The EMBO Journal vol.9 no.11 pp.3603-3609, 1990

Drosophila neurotactin mediates heterophilic cell adhesion

Yves Barthalay[1], Régine Hipeau-Jacquotte[1], Santiago de la Escalera[2], Fernando Jiménez[2] and Michel Piovant[1,3]

[1]Laboratoire de Génétique et Biologie Cellulaire, CNRS, Case 907, 13288 Marseille Cedex 9, France and [2]Centro de Biologia Molecular, CSIC Universidad Autonoma de Madrid, 28049 Madrid, Spain

[3]Corresponding author

Communicated by J.Campos-Ortega

Neurotactin is a 135 kd membrane glycoprotein which consists of a core protein, with an apparent molecular weight of 120 kd, and of N-linked oligosaccharides. *In vivo*, the protein can be phosphorylated in presence of radioactive orthophosphate. Neurotactin expression in the larval CNS and in primary embryonic cell cultures suggests that it behaves as a contact molecule between neurons or epithelial cells. Electron microscopy studies reveal that neurotactin is uniformly expressed along the areas of contacts between cells, without, however, being restricted to a particular type of junction. Its putative adhesive properties have been tested by transfecting non adhesive *Drosophila* S2 cells with neurotactin cDNA. Heat shocked transfected cells do not aggregate, suggesting that neurotactin does not mediate homophilic cell adhesion. However, these transfected cells bind to a subpopulation of embryonic cells which probably possess a related ligand. The location at cellular junctions between specific neurons or epithelial cells, the heterophilic binding to a putative ligand and the ability to be phosphorylated are consistent with the suggestion that neurotactin functions as an adhesion molecule.

Key words: cell adhesion/*Drosophila*/neurogenesis/phosphorylation/transfection

Introduction

The importance of cell adhesion in morphogenetic processes has long been appreciated by developmental biologists and neurobiologists. Models involving specific forms of adhesion can explain the sorting out of cells within aggregates, the release of mesenchymal cells from epithelial structures during the formation of the neural crest, the directed migration of cells, the selective fasciculation of axons and the guidance of growth cones by cellular or substratum bound cues. The recent detailed analysis of the structure of these molecules has shown that they are generally more complex than one could have anticipated (Lander, 1989).

First, the extracellular domain of adhesion molecules has multifunctional binding specificities. Second, cell surface adhesion molecules also have cytoplasmic domains whose size is variable, generally shorter than 100 amino acids (except for the β4 integrin, Hogervorst *et al.*, 1990) and which are very well conserved within one class of molecules (cadherins). These domains are not simple devices to anchor the extracellular domain into the lipid bilayer, since for example, the removal of the cytoplasmic domain suppresses the adhesive properties of E-cadherin (Nagafuchi and Takeichi, 1988). Moreover, they are generally under precise developmental control in specific tissues as is the case for N-CAM (Barbas *et al.*, 1988). All these observations lead to the conclusion that these molecules have roles other than mediating adhesion. For instance, N-CAM molecules are able to modify the levels of choline acetyltransferase activity in chick embryonic sympathetic neurons (Acheson and Rutishauser, 1988). They can also modulate the intracellular levels of the inositol phosphates IP2 and IP3 in PC12 rat pheochromocytoma cell line (Schuch *et al.*, 1989). All these processes might be promoted either by cell surface molecules via linker proteins (Ozawa *et al.*, 1989; Nagafuchi and Takeichi, 1989) or directly by the cytoplasmic domain of cell adhesion molecules (Hogervorst *et al.*, 1990), both events resulting in the triggering of a cascade of reactions that lead to the final response. Investigation of molecules that mediate cell-cell and cell-substratum interactions is one major approach to the understanding of the developmental biology and it is useful to add these molecules to our repertoire even if their physiological functions are not always clear.

As described in the preceding article by De la Escalera *et al.* (1990) and as also reported by M.Hortsch and co-workers (submitted), neurotactin is an illustration of a new class of transmembrane glycoprotein which consists of a 503 amino acid extracellular domain related to cholinesterases and a 323 amino acid cytoplasmic domain containing several putative phosphorylation sites and exhibiting no significant homology with any known protein. The expression pattern of neurotactin during embryogenesis, in larval CNS and in *in vitro* cultures is consistent with the hypothesis that neurotactin could play a role in cell adhesion.

In order to demonstrate this hypothesis, we transfected *Drosophila* S2 cells with neurotactin cDNA under the control of a heat shock promoter. We show that after induction, neurotactin is capable of mediating cell adhesion in a heterophilic manner. Therefore neurotactin is the first example of a cell-cell contact molecule related to the serine esterase family which could play a role in cell adhesion as demonstrated by using cell transfection and *in vitro* binding assays.

Results

Neurotactin is an integral membrane protein

Antibody staining of tissue sections with MAbE1C which is specific for neurotactin, and the reactivity of the immunopurified antigen with concanavalin A (conA) had previously suggested that neurotactin is a membrane glycoprotein (Piovant and Léna, 1988). In order to confirm this property, embryos were fractionated according to Venkatesh *et al.*

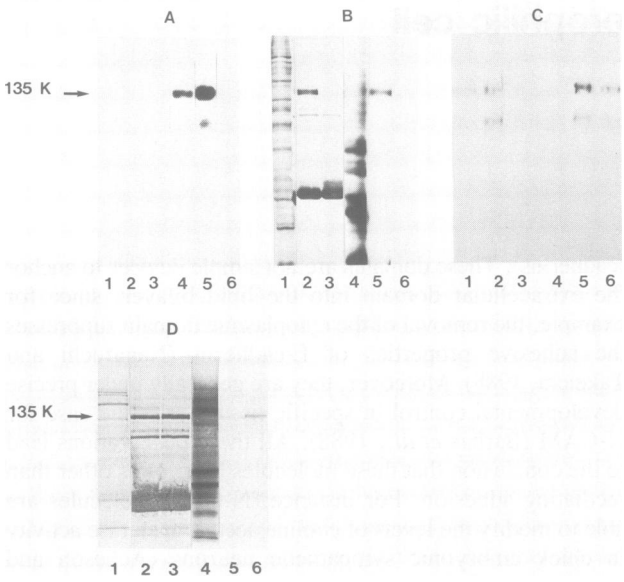

Fig. 1. Neurotactin is a phosphorylated integral membrane protein. (**A**) Western blot assays carried out on membrane fractions. Crude membranes (lanes 1 and 4), purified membrane fraction after ultracentrifugation on sucrose gradient (lanes 2 and 5), 100 000 *g* supernatant fraction (lanes 3 and 6). Lanes 1, 2 and 3 were incubated without primary antibody. Specific binding of MAbE1C is shown in lanes 4, 5 and 6. (**B**) Intact embryonic cells (10^8) were iodinated with lactoperoxidase, washed, solubilized and immunoprecipitated. Precipitates were analyzed by SDS−PAGE, blotted onto nitrocellulose and the blots were exposed to X-ray film. Lane 1: total proteins, lane 2: immunopurified neurotactin, lane 3: control showing primary antibody molecules. In lanes 4 and 5 the same blots as in 1 and 2 respectively were stained with conA−HRP. Lane 6: control without primary antibody. (**C**) Membrane fractions were homogenized and extracted in Triton X-114 as described in Materials and methods. Aliquots of the different phases were TCA precipitated, analyzed by SDS−PAGE, blotted onto nitrocellulose and probed with MAbE1C. Lane 1: remaining aqueous phase after all the extractions, lanes 2, 3 and 4: first, second and third extractions with 1% Triton X-114, lanes 5 and 6, first and second extractions with 2% Triton X-114. (**D**) Phosphorylation of neurotactin in intact embryonic cells. Lane 1: total proteins, lane 2: alkaline phosphatase treated immunoprecipitate, lane 3: untreated immunoprecipitate (4×10^7 cells per lane). Lanes 1, 2 and 3 are stained with con A−HRP and lanes 4, 5 and 6 represent the same blots exposed to X ray film.

(1980) and membrane fractions were probed on Western blots with MAbE1C. Neurotactin was predominantly found in the purified membrane fraction and could not be detected in the 100 000 *g* supernatant fraction containing soluble proteins (Figure 1A).

This fractionation procedure however, does not distinguish between peripheral and integral membrane proteins. Membrane fractions were therefore extracted with Triton X-114. This detergent is water soluble at temperatures below 22°C but above this temperature it will form a detergent rich pellet phase after centrifugation (Bordier, 1981). Integral membrane proteins will partition into the detergent rich pellet whereas soluble proteins remain in the upper aqueous phase. Neurotactin was collected into the detergent phase (Figure 1C), indicating that it has a hydrophobic domain and is therefore probably a transmembrane protein. By contrast, neurotactin was not released from membrane vesicles by washing either at high pH or with high salt buffers (data not shown), suggesting that it is not a typical peripheral membrane protein. Finally, intact embryonic cells were prepared from gastrulating embryos and surface proteins of these cells were labeled with [^{125}I] iodine. Immunoprecipitation experiments carried out on these cells show that the precipitate contains labeled neurotactin leading to the conclusion that the molecule has an extracellular domain (Figure 1B).

Neurotactin is phosphorylated in intact embryonic cells

The analysis of the cDNA sequence reveals the presence of several potential phosphorylation sites in the neurotactin cytoplasmic domain (De la Escalera *et al.*, 1990). Metabolic labeling of the protein was attempted by incubating embryonic cells in the presence of [^{32}P]orthophosphate. The analysis of immunoprecipitation products by SDS−PAGE, followed by electroblotting and autoradiography shows that the only ^{32}P-labeled protein is the mature neurotactin (Figure 1D). This label is sensitive to alkaline phosphatase suggesting that the phosphate is covalently bound. The characterization of a phosphorylated form(s) suggests that the function of neurotactin is regulated and may occur in a cascade of events dealing with cell−cell contacts.

Neurotactin is preferentially expressed at junctions between cells

To study the localization of neurotactin in the membrane, embryonic cells and third instar larval CNS were examined by light and electron microscopy (LM and EM). The ventral surface of the thoracic ganglion and the optic lobes are strongly immunoreactive (Figure 2A). They correspond to superficial clusters of cells, each of them constituted by an apical neuroblast and its progeny (Figure 2B,C). Each cluster projects axons towards the neuropile which itself is not stained. EM study confirms that neurotactin is localized in the plasma membrane, more intensely at the contacts between cells where it exhibits a rather uniform distribution without being restricted to a specific type of junction (Figure 2D,E). In an immunoreactive junction, membranes are closely apposed and exhibit a heavy staining (Figure 2E) while in the same section, a non-reactive junction shows a wider intercellular space (Figure 2F). The antigen is also detected in the cytoplasm, probably associated with membrane vesicles especially around the RER and the Golgi.

Since the antigen is expressed rather uniformly at gastrula stage, embryonic cells were used to study the cellular location of neurotactin in two *in vitro* systems. Embryonic cells, obtained by mechanical dissociation of gastrula stage embryos can either undergo differentiation or aggregation depending on the culture conditions. When cells are allowed to attach to a substrate, they divide and differentiate after 18 h mainly in two cell types: neurons and myocytes (Salvaterra *et al.*, 1987). Neurotactin exhibits a tissue specificity for the neuronal type cells, and is also expressed on cellular bodies and neurites. Within a group of neurons the antigen segregates preferentially at the intercellular junctions (Figures 3A and 4). The staining appears at contact points when membranes are closely apposed within a neuronal type cluster (Figure 4A,B). By contrast if the embryonic cells are maintained in suspension on a roller, they aggregate within 30 min and this phenomenon is calcium dependent (Gratecos *et al.*, 1990). Neurotactin is again preferentially accumulated at intercellular junctions in the aggregates whereas in isolated cells it is expressed all around the surface. Patches of labeling at one pole of the cell could also be observed occasionally (Figure 3B,C).

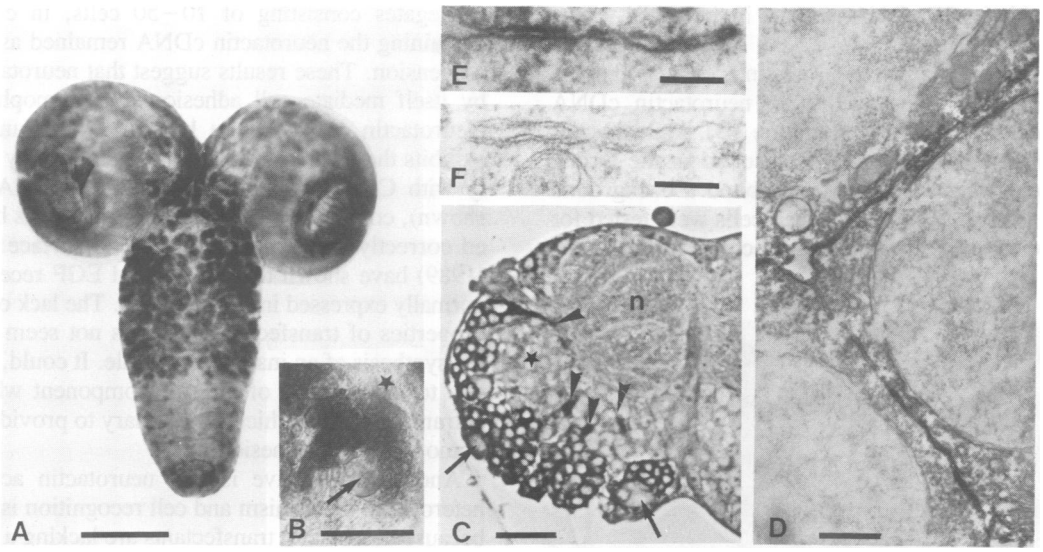

Fig. 2. Ultrastructural localization of neurotactin in third instar larval CNS. **(A)** Ventral view of the CNS (whole mount). Prominent labeling of cell clusters on thoracic and optic lobes regions. Bar represents 100 μm. **(B)** Enlarged view of (A). A large neuroblast (arrow) and its progeny curving around it. Unstained neurons are marked with a star. Bar represents 10 μm. **(C)** Semi-thin cross section of the ventral ganglion in the thoracic region. Ventral and latero-ventral superficial clusters of cells are each associated with an apical neuroblast (arrows). From each cluster protrudes an axon fascicle (arrowheads) in the direction of the neuropile (n) where these axons separate from each other and do not express neurotactin anymore. Unstained neurons are marked with a star. (Signal intensity was increased by TCH−osmium reaction.) Bar represents 20 μm. **(D)** Ultrathin section through the ventral ganglion. Three cells issuing from the same parent neuroblast. Bar represents 0.5 μm. **(E,F)** Comparison of junctions between two reactive (E) and two non reactive (F) neuronal cells of the same section. Bar represents 0.2 μm. The antibodies used were MAbE1C (A−C) and anti-neurotactin polyclonal antibody (D−F).

Previous results have shown that the antigen is associated with the differentiation of the precursors of the photoreceptor cells which occurs behind the morphogenetic furrow in the eye imaginal disc (Piovant and Léna, 1988). During this process the antigen is preferentially localized between the cells when they reach their final position within the epithelium, suggesting that it could accumulate at the cellular junctions. EM immunolocalization reveals that neurotactin is not restricted to specific junctions such as adherens junctions or septate junctions which are very abundant in this tissue (data not shown).

In conclusion, the preferential localization of neurotactin within intercellular junctions is observed *in vitro* (embryonic cell aggregates and differentiated neuron clusters) and is comparable with that observed *in vivo* (larval CNS and in the eye disc).

Neurotactin mediates heterophilic cell−cell recognition between transfected S2 cells and embryonic cells

Several cell lines were analyzed for the expression of the antigen by Western blot analysis and immunoprecipitation assays. 1182 haploid (Debec and Abbadie, 1989) and Kc (Echalier and Ohanessian, 1969) lines show relatively high levels of neurotactin, although they do not display any adhesive properties. By contrast, neurotactin is not detected in S2 cells. We have used a neurotactin cDNA to study the function of the protein by gene transfection techniques (Snow *et al.*, 1989). Briefly, we examined the effects of neurotactin expression on the behavior of a transfected S2 *Drosophila* cell line. The cDNA encoding neurotactin was inserted in the pHT4 vector (Schneuwly *et al.*, 1987) in both translational orientations relative to the heat shock promoter. These constructs were cotransfected into the S2 cell line with the pPC4 plasmid which encodes a *Drosophila* α-amanitin resistant RNA polymerase II gene (Jokerst *et al.*, 1989).

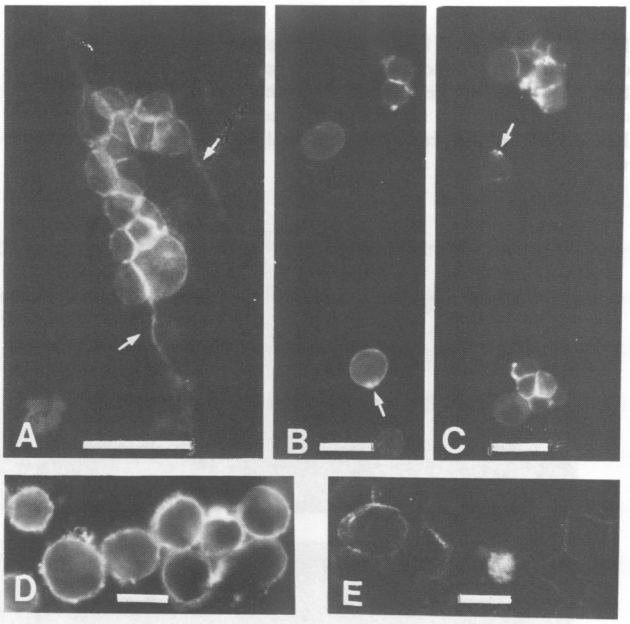

Fig. 3. *In vitro* expression of neurotactin in embryonic and transfected cells. **(A)** *In vitro* neuronal differentiation of embryonic cells after 18 h of culture. Anti-neurotactin polyclonal antibody stains cell bodies and neurites (arrows). **(B,C)** Polarized labeling by MAbJB10 occurs in some isolated embryonic cells (arrows) and displays an intensification at the junctions between the embryonic cells in aggregates. **(D,E)** MAbJB10 staining of heat shocked transfected S2 cells after recovery. Transfections were performed with neurotactin cDNA inserted in the correct (D) or the incorrect (E) transcriptional orientations into the pHT4 vector. Bar represents 5 μm.

Cells expressing this polymerase and presumably neurotactin were then selected by adding α-amanitin to the transfected cells. As a control assay we performed a transfection

experiment with fascIII cDNA inserted in the pHT4 vector (Snow *et al.*, 1989).

After α-amanitin selection, neurotactin is expressed at the surface of S2 cells transfected with neurotactin cDNA inserted in the correct orientation (Figure 3D), while S2 cells transfected with neurotactin cDNA inserted in the wrong orientation (control transfectants) exhibited a background fluorescence (Figure 3E). Transfected cells were tested for their adhesive properties: fascIII transfectants formed large aggregates consisting of 10–50 cells, in contrast, cells containing the neurotactin cDNA remained as a single cell suspension. These results suggest that neurotactin does not by itself mediate cell adhesion in a homophilic manner. Neurotactin produced by heat shocked transfected cells exhibits the expected electrophoretic mobility and reactivity with ConA and with anti-neurotactin MAbs (data not shown), confirming that the glycoprotein has been processed correctly and exported at the cell surface. Snow *et al.* (1989) have shown that fascIII and EGF receptor are also normally expressed in such systems. The lack of aggregative properties of transfected cells does not seem to be due to the synthesis of an inactive molecule. It could, however, be due to the absence of another component which was not cotransfected and which is necessary to provide an efficient homophilic cell adhesion.

Another alternative is that neurotactin acts through a heterophilic mechanism and cell recognition is not achieved because neurotactin transfectants are lacking a ligand which is not expressed in S2 cells. To test this hypothesis, cellular binding assays were performed with embryonic cells which may possess such a ligand. Transfectants were grown to confluency on slideflasks and heat shocked. After recovery, the transfected cells were incubated for 30 min with embryonic cells stained with methylene blue. On the one hand, the amount of unbound cells was evaluated in the medium and washes: 40% of the original embryonic cells exhibit a binding activity to neurotactin transfectants while in the case of control transfectants this value drops to < 10% (in this latter case, most embryonic cells were bound to the plastic, exhibiting no specificity for neurotactin transfectants). On the other hand, several fields were examined in each case with an image analysis program, in order to measure the average number of embryonic blue stained cells among the total number of cells present in a given field

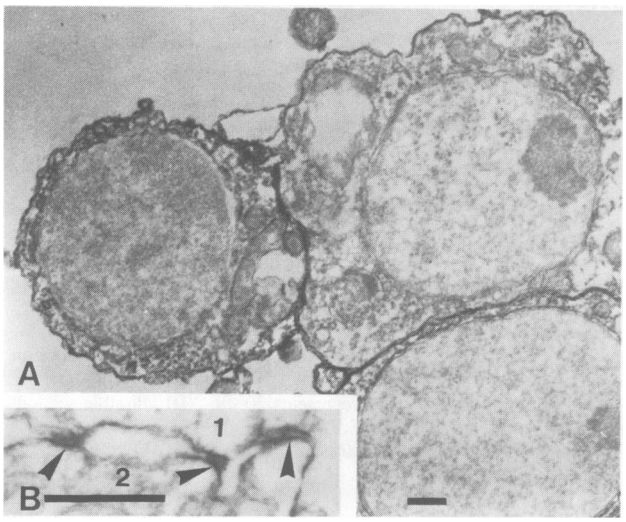

Fig. 4. Ultrastructural localization of neurotactin in primary embryonic cell clusters. (**A**) Cluster of neuronal type cells in close contact. Bar: 1 μm. (**B**) Two cells (1 and 2) loosely bound within another cluster, are expressing neurotactin in three contact points (arrowheads). Bar: 0.5 nm. In both cases the polyclonal antibody against neurotactin was used.

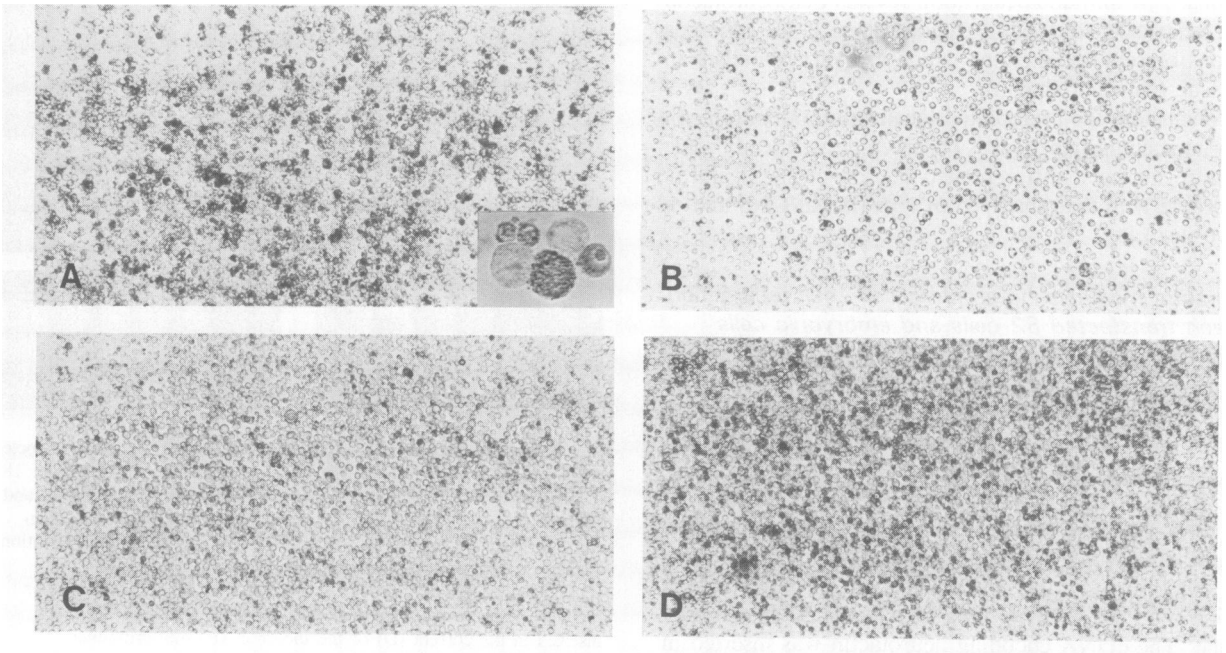

Fig. 5. Heterophilic binding of embryonic cells to neurotactin transfectants. (**A**) Methylene blue stained embryonic cells bound to S2 cells transfected with neurotactin cDNA in the correct orientation. Insert: an example of heterophilic junctions between two S2 and four embryonic cells. (**B**) Control experiment for (A) with S2 cells transfected with neurotactin cDNA in the reverse orientation. (**C**) Inhibition of heterophilic adhesion by anti-neurotactin antibodies. Neurotactin transfectants were incubated for 1 h with polyclonal antibodies raised against neurotactin electroeluted from a polyacrylamide gel then embryonic cells stained with methylene blue were added to the incubation medium. (**D**) Control experiment for (B) using non-immune polyclonal antibodies.

(Figure 5). In these conditions the percentage of embryonic cells bound on neurotactin transfectants was 14.1 ± 1.4% (n = 20) (Figure 5A) while it was only 2.7 ± 0.5% (n = 15) (Figure 5B) for control transfectants. A longer incubation time (1 h) or gentle shaking did not increase those percentages indicating that the amount of the ligand expressed by embryonic cells is controlling the interaction with the transfectants. Moreover, the unbound cells recovered in the supernatant were not able to bind to newly transfected cells when incubated a second time with such cells. This result indicates that only a subset of the embryonic cell population expresses the ligand. This heterophilic recognition is mediated by neurotactin since it is specifically inhibited by the presence of anti-neurotactin antibodies (Figure 5C,D). Preliminary experiments using EDTA or temperatures below 10°C suggest that this process is independent from the presence of Ca^{2+} and requires membrane fluidity.

Discussion

In this paper we have described the biochemical characterization and the cellular localization of neurotactin, a cell contact molecule which mediates adhesion between embryonic cells. Neurotactin is a cell surface glycoprotein that is expressed in membranes during embryogenesis and then accumulates in CNS and PNS (Piovant and Léna, 1988; De la Escalera *et al.*, 1990). Neurotactin represents a novel cell adhesion molecule that does not belong to any of the previously described families of vertebrate molecules although its extracellular domain is related to cholinesterases (De la Escalera *et al.*, 1990). This homology may define a new class of molecules in which thyroglobulin (Swillens *et al.*, 1986) and glutactin (Olson *et al.*, 1990) may be included.

The biochemical properties of neurotactin are in good agreement with the amino acid sequence deduced from the cDNA (De la Escalera *et al.*, 1990). Taken together all these data demonstrate that neurotactin is an integral membrane glycoprotein with only one transmembrane spanning domain. Chemical deglycosylation shows that neurotactin consists of a core protein of an apparent mol. wt of 120 kd (data not shown) which is compatible with the mol. wt of 93 kd given by the sequence data. The electrophoretic migration of the molecule in the absence of reducing agents suggests that some of the cysteine residues must participate in the formation of intrachain disulfide bonds. As predicted from the amino acid sequence which contains several putative phosphorylation sites, neurotactin is phosphorylated in embryonic cells. The study of the tissue expression of neurotactin in the embryo and in larvae (Piovant and Léna, 1988; De la Escalera *et al.*, 1990) suggested that the protein may be involved in cell−cell contacts in several tissues.

By transfecting S2 non adhesive cells with neurotactin cDNA it has been demonstrated that neurotactin expression in S2 cells is not able to promote aggregation in the same manner as the fasciclin III molecule (Snow *et al.*, 1989). This probably means that neurotactin does not interact with itself in a homophilic manner. The lack of ability of the cells to aggregate does not seem to be due to the synthesis of a defective protein by the transfected cells, and/or to the absence of another component required for the proper adhesive function which would not have been co-transfected. The possibility that neurotactin could be associated with another protein to perform S2 cell homophilic aggregation is not likely since 30% of embryonic cells prepared from gastrula stage embryos are able to bind to neurotactin transfected cells. In fact this result suggests that neurotactin binds, in an heterophilic manner to a putative ligand which is expressed by a subpopulation of embryonic cells. This is another example of an invertebrate heterophilic cell−cell recognition molecule. Recently Fehon *et al.* (1990) have demonstrated by using inducible expression in S2 cells that notch and delta transmembrane proteins bind to one another via their extracellular domains. Ligand−receptor associations of a cell adhesion molecule containing Ig like domains with an integrin have already been described in vertebrates (Marlin and Springer, 1987; Stauton *et al.*, 1989; Elices *et al.*, 1990). The fact that neurotactin does not belong to any type of cell adhesion molecule previously described makes the comparison with vertebrate molecules difficult. The recent characterization of a new β4 integrin chain (Suzuki and Naitoh, 1990; Hogervorst *et al.*, 1990) shows that cell adhesion molecules may have a large cytoplasmic domain instead of a short cytoplasmic tail. Neurotactin might be another example of such a molecule with a large cytoplasmic domain.

In the view of a heterophilic interaction, a putative ligand might be searched for amongst the molecules whose developmental expression patterns have already been described and are superimposable on the neurotactin pattern of expression. In this respect, De la Escalera *et al.* (1990) pointed out two candidates which are the notch protein, recently analyzed by two groups (Kidd *et al.*, 1989 and Johansen *et al.*, 1989) and the amalgam protein whose embryonic expression pattern (Seeger *et al.*, 1988) overlaps with neurotactin expression. These two candidates may be tested in a binding assay consisting of neurotactin transfected cells and amalgam or notch transfected cells. In the case of notch protein, the heterophilic binding with delta protein does not exclude the involvement of a third component. Otherwise a more extensive screening should be carried out by direct expression cloning using cell−cell recognition as a functional assay (Osborn *et al.*, 1989).

An interaction with a putative ligand which occurs at the cell surface may induce a conformational change of the cytoplasmic domain which may interact with a cytoplasmic component (cytoskeleton or soluble protein). The signal may also travel in the reverse direction. This has been suggested for the β4 integrin chain whose large cytoplasmic domain might play the role of a linker protein (Hogervorst *et al.*, 1990). Transfection experiments with truncated cDNAs might be useful for understanding whether these domains can function separately and/or whether the recognition and the signal transduction may be dissociated. Work is in progress in these directions to get more insights into the intimate function of the neurotactin molecule.

Materials and methods

Gel electrophoresis, protein blotting and detection of neurotactin

All these techniques were performed according to Piovant and Léna (1988). Alkaline phosphatase conjugated secondary antibodies (Promega) were also used.

Membrane preparation

Membranes were prepared according to Venkatesh *et al.* (1980). Unstaged embryos were homogenized in 10 mM Tris−HCl buffer pH 7.5, containing 0.5 mM $CaCl_2$, 0.25 M sucrose, 0.2% aprotinin and 0.5 mM PMSF (phenylmethylsulfonyl fluoride). The homogenate was filtered through a metallic grid and centrifuged at low speed to remove large debris. The

supernatant was centrifuged at 25 000 *g* to pellet a crude membrane fraction. This fraction was purified by centrifugation at 35 000 r.p.m. for 18 h on a discontinuous sucrose gradient in a SW41 swinging bucket rotor in a Beckman centrifuge. The efficiency of the fractionation procedure was followed by SDS−PAGE analysis.

Partitioning of neurotactin into a non ionic detergent phase

Partitioning of the antigen was carried out according to Bordier (1981). Membrane fractions were homogenized in a solution composed of 10 mM Tris, 0.5 mM $CaCl_2$ and 1% Triton X-114, incubated for 1 h at 0°C and layered on top of a 6% sucrose cushion containing 10 mM Tris, 0.5 mM $CaCl_2$, 0.05% Triton X-114. The mixture was equilibrated for 3 min at 0°C and the temperature was raised to 37°C for 3 min. The detergent and aqueous phases were separated by centrifugation and the aqueous phase could be extracted a second time with 2% Triton X-114. Aliquots of the different phases were precipitated with TCA, analyzed by SDS−PAGE and blotted onto nitrocellulose. The blots were probed with MAbE1C.

Cell surface labeling

For lactoperoxidase-catalyzed [^{125}I]-iodination of surface proteins, 10^8 embryonic cells were resuspended in 1 ml of Shield medium containing 10 mM glucose (Gratecos *et al.*, 1990). [^{125}I]Na (100 mCi/ml carrier free, Amersham, England) was added to a final concentration of 3−4 mCi/ml, followed by lactoperoxidase (from cow milk, Boehringer-Mannheim) and glucose oxidase (Grade I, Boehringer-Mannheim) to the final concentrations of 0.2 mg/ml and 4 μg/ml, respectively. The reaction mixture was incubated for 30 min at 4°C and the cells were washed three times with Shield medium containing 5 mM NaI and processed for immunoprecipitation assay.

Phosphorylation of neurotactin in intact embryonic cells

Embryonic cells were washed twice in buffer A (50 mM HEPES, 150 mM NaCl, pH 7.6) and resuspended in 2 ml of the same buffer (2×10^8 cells/ml). The intracellular ATP pool labeling was carried out by addition of 6 mCi of [^{32}P]orthophosphate (Amersham 370 MBq/ml, 1 mCi/ml) for 3 h at 25°C. The cells were washed once in buffer A containing phosphatase inhibitor (3 mM *o*-vanadate) and resuspended in lysis buffer (1.5% Triton X-100 in buffer A containing 0.1 mM PMSF, 100 mM NaF, 20 mM EDTA, and 3 mM *o*-vanadate). Immunoprecipitation was carried out as described in Piovant and Léna (1988). The immunoprecipitates were analyzed by SDS−PAGE and blotted onto nitrocellulose. Western blots were stained with con A and exposed to X-ray film.

Immunocytochemical localization

Three types of antibodies were used: MAbE1C (Piovant and Léna, 1988) which requires methanol and detergent permeabilization, MAbJB10 which reacts without permeabilization and a polyclonal serum directed against the crude immunoprecipitate.

The localization of the antigen was performed in the CNS from third instar larvae and on embryonic cells. Gastrula stage embryos (3.5−5 h) were mechanically dissociated; the cells were collected by centrifugation and resuspended in Schneider's *Drosophila* medium (Gibco) supplemented with 12% heat inactivated fetal calf serum (Seromed) and 100 μg/ml gentamicin (Gibco). For light microscopy, 10^6 cells in 2.5 ml of culture medium were plated on 9 cm^2 culture slideflasks (Nunc) and incubated at 25°C with air as the gas phase. After 18 h, these cells differentiated into two main cellular types: myocytes and neurons. For electron microscopy, 5×10^5 cells in 1 ml of culture medium were plated in each well of a 12-well microtiter plate (Flow Labs) and incubated at 25°C. After 18 h of differentiation the immunocytochemical staining was performed directly in the culture wells.

Light microscopy. Larval brains were dissected in Ringer, fixed either in 2 or 4% paraformaldehyde or in PLP (see below), permeabilized or not with methanol (1 min) and equilibrated in Dulbecco's modified Eagle medium. All following incubations and washes were carried out in the presence of 0.1% Triton X-100 or 0.01% saponin. After incubation in hybridoma supernatants or ascites fluid dilutions, the tissues were incubated in a biotinylated horse anti-mouse IgG (H + L) then in a preformed avidin-biotinylated horseradish peroxidase complex (Vector Labs) and finally in a peroxidase substrate solution to visualize the antigen (for details see Piovant and Léna, 1988). In a few cases, the intensity of the signal was increased by the thiocarbohydrazide (TCH)−osmium interaction (Hanker *et al.*, 1966) as described by Tomlinson and Ready (1987). Gastrula embryonic cells were incubated with a primary antibody: a rabbit polyclonal serum (1/500) or a mouse monoclonal antibody (ascites fluid, 1/500) followed by the secondary antibody: FITC-conjugated goat anti-rabbit IgG (H + L) antibody (Kirkegaard and Perry Labs) or a FITC-conjugated sheep anti-mouse IgG (H + L) antibody (Biosys) used at a dilution of 1/100.

Electron microscopy. A protocol of pre-embedding for ultrastructural immunocytochemistry was used. The periodate−lysine−paraformaldehyde fixative (PLP: 2% paraformaldehyde, 0.01 M $NaIO_4$, 0.075 M lysine, 0.037 M phosphate buffer) was chosen because it alters neither the antigenicity nor the ultrastructure (McLean and Nakane, 1974).

The primary antibody was the polyclonal serum with an immunoperoxidase staining (biotinylated sheep anti-rabbit IgG (H + L) and avidin peroxidase from Vector labs). After PLP fixation, larval brains or primary embryonic cell cultures were incubated and washed with 0.03% saponin as detergent. After dehydration, tissues or culture cells were embedded in Epon. Sections were examined in the electron microscope without further counterstaining. The detailed protocol used for the brains was similar to that described by Vila-Porcile *et al.* (1987) except for the fixative and the secondary antibody.

The staining of embryonic cells in culture was performed directly in the culture wells according to Tougard and Picart (1986). The method was modified by using the PLP fixative and a biotinylated secondary antibody.

Vector plasmid construction

The heat shock expression vector corresponds to the pHT4 vector constructed by Schneuwly *et al.* (1987) in which the unique *Kpn*I site was changed into a unique *Not*I site. The complementary DNA encoding neurotactin (neurotactin cDNA) was placed under the control of the heat shock (*hsp70*) promoter. The 3.1 kb *Eco*RI fragment from neurotactin cDNA containing the whole open reading frame (De la Escalera *et al.*, 1990) was made blunt ended with the Klenow fragment of *E.coli* DNA polymerase I and cloned into the vector pHT4, which was linearized with *Not*I, made blunt ended with the Klenow fragment and recircularized using T4 DNA ligase.

Drosophila cell culture and transfection

S2 cells (Schneider, 1972) were grown at 25°C with air as gas phase in complete Schneider's *Drosophila* medium. Transformations were carried out using DNA−Ca^{2+} coprecipitates prepared as described by Wigler *et al.* (1979). The procedure used was described by Snow *et al.* (1989); the plasmid pPC4 (Jokerst *et al.*, 1989) was the selectable marker confering α-amanitin resistance. For transformation assays, 10^6 cells/ml in 2.5 ml of complete medium were plated onto 25 cm^2 tissue culture flasks (Falcon) and incubated overnight.

One ml of DNA−Ca^{2+} phosphate coprecipitate containing 10 μg each of plasmid pPC4 and pHT4−neurotactin (see plasmid construction) was added dropwise to each flask and incubated for 15−18 h. For the following steps the protocol used was similar to that described by Snow *et al.* (1989).

Cellular binding assay

Neurotactin transfected cells were grown to confluence in complete Schneider's medium on slide flasks, heat shocked at 37°C for 15 min and allowed to recover for 1 h at 25°C. Visualization of neurotactin expression was carried out as described in the light microscopy section. Embryonic cells were stained with methylene blue (1 mg/ml in Shield medium free of Ca^{2+} and Mg^{2+}) for 10 min at 25°C, centrifuged and resuspended in Schneider's medium. This dye is not lethal, since stained cells undergo differentiation as well as unstained cells. 4×10^6 stained embryonic cells were spread onto neurotactin transfected cells and incubated for 30 min at 25°C without agitation. The medium was removed and the slide flasks were gently rinsed twice with Schneider medium. All the solutions were pooled and the number of unbound cells was evaluated. Cell fixation was carried out as described above and the slides were washed several times before mounting in 90% glycerol in PBS. The number of bound cells was evaluated by image analysis processing. The program used parameters dealing with the density of cells in a given field, the cell shapes, sizes, contrasts and colors. Control experiments were carried out with heat shocked S2 cells transfected with the neurotactin cDNA in the reverse orientation.

Acknowledgements

We thank C.Moretti for his expert technical assistance in parts of this project. We also thank M.Horstch for sharing sequence data before publication, A.Bieber for sending protocols concerning transfection experiments (originally from Dr Greenleaf), pHT4 and pHT4-fascIII plasmids, D.Gratecos and R.Rosset for their comments on the manuscript and E.Krejci and M.Sémériva for helpful and stimulating discussions. This work was supported by the Centre National de la Recherche Scientifique (CNRS), grant no. 862023 from the Institute National de la Santé et de la Recherche Médicale (INSERM) and grant no. 6450 from the Association de la Recherche sur le Cancer (ARC) to M.P., by grants of the Direccion general de Investigacion Cientifica (PB87-0433-CO2-02) to F.J. and by the scientific cooperation program between France and Spain.

References

Acheson,A. and Rutishauser,U. (1988) *J. Cell. Biol.*, **106**, 479–486.

Barbas,J.-A., Chaix,J.-C., Steinmetz,M. and Goridis,C. (1988) *EMBO J.*, **7**, 625–632.

Bordier,C. (1981) *J. Biol. Chem.*, **256**, 1604–1607.

Debec,A. and Abbadie,C. (1989) *Biol. Cell*, **67**, 307–311.

De la Escalera,S., Bockamp,E.-O., Moya,F., Piovant,M. and Jiménez,F. (1990) *EMBO J.*, **9**, 3593–3601.

Echalier,G. and Ohanessian,A. (1969) *C.R. Hebd. Séances Acad. Sci. Paris*, **268**, 1771–1773.

Elices,M.J., Osborn,L., Takada,Y., Crouse,C., Luhowskyj,S., Hemler,M.E. and Lobb,R.R. (1990) *Cell*, **60**, 577–584.

Fehon,R.G., Kooh,P.J., Rebay,I., Regan,C.L., Xu,T., Muskavitch,M.A.T. and Artavanis-Tsakonas,S. (1990) *Cell*, **61**, 523–534.

Gratecos,D., Krejci,E. and Sémériva,M. (1990) *Roux's Arch. Dev. Biol.*, **198**, 411–419.

Hanker,S.J., Deb,C., Wasserkrug,H.L. and Seligman,A.M. (1966) *Science*, **152**, 1631–1634.

Hogervorst,F., Kuikman,I., von dem Borne,A.E.G.Kr. and Sonnenberg,A. (1990) *EMBO J.*, **9**, 765–770.

Johansen,K.M., Fehon,R.G. and Artavanis-Tsakonas,S. (1989) *J. Cell. Biol.*, **109**, 2427–2440.

Jokerst,R.S., Weeks,J.R., Zehring,W.A. and Greenleaf,A.L. (1989) *Mol. Gen. Genet.*, **215**, 266–275.

Kidd,S., Baylie,M.K., Gasic,G.P. and Young,M. (1989) *Genes Dev.*, **3**, 1113–1129.

Lander,A.D. (1989) *Trends Neurosci.*, **12**, 189–195.

McLean,I.W. and Nakane,P.K. (1974) *J. Histochem. Cytochem.*, **22**, 1077–1083.

Marlin,S.D. and Springer,T.A. (1987) *Cell*, **51**, 813–819.

Nagafuchi,A. and Takeichi,M. (1988) *EMBO J.*, **7**, 3679–3684.

Nagafuchi,A. and Takeichi,M. (1989) *Cell Regulation*, **1**, 37–44.

Olson,P.F., Fessler,L.I., Nelson,R.E., Sterne,R.E., Campbell,A.G. and Fessler,J.H. (1990) *EMBO J.*, **9**, 1219–1227.

Osborn,L., Hession,C., Tizard,R., Vassalo,C., Luhowskyj,S., Chi-Rosso, G. and Lobb,R. (1989) *Cell*, **59**, 1203–1211.

Ozawa,M., Baribault,H. and Kemler,R. (1989) *EMBO J.*, **8**, 1711–1717.

Piovant,M. and Léna,P. (1988) *Development*, **103**, 145–156.

Salvaterra,P., Bournias-Vardiabasis,N., Nair,T., Hou,G. and Lieu,C. (1987) *J. Neurosci.*, **7**, 10–22.

Schneider,I. (1972) *J. Embryol. Exp. Morph.*, **27**, 353–365.

Schneuwly,S., Klemenz,R. and Gehring,W.J. (1987) *Nature*, **325**, 816–818.

Schuch,U., Lohse,M.J. and Schachner,M. (1989) *Neuron*, **3**, 13–20.

Seeger,M.A., Haffley,L. and Kaufman,T.C. (1988) *Cell*, **55**, 589–600.

Snow,M.S., Bieber,A.J. and Goodman,C.S. (1989) *Cell*, **59**, 313–323.

Stauton,D.E., Dustin,M.L. and Springer,T.A. (1989) *Nature*, **339**, 61–64.

Suzuki,S. and Naitoh,Y. (1990) *EMBO J.*, **9**, 757–763.

Swillens,S., Ludgate,M., Mercken,L., Dumont,J.E., Vassart,G. (1986) *Biochem. Biophys. Res. Commun.*, **137**, 142–148.

Tomlinson,A. and Ready,D.F. (1987) *Dev. Biol.*, **120**, 366–376.

Tougard,C. and Picart,R. (1986) *Am. J. Anat.*, **175**, 161–177.

Venkatesh,T.R., Zingde,S. and Krishnan,K.S. (1980) In Siddiqi,O., Babu,P., Hall,L.M. and Hall,J.C. (eds), *Development and Neurobiology of Drosophila*. Basic Life Sciences. Vol. 16, Plenum Press, New York and London, pp. 313–321.

Vila-Porcile,E., Picart,R., Tixier-Vidal,A. and Tougard,C. (1987) *J. Histochem. Cytochem.*, **35**, 287–299.

Wigler,M., Pellicer,A., Silverstein,S., Axel,R., Urlaub,G. and Chasin,L. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1373–1376.

Received on June 19, 1990; revised on July 31, 1990