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Neuropeptides Are Chemoattractants for Human Tumor Cells and Monocytes: A Possible Mechanism for Metastasis

MICHAEL RUFF, ELLIOTT SCHIFFMANN,* VICTOR TERRANOVA,* AND CANDACE B. PERT†,[1]

*Cellular Immunology Section, Laboratory of Microbiology and Immunology, *Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, NIH and †Section on Brain Biochemistry, Clinical Neuroscience Branch, National Institute of Mental Health, ADAMHA, Bethesda, Maryland 20205*

Bombesin (BN), a tetradecapeptide neuropeptide growth factor, is shown to be a potent (ED_{50} of 5×10^{-12} *M*) chemoattractant for human monocytes and small cell lung carcinoma cells (SCCL). These effects are BN receptor-mediated since potencies of several BN analogs to induce chemotaxis and to inhibit [^{125}I-tyr^4] BN binding activity correlate well ($P < 0.001$). As has been demonstrated for other BN receptor-mediated effects, carboxy-terminal amino acids are required for optimum biological activity. BN is not an exclusive chemoattractant for SCCL cells but was also active in promoting migration of other, but not all, lung tumor cells. Other neuropeptides, such as β-endorphin, substance P, and arg-vasopressin, are also shown to be chemoattractants for SCCL cells, with EC_{50}'s also in the 10^{-12} *M* range. The ability of these ligands to effect monocyte and some tumor cell migration suggest a role for neuropeptides in inflammation and metastasis. In the latter case, tumor cells, in response to neuropeptide chemical gradients, may become localized at specific body sites. Neuropeptide release, in response to cognitive or other stimuli, may thereby modify cell migratory patterns. Additionally, such hormones may influence early developmental events such as tissue organization and histogenesis.

INTRODUCTION

Neuropeptides, short chains of amino acids present in brain as well as non-neural tissues, are primarily known for their role as neurotransmitters (1). It is clear that these compounds, far from acting solely within the nervous system, are localized throughout the brain and body where they act as transmitters, growth hormones, and signal agents for many body systems (2, 3). Neuropeptides, including opiate peptides (4, 5) and substance P (6), as well as the benzodiazepines (7) [whose endogenous ligand is a neuropeptide (8)] have also been shown to be monocyte chemoattractants, suggesting additional roles for these ligands in immune system function.

Recently, multiple monocyte specific antigens have been reported to be present

[1] To whom reprint requests should be addressed: Section on Brain Biochemistry, NSB National Institute of Mental Health, Building 10, Room 3N256, 9000 Rockville Pike, Washington, D.C. 20205-1000.

[2] Abbreviations used: BN, bombesin; FMLP, *N*-formyl-L-methionyl-L-leucine-L-phenyl-alanine; PDGF, platelet derived growth factor, SP, substance P; VP, vasopressin; β-ED, β-endorphin; OX, oxytocin; SCCL, small cell carcinoma of the lung, PBS, phosphate-buffered saline.

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on small cell lung carcinoma (SCCL) cells (9, 10) suggesting that this disease may arise from hemopoietic precursors when the normal macrophage-mediated repair of lung tissue is deranged by continuous heavy smoking (9). SCCL is a major cause of cancer death with high metastatic propensity whose tumors store and secrete numerous neuropeptides, including ACTH (11), β-endorphin (12), and bombesin (BN) (13–15). The presence of the tetradecapeptide bombesin, in particular, has been reported to characterize this tumor (13) and BN has been suggested to be a growth factor for these (16) cells. In an attempt to define further how monocytes and SCCL cells become localized to various body sites we have examined the role of BN and other neuropeptides as chemoattractants for SCCL cells and human monocytes.

MATERIALS AND METHODS

Cells. Human buffy coat mononuclear cells were prepared according to our previous report (6) by partial purification on Ficoll–Paque (Pharmacia). Lung tumor cell lines [Calu-3 (17), SK-MES-1 (17), NCI-H69 (18)] were obtained from the American Type Collection (Rockville, Md.). The alveolar carcinoma cell line A549 (19) was the gift of R. Bankert. All cells were grown in RPMI-1640 supplemented with 10% FCS (growth medium). All of these cells grew attached to plastic substrata with the exception of the SCCL line (NCI-H69) which, as received, grew in suspension. In order to perform chemotaxis studies we adapted this line to monolayer growth by allowing adherence to occur over a 3- to 5-week period during which time the nonadherent cells were passaged weekly at a split ratio of 1:4. When the adherent cells had attained an area of roughly 25% of a flask (75 cm^3) they were removed with 0.1% trypsin/0.1% EDTA for 10 min at 37°C. Cells were then replated into T-75 flasks with growth medium where they grew as stable monolayers. These cells continue to express the OKM1 surface antigen of the parent line (9) although they no longer contain detectable BN immunoreactivity (data not shown).

Chemotaxis. Chemotaxis assays were performed by using modified Boyden chambers as described (4, 5). Nucleopore filters of 5 μm diameter pore size were used for the monocyte chemotaxis assays (4). Filters of 8 μm diameter pore size, coated with type IV collagen (20), 50 μg/filter, in 0.1 *M* acetic acid were used with the tumor cells. Coated filters were air dried overnight. For all assays, peptides, dissolved in buffer consisting of MEM, Hanks salts (GIBCO) containing 1% BSA, 20 m*M* Hepes, were added to lower chambers. Freshly isolated monocytes (50,000/assay) or freshly removed [10 m*M* EDTA/PBS] tumor cells (25,000/assay) in buffer were added to the upper chamber. The chambers were incubated for 90 min (monocytes) or 4 hr (tumor cells) at 37°C in a 5% CO_2 incubator. Subsequently, the filters were removed, fixed, stained with hematoxylin, and the number of migrating cells were quantitated utilizing an image analyzer by microscopically counting the number of cells in three high power (200X) fields from each of triplicate determinations. Data are expressed as a chemotactic index, which is the ratio of migration toward test attractants and buffer only control. The number of migrating cells in the buffer alone control ranged from 20 to 60

cells per field in these experiments. The percentage of migrating cells was determined to be approximately 2–4% for the SCCL cells and 3–6% for the monocytes.

Peptides. Peptides were obtained from Peninsula Laboratories (San Carlos, Calif.) or were gifts from T. Moody [Tyr^{4}BN, D-leu^{13}BN, (8–14) BN, (10–14) BN, BN-OH]. Peptides were stored as 10^{-4} *M* stocks in 0.02 *M* acetic acid at −20°C and were diluted just prior to use.

Karyotyping. Chromosome analysis was performed on SCCL (NCI-H69) cells growing in monolayer culture which had been passaged 48 hr previously. Cultures were washed with PBS to remove loose or nonadherent cells. Colcemid (0.2 μg/ml) was added for 45 min and air-dried preparations were made, which were then Giemsa stained and banded using trypsin-Giemsa techniques, as described (21).

RESULTS

Bombesin Receptor-Mediated Chemotaxis of Human Monocytes and SCCL Cells

A simple selection process enabled us to derive a culture of SCCL cells which grew as stable monolayers and which were suitable for chemotaxis studies. These cells are derived from their parental non-adherent line, NCI-H69, since karyotypic analysis confirms the presence of several deletions and rearrangements which were reported to typify this cell line (21). In particular the deletion in chromosome 3 is observed (Fig. 1A, arrows) as well as rearrangements on chromosomes 13, 16, and 19 (Fig. 1A) and the XXX p-q-Y complement.

The adherent nature of these cells can be observed; Fig. 1B shows a subconfluent culture comprised of individual well-spread cells as well as some colonies of tightly clustered adherent cells, although loose aggregates of nonadherent cells can also be observed.

Utilizing a Boyden chamber chemotaxis assay method we are able to show that human buffy coat monocytes will respond chemotactically to the tetradecapeptide bombesin (Fig. 2A). The apparent ED_{50} (concentration required for half maximum effect) for BN's chemotactic effect is approximately 5×10^{-12} *M*. High concentrations of BN resulted in a diminished response which may be due to receptor desensitization and/or gradient breakdown. Gastrin releasing peptide (GRP), which contains a C-terminal sequence very similar to BN, is also active in this assay although its rank order of potency is shifted to an ED_{50} of approximately 10^{-11} *M*. The BN analog, D-Leu^{13}BN was much less active in this assay, EC_{50} approximately 2×10^{-8} *M*. These results extend earlier reports revealing a role for neuropeptides in macrophage chemotaxis (4–7). SCCL cells also share the ability to migrate chemotactically to BN and its analogs (Fig. 2B) with a similar rank order of potency. Thus, for both cell types the activity was BN > GRP > (D-Leu^{13})BN. That these chemotactic effects are BN receptor-mediated processes can be demonstrated by structure-function analysis comparing the efficacies of a larger series of BN analogs to induce chemotaxis in SCCL cells or displace binding of ^{125}I-tyr^{4}BN to SCCL cells (Fig. 3). For the purposes of this analysis EC_{50} values for chemotactic effects, generated utilizing the methods described here, were compared with binding data taken from a report by Moody *et al.* (22)

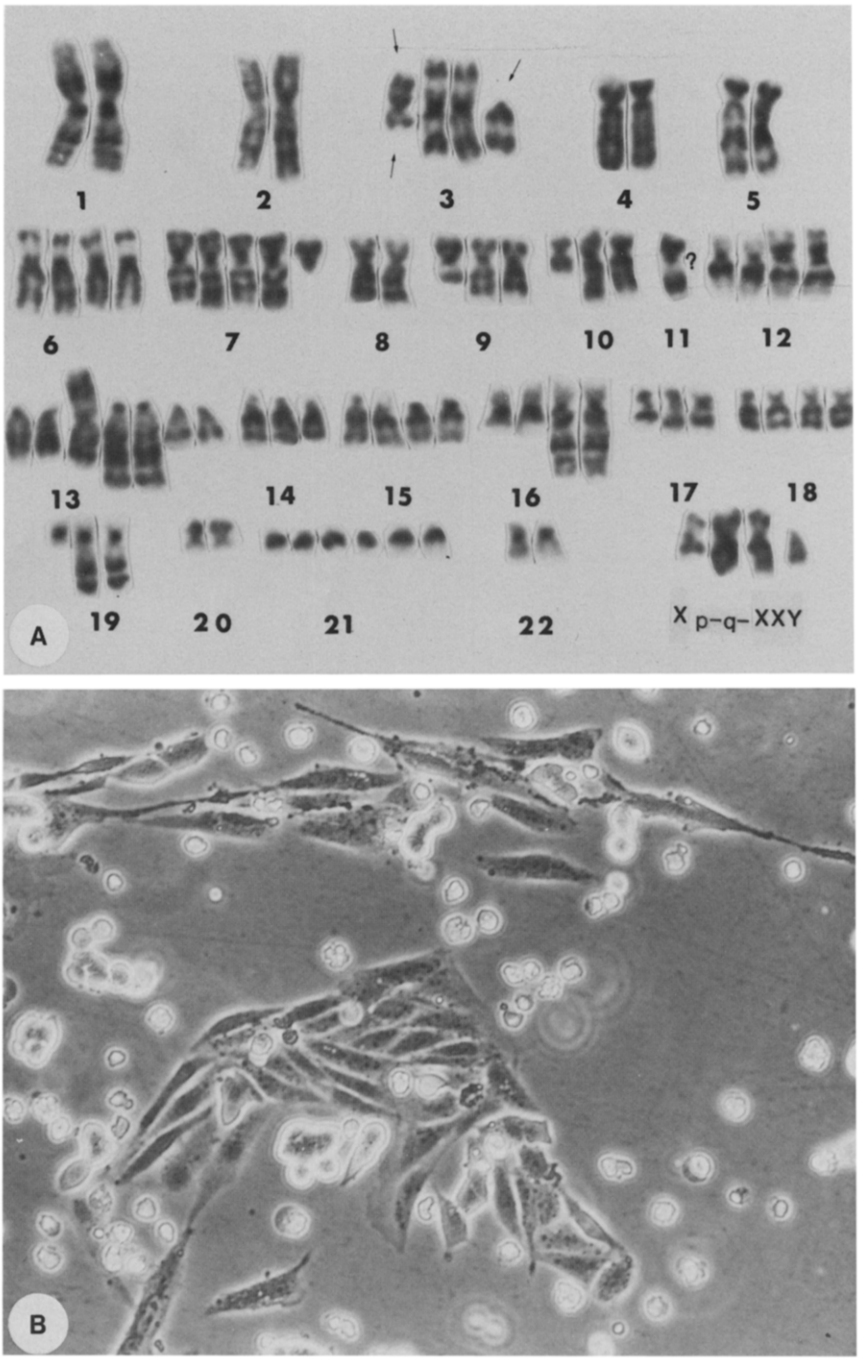

FIG. 1. (A) Karyotype of a near-triploid adherent cell isolated from the SCCL line NCI-H69 with 76 chromosomes and showing the specific deletion at chromosome 3 (arrow) which typifies these cells, (B) adherent cells (X210) isolated from cultures of the SCCL line NCI-H69.

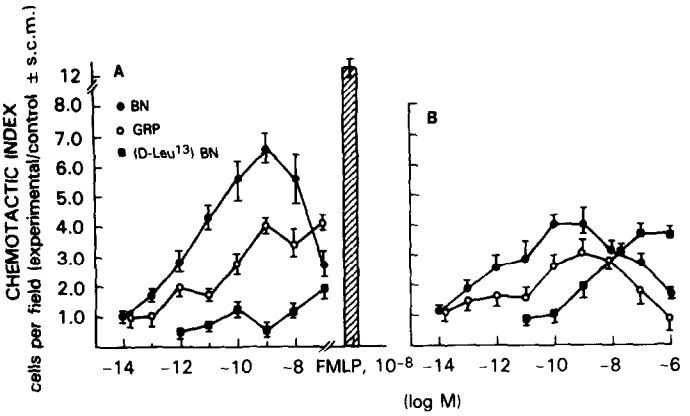

FIG. 2. Human monocyte (A) and small cell lung carcinoma (SCCL) tumor cell (B) chemotaxis in response to bombesin (BN), gastrin releasing peptide (GRP), and D-Leu^{13} bombesin (D-Leu^{13}BN). Mean and standard error are shown, $n = 3$. Background cell migration was 23 for the monocyte and 5 for the SCCL experiments.

which characterized the receptor on these cells. Increasingly N-terminus deleted BN analogs revealed decreasing potency [e.g., BN(8–14) > BN(10–14)] in both assays, and deamidated BN, BN-OH, was largely inert, indicating that C-terminal amino acids are required for maximum activity. As can be seen, there is a close correlation ($P < 0.001$) between the abilities of BN and its analogs to induce chemotaxis in SCCL cells and to bind to the receptor previously defined on these cells by radioligand binding studies, suggesting that chemotaxis is mediated by a specific BN receptor.

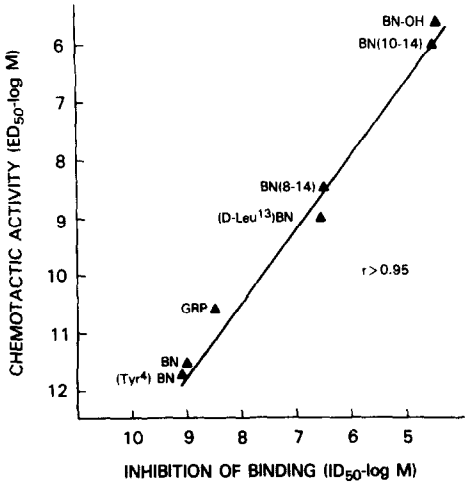

FIG. 3. Relative potencies of a series of BN fragments and analogs in displacing specifically bound ^{125}I-tyr^{4}BN from SCCL cells versus the relative potencies of these compounds at inducing SCCL cell chemotaxis ($r = 0.98$, $P < 0.001$). Binding data was taken from Moody *et al.* (22). ID_{50} refers to the concentration of peptide required to inhibit binding of ^{125}I-tyr^{4}BN to cells by 50%, while ED_{50} refers to the concentration of peptide required to elicit 50% of maximal response.

Chemotactic Nature of the Response

The enhanced migration we have observed in the presence of BN could be explained as an increase of cell attachment to the chemotaxis filters or an increase in the random migration (chemokinesis) of these cells. Table 1 shows that migration occurs primarily by directed migration in response to a gradient and thus is truly chemotactic. The "checkerboard" analysis (23) which incudes test attractants in both the upper and lower compartments of the chambers indicates that both the monocytes, and the SCCL cells are exhibiting primarily chemotactic with some chemokinetic activity to BN as an attractant.

Chemotaxis of Other Lung Tumor Lines

We were interested in the relevance of monocyte and SCCL cell chemotaxis to BN with regard to the apparent specificity of this peptide as a marker for SCCL and not other lung neoplasias in general. BN is present in specific cell bodies in normal adult lung as well as in high concentration during fetal development and its secretion could be potentially relevant to the trafficking of normal or tumor cells. We tested several lung tumor lines for their ability to migrate to this peptide. As is shown (Fig. 4), chemotactic responsiveness to BN is not solely a characteristic of SCCL cells or monocytes but other tumor lines such as the lung alveolar carcinoma, A-549, also respond. Although lung squamous cell lines, such as Calu-3 or SK-MES-1, did not migrate in this type of assay, we may not have defined appropriate conditions to detect their responsiveness. Attachment to the filters was comparable for all lines.

SCCL Chemotaxis to Other Neuropeptides

We wished to test the generality of those observations which indicated that macrophages express chemotactically active, specific receptors for a variety of neuropeptides (4–7) with regard to the SCCL cells. Results (Fig. 5) indicate that SCCL cells also chemotax to other neuropeptides in addition to those for BN. Arg-vasopressin, in addition to BN, is a potential autocrine growth factor for these cells which is also a chemoattractant. The closely related nonapeptide oxytocin was not active in promoting migration (Fig. 5). Active peptides, such as substance P, arg-vasopressin, β-endorphin, and BN, although yielding results similar to those in Fig. 5, upon repeated testing, did not always follow these same potency orders. Thus, all of these peptides appear to be similarly active (EC_{50} of 10^{-12} *M*, maximal stimulation of 4.2–4.8) in inducing chemotaxis and are comparable to BN (Fig. 2) in magnitude and potency of effect. Interestingly, the well-studied leukocyte attractants, FMLP (Fig. 5) and FMLPP (data not shown) were also attractants for SCCL cells with EC_{50}'s of 10^{-9} and 5×10^{-10} *M*, respectively, potencies similar to those obtained in eliciting macrophage chemotaxis or smooth muscle contraction (24). The peptides neurotensin and melanocyte-stimulating hormone (MSH) were not active as SCCL attractants in this assay (data not shown) further indicating the specificity of these responses and supporting the concept that unique neuropeptide receptors are responsible for these effects. As further evidence for this, the well-studied formyl-peptide antagonist Boc–

TABLE 1
MONOCYTE AND SCCL CHEMOTAXIS TO BOMBESIN

	Bombesin (M), upper chamber					Bombesin (M), upper chamber			
Monocytes	0	10^{-12}	10^{-10}	10^{-8}	SCCL	0	10^{-12}	10^{-10}	10^{-8}
0	19 ± 9	18 ± 6	20 ± 7	21 ± 2	0	6 ± 2	7 ± 2	13 ± 4	9 ± 3
10^{-12}	73 ± 11	20 ± 7	17 ± 3	13 ± 2	10^{-12}	14 ± 2	16 ± 2	10 ± 3	6 ± 4
10^{-10}	136 ± 5	82 ± 8	37 ± 3	16 ± 2	10^{-10}	28 ± 3	22 ± 3	12 ± 2	8 ± 2
10^{-8}	128 ± 14	145 ± 15	96 ± 11	14 ± 3	10^{-8}	32 ± 4	17 ± 3	20 ± 4	9 ± 1

Note. Directed cell movement in response to an attractant gradient, a measure of chemotaxis, was demonstrated for monocytes and SCCL cells (23). Data are expressed as cells per high power field ± SEM, $n = 2$.

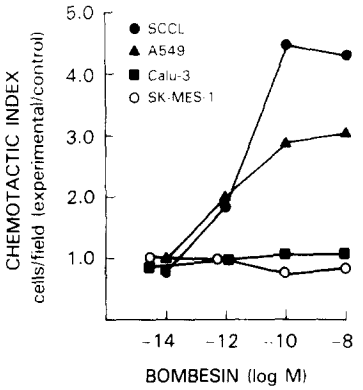

FIG. 4. Chemotaxis of human lung tumor lines in response to bombesin. Background cell migration for these experiments was: SCCL, 6 ± 2; A-549, 8 ± 3; Calu-3, 2 ± 1; SK-MES-1, 4 ± 3. Standard errors ranged from 3 to 14% (n = 2).

Phe–Leu–Phe–Leu–Phe, at 10^{-5} M was able to block FMLP (10^{-8} M)-induced SCCL cell chemotaxis by 90% yet had little effect on BN (10^{-9} M)-induced chemotaxis (data not shown.)

DISCUSSION

We have investigated whether BN and other neuropeptides are chemoattractants for monocytes and adherent SCCL tumor cells (Fig. 1). Such results would provide a functional link to support the antigenic relatedness recently described for these cell types (9) and could provide a basis for both macrophage and tumor localization to specific body sites. BN, chosen because of its high content in SCCL cells and tumors, was a potent chemoattractant for human monocytes and

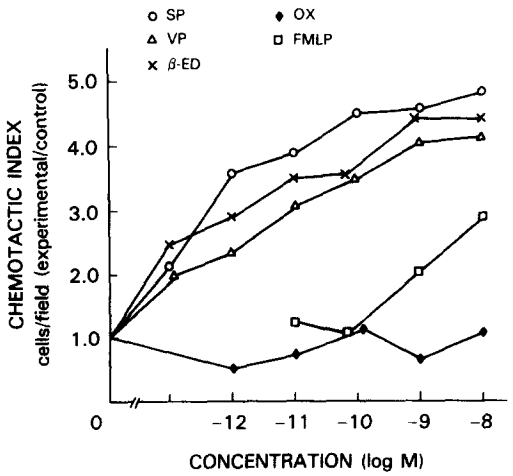

FIG. 5. Chemotaxis of SCCL cells to various neuropeptides: substance P, SP; vasopressin, VP; β-endorphin, β-ED; oxytocin, OX; and formyl-Met–Leu–Phe, FMLP. Standard errors ranged from 6 to 15% and this experiment was repeated several times with similar results.

SCCL tumors (Fig. 2). These chemotactic responses appear to be mediated by a specific BN receptor since a linear correlation ($r > 0.95$) between the ability of BN and a series of analogs to displace ^{125}I-tyr^{4}BN binding to SCCL cells (22) and to mediate chemotaxis was demonstrated (Fig. 3). These results also are in agreement with studies on BN binding to rat brain receptors which show that activity primarily resides in the $CONH_2$ terminal (25).

We questioned whether bombesin might function as a specific chemoattractant for SCCL cells compared to other lung tumors. In a strict sense this did not appear to be the case since at least one other transformed lung tumor line, A-549, could migrate in response to BN (Fig. 4), although the magnitude of this response was lower compared to the SCCL line. These differences were not due to varied cell attachment to the filters (data not shown) but could reflect other differences in the migratory potential of these cell types. The squamous lung lines, Calu-3 and SK-MES-1, in accord with their generally low motility and metastatic potential were not active in this system. Further results show that not only BN but a number of other neuropeptides, such as substance P and β-endorphin, known to be secreted by these tumors (13, 14), are active in inducing chemotaxis of SCCL cells (Fig. 5) and may therefore also be relevant to tumor spread or growth. Arg-vasopressin and bombesin, potential autocrine growth factors for SCCL cells (16), have now been shown to act as chemoattractants for responder cells. As was first described for PDGF (26), this is another example of a peptide having dual functions in cell replication and motility. The observation that SCCL cells both secrete and chemotax to BN raises the speculative possibility of an autocrine response whereby tumor cells are recruited to a metastatic site.

These results may also be quite relevant to the metastatic process per se. Cells that have detached from the primary tumor mass could be responding to organ-specific attractants that the cells, e.g., in the circulation, sense in the vicinity of the target. The cells would then arrest on the subendothelial basement membrane and invade this structure following the gradient of chemical signal from the target. CNS, bone marrow, and node metastases are a frequent complication of SCCL and other malignancies, such as breast and hemopoietic cancers; tumor spread to these and other body sites may be controlled, in part, by local neuropeptide release. Wound (27) and inflammatory (28) sites, such as occur in smokers' lung, also seem to be preferential metastatic sites and local neuropeptide release are features of these lesions as well. Experimental *in vivo* administration of chemotactic peptides (29) to animals bearing circulating tumor cells resulted in localization of tumors to the injection site.

Neuropeptides are shown by these results, and others, to mediate macrophage and some tumor cell migration. We propose that neuropeptides, therefore, may have a general role in histogenesis and tissue organization, serving to recruit and/or maintain resident marcrophage and other cell populations. Disseminated neoplastic diseases such as SCCL may, to some extent, develop as a result of dysfunctions in neuropeptide regulated cell trafficking. Neuropeptides, released in the CNS or the body, in response to cognitive or other stimuli may modify these migratory patterns and thereby alter the course and progression of disease (30).

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