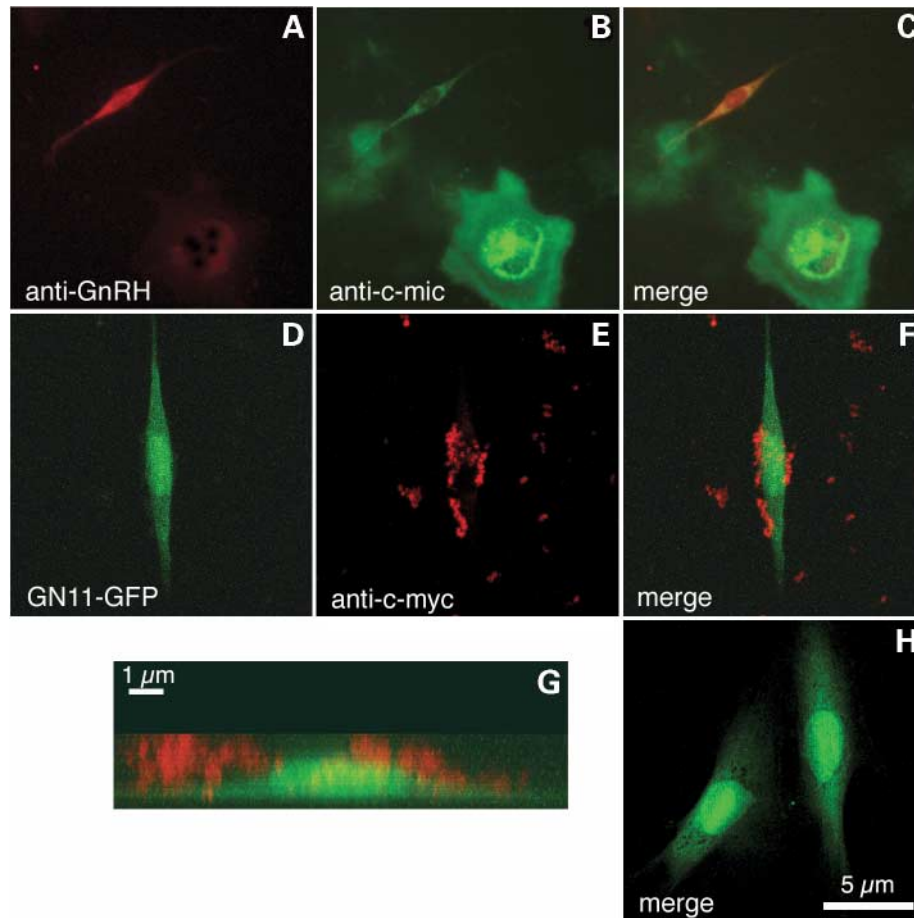
the cultures in the absence of primary antibodies or with fluorescent secondary antibodies alone did not reveal any fluorescent signal (data not shown). According to the hypothesis of an interaction of anosmin-1 with HSPGs, a pretreatment of the cultures with heparin strongly reduces or abolishes the anosmin-1-immunoreactive signal (Fig. 6H).

### The migration of GN11 into a three-dimensional collagen gel matrix is also stimulated by anosmin-1

A preliminary series of experiments devoted to evaluate the effect of anosmin-1 on the migration of GN11 in a three-dimensional matrix of collagen gel were inconclusive because of the bias due to the effect of the control CM obtained from COS-7 cells on the chemomigration of GN11 cells. Consequently, we analysed the CM of other cell lines for their basal chemoattractant activity. We found that CM derived from CHO cells showed a chemotactic effect much lower than the CM obtained from COS-7 cells. CHO cells have been already successfully used by other authors to produce anosmin (45). Consequently, we transfected CHO cells with pMT21-*KALI*<sub>myc</sub> and we confirmed by immunocytochemistry and immunoblotting analysis the expression and the secretion of the *KALI*-encoded protein also in our experimental conditions (data not shown). We also verified the ability of anosmin-1-enriched CM derived from CHO cells to induce the chemotaxis of GN11 cells, and the results have shown that the exposure to control CM and CM<sub>myc</sub> obtained from CHO cells led to a very low basal chemomigration, whereas the CM-enriched anosmin-1 induced a remarkable chemotactic response of the GN11 cells (Fig. 7A). Taking advantage of the strong difference between the effects of control and anosmin-1-enriched CM, we then performed a migration test in collagen gel matrix. Aggregates of GN11 cells were placed in a double layer of collagen gel and cultured for 3 days in the presence of either CM or anosmin-1. As also shown in Figure 6, cells maintained in control CM were unable to move significantly from their aggregate form (Fig. 7B); on the other hand, the exposure of the aggregates to anosmin-1 induced a migration of a number of GN11 cells into the collagen matrix (Fig. 7C and D).

## DISCUSSION

The present study provides the first experimental evidence of a direct stimulatory action of the *KALI*-encoded protein on the migration of GnRH-expressing neurons *in vitro*. This demonstration underlines a possible physiological role exerted by KAL/anosmin-1 on the migration of GnRH neurons during brain development. This new action of anosmin-1 was assessed by microchemotaxis experiments in which GN11 cells (a cell line of immortalized GnRH neurons found to exhibit migratory activity '*in vitro*') (41–43) were exposed to COS-7 or CHO cell CM enriched in human anosmin-1 protein. The attribution to anosmin-1 of the effects observed by using CM has been confirmed by different experimental approaches. At first, we found that immunoneutralization of the anosmin-1<sub>myc</sub> construct present in the CM with the 9E10 anti-myc antibody abolishes the effect of anosmin-1-enriched



**Figure 6.** Immunocytofluorescence analysis of anosmin-1 distribution in co-cultures of transfected COS-7 cells producing the protein, with GN11 neurons. (A–C) COS<sub>KAL</sub> and GN11 cells were double labelled with monoclonal 9E10 anti-myc antibody (green) and polyclonal LR1 anti-GnRH antibody (red) in permeabilized conditions. It is evident that anosmin-1 signal co-localizes with GnRH-immunoreactive GN11 cells. (D–F) Confocal microscopy analysis of GN11 cells expressing green fluorescent protein co-cultured with COS<sub>KAL</sub> cells and immunolabelled with 9E10 anti-myc antibody (red) in unpermeabilized conditions. A three-dimensional reconstruction of the full scanning of GN11 cell reported in (F) was generated from a series of *xz*-views (G). The pictures show that anosmin-1 is associated to the surface of GN11 cells. The pretreatment of the cultures with heparin eliminate the anosmin-1-immunoreactive signal (H).

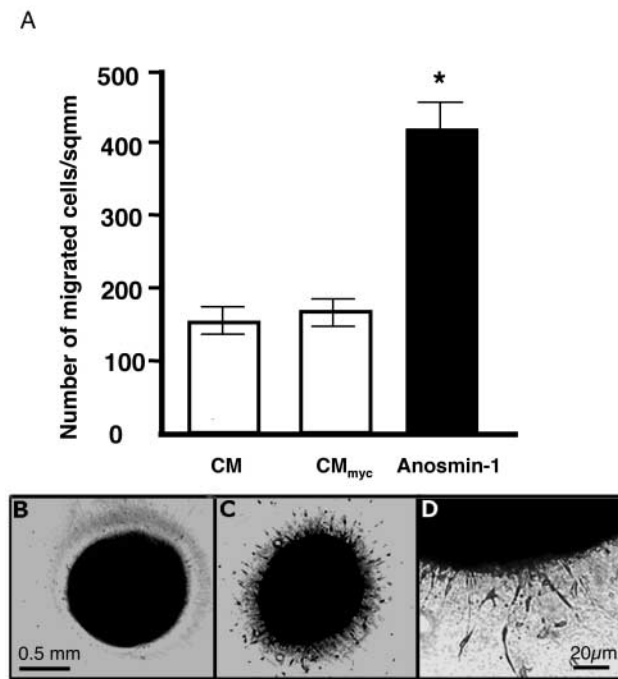
media on the chemotaxis of GN11 cells; even if the antibody does not recognize directly the protein, it might affect anosmin-1 function by steric shielding.

Furthermore, we have investigated the function of three forms of anosmin-1 carrying mutations found in Kallmann's affected patients (46). We found that the mutations do not affect synthesis, processing and release of the protein encoded by mutated *KALI*. However, all of the three anosmin-1 mutants proved unable to induce the migration of immortalized GnRH neurons. This finding leads once again to the conclusion that wild-type anosmin-1 is responsible for the observed chemotactic effects and that mutations induce an almost complete loss of this function. These last results are in agreement with a previous study (48) which reported that N267K and E514K substitutions may affect the adhesion of other cell models.

The action of anosmin-1-enriched media appears specific for GN11 neurons since the motility of more differentiated immortalized GnRH neurons (GT1-7 cell line), and that of metastatic human neuroblastoma cells, was not affected by the exposure to the recombinant protein. It is of interest that

GT1-7 cells, while being responsive to the adhesive properties of anosmin-1 (17), do not respond to the chemotactic effect of anosmin-1. Previous observation that GT1-7 cells are representative of mature postmigratory GnRH neurons (43) may justify the insensitivity of these cells to the chemotactic effect of anosmin-1. Further support to the specificity of the effects presented here comes from observation that GN11 cells are not responsive to anosmin-1 in the absence of a concentration gradient of the protein (chemokinesis), but react to a gradient of anosmin-1 either in soluble form (chemotaxis) or absorbed on a solid matrix (haptotaxis).

In agreement with the data reported by others previously (17,48), we have also found that anosmin-1 binds to the surface of GN11 cells in an heparin-dependent manner and that the chemotactic effect of anosmin-1 on GN11 neurons is prevented by heparin as well as heparitinase treatment. These pieces of evidence indicate that anosmin-1 also exerts its chemotactic effect on GnRH neurons by interacting with HSPG. HSPG play a key role in mediating the development of the olfactory bulb (49), and many of the point mutations detected in X-KS patients have been found in the fibronectin-like



**Figure 7.** Chemomigration of GN11 cells in response to the media conditioned by CHO cells expressing anosmin-1. **(A)** Microchemotaxis experiments were performed in Boyden's chamber using CM from untransfected CHO cells (CM), CHO cells transfected with the empty pMT21 vector (CM<sub>myc</sub>) or with pMT21-*KALI*<sub>myc</sub> (anosmin-1). The results are expressed as number of migrated cells per mm<sup>2</sup> of the porous membrane in 3 h. Mean  $\pm$  SD of five independent experiments. \*Significant ( $P < 0.05$ ) versus respective CM-CHO. **(B, C)** Collagen gel invasion assay. GN11 cells have been aggregated by the hanging drop technique and incubated in collagen gel in the presence of CM **(B)** or of anosmin-1-enriched CM (anosmin-1) **(C)**, and **D** at higher magnification. The preparations were observed daily and the pictures were taken after incubation of cell aggregates for 3 days.

domains, where the interaction with HSPG occurs (46,48). It has been proposed that anosmin-1 binds to its receptor, or other extracellular molecules or domains, by means of a HSPG to induce axonal branching and axon misrouting (21). In addition, one of the genetic loci that either suppresses or enhances the *KALI*-induced axonal defects in *C. elegans* codes for the enzyme heparan-6O-sulphotransferase, which modifies specific residues in HSPG (21). Most important in the context of the present results is the evidence that HSPG are detected along the migratory route of cells coming from the rat olfactory placode; in particular, immunoreactivity for both heparan sulphate and *N*-syndecan was localized in and around the cell clusters formed by migrating GnRH neurons (50). The molecular modelling of anosmin-1 has shown that the missense mutations N267K, E514K and F517L lay in the FNIII domains 1 and 3, and that large basic surfaces, containing clusters of conserved predicted heparan sulphate-binding residues, are adjacent to these sites (48).

Finally, the results presented here indicate that, according to results obtained on rat tissues (22), the human anosmin-1 is effective also on mouse neurons. This is confirmatory of the functional evolutionary conservation of the protein (18), and could be indicative of a similarity of the structures of the

putative mouse and human anosmin, even if an homologous to *KALI* gene has not been identified so far in mouse. The reported observation that human *KALI* cDNA expression can compensate for the loss of the *C. elegans* homologue of *KALI* supports the present hypothesis (18).

No data have been provided so far on a direct action of anosmin-1 on GnRH neurons, but the involvement of an impaired migration of GnRH neurons in X-KS has been postulated since a 19-week-old affected fetus was analysed (7). In this fetus, the findings were consistent with previous arrest of GnRH neurons and olfactory axons in a tangle over the cribriform plate, just below the telencephalon. Because of this, it has been hypothesized that the disruption of the central roots of the terminal nerves would account for the arrest of the GnRH cells, which, in normal conditions, migrate along these nerve fibres, at least up to their entry into the forebrain (51). However, the finding reported in the present paper clearly indicates that anosmin-1 can be involved in the direct control of the migration of GnRH neurons and strongly supports a pleiotropic role of anosmin-1 on neuronal cell functions (axonal elongation and fasciculation as well as neuronal migration) during development.

The distribution of anosmin-1 immunoreactivity in human and animal brains strongly supports our finding that GnRH neurons are one of the anosmin-1 physiological targets. In the human embryo, anosmin-1 is detected in the olfactory region as an extracellular matrix component (12). Anosmin-1 immunoreactivity was found in the rostral brain region, including the anlage of the olfactory bulb, as well as in the adjacent area, where fibres of the terminal nerve and GnRH cells penetrate the forebrain; anosmin-1 is also found in the medial wall of the primitive cerebral hemisphere, a part of the migratory pathway of GnRH neurons. This finding supports a role, permissive or instructive, in the terminal elongation and/or targeting of the olfactory axons, and also in the tangential navigation of the central roots of the terminal nerve and of GnRH neurons. In conceptual agreement with the present paper is also the observation that *C. elegans KALI* may influence both the migration and the adhesion of epidermal cells undergoing morphogenesis during ventral enclosure and male tail formation (18). The observation that in the X-KS fetus both GnRH neurons and olfactory axons had left the olfactory epithelium, but they had accumulated just over the cribriform plate (7), is suggestive of an action of anosmin-1 spatially restricted to the nasal-forebrain junction (12). The nasal-forebrain junction is a critical region where migratory GnRH neurons are induced to turn ventrally toward the forebrain; this is confirmed by several experimental paradigms of altered migration of GnRH (52-56).

The evidence that anosmin-1 may positively affect the migratory activity of GnRH neurons adds new insight on the understanding of Kallmann disease and of other forms of HH. Actually, non-idiopathic HH and anosmia can occur both together and separately within the same autosomal pedigree, suggesting that solitary impairment of GnRH neurons migration/development could be in part responsible for HH (2,3,46). In the last few years, different factors have been shown or postulated to control the migration of GnRH neurons; among these are the nasal embryonic LHRH factor (57), Gas6-Ark system (58), netrin-1 (54,56),

the hepatocyte growth factor (42) and the Ebf2 transcription factor (55). Moreover, mutation of FGF receptor was detected in autosomal dominant KS patients (59) and, more recently, also the loss of function of the KiSS-derived peptide GPR54 was found to be a cause of HH (60). The availability of a model of migrating GnRH neurons will allow for screening, under controlled conditions, of the single actions of each of these factors before it will be confirmed by technically and ethically more complex *in vivo* or *ex vivo* experiments.

In conclusion, the results provided in the present paper strongly suggest that anosmin-1 may have a direct effect (instructive or permissive) on the migratory activity of GnRH neurons; moreover, the mouse immortalized GnRH neurons utilized in this study could provide a useful tool to also investigate other functions of anosmin-1.

## MATERIALS AND METHODS

### Cell lines and treatments

COS-7 cells and CHO cells (American Type Culture Collection, ATCC, Manassas, VA, USA) were routinely grown in a monolayer at 37°C in a humidified CO<sub>2</sub> incubator in Dulbecco's minimum essential medium (DMEM) containing 1 mM sodium pyruvate, 100 µg/ml streptomycin, 100 U/ml penicillin and 10 mg/l of phenol red (Biochrom KG, Berlin, Germany) and supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). The medium was replaced at 2 day intervals. Subconfluent cells were routinely harvested by trypsinization and cultured in 57 cm<sup>2</sup> dishes (2.5 × 10<sup>5</sup> cells).

GN11 and GT1-7 cells were generously provided by S. Radovick (University of Chicago, Chicago, IL, USA) and R.I. Weiner (San Francisco, CA, USA), respectively. The cells were routinely grown in a monolayer at 37°C in a humidified CO<sub>2</sub> incubator in DMEM containing 1 mM sodium pyruvate, 100 µg/ml streptomycin, 100 U/ml penicillin and 10 mg/l of phenol red (Biochrom KG) and supplemented with 10% FBS (Gibco). The medium was replaced at 2 day intervals. Subconfluent cells were routinely harvested by trypsinization and seeded in 57 cm<sup>2</sup> dishes (1 × 10<sup>5</sup> for GN11 cells and 3.5 × 10<sup>6</sup> for GT1-7 cells). Cells within six passages were used throughout the experiments.

SK-N-SH cells (ATCC) were grown at 37°C in a humidified CO<sub>2</sub> incubator in a monolayer in MEM containing non-essential aminoacids, 1 mM sodium pyruvate, 100 µg streptomycin/ml, 100 U/ml penicillin and 10 mg/l of phenol red (Biochrom KG). The medium was supplemented with 10% FBS (Gibco). Confluent cells were harvested with 0.05/0.02% trypsin/EDTA and 1 × 10<sup>6</sup> cells were seeded in 57 cm<sup>2</sup> dishes. The medium was replaced at 2 day intervals.

### Transfection of *KALI* constructs and preparation of anosmin-1- enriched CM

pMT21-*KALI*<sub>myc</sub> expression vector was obtained as previously described (44). GFP-tagged *KALI* was constructed by inserting the coding region of human *KALI* into the MCS region of pEGFP-N<sub>2</sub> vector. In these constructs, the

myc and GFP tags were positioned at the C-terminal of the *KALI*-coding region.

To introduce the point mutations N267K, E514K and F517L into the anosmin-1<sub>myc</sub> construct, *in vitro* mutagenesis was performed using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and according to the manufacturer's instructions. Primers' sequences are available upon request. The presence of the point mutation was then confirmed by DNA sequencing. For transfection, COS-7 and CHO cells (at 80% confluence) were grown in culture plates in complete culture medium for 24 h and incubated for 3 h with the selected expression vector (1 µg/ml) in the presence of the LipofectAMINE<sup>TM</sup>-Plus Reagent (Life Technologies, MD, USA) according to the manufacturer's instructions. The expression of the different constructs was verified by immunofluorescence.

Anosmin-1-enriched CM were obtained as follows: transfected COS-7 (COS<sub>KAL</sub>) or CHO (CHO<sub>KAL</sub>) cells were left in culture for 48 h in complete medium; this was then replaced with half volume of serum-free medium and the cells incubated for 16–18 h. The medium (CM) was decanted in the presence of protease inhibitor aprotinin (0.5 µg/ml), centrifuged at 3000g for 5 min and immediately used for microchemotaxis assays. In preliminary experiments, we found that anosmin-1-enriched CM can be stored at –20° for 15 days without loss of chemotactic activity.

Western immunoblot analysis was routinely performed to verify the presence of anosmin-1<sub>myc</sub> in cell extracts or CM preparations. Dishes containing cell monolayers were put on ice bath and harvested using phosphate buffered saline (PBS) without Ca<sup>2+</sup> and Mg<sup>2+</sup> with the addition of 0.5 µg/ml of aprotinin; the cell extracts were centrifuged for 10 min at 9000 g at 4°C and the pellets were resuspended in Laemmli sample buffer (Tris 225 mM pH 6.8, SDS 8%, glycerol 40%, β-mercaptoethanol 8%, bromophenol blue 0.02%) in order to get a final concentration of 10–50 µg of the proteins. The CM were concentrated by diafiltration with Microcon YH30 tubes (Millipore, Milano, Italy) and diluted 1:2 in Laemmli sample buffer. A constant volume of samples (20 µl) were assayed. Proteins were separated by SDS-PAGE on a 12% acrylamide/bis gel (Biorad, Hercules, CA, USA) and transferred to nitrocellulose membranes (Biorad) using a 25 mM Tris, 192 mM Glycine, 20% (v/v) methanol buffer pH 8.3. The membrane was washed in 10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20 (TBST) for 30 min, blocked with 5% powder milk in TBST for 30 min and then incubated overnight with shaking, at 4°C with 9E10 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:50 for cell extracts and 1:25 for CM. Membranes were washed three times with TBST and incubated for 2 h, with shaking, at room temperature with anti-mouse IgG-HRP (Santa Cruz Biotechnology, 1:40,000). Membranes were washed three times with TBST and secondary antibody was revealed with a chemiluminescent system using SuperSignal West Pico Substrate (PIERCE, Rockford, IL, USA) according to manufacturer's instructions.

### Immunofluorescence

Cells were grown on coverslips and fixed 2 days later in 4% buffered paraformaldehyde (20 min at 4°C), permeabilized



with 0.2% Triton X-100 for 30 min, blocked for 20 min with 1.5% horse normal serum in PBS, washed twice with PBS and incubated at 4°C overnight with 1% normal horse serum in PBS, 5% bovine serum albumin (BSA) and the different antibodies. C-myc-tagged anosmin-1<sub>myc</sub> was detected with 9E10 Mab (Santa Cruz Biotechnology); GnRH was detected with polyclonal antibody LR1 (generous gift from Dr R. Benoit, Montreal) at a dilution 1:5000 at 4°C overnight. The cells were then washed in PBS and incubated for 1 h with FITC- or TRITC- conjugated isotype-specific secondary antibody (1:200, Jackson Immunoresearch, West Grove, PA, USA) For immunofluorescence analysis on unpermeabilized cells, the Triton X-100 treatment was omitted. Finally, after wash in deionized water, the coverslips were mounted with DABCO and viewed on a Zeiss Axioscope 2 Plus microscope under epifluorescence light.

Samples were also observed under a confocal laser scanning microscope (MicroRadiance 2100 BioRad) mounted on an inverted microscope (Nikon Eclipse 300). Illumination sources were an argon laser (488 nm) and He-Ne (543 nm). Single channels were collected sequentially using a selective barrier filter detecting FITC fluorescence to cut-off the possible emission of the second peak of TRITC at about 515 nm. All images were collected using a PlanApo 60X oil immersion lens and the scanning resolution was 512 × 512 points. Samples were sectioned on the Z axis with steps of 0.5 μm. Projection images were obtained from serial optical sections using LaserSharp software (BioRad).

### Microchemotaxis assay

The assay was performed using a 48-well Boyden's microchemotaxis chamber according to the instructions (Neuroprobe, Cabin John, MD, USA). Briefly, the lower compartment of the chamber was loaded with the chemoattractants (CM) or control medium; the cells, grown in complete medium until subconfluence, were suspended (10<sup>5</sup> cells/50 μl) in serum-free culture medium and placed in the open-bottom wells of the upper compartment of the Boyden's chamber. Each pair of facing wells was separated by a polyvinylpyrrolidone-free polycarbonate porous membrane (8 μm pores) precoated with gelatine (0.2 mg/ml in PBS). To measure chemotaxis, the control or anosmin-1-enriched CM were placed only into the wells of the lower compartment of the chamber; haptotaxis was measured by coating the lower surface of the gelatine-treated porous membrane for 24 h with control or anosmin-1-enriched CM. Finally, the chemokinesis was assessed by placing the same concentration of chemoattractant in both the upper and the lower wells of the Boyden's chamber, thereby eliminating the chemical gradient.

Immunoneutralization of anosmin-1<sub>myc</sub> was performed by incubating CM in presence of monoclonal 9E10 anti-myc antibody (1:100 dilution; Santa Cruz Biotechnology) for 30 min at 37°C before microchemotaxis experiments. HSPG competition was assayed by incubating the CM at 37°C with 30 μg/ml heparin (sodium salt H9399, Sigma, St Louis, MA, USA), for 30 min or GN11 cells with 1U/ml of heparitinase (EC 4.2.2.8; H8891, Sigma) for 55 min before microchemotaxis assay.

The chamber was then kept in the cell culture incubator for 3 h; this time interval was selected on the basis of previous experiments as the minimum time to attain a highly significant migratory activity of GN11 neurons (61). After incubation, the cells migrated through the pores, and adhered to the underside of the membrane; they were then fixed and stained using the Diff-Quick stain kit (Biomap, Milano, Italy) and mounted onto glass slides. For quantitative analysis, the membranes were observed on a Leitz microscope using a 20× objective. Three random fields of stained cells were counted for each well, and the mean number of migrating cells/mm<sup>2</sup> for each chemoattractant condition was calculated. Statistical analysis was performed by ANOVA and adequate *post hoc* test.

### Cell aggregates and collagen gel assay

Cell aggregates were prepared by the 'hanging drop' technique (62); subconfluent cells were collected by trypsinization, recovered in complete culture medium and seeded in 20 μl drops (2 × 10<sup>5</sup> cells) on the lid of a culture dish; the lid was then placed by a dish filled with 2 ml of culture medium and incubated at 37°C for 48 h. Rat tail collagen solution was prepared as described by Tessier-Lavigne *et al.* (63). Collagen gel was obtained by addition to an aliquot of collagen stock solution of 10% (v/v) of 10× concentrated DMEM (without phenol red) and of 0.8 M bicarbonate. This solution (20 μl) was pipetted onto the bottom of a well of a 24-wells culture dish, and left to set at room temperature. Cell aggregates were transferred over the dish-lid and then overlaid with additional 20 μl of collagen. After the overlaid collagen set, it was covered with 400 μl of control CM or anosmin-1-containing CM. The aggregates were observed daily under a light microscope; at the end of the incubation period aggregates were fixed in 4% paraformaldehyde, stained with a 0.5% cresyl violet solution.

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