The EMBO Journal vol.9 no.11 pp.3593-3601, 1990

Characterization and gene cloning of neurotactin, a *Drosophila* transmembrane protein related to cholinesterases

Santiago de la Escalera[1], Ernst-Otto Bockamp[1], Fernando Moya[1,3], Michel Piovant[2] and Fernando Jiménez[1,4]

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

[3]Present address: Departamento de Genética Molecular y Microbiología, Universidad de Alicante, San Juan, Alicante, Spain

[4]Corresponding author

Communicated by J.Modolell

Monoclonal antibodies have served to characterize neurotactin, a novel *Drosophila* protein for which a role in cell adhesion is postulated. Neurotactin is a transmembrane protein, as indicated by epitope mapping and amino acid sequence. Similarly to other cell adhesion molecules, neurotactin accumulates in parts of the membrane where neurotactin-expressing cells contact each other. The protein is only detected during cell proliferation and differentiation, and it is found mainly in neural tissue and also in mesoderm and imaginal discs. Neurotactin has a large cytoplasmic domain rich in charged residues and an extracellular domain similar to cholinesterases that lacks the active site serine required for esterase activity. The extracellular domain also contains three copies of the tripeptide leucine-arginine-glutamate, a motif that forms the primary sequence of the adhesive site of vertebrate s-laminin.

Key words: cell adhesion/cholinesterases/*Drosophila*/LRE motif/neurotactin

Introduction

The ability of cells to adhere selectively to other cells is a key factor in the regulation of the morphogenetic events that take place during embryogenesis. These events range from changes in cell shape to segregation of different cell types, cell rearrangements and migration, selective fasciculation of axons, etc. Cell adhesion is a complex phenomenon, both in its structural and functional aspects, mediated by specialized molecules of different classes that are known under the generic name of adhesion molecules. Most of these molecules display complex dynamic spatio-temporal patterns of expression; they are usually expressed in a variety of tissues and any given cell type may simultaneously express more than one type of them. The emerging concept from the studies on vertebrate adhesion molecules is that the adhesive properties of individual cells result from specific combinations of several of those molecules that may change in the course of development. It is also generally believed that the activity of adhesion molecules goes beyond the mere provision of external contact sites, and that cell adhesion can trigger regulatory mechanisms operating possibly through the cytoplasmic domains present in some cell adhesion molecules (for reviews see, Edelman, 1986; Jessell, 1988; Lander, 1989; Rutishauser and Jessell, 1988; Takeichi, 1988).

Structural homologs of all major classes of known vertebrate adhesion molecules have already been identified in invertebrates, and in some instances the corresponding genes have been isolated. Thus, for example, homologs have been found in *Drosophila* for vertebrate laminin B1 (Montell and Goodman, 1988), the α and β subunits of integrins (Bogaert *et al.*, 1987; MacKrell *et al.*, 1988) and neural adhesion molecule L1 (Bieber *et al.*, 1989). Fasciclin III, a *Drosophila* membrane glycoprotein that mediates cell adhesion has, on the contrary, no significant homology to any known vertebrate adhesion molecule (Snow *et al.*, 1989). *Drosophila* provides a powerful experimental system to study cell adhesion and its regulation within the intact animal by means of genetic manipulation. In fact, the analysis of mutations in genes encoding adhesion molecules has been initiated (Elkins *et al.*, 1990).

In the present study we describe the characterization and gene cloning of neurotactin, a novel transmembrane protein of *Drosophila*. Several of its properties, particularly aspects of the expression pattern and of the amino acid sequence, indicate that neurotactin can be considered a cell adhesion molecule. The accompanying article by Barthalay *et al.* (1990) further demonstrates that neurotactin is able to mediate heterophilic cell-cell adhesion *in vitro*.

Results

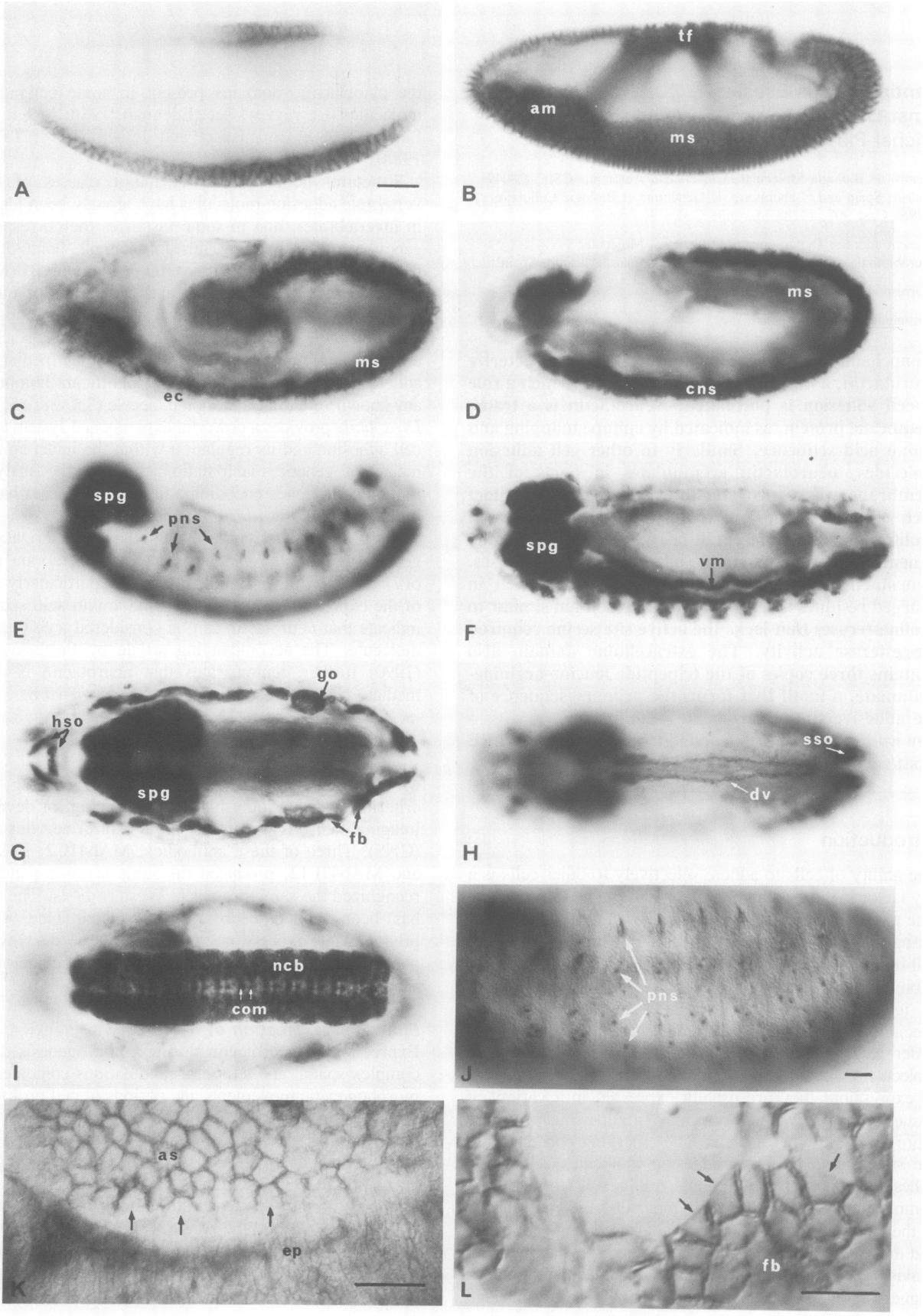
Monoclonal antibodies were isolated according to the criterion that they only bind to the fraction of developing imaginal neurons within the larval central nervous system (CNS). Three of these antibodies, MAb41C2, MAb43B5 and MAb47F12, produced the same staining pattern and recognized the same protein in Western blots. The protein has been named neurotactin, in accordance with its predominant expression in neural tissue and with its postulated function in cell adhesion (see below and accompanying paper by Barthalay *et al.*, 1990).

Embryonic expression of neurotactin

Expression of neurotactin during embryogenesis displays complex spatial and temporal modulations coincident with morphogenetic movements and periods of cell proliferation and differentiation of certain tissues. Neurotactin is first detected in the cell membrane at two regions in the blastoderm [stage 5 according to Campos-Ortega and Hartenstein (1985)] (Figure 1A). By the onset of gastrulation, its expression extends throughout the whole embryo, with the exception of the pole cells (Figure 1B). High levels persist in mesoderm and mesectoderm during stages 9-10 (Figure 1C), to decay later until a new wave of expression appears in the visceral mesoderm during stage 13. Neuro-

tactin accumulates strongly in the proliferating CNS during stages 10 and 11 (Figure 1D). Both neuroblasts and their progeny express it, although the latter do so more intensely. We frequently observed a preferential accumulation of the protein in neuroblast surfaces that are in contact with adjacent neural cells, as compared to those in contact with the underlying epidermis (not shown).

As mesodermal differentiation proceeds, the protein is



expressed in this layer in a differential manner. Whereas the somatic muscles never show it at detectable levels, a transient accumulation is observed in the visceral musculature during stage 13, to decrease gradually until it is barely detectable at stage 16 (Figure 1F). Other mesodermal derivatives, such as the fat body and the dorsal vessel, also exhibit an accumulation of neurotactin during their differentiation that persists until larval hatching (Figure 1G,H). Similarly to the situation in neuroblasts, neurotactin concentrates on the membrane of fat body cells where these cells contact one another (Figure 1L). Neurotactin is present in the amnioserosa from early on. When the amnioserosa stretches out, following germ band shortening, it can be seen that amnioserosa cell membranes in contact with adjacent epidermal cells are not labeled (Figure 1K).

In the CNS, high levels of neurotactin expression continue during neural cytodifferentiation (Figure 1E–G,I). However, staining of motor axons, as they leave the ventral cord, is rather weak. The presence of neurotactin in the CNS remains unchanged until the first larval instar. During stage 13, the early uniform staining in the ectoderm disappears and now concentrates in several structures of the peripheral nervous system. These comprise the head sensory organs, the spiracle sensory organ in the tail region, and a single cell cluster per segment in the trunk (Figure 1E,G,H). Closer observation reveals the preferential accumulation of neurotactin on apposed cell membranes within the clusters (not shown). By stage 16, weak staining of other sensory organs can be detected in thoracic and abdominal segments (Figure 1J).

It is worth mentioning that the embryonic expression pattern of neurotactin is very reminiscent of that reported for amalgam, a *Drosophila* membrane protein structurally homologous to the neural adhesion molecules of the immunoglobulin superfamily (Seeger *et al.*, 1988). Neurotactin and amalgam share the same expression pattern in mesoderm, peripheral and central nervous systems. However, early expression of amalgam seems to be restricted to the mesoderm. Also, it has not been detected in neuroblasts.

Postembryonic expression of neurotactin

Neurotactin decays rapidly after larval hatching, but appears again soon in the CNS. It is first detected in the proliferating optic centers (Figure 2A); later, it is also found in neuron clusters and their associated axons in the ventral cord (Figure 2B). According to their location and time course of staining, these clusters should correspond to the cells resulting from the mitotic activity of neuroblasts during larval life (Truman and Bate, 1988). Thus neurotactin after disappearing from neurons with embryonic origins, is expressed again in dividing imaginal neuroblasts and their progeny. Neurotactin persists in imaginal neurons during the first half of the pupal period, as they complete their maturation, and then disappears gradually, with no detectable levels of staining found in the brain of the adult fly (not shown).

Imaginal discs also express neurotactin during the third larval instar. For example, it is expressed in the photoreceptors of the eye disc (Figure 2B). Furthermore, the number of stained photoreceptors within each ommatidium increases sequentially behind the morphogenetic furrow (not shown, but see Piovant and Léna, 1988), mirroring the ordered sequence in which they adopt their final developmental pathways and initiate cytodifferentiation (Tomlinson and Ready, 1987). In the wing disc, high levels of neurotactin are observed in a row of cells which will give rise to the wing margin (Figure 3A). Alternate positive and negative territories of staining can also be detected at both sides of the margin. Their nature can be determined by following the staining pattern in the everting wing (Figure 3B–D). Thus, territories devoid of staining correspond to the prepupal veins (Waddington, 1940), whereas the intervein regions express neurotactin.

Biochemical characterization of neurotactin

The three monoclonal antibodies used for the histological characterization of neurotactin recognize a single protein of 135 kd on Western blots, as determined by SDS–PAGE in the presence of reducing agents (Figure 4). Under non-reducing conditions, the protein migrates somewhat faster (not shown), indicating that it is not covalently linked to other subunits or membrane components, and that it contains intramolecular disulfide bonds (Wilcox, 1986). The temporal expression pattern of neurotactin, as deduced from Western blots, is in agreement with that found by immunocytochemistry, with two peaks of expression at embryogenesis and early pupation, accumulation in late third instar larval heads and low levels of expression in adults.

Cloning and chromosome mapping of the neurotactin gene

A collection of homologous cDNA clones was isolated from two expression libraries. The insert of clone HD9 (2.3 kb) hybridized to larval polytene chromosomes at cytological location 73C1-2 on the third chromosome. Analysis of

Fig. 1. Embryonic expression of neurotactin. Embryos were stained with MAb41C2, which binds to a cytoplasmic epitope. Anterior is to the left and dorsal is at the top. (A–E) and (J–L) are sagittal, (G–I) horizontal and (F) parasagittal views. (**A**) Cellular blastoderm shortly before ventral furrow formation. Neurotactin is expressed along a ventral stripe which will invaginate to produce the mesoderm, and dorsally in the prospective transversal furrows. (**B**) Stage 7 embryo. The staining is now more broadly distributed, but exhibits the highest levels in mesoderm (ms), anterior midgut (am) and transversal furrows (tf). (**C**) Stage 9 embryo. Higher levels of protein are still found in mesoderm, as compared with ectoderm (ec). (**D**) An embryo at the beginning of germ band shortening. Accumulation of neurotactin in the proliferating central nervous system (cns) is evident, and its presence in mesoderm has substantially decreased as compared with stages 9–10. (**E**) Stage 13 embryo (shortened germ band) focused on the lateral surface. Staining is found in the supraesophageal ganglia (spg) and in a single set of sensory organs in the peripheral nervous system (pns), along the trunk. Note that the labeled sensory organ in the second and third thoracic segments is located more ventrally, as compared with other segments. (**F**) An embryo of a similar age to that in (E) showing the transient expression of neurotactin in the visceral mesoderm (vm) that surrounds the gut. (**G**)–(**I**) are stage 16 embryos at different levels of focusing. A medial plane in (G) allows observation of the accumulation of the protein in head sensory organs (hso), supraesophageal ganglia (spg) and fat body (fb). The latter is seen as two lateral bands of tissue that run from head to tail and surround the gonads (go). A dorsal plane in (H) shows the stained dorsal vessel (dv) and spiracle sensory organ (sso). A ventral plane in (I) shows the strong staining of neural cell bodies (ncb) and commissures (com) in the ventral cord of the CNS. (**J**) Superficial view of a stage 16 embryo showing expression of neurotactin in different sensory organs of every segment in the trunk. (**K**) A stage 13 embryo, beginning dorsal closure. Neurotactin is found in the membrane of amnioserosa cells (as), except in those parts of the membrane (arrows) that contact cells of the dorsal epidermis (ep). (**L**) A stage 16 embryo showing that fat body cells (fb) express the protein in their membrane, only where they contact one another. Portions of the membrane that surround cavities found within the tissue are devoid of staining (arrows). Magnification: in A–I, bar = 50 μm, in J–L, bar = 25 μm.

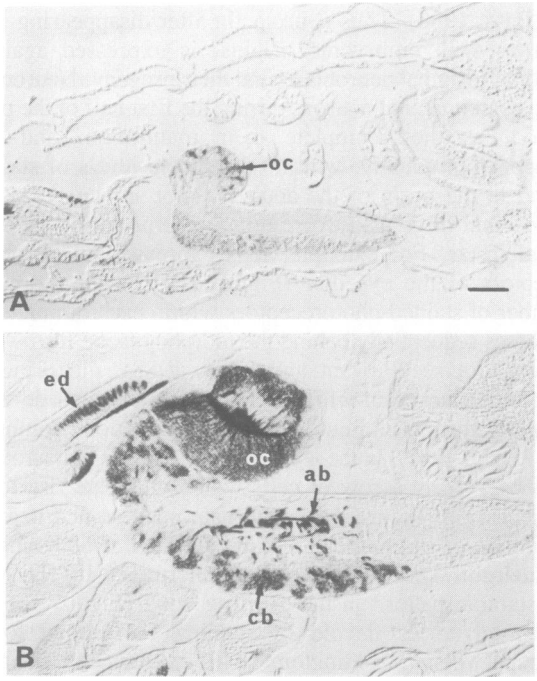


Fig. 2. Expression of neurotactin in the CNS of the larva revealed with MAb43B5. (**A**) The protein, which had disappeared from the CNS after larval hatching, is detected again in the prospective optic centers (oc), which begin to proliferate in the supraesophageal ganglia of the second instar larva. (**B**) In the CNS of the late third instar larva, staining is found in the optic centers, which now occupy most of the brain lobes, and in groups of neuronal cell bodies (cb) and their corresponding axon bundles (ab) in the ventral cord. These neurons are produced by the imaginal neuroblasts. Neurotactin is also found in the differentiating photoreceptors in the eye disc (ed). Same magnification in both pictures (bar in A = 50 μm).

embryos homozygous for some chromosome deficiencies of the 73 region shows that they do not stain with any of the three MAbs when the 73C1-2 double band is deleted (Figure 5), indicating that we have cloned *neurotactin*. This conclusion is further confirmed with the analysis of transcription of the 73C1-2 gene by *in situ* hybridization. The transcription pattern of the gene (not shown) coincides with the expression pattern of the protein (Figure 1).

The developmental effects of the absence of neurotactin cannot be directly assessed because of the lack of specific mutations of the gene. However, phenotypic comparison between deficiencies of the 73 region indicates that the removal of *neurotactin* does not grossly disrupt the embryonic CNS. Deficiencies of the region that do not delete the gene produce characteristic defects in the sterotyped axonal pattern, because of the lack of the *Abelson tyrosine kinase* (*abl*) and *disable* (*dab*) genes which map in 73B (Gertler *et al.*, 1989). Further removal of *neurotactin* does not introduce appreciable changes in that mutant phenotype (Figure 5B).

Transcription of neurotactin

To identify transcripts of the gene, clone HD9 was hybridized to Northern blots of staged *Drosophila* poly(A)$^+$ RNA. Two main transcripts of 4.7 and 3.7 kb were identified, with two peaks of expression during the first half of embryogenesis and the early pupal stage (Figure 6A). The 3.7 kb RNA is predominant in the embryo but both transcripts are expressed at similar levels during pupation. A third, minor 3.4 kb RNA is detectable only during embryogenesis. Because our *in situ* hybridization analysis was performed with the HD9 clone, which is homologous to the three RNAs, we do not presently know whether or not the transcripts share a common spatial pattern of expression.

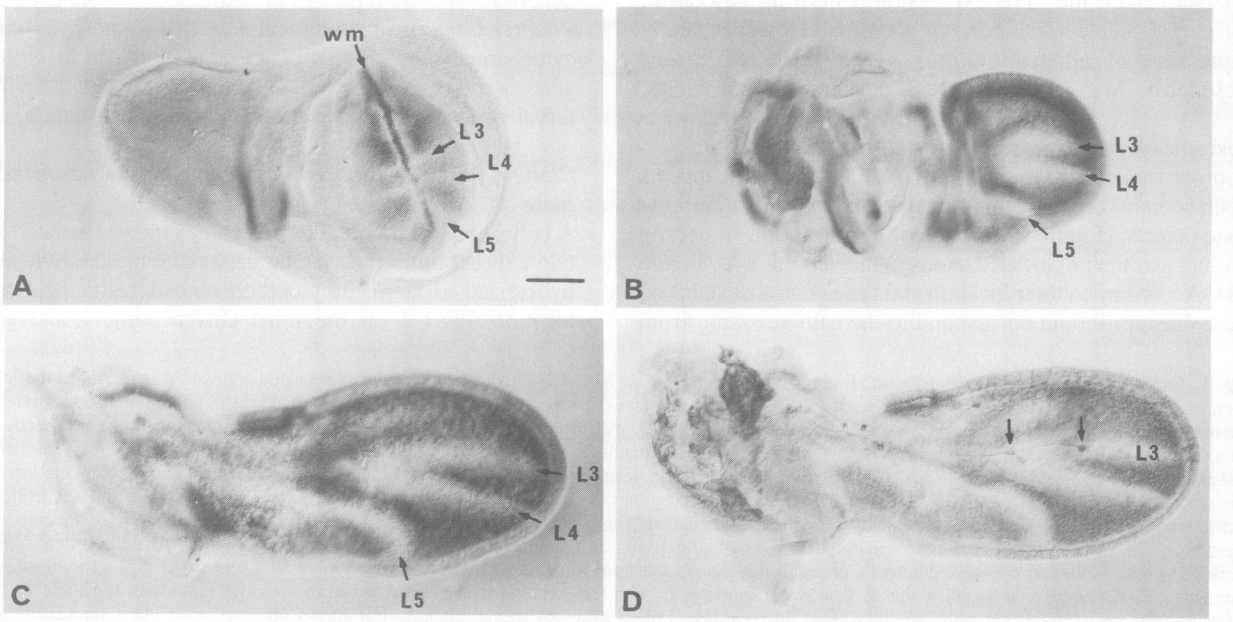


Fig. 3. Expression of neurotactin during eversion of the wing disc. (**A**) The wing disc of the late third instar larva, stained with MAb43B5, shows accumulation of the protein in a row of cells, preferentially in its anterior part, which will form the wing margin (wm). Alternating territories of positive and negative staining are seen at both sides of the margin. They correspond to intervein and vein territories of the future wing, respectively. The correspondence can be followed in the everting wing, at 3 h (**B**) and 6 h (**C**) after puparium formation, where veins L3, L4 and L5 are best recognized. (**D**) A wing of the same age as that in (C), double stained with MAb22C10, which labels differentiating sensory neurons, and MAb43B5. Arrows point to neurons initiating axonogenesis along the L3 vein. These neurons belong to the campaniform sensilla found on vein L3 in the adult wing. Same magnification in all pictures (bar in A = 100 μm).

Sequence of a neurotactin cDNA

The HD9 insert was used to probe two embryonic cDNA libraries (Brown and Kafatos, 1988), to isolate full-length cDNA clones. The sequence of one of these clones (cDNA B41), of the same size as the major embryonic transcript, contains one long open reading frame encoding a core protein of 846 amino acids and 92.8 kd (Figure 7). Post-translational modifications and abnormal electrophoretic migration may explain the difference between the size of the core protein and that estimated from its mobility in gels. A single long hydrophobic region, with the appropriate size to be a transmembrane domain, is found between amino acid positions 325 and 346. MAb47F12 recognizes λgt11 clones that express the C-terminal region of neurotactin and stains embryos fixed under conditions that preserve the integrity of the membrane (Thomas *et al.*, 1984), indicating that it recognizes an extracellular epitope. On the contrary, MAbs 41C2 and 43B5 are only reactive with clones expressing the N-terminal region of the protein and recognize intracellular epitopes, for they can only stain embryos that have been treated with Triton X-100 to permeabilize the cell membrane (not shown). These results indicate that neurotactin is inserted in the cell membrane with the N-terminus localized in the cytoplasm and the C-terminus extracellularly. The cytoplasmic domain consists of 324 amino acids and is highly hydrophilic, with a 40% content of charged residues. It also contains one potential site for phosphorylation by cAMP-dependent protein kinase (Cohen, 1985) and up to five potential sites for phosphorylation by protein kinase C (Ferrari *et al.*, 1985). Six potential acceptor sites for N-linked glycosylation (Bause, 1983) are found on the 500 amino acid extracellular domain. This domain also contains three repeats of the tripeptide leucine-arginine-glutamate (LRE), previously identified as the primary sequence of the adhesive site of s-laminin (Hunter *et al.*, 1989).

A *neurotactin* cDNA clone, which by its size presumably corresponds to the larger transcript, has been isolated and sequenced by M.Horstch (personal communication). It differs from our B41 clone by the presence of a longer 3′ non-translated region. Hence, the use of different poly-adenylation cleavage sites seems to be the origin of the two main mRNAs.

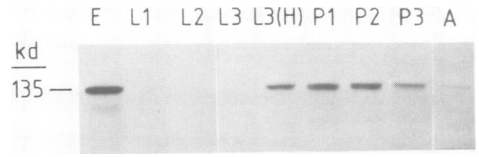


Fig. 4. Western blot analysis. Proteins from homogenates prepared at different stages were separated by electrophoresis under reducing conditions, transferred to nitrocellulose and treated with MAb43B5, as indicated in Materials and methods. The size of neurotactin was estimated using standards of known molecular weight. E: 0−20 h embryos, L1−L3: 1st, 2nd and 3rd instar larvae; L3(H): dissected heads from late 3rd instar larvae; P1−P3: 1, 2 and 3 day old pupae; A: adults.

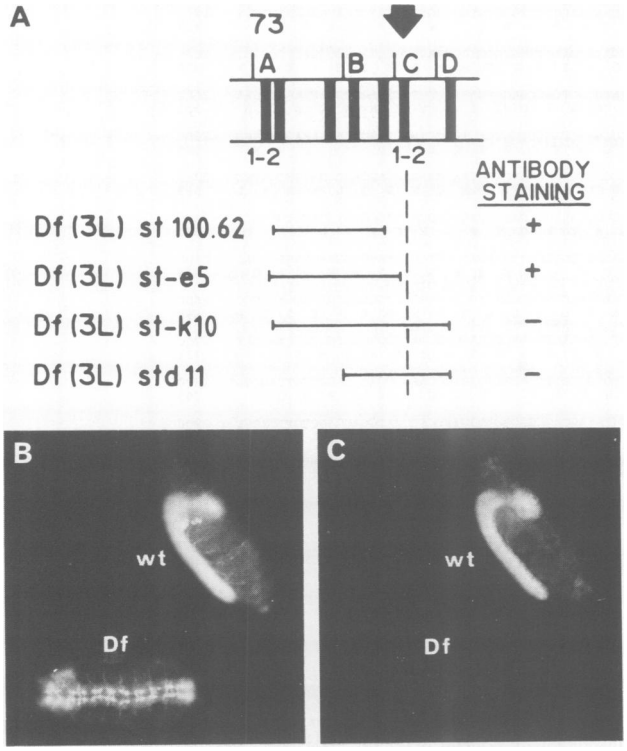


Fig. 5. Cytological localization of the *neurotactin* gene. (**A**) *In situ* hybridization to polytene chromosomes, using the HD9 cDNA insert, served to map *neurotactin* to position 73 C1−2 on the left arm of the third chromosome (arrow). Embryos from several deficiency stocks for the 73 region were treated with each of the three MAbs used in this work. About one-fourth of the embryos are not stained when the deficiency eliminates the 73C1-2 double band (the extent of the deficiency is indicated in each case by a horizontal line). (**B**) and (**C**) correspond to wild type (wt) and deficiency (Df) mutant embryos from the Df(3L)std11 stock, double stained with anti-HRP antibodies, which preferentially label the CNS, and MAb43B5. Embryos treated with anti-HRP antibodies, revealed with a rhodamine-labeled secondary antibody, are shown in (B). The mutant embryo, viewed horizontally, shows the characteristic disorganization of the axon bundles due to the lack of the *abl* and *dab* genes, which map in the 73B region and are also deleted in the deficiency. (**C**) The same embryos as in (B), but treated with MAb43B5 and revealed with a FITC-labeled secondary antibody. The mutant embryo lacks neurotactin expression, whereas the wild type embryo, in a lateral view, presents a similar level of CNS staining to that in (B).

The extracellular domain of neurotactin is related to cholinesterases

Comparison with the NBRF sequence data bank has not revealed global homologies between neurotactin and any

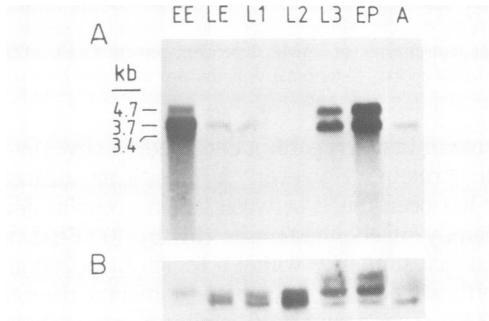


Fig 6. Transcription of the *neurotactin* gene. Poly(A)$^+$ RNA from different stages was size fractionated, transferred to nitrocellulose and probed with the HD9 cDNA insert (**A**). This insert encompasses nucleotides 885−3448 of the sequence of the B41 cDNA clone shown in Figure 7. The size of the RNAs was determined using appropriate standards. EE: 0−12 h embryos; LE: 12−24 h embryos; L1−L3: 1st, 2nd and 3rd instar larvae; EP: 0−2 day old pupae; A: adults. (**B**) The same blot probed with a *Drosophila* actin clone to indicate the relative amounts of RNA per lane.

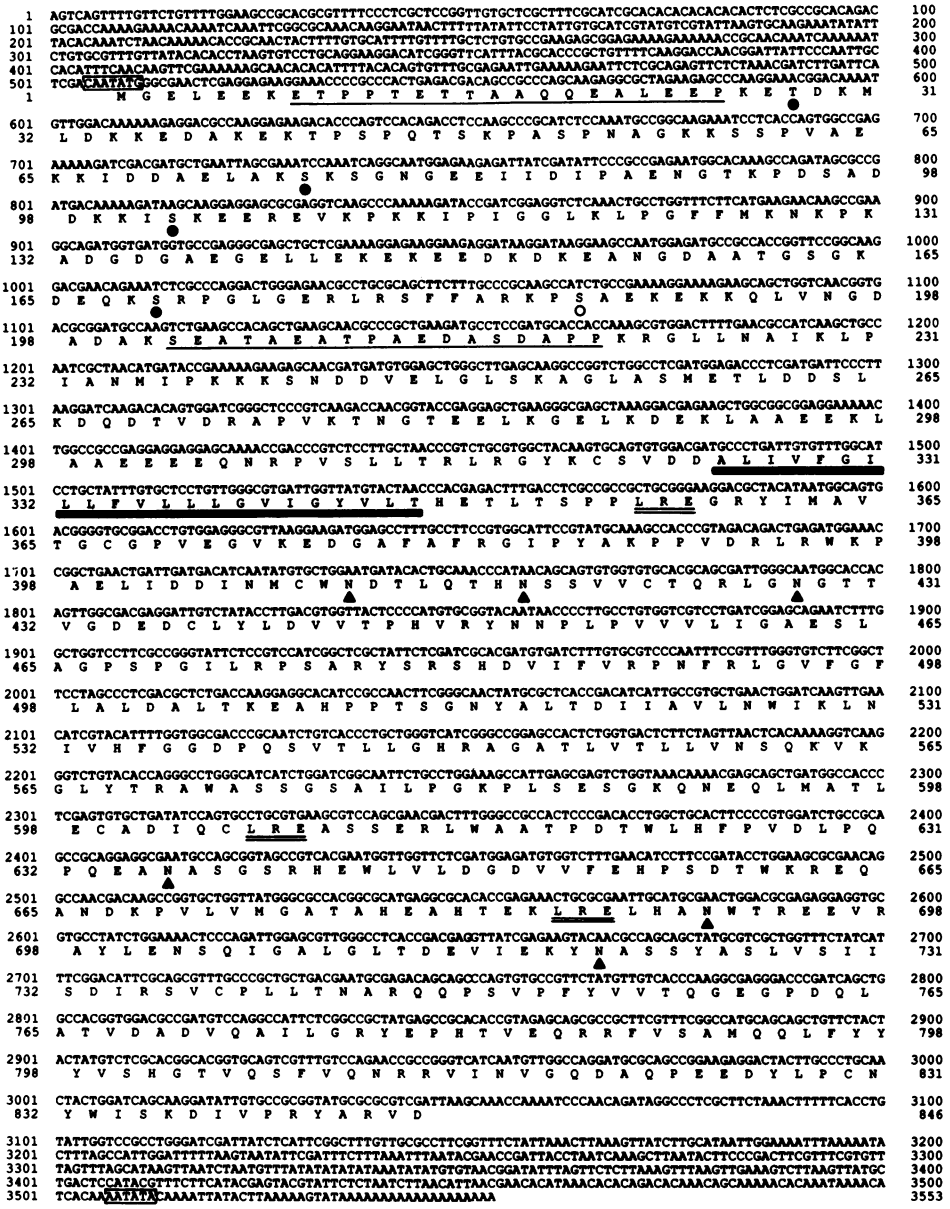


Fig. 7. Nucleotide and deduced amino acid sequence of *neurotactin* cDNA clone B41. Translation initiation and polyadenylation signal consensus sequences are boxed. Microsequencing data of the N-terminus of the immunopurified protein has confirmed the initiation of translation. Underlined are two PEST sequences, characteristic of proteins that are rapidly degraded (Rogers *et al.*, 1986), found in the cytoplasmic, N-terminal domain. Within the same domain, closed circles indicate potential phosphorylation residues by protein kinase C and the open circle a potential phosphorylation site by either that enzyme or cAMP dependent protein kinase. The putative transmembrane domain is thickly underlined. Potential N-glycosylation sites in the extracellular, C-terminal domain, are indicated by triangles. Three LRE (leucine-arginine-glutamate) motifs in that domain are double underlined.

known protein. In this regard, it constitutes a novel integral membrane protein. However, a significant degree of similarity has been found between its extracellular domain and the family of cholinesterases (Figure 8). First, there exists sequence similarity within a region of ~200 amino acids, shortly after the transmembrane domain of neurotactin (Figure 8A). The degrees of similarity range between 28% amino acid identity with *Drosophila* acetylcholinesterase (AChE) and 37% with human butyrylcholinesterase (BuChE). These values rise to 45 and 54% similarity, respectively, when conservative amino acid substitutions are considered. The homologous region contains the catalytic center of cholinesterases, although the active site serine is replaced by arginine, both in neurotactin and thyroglobulin. Comparison of protein sequences by dot matrix analysis (Figure 8B) reveals that the region of *Torpedo* AChE that is similar to neurotactin also presents the highest similarity to thyroglobulin and to glutactin, a *Drosophila* glycoprotein located in basement membranes (Olson *et al.*, 1990). A second level of similarity is shown by the conservation in neurotactin of the relative positions of six cysteines involved in the formation of intramolecular disulfide bonds in cholinesterases (Figure 8C). This suggests that the tertiary structure of the extracellular domain of neurotactin is similar to that of cholinesterases.

Discussion

We have reported in this article the characterization and gene cloning of neurotactin, a novel membrane protein of

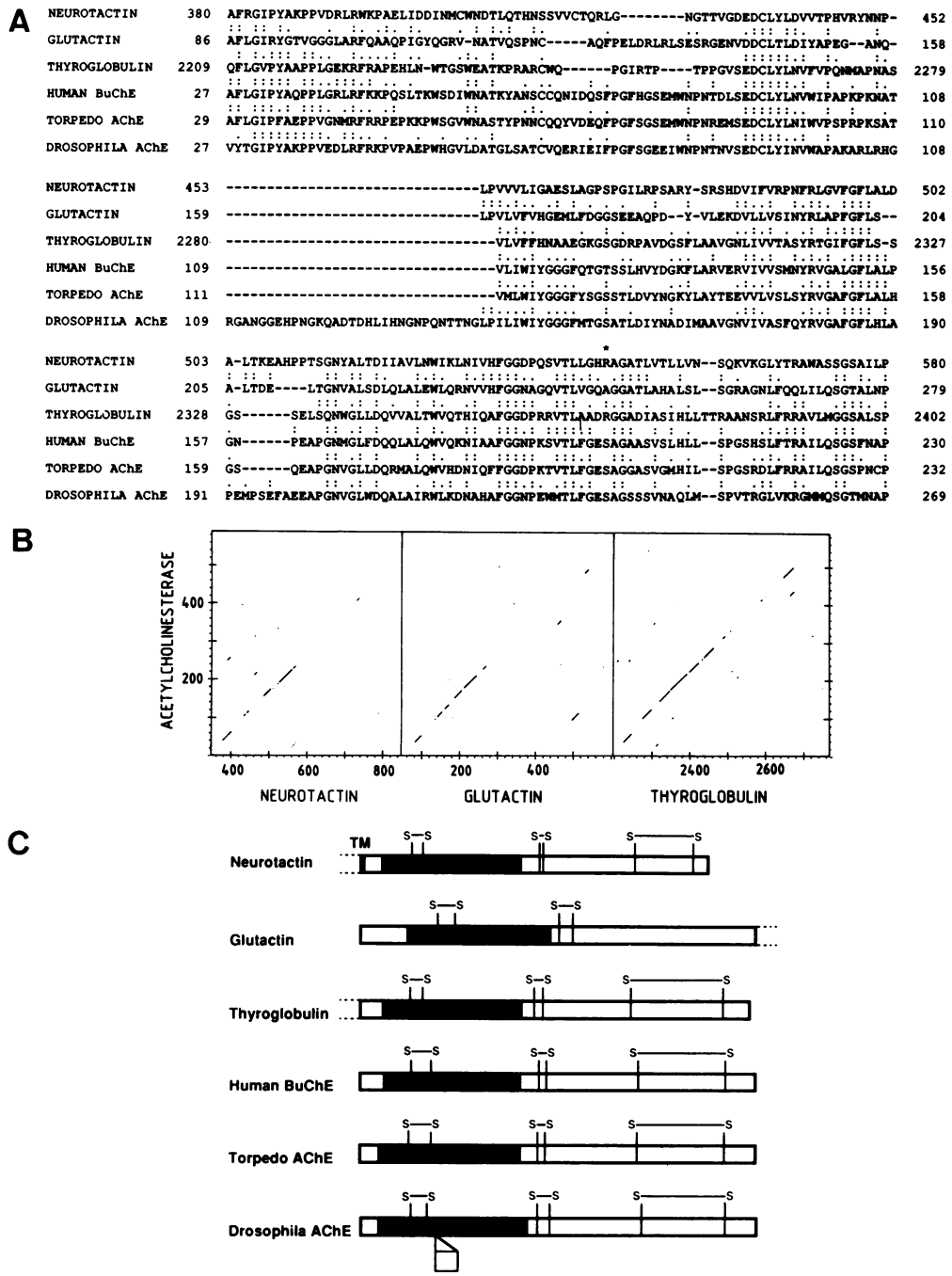


Fig. 8. Similarities between neurotactin and the cholinesterase family. (**A**) Alignment of the domain of sequence similarity between neurotactin, glutactin (Olson *et al.*, 1990), bovine thyroglobulin (Mercken *et al.*, 1985), human BuChE (Lockridge *et al.*, 1987), *Torpedo* AChE (Schumacher *et al.*, 1986), and *Drosophila* AChE (Hall and Spierer, 1986). Numbers indicate amino acid positions within the corresponding protein sequence. Amino acid residues in the cholinesterase sequences are indicated by double dots when identical to those in the neurotactin sequence. Those corresponding to conservative changes are indicated by single dots. The asterisk indicates the position of the active site serine in cholinesterases, substituted by arginine in neurotactin and thyroglobulin. (**B**) Dot matrix analysis of the *Torpedo* AChE protein sequence and selected portions of neurotactin, glutactin and thyroglobulin sequences, obtained with the program COMPARE using the following settings: window, 21; stringency, 13.0 (Maizel and Lenk, 1981). The axes are calibrated in residue numbers. (**C**) Schematic diagrams of the extracellular domain of neurotactin [following the transmembrane domain (TM)]; the N-terminal region of glutactin; the C-terminal region of thyroglobulin; three cholinesterases. The regions of sequence similarity shown in (A) are indicated here as a dotted interval. Only the large insertion in *Drosophila* AChE has been indicated. Disulfide bonds (S−S) for human BChE and *Torpedo* AChE have been previously determined (Lockridge *et al.*, 1987; MacPhee-Quigley *et al.*, 1986). The location of presumptive disulphide bonds in other proteins has been deduced by homology.

Drosophila. The analysis of its structure and pattern of expression provide several lines of evidence compatible with the idea that neurotactin may mediate or modulate cell adhesion during development. Further evidence supporting this role is presented in the accompanying paper by Barthalay *et al.* (1990).

Structure of neurotactin

Two main lines of evidence suggest that neurotactin is a transmembrane protein. First, epitopes have been detected on both sides of the cell membrane. Second, its sequence contains a single hydrophobic region with the characteristics of a transmembrane domain. The N-terminal domain of the

protein, localized in the cytoplasm, contains several potential phosphorylation sites, in accordance with the observation that neurotactin is phosphorylated in intact cells (Barthalay *et al.*, 1990). Phosphorylation might modulate transduction of signals elicited after the establishment of contact between cells. The C-terminal domain, localized extracellularly, contains three copies of the motif LRE, a tripeptide present in the adhesive site of s-laminin and also in many neuronal adhesion molecules (Hunter *et al.*, 1989). It is then possible that LRE sequences mediate adhesive functions in all these proteins, including neurotactin. The domain also shows similarity with cholinesterases, suggesting that neurotactin arose by gene fusion, involving an ancestral cholinesterase precursor that contributed to the entire extracellular domain. We do not presently know the functional significance of the presence in neurotactin of a cholinesterase-like structure, lacking the esterase active site.

Expression of neurotactin

Expression of neurotactin is restricted to embryogenesis and to the late larval–early pupal period, indicating that its function seems to be required for larval and imaginal development. The distribution of neurotactin on the cell membrane is not always homogeneous, usually accumulating in those regions where neurotactin-positive cells contact one another (Figure 1K and L), an expected property of molecules involved in processes of differential cell adhesion. Thus, for example, an uneven distribution of the *Drosophila* adhesion molecule fasciclin III on the surface of different embryonic cell types has been reported (Patel *et al.*, 1987). Furthermore, cells transfected either with fasciclin III (Snow *et al.*, 1989) or with mouse E-cadherin (Nagafuchi *et al.*, 1987; Takeichi, 1988) aggregate upon gene induction and, in both cases, the induced protein appears concentrated at intercellular junctions. The spatial distribution of neurotactin during embryogenesis is strikingly similar to that of amalgam, a *Drosophila* protein believed to play a role in cell surface recognition events (Seeger *et al.*, 1988). Therefore it is possible that the apparent coexpression of neurotactin and amalgam in the same cells may reflect their participation in common processes of cell adhesion in the embryo.

In view of the presumed function of neurotactin in cell–cell adhesion we can suggest a functional interpretation of many features of its expression. The initial accumulation of neurotactin in regions of the blastoderm that will undergo morphogenetic movements during gastrulation may reflect its requirement for preferential adhesiveness between cells involved in those processes. Later, expression of neurotactin in the CNS, or during formation and differentiation of mesodermal derivatives, may provide affinity between cells to maintain tissue cohesion. In the same way, its expression in the retinal photoreceptors, once they become determined, may mediate their clustering. Finally, the appearance of neurotactin in cells of the wing interveins may contribute to the attachment of dorsal and ventral wing surfaces, which are connected only at the intervein regions, and/or favor their aggregation, preventing their mixing with cells of the vein territories. Note, however, that the function of neurotactin during development does not seem to be essential. Thus, the comparative analysis of the embryonic neural phenotype produced by deficiencies of the 73 region indicates, albeit indirectly, that the loss of neurotactin does not disrupt the general morphology of the CNS considerably. This probably results from the functional redundancy postulated in systems of cell adhesion molecules, both in *Drosophila* (Seeger *et al.*, 1988; Bieber *et al.*, 1989; Elkins *et al.*, 1990) and in vertebrates (Bixby *et al.*, 1987).

Finally, we should mention that neurotactin is identical to a membrane glycoprotein previously identified with MAbE1C by Piovant and Léna (1988), as demonstrated by several lines of evidence. Among them are the facts that both proteins share the same expression pattern (R.Hipeau-Jacquotte, personal communication), that MAbE1C recognizes cDNA clones expressing neurotactin, and that it does not stain embryos lacking the 73C1-2 double band.

Materials and methods

Drosophila stocks

Drosophila deficiency strains were obtained from B.Baker and F.M.Hoffman. They are described in Lindsley and Zimm (1985) and Tearle *et al.* (1989).

Production of monoclonal antibodies

Hybridoma cell lines were derived by standard methods from a mouse immunized with homogenates of CNS and imaginal discs dissected from third instar larvae. Supernatants from the hybridoma cultures were screened by indirect immunofluorescence on cryostat sections from third instar larvae. Ascites fluid, purified by ammonium sulfate precipitation, was used as a source of monoclonal antibodies for further experiments.

Immunocytochemistry

Embryos were fixed for 10 min in heptane, saturated with 4% paraformaldehyde in PBS, and their vitelline envelopes removed as described by Mitchison and Sedat (1983). They were further processed as whole mounts. Other stages were cut at the posterior end and treated for 2 h with Carnoy's fixative. After dehydration, they were embedded in Paraplast and sectioned at 8 μm. Hand-dissected discs and pupal wings were fixed by methanol treatment for 5 min.

Following an overnight (whole mounts) or 30 min (sections) incubation with primary antibodies in PBGT (PBS, 10% goat serum, 0.3% Triton X-100), the material was washed with PBGT and reacted with the appropriate (HRP-, FITC-, or Rhodamine-) labeled secondary antibody for 2 h. After washing, HRP was developed with diaminobenzidine and H_2O_2. Samples for immunofluorescence were embedded in 90% glycerol in PBS. Those stained for HRP were dehydrated and embedded in Epon.

Western blotting

Homogenates from different stages were prepared with a Dounce homogenizer in Laemmli's gel loading buffer. 200 μg of protein from each homogenate were electrophoresed on a 7.5% polyacrylamide gel (Laemmli, 1970). Transfer to a nitrocellulose filter was performed according to Towbin *et al.* (1979) in the presence of 0.01% SDS. The filter was preincubated for 30 min in PBS containing 5% goat serum and 0.05% Tween 20, and monoclonal antibodies were then added to an appropriate dilution, followed by an overnight incubation at room temperature. The filter was washed, incubated with an alkaline phosphatase (AP)-labeled secondary antibody for 2 h and, after washing, AP was developed by standard procedures.

Protein microsequencing

The antigen extracted from 200 mg of embryos by immunoprecipitation with MAbE1C (Piovant and Léna, 1988) was purified by SDS–PAGE and blotted onto a Millipore Immobilon PDVF membrane. Electroblotting was carried out according to Matsudaira (1987) in 10 mM CAPS (3-cyclohexylamino-1-propane sulfonic acid) pH 11, for 1 h at room temperature. Approximately 30–40 pmol of antigen bound to the membrane were allowed to perform 11 cycles in the sequenator apparatus of the Analysis Central Service, CNRS, Vernaison, France.

Isolation of cDNAs

The monoclonal antibodies were used for expression cloning from the Crews 10–13 h embryonic CNS λgt11 cDNA library (Patel *et al.*, 1987) and from a 0–20 h embryonic λgt11 cDNA library, prepared by random priming

(a gift from B.Hovemann). The libraries were screened essentially as described by Huynh *et al.* (1985), using an AP-labeled secondary antibody. To obtain full-length cDNAs, the insert from clone HD9, isolated from the random primed expression library, was labeled with ^{32}P by nick-translation and used to probe two additional cDNA libraries. These libraries were constructed with poly(A)$^+$ RNAs from 4–8 and 8–12 h embryos, cloned in the pNB40 vector (Brown and Kafatos, 1988).

DNA and RNA techniques

Subcloning, DNA labeling, Southern blotting and other molecular biological techniques followed standard procedures (Maniatis *et al.*, 1982). Poly(A)$^+$ RNA from different developmental stages of the wild type Oregon-R strain was prepared as indicated previously (Meisen *et al.*, 1988). For Northern blots, 5 μg of poly(A)$^+$ RNA from each stage were fractionated on a 1.2% agarose–formaldehyde gel and transferred to nitrocellulose. The filter was hybridized with random primed ^{32}P-labeled HD9 insert under standard conditions.

In situ hybridization

Hybridization to polytene chromosomes was according to Pardue (1985), as modified by Weigel *et al.* (1987). Hybridization to whole embryos with digoxigenin-labeled probes was as described by Tautz and Pfeiffe (1989).

DNA sequencing

DNA sequencing was performed by the dideoxy method (Sanger *et al.*, 1977) using ^{35}S-labeled d(thio)ATP and Sequenase (Stratagene). Subclones of the B41 cDNA were generated by partial exonuclease III digestion (Henikoff, 1984) and using the Bluescript plasmid system. When necessary, custom-made oligonucleotide primers were used. The sequence was determined for both DNA strands. Sequences were constructed and analyzed with the help of the University of Wisconsin GCG software package (Devereux *et al.*, 1984). Similarities were searched for with the WORDSEARCH program.

Acknowledgements

We are most grateful to G.Ramirez for his encouragement and for providing facilities during most of this work, to D.Bachiller, E.Krejci and D.Pulido for help in sequence analysis and to members of J.Modolell's group for their continuous help in the course of this work. We thank M.Horstch and R.Hipeau-Jacquotte for sharing their unpublished results, B.Baker and F.M.Hoffman for mutant stocks, S.Crews, B.Hovemann and M.Wilcox for cDNA libraries, A.Ferrús for MAb22C10 and A.Martínez Arias and J.Modolell for comments on the manuscript. S.de la E. holds a predoctoral fellowship from Ministerio de Educación y Ciencia. This work was supported by grants of the Dirección General de Investigación Científica y Técnica (PB87-0433-CO2-02) to F.J. and of the Centre National de la Recherche Scientifique to M.P. The institutional help of the Fundación Ramón Areces to the Centro de Biología Molecular is acknowledged.

References

Barthalay,Y., Hipeau-Jacquotte,R., de la Escalera,S., Jiménez,F. and Piovant,M. (1990) *EMBO J.*, **9**, 3603–3609.
Bause,E. (1983) *Biochem. J.*, **290**, 331–336.
Bieber,A.J., Snow,P.M., Hortsch,M., Patel,N.H., Jacobs,J.R., Traquina,Z.R., Schilling,J. and Goodman,C.S. (1989) *Cell*, **59**, 447–460.
Bixby,J.L., Pratt,R.S., Lilien,J. and Reichardt,L.F. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 2555–2559.
Bogaert,T., Brown,N. and Wilcox,M. (1987) *Cell*, **51**, 929–940.
Brown,N. and Kafatos,F. (1988) *J. Mol. Biol.*, **203**, 425–537.
Campos-Ortega,J.A. and Hartenstein,V. (1985) *The Embryonic Development of Drosophila melanogaster*. Springer-Verlag, Heidelberg.
Cohen,P. (1985) *Eur. J. Biochem.*, **151**, 439–448.
Devereux,J., Haeberli,P. and Smithies,O. (1984) *Nucleic Acids Res.*, **12**, 387–395.
Edelman,G.M. (1986) *Annu. Rev. Cell Biol.*, **2**, 81–116.
Elkins,T., Zinn,K., McAllister,L., Hoffmann,F.M. and Goodman,C.S. (1990) *Cell*, **60**, 565–575.
Ferrari,S., Marchiori,F., Borin,G. and Pinna,L.A. (1985) *FEBS Lett.*, **184**, 72–77.
Gertler,F.B., Bennett,R.L., Clark,M.J. and Hoffmann,F.M. (1989) *Cell*, **58**, 103–113.
Hall,L.M.C. and Spierer,P. (1986) *EMBO J.*, **5**, 2949–2954.
Henikoff,S. (1984) *Gene*, **28**, 351–359.
Hunter,D.D., Porter,B.E., Bulock,J.W., Adams,S.P., Merlie,J.P. and Sanes,J.R. (1989) *Cell*, **59**, 905–913.
Huynh,T.V., Young,R.A. and Davis,R.W. (1985) In Glover,D.M. (ed.), *DNA Cloning—A Practical Approach*. IRL Press, Oxford, Vol. 1, pp. 49–78.
Jessell,T.M. (1988) *Neuron*, **1**, 3–13.
Laemmli,V.K. (1970) *Nature*, **227**, 680–685.
Lander,A.D. (1989) *Trends Neurosci.*, **12**, 189–195.
Lindsley,D.L. and Zimm,G. (1985) *Drosophila Inf. Serv.*, **62**.
Lockridge,O., Bartels,C.F., Vaughan,T.A., Wong,C.K., Norton,S.E. and Johnson,L.L. (1987) *J. Biol. Chem.*, **262**, 549–557.
MacKrell,A.J., Blumberg,G., Haynes,S.R. and Fessler,J.M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2633–2637.
MacPhee-Quigley,K., Vedvick,T., Taylor,P. and Taylor,S.S. (1986) *J. Biol. Chem.*, **261**, 13565–13570.
Maizel,J.V. and Lenk,R.P. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7665–7669.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Matsudaira,P. (1987) *J. Biol. Chem.*, **262**, 10035–10038.
Meisen,N., Baars,S. and Jiménez,F. (1988) *Gene*, **74**, 457–464.
Mercken,L., Simons,M.J., Swillens,S., Massaer,M. and Vassart,G. (1985) *Nature*, **316**, 647–651.
Mitchison,T. and Sedat,J. (1983) *Dev. Biol.*, **99**, 261–264.
Montell,D.J. and Goodman,C.S. (1988) *Cell*, **53**, 463–473.
Nagafuchi,A., Shirayoshi,Y., Okazaki,K., Yasuda,K. and Takeichi,M. (1987) *Nature*, **329**, 341–343.
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.
Pardue,M.L. (1985) In Hames,B.D. and Higgins,S.L. (eds), *Nucleic Acid Hybridization—A Practical Approach*. IRL Press, Oxford, pp. 179–202.
Patel,N.H., Snow,P.M. and Goodman,C.S. (1987) *Cell*, **48**, 975–988.
Piovant,M. and Léna,P. (1988) *Development*, **103**, 145–156.
Rogers,S., Wells,R. and Rechsteiner,M. (1986) *Science*, **234**, 364–368.
Rutishauser,V. and Jessell,T.M. (1988) *Physiol. Rev.*, **68**, 819–857.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Schumacher,M., Camp,S., Maulet,Y., Newton,M., MacPhee-Quigley,K., Taylor,S.S., Friedmann,T. and Taylor,P. (1986) *Nature*, **319**, 407–409.
Seeger,M.A., Haffley,L. and Kaufman,T.C. (1988) *Cell*, **55**, 589–600.
Snow,P.M., Bieber,A.J. and Goodman,C.S. (1989) *Cell*, **59**, 313–323.
Takeichi,M. (1988) *Development*, **102**, 639–655.
Tautz,D. and Pfeiffe,C. (1989) *Chromosoma*, **98**, 81–85.
Tearle,R.G., Belote,J.M., McKeown,M., Baker,B.S. and Howells,A.J. (1989) *Genetics*, **122**, 595–606.
Thomas,J.B., Bastiani,M.J., Bate,C.M. and Goodman,C.S. (1984) *Nature*, **310**, 203–207.
Tomlinson,A. and Ready,D. (1987) *Dev. Biol.*, **120**, 366–376.
Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
Truman,J.W. and Bate,M. (1988) *Dev. Biol.*, **125**, 145–157.
Waddington,C.H. (1940) *J. Genet.*, **41**, 75–139.
Weigel,D., Knust,E. and Campos-Ortega,J.A. (1987) *Mol. Gen. Genet.*, **207**, 374–384.
Wilcox,M. (1986) In Roberts,D.B. (ed.), *Drosophila—A Practical Approach*. IRL Press, Oxford, pp. 243–274.

Received on June 19, 1990; revised on July 25, 1990

Notes added in proof

The characterization and gene cloning of neurotactin have been independently carried out by M.Hortsch, N.H.Patel, A.J.Bieber, Z.R.Traquina and C.S.Goodman; the corresponding publication is now in press in *Development*.

The mRNA sequence from neurotactin has been deposited in the EMBL Data Library with the accession number X53837.