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Teneurins: A Novel Family of Neuronal Cell Surface Proteins in Vertebrates, Homologous to the *Drosophila* Pair-Rule Gene Product Ten-m

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We have characterized chicken teneurin-1 and teneurin-2, two homologues of the *Drosophila* pair-rule gene product Ten-m and *Drosophila* Ten-a. The high degree of conservation between the vertebrate and invertebrate proteins suggests that these belong to a novel family. We propose to name the vertebrate members of this family teneurins, because of their predominant expression in the nervous system. The expression of teneurin-1 and -2 was investigated by *in situ* hybridization. We show that teneurin-1 and -2 are expressed by distinct populations of neurons during the time of axonal growth. The most prominent site of expression of chicken teneurins is the developing visual system. Recombinant teneurin-2 was expressed to assay its molecular and functional properties. We show that it is a type II transmembrane protein, which can be released from the cell surface by proteolytic cleavage at a furin site. The expression of teneurin-2 in neuronal cells led to a significant increase in the number of filopodia and to the formation of enlarged growth cones. The expression pattern of teneurins in the developing nervous system and the ability of teneurin-2 to reorganize the cellular morphology indicate that these proteins may have an important function in the formation of neuronal connections.

Key Words: **nervous system; transmembrane; filopodia; axon guidance; proteolytic processing.**

INTRODUCTION

Studies in *Drosophila* have led to the discovery of two closely related genes named *ten-a* and *ten-m/odd Oz* (Baumgartner and Chiquet-Ehrismann, 1993; Baumgartner *et al.*, 1994; Levine *et al.*, 1994). Mutational analysis showed that *ten-m* belongs to the pair-rule gene family. In this class of genes it is the first member identified that does not encode a DNA-binding protein, but an extracellular protein, which could be involved in signaling between cells. In the case of *ten-a* no mutational analysis has been performed yet. It is expressed mainly in the developing nervous system and at muscle attachment sites (Baumgartner and Chiquet-Ehrismann, 1993). Ten-m protein has been shown to be expressed during early embryonic development before the onset of gastrulation in a seven-stripe pattern characteristic for a pair-rule gene (Baumgartner *et al.*, 1994). In agreement with the expression pattern, *ten-m*-mutants show an aberrant segmentation and can be used to classify *ten-m* as a secondary pair-rule gene. Interestingly, Ten-m/odd Oz is also expressed during larval development in the eye imaginal disc, within the morphogenetic furrow in cells just undergoing differentiation, and in fully differentiated R7 photoreceptor cells, as well as the optic stalk and the optic lobe (Levine *et al.*, 1997). The ubiquitous expression of Ten-m in cells undergoing early gastrulation as well as in cells undergoing final differentiation suggests that it has a rather basic role in determining cell fate.

We recently cloned chicken teneurin-1 (Minet *et al.*, 1999); based on this sequence four independent mouse clones termed Ten-m 1–4 were isolated and shown to be expressed mainly in the central nervous system (Oohashi *et al.*, 1999). In an independent study, DOC4 of mouse, which by sequence comparison can be shown to correspond to mouse Ten-m 4, has been cloned (Wang *et al.*, 1998). DOC4 protein is found at the midbrain–hindbrain junction, a site where several vertebrate homologues of *Drosophila* segmentation genes are expressed and which is important in early patterning of the vertebrate brain (Joyner, 1996). DOC4 is also known to be expressed under the control of a stress-specific transcription factor named CHOP in response to perturbation of protein folding in the endoplasmic

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reticulum (Wang *et al.*, 1998). However, this role seems to be independent of the one in the nervous system, as its expression at the midbrain–hindbrain junction is not altered in the absence of CHOP (Wang *et al.*, 1998).

Here we show that chicken teneurin-1, chicken teneurin-2, mouse DOC4/Ten-m 4, *Drosophila* Ten-a, and *Drosophila* Ten-m are members of a phylogenetically conserved family of proteins. In the developing nervous system, chicken teneurin-1 and teneurin-2 are found to be expressed by populations of neurons establishing connections with each other. Our findings suggest that teneurins are transmembrane proteins and that the extracellular domain of some of their members can be released from the cell surface by proteolytic cleavage. The expression of teneurin-2 was shown to strongly induce the formation of filopodia and enlarged growth cones in neuronal cells. Taken together, these results suggest that the teneurins might have an important function in the development of the nervous system.

MATERIALS AND METHODS

Cloning of Chicken Teneurin-1 and Teneurin-2

A sequence alignment of *Drosophila* Ten-a and Ten-m and *Caenorhabditis elegans* Ten-m was used to identify conserved peptide sequences from which degenerate primer sets were derived to amplify chicken teneurin-1and chicken teneurin-2 (see also Minet *et al.*, 1999). Four primers each were selected within the EGF-like repeat region. They were used in two consecutive nested polymerase chain reactions (PCR) of cDNA reverse transcribed from 11-day chick embryo brain mRNA. The first primer set was 5′-GGNTKBAARGGNAARGARTGY-3′/5′-CCNGCRCANSWRTTNGGRCANCC-3′ and the second one was 5′-TGYGARGTNGCNGAYTG-3′/5′-CARTGYTTNCCRTTCCANCC-3′. A 387-bp product encoding 129 amino acids of chicken teneurin-2 covering the sequence from amino acid 695–823 is shown in Fig. 1. This clone was used to prepare digoxigenin-labeled DNA probes (PCR DIG Probe Synthesis Kit; Boehringer Mannheim) to screen a cDNA library prepared from 11-day chick embryo brain mRNA (Zap Express cDNA/Gigapack cloning kit; Stratagene, La Jolla, CA). Multiple rounds of screening of the library described above, as well as of cDNA libraries of embryonic day 14 chicken brain (Zuellig *et al.*, 1992), of embryonic day 11 chicken retina (kindly provided by Dr. Peter Sonderegger), and of adult chicken brain (chicken brain 5′-STRETCH cDNA; Clontech, Palo Alto, CA), were performed, as described by Hagios *et al.* (1996). The isolated clones were assembled into the coding sequence for chicken teneurin-2. The DNA sequence data have been submitted to the DDB/EMBL/GenBank databases under Accession No. AJ245711 for chicken teneurin-2. The accession number of chicken teneurin-1 is AJ238613.

Cloning of Mouse Teneurin-2

A DIG-labeled chicken teneurin-2 probe (PCR DIG probe synthesis kit; Boehringer, Mannheim, Germany) was used under low stringency conditions (McGinnis *et al.*, 1984) to screen a newborn mouse brain cDNA library (Stratagene). In a second round of screening a mouse teneurin-2 probe derived from the first screen was applied under regular hybridization conditions, as described by Hagios *et al.* (1996). In this way it was possible to assemble a 687-bp sequence encoding the EGF-like repeats. This sequence has been submitted to the DDB/EMBL/GenBank databases under Accession No. AJ245710.

Expression of Recombinant Teneurin-2 Carrying a Viral Tag

To the 3′ end of the complete chick teneurin-2 cDNA a VSV (vesicular stomatitis virus) tag encoding the amino acid sequence *YTDIEMNRLGK* was added by PCR. This construct was inserted into the eukaryotic expression vector pcDNA3/Neo (Invitrogen, De Schelp, The Netherlands) and expressed under the control of the cytomegalovirus promotor. The plasmid DNA was transiently transfected into COS-7 green monkey kidney cells or Nb2a mouse neuroblastoma cells using either the transfection reagent lipofectamine (Gibco BRL, Paisley, Great Britain) or fugene 6 (Boehringer), following the manufacturer's instructions. Twelve hours following the start of the transfection Nb2a cells were induced to acquire a neuronal phenotype by replacing the serum containing medium with medium containing a serum-free supplement, as described in Suidan *et al.* (1992).

Immunohistochemistry and Western Blots of Cells Transfected with Teneurin-2

Twenty-four to 72 h after transfection the cells were rinsed once with Ca^{2+}- and Mg^{2+}-free PBS (PBS CMF) and either fixed with 4% formaldehyde in PBS CMF for immunohistochemistry or scraped off the dish and solubilized in SDS–PAGE sample buffer for Western blot analysis.

To permeabilize the fixed cells 0.2% Triton X-100 in PBS CMF was applied for 15 min. Following this step the permeabilized cells, like the intact cells, were rinsed with PBS CMF and incubated in blocking buffer (3% BSA in PBS CMF) for 15 min. The primary antibodies (anti-teneurin-2 described below; anti-VSV tag, affinity-purified peptide antibody, from A. Matus, Friedrich Miescher-Institute), as well as the fluorescein-coupled, secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), were diluted in the blocking buffer. Along with the secondary antibody phalloidin coupled to rhodamine was incubated (Sigma, Buchs, Switzerland). After each of the incubations, cells were rinsed in PBS CMF. For viewing cells were mounted in Mowiol (Calbiochem, La Jolla, CA).

For Western blot analyses samples were separated on a 10% SDS–PAGE, transferred onto a nylon membrane, and treated as described by Hagios *et al.* (1996).

Confocal Microscopy

An inverted Leica microscope equipped with the TCS confocal system and an Ar/Kr laser was used to generate images of immunostained cells through a 63× or 100× objective with a numerical aperture of 1.4. Stacks of images were analyzed on a Silicon Graphics Computer (Indy) with the Imaris program (Bitplane AG, Zürich, Switzerland).

Quantification of the Nb2a Cell Morphology

Video images of teneurin-2 and vector-transfected control cells (50 cells each) were analyzed for the number of actin-containing filopodia which had formed along their neurites. For this analysis

neurites had to have a length corresponding to at least twice that of the cell body diameter. To be considered as filopodium the minimum length of a filamentous actin-containing protrusion was 7 μm.

For growth cone measurements, the outline corresponding to the first 15 μm from the tip of each neurite was traced manually on video images, and the area was quantified with the help of the NIH Image software.

Expression of the Tenascin-C/Teneurin-2 Fusion Protein (TN/ten-2)

The N-terminal part of chicken tenascin-C (nucleotides 1–794 tenascin-C EMBL database entry M23121), which contains the epitope for the monoclonal antibody anti-TN60 (Pearson *et al.*, 1988), was fused to the extracellular half of chicken teneurin-2 (amino acids 429–831). For this purpose the two coding sequences were joined in a PCR using partially overlapping templates by an approach called "splicing by overlap extension" (Horton *et al.*, 1989). After stable transfection into HT1080 human fibrosarcoma cells the fusion protein-encoding sequence was expressed under the control of a cytomegalovirus promotor using the expression vector pcDNA3/Neo (Invitrogen) as previously described (Fischer *et al.*, 1995). The HT1080 cells were grown to saturation in suspension cultures in Joklik's MEM (Gibco BRL)/5% fetal calf serum (Gibco BRL)/10^{-7} M dexamethasone. The conditioned medium was harvested and the fusion protein was purified by ammonium sulfate precipitation, followed by dialysis and affinity purification as described (Fischer *et al.*, 1995). The purity of the protein fraction was analyzed by SDS–PAGE.

Production of the Anti-teneurin-2 Antibody

The affinity-purified protein TN/ten-2 was injected into rabbits for antibody production. The resulting antisera were passed over a tenascin-C affinity column prepared as described by Fischer *et al.* (1995), to eliminate the antibodies directed against the tenascin-C moiety of the protein. The specificity of the antiserum was confirmed by Western blots using recombinant tenascin-C and teneurin-2 as antigens.

Trypsin Digest of TN/ten-2 and Protein Sequencing

Affinity-purified TN/ten-2 (120 μg/ml) was incubated for 1 hour, at room temperature with 0.01 or 0.1 μg/ml trypsin (Bovine Pancreas, Type III, Sigma) in PBS CMF with 0.01% Tween 20. At the end of the reaction SDS–PAGE sample buffer was added and the mixture was incubated at 95°C for 5 min.

For N-terminal sequencing affinity-purified TN/ten-2 was separated on SDS–PAGE and transferred to Immobilon P membrane (Millipore Corp., Bedford, MA). The bands of interest were cut out and analyzed on a protein sequencer (Model 477 A, Applied Biosystems, Foster City, CA) according to the recommendations of the manufacturer.

Northern Blots

RNA was isolated from the tissues indicated according to the method described by Chomczynski and Sacchi (1987), separated on 1% agarose/formaldehyde gels, blotted, and probed as described (Hagios *et al.*, 1996). A DIG-labeled RNA probe (DIG RNA labeling kit; Boehringer), encompassing 850 bp of the EGF-like repeat encoding sequence of teneurin-2. was used. Poly(A)+ RNA was isolated with the QuickPrep Micro mRNA purification kit (Pharmacia, Uppsala, Sweden).

Reverse Transcription PCR

RNA of embryonic tissue of different stages was isolated by the method of Chomczynski and Sacchi (1987). cDNA was reverse transcribed from RNA following the manufacturer's instructions (Advantage RT-for-PCR kit, Clontech). An aliquot of these cDNAs was incubated with teneurin-2-specific primers in a PCR (30 cycles, 55°C annealing temperature) to generate a 351-bp fragment located at the 3′ end of the coding sequence, comprising amino acids 685–802. As a control a 140-bp fragment of the coding sequence of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified in the same reaction.

In Situ Hybridization

For nonradioactive *in situ* hybridization we used the DIG-labeled teneurin-2 RNA probe, described above, in a protocol described by Schaeren-Wiemers and Gerfin-Moser (1993). The DIG-labeled teneurin-1 RNA probe corresponded to a 1332-bp sequence encoding amino acids 177–621.

Radioactive *in situ* hybridization with [α-^{35}S]dCTP-labeled teneurin-1 cDNA (encoding the region from amino acid 868 to amino acid 1330), [^{35}S]dCTP-labeled teneurin-2 cDNA (encoding the EGF-like repeats from amino acid 695 to amino acid 823), or a pUC control DNA was performed as described previously (Tucker *et al.*, 1997). The slides were dried and exposed to high-resolution X-ray film (Amersham) and then dipped in autoradiographic emulsion (Kodak). Before viewing with dark-field illumination, the slides were mounted using 50% glycerol/PBS with 0.001% H33258 (Hoechst nuclear stain, Boehringer Mannheim).

RESULTS

The Teneurin Family Is Conserved from Invertebrates to Vertebrates

In an attempt to identify the vertebrate homologues of *Drosophila ten-a* and *ten-m*, cDNAs were isolated by PCR using degenerate primers derived from the EGF-like repeats of *Drosophila ten-a* and *ten-m* on an embryonic-day-11 chicken brain library. Two types of clones were identified and named chicken teneurin-1 (Minet *et al.*, 1999) and chicken teneurin-2. Specific probes for each were used for screening different cDNA libraries representing mRNA of the developing and adult chicken brain (see Material and Methods). The isolated clones were assembled and the coding sequences were deduced. Structure analysis based on the amino acid sequence (Nakai and Kanehisa, 1992; http://psort.nibb.ac.jp) predicts that all teneurins are type II transmembrane proteins with a cytoplasmic tail at the N-terminus, which comprises 300 amino acids in the case of teneurin-1 and 375 amino acids in the case of teneurin-2 (Figs. 1A and 1B). The intracellular domain contains potential calcium-binding sites (EF-hand motifs) (Kawasaki and Kretsinger, 1994) and putative docking sites for proteins carrying SH3-domains (proline-rich stretches) (Mayer and

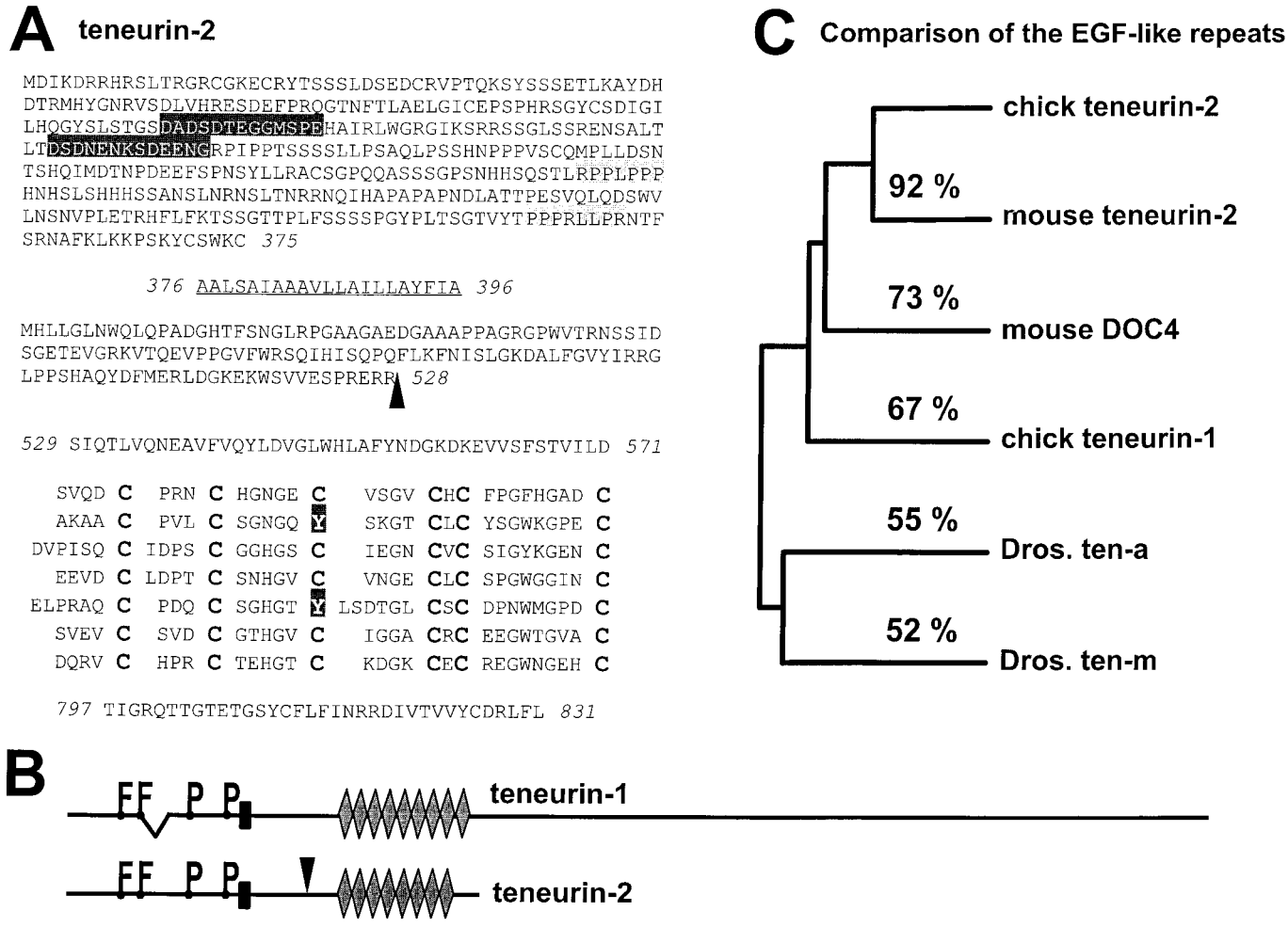


FIG. 1. Primary structure of chicken teneurin-1 and -2 and the phylogenetic tree of the teneurin family. The cDNA sequence of chicken teneurin-1 encodes a protein of 2705 amino acids (Accession No. AJ238613), and the sequence of teneurin-2 corresponds to 831 amino acids (Accession No. AJ245711). The complete sequence of teneurin-2 is given in (A) and compared schematically to that of teneurin-1 in (B). The intracellular parts of both proteins comprise the first 300 and 375 amino acids, respectively. The positions of the 75 amino acids present in teneurin-2, but absent in teneurin-1, are marked in the model in (B). The intracellular domains contain two conserved, potential calcium-binding sites (highlighted with a light gray box in A, marked by an "F" in B) and two conserved, proline-rich domains (highlighted with a dark gray box in A, marked by a "P" in B). The intracellular domains are followed by putative transmembrane domains, each 20 amino acids long (underlined in A, represented by a black rectangle in B). Amino acid 397 to amino acid 528 constitute the first part of the extracellular half of the teneurin-2, which would remain on the cell surface following proteolytic cleavage by furin after the dibasic motif RERR at amino acid 528 (marked by a black triangle in A and B). This proteolytic site is not conserved in teneurin-1. The seven EGF-like repeats of teneurin-2 (drawn as black diamonds in B) have been aligned according to the conserved cysteines in (A). In the second EGF-like repeat of teneurin-1 the third cysteine has been replaced by a tyrosine, and in the fifth EGF-like repeat, by phenylalanine. The same cysteines have been replaced by tyrosines in teneurin-2 (highlighted Y's). The EGF-like repeats of teneurin-2 terminate at amino acid 796 and are followed by a short C-terminal sequence from amino acid 797 to amino acid 831. The C-terminal sequence of teneurin-1 contains an additional EGF-like repeat and extends for another 1980 amino acids, which are homologous to *Drosophila* Ten-m and mouse DOC/Ten-m 4. (C) The dendrogram establishing the degree of homology between different members of the teneurin/ten family was created by the multiple alignment program "Pileup" (Genetics Computer Group, University Research Park, Madison, WI). The amino acid sequences comprising the first seven EGF-like repeats present in all known teneurin members were compared. The percentages of identical amino acids between each of the members and teneurin-2 are given.

Eck, 1995) (Fig. 1A). These motifs are highly conserved between chicken teneurin-1, teneurin-2, and mouse DOC4/Ten-m 4 (Fig. 1B). The cytoplasmic domain is followed by a 20 amino acid long hydrophobic stretch that has the characteristics of a transmembrane domain. The extracellular C-terminal half of the teneurin-2 is 435 amino acids in

length, similar to that of *Drosophila* Ten-a. It consists of a stretch of 156 amino acids, containing a furin recognition site, conserved in *Drosophila* Ten-a and seven EGF-like repeats, which are present in all of the teneurins and the *Drosophila* genes (Fig. 1B). The first part of the extracellular domain of teneurin-1 resembles closely that of teneurin-2; it does however not contain a furin recognition site and it has one additional EGF-like repeat. The C-terminal half of the extracellular domain of teneurin-1 comprises 1980 amino acids, which are about 70% identical to the C-terminal part of mouse DOC4/Ten-m 4 (Wang *et al.*, 1998; Oohashi *et al.*, 1999) and 32% identical to the C-terminal part of *Drosophila* Ten-m (Baumgartner *et al.*, 1994). The C-terminal third of the different proteins contains a novel type of domain, which has a characteristic amino acid motif consisting of tyrosine and aspartic acid. It has been shown to be involved in carbohydrate binding and neurite outgrowth (Minet *et al.*, 1999).

We have in addition isolated mouse teneurin-2 cDNAs covering the sequence coding for the EGF-like repeats by screening a mouse brain cDNA library with the help of chicken teneurin-2 probes.

To establish the relationship between all teneurins known we compared the amino acid sequences representing the EGF-like repeats one to seven in a "pileup" analysis (Genetics Computer Group, University Research Park, Madison, WI) (Fig. 1C). Chicken teneurin-2, chicken teneurin-1, mouse DOC4/Ten-m 4 (Wang *et al.*, 1998; Oohashi *et al.*, 1999), mouse teneurin-2, and the two *Drosophila* proteins Ten-a and Ten-m (Baumgartner and Chiquet-Ehrismann, 1993; Baumgartner *et al.*, 1994) were included in the sequence comparisons.

The proportion of identical amino acids between the chicken teneurin-2 sequence and all related sequences is indicated in Fig. 1C. Mouse teneurin-2 is 92% identical to chicken teneurin-2 at the amino acid level. This value is distinctly higher than that observed for the level of identity between the different vertebrate members of the family, which is around 70% in this part of the protein. In agreement with the high degree of conservation between proteins of different vertebrate species, the number of amino acids conserved between invertebrates and vertebrates was also relatively high, ranging between 50 and 60% identity. By this sequence comparison it was, nevertheless, not possible to identify unambiguously which of the vertebrate homologues corresponds to *Drosophila* Ten-a or Ten-m, respectively. However, judging from the overall domain organization and size of the proteins, teneurin-1 and mouse DOC4/Ten-m 4 resemble more *Drosophila* Ten-m, while teneurin-2 corresponds more to *Drosophila* Ten-a. This is also reflected in another distinctive feature of all teneurins: the replacement of the third cysteine in the second and fifth EGF-like repeat by either tyrosine or phenylalanine. In *Drosophila* Ten-a and teneurin-2 the two cysteines have been substituted by two tyrosines, and in *Drosophila* Ten-m, mouse DOC4/Ten-m 4 and teneurin-1 the second EGF-like repeat contains a tyrosine and the fifth one contains a phenylalanine instead of a cysteine.

Teneurin-2 Is Present at the Cell Membrane

For the molecular characterization we have focused on teneurin-2. To determine its subcellular localization a vector containing the entire chicken teneurin-2, to which a VSV tag had been added at the 3′ end, was introduced into COS-7 and Nb2a cells. The transfected COS-7 cells were fixed and incubated with a polyclonal antibody specific for teneurin-2 and a fluorescently labeled secondary antibody, either with or without being permeabilized prior to immunostaining. In the permeabilized cells, in addition to the cell membrane, intracellular compartments were revealed, probably corresponding to the Golgi apparatus and the endoplasmic reticulum (Fig. 2A). On intact cells a very strong cell-surface staining was retained (Fig. 2B). These results suggest that teneurin-2 is either tightly bound to the cell surface or constitutes an integral membrane protein, which would be in agreement with the sequence analysis predicting the existence of a single transmembrane domain.

When an extract of the transfected COS-7 cells was analyzed on a Western blot either with a VSV-tag-specific or a teneurin-2-specific antibody, a band of 120 kDa was detected by both antibodies (Fig. 2C, lanes 2 and 3). A band of the same size was also detected by the teneurin-2-specific antibody in an extract of Nb2a cells transfected with the teneurin-2-encoding plasmid (Fig. 2C, lane 5), but was completely absent in nontransfected Nb2a cells (Fig. 2C, lane 4). In addition to the band of about 120 kDa a signal of variable strength was detected by the teneurin-2-specific antibody at the interface between stacking and separating gel (compare lanes 3 and 5, the signal on top of the blot). As this signal was absent in nontransfected cells (Fig. 2C, lane 4) this band probably represents different amounts of partially solubilized recombinant teneurin-2 protein collecting at the interface between the two gel parts. Therefore, the Western blot data confirmed the specificity of the immunostaining we had observed. The calculated relative molecular mass of teneurin-2 is 94 kDa. The higher apparent relative molecular mass of the recombinant protein of 120 kDa could be explained by glycosylation, as well as by aberrant migration due to the denaturation of the EGF-like repeats under reducing conditions. A comparable difference in apparent and calculated size has been observed with recombinant tenascin-C variants of similar structure (Fischer *et al.*, 1997).

Teneurin-2 Is Cleaved at a Furin-like Site

We identified a dibasic amino acid motif characteristic for cleavage by furin-like proteases located in the extracellular half of teneurin-2, at amino acid 528 (RXRR; Hosaka *et al.*, 1991) (Figs. 1A and 3B). Due to the limited amount of recombinant teneurin-2 protein produced it was not possible to isolate and analyze cleavage products by protein sequencing. Therefore, a tenascin C/teneurin-2-fusion pro-

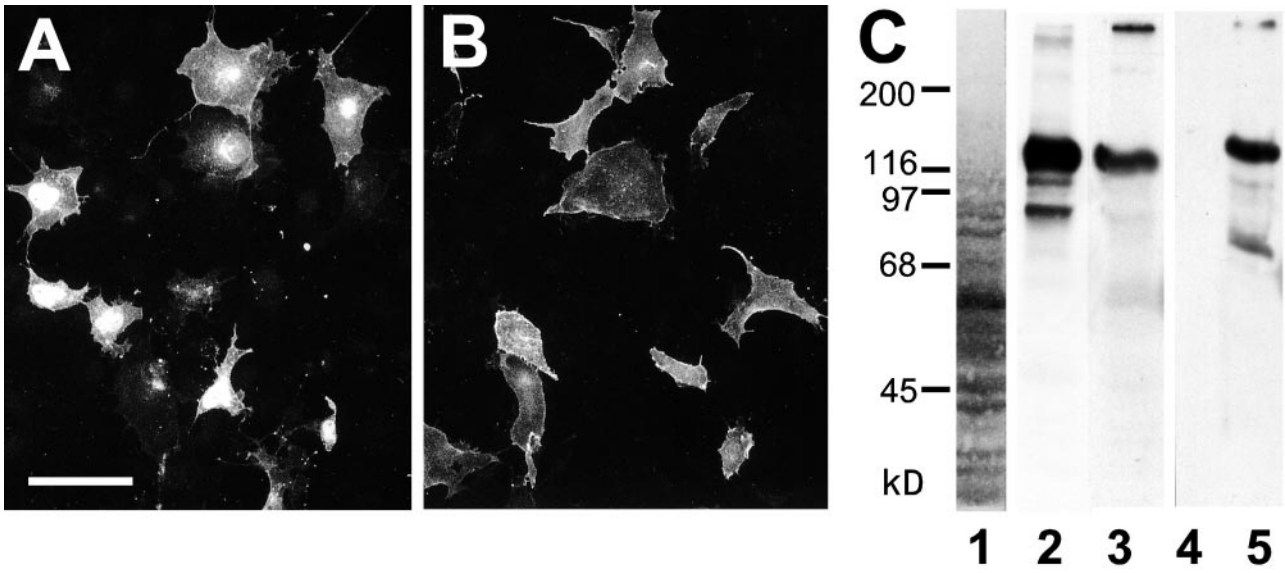


FIG. 2. Expression of recombinant teneurin-2 in COS-7 cells. COS-7 cells were transfected with a plasmid encoding full-length teneurin-2 carrying a VSV tag at its C-terminus. Following transfection the cells were fixed and either briefly treated with detergent (A) or stained directly (B) using a polyclonal antibody directed against the extracellular part of teneurin-2 and a fluorescein-coupled secondary antibody. (C) An extract of the transfected COS-7 cells was separated by SDS–PAGE and immunoblotted. Lane 1, the protein extract stained with amido black. In Lane 2 the signal obtained with a polyclonal VSV-tag-specific antibody and in lane 3 the signal obtained by the teneurin-2-specific antibody are shown. In Lane 4 an extract of nontransfected Nb2a cells and in lane 5 an extract of Nb2a cells transfected with the teneurin-2 plasmid described above was separated by SDS–PAGE and immunoblotted with the teneurin-2-specific antibody. Bar, 50 μm.

tein (TN/ten-2) was constructed. This protein included the N-terminal domain of the tenascin-C subunit (the central globe and the heptad repeats) joined to the entire extracellular part of teneurin-2. The fusion protein was secreted into the cell culture medium and could be affinity purified with the help of a monoclonal antibody (anti-TN60) directed against the tenascin-C moiety. The affinity-purified fraction not only contained the intact TN/ten-2 protein, but also two distinct cleavage products of 49 and 53 kDa relative molecular mass (Fig. 3A, lane 1). N-terminal peptide sequence, which was obtained from the 49-kDa cleavage product, confirmed that the two fragments were generated by proteolytic cleavage at the furin recognition site: RERR. Further evidence that this site is specifically accessible to cleavage by proteases recognizing basic residues was the complete conversion of the intact fusion protein into the two degradation-resistant cleavage products by trypsin. (Fig. 3A, lane 2 and 3). In *Drosophila* Ten-a and mouse DOC4 a site corresponding to the furin consensus motif has been identified as well, but not shown to be functional (Wang *et al.*, 1998).

The results described above are in agreement with those of Schaefer *et al.* (1997), who report the proteolytic cleavage of γ-heregulin, a novel member of the neuregulin family of proteins. The N-terminal halves of teneurin-2 and γ-heregulin are highly homologous and the dibasic site in the extracellular domain of teneurin-2 can be aligned to a basic cleavage site in γ-heregulin, close to the transmembrane domain (see motif and schematic representation in Fig. 3B). In the case of γ-heregulin, the C-terminal part which confers the growth-promoting properties to the protein consists of the immunoglobulin domain and the single EGF-like repeat characteristic for the neuregulin family. This part of the protein is released into the medium by proteolytic cleavage (Schaefer *et al.*, 1997). These findings suggest that the activity of teneurins and related proteins might be regulated by proteolytic cleavage.

Teneurin Expression in Nb2a Cells Leads to Filopodia Formation and Enlarged Growth Cones

By *in situ* analysis we found teneurin-1 and teneurin-2 to be expressed by neurons at different sites in the developing chick central nervous system (see below). To test the effect of teneurin-2 on neuronal cells *in vitro*, a neuroblastoma cell line (Nb2a cells) was transfected with a plasmid encoding full-length teneurin-2. Following the transfection, serum was withdrawn in order to induce the cells to acquire a neuron-like phenotype (Suidan *et al.*, 1992). After 48 to 72 h in culture the cells were fixed and stained with an antibody specific for teneurin-2 (green, fluorescein signal) and phalloidin (red, rhodamine signal) to reveal filamentous actin. Confocal (Figs. 4a–4f and 4i) and conventional light microscopy (Figs. 4g and 4h) were used to analyze the

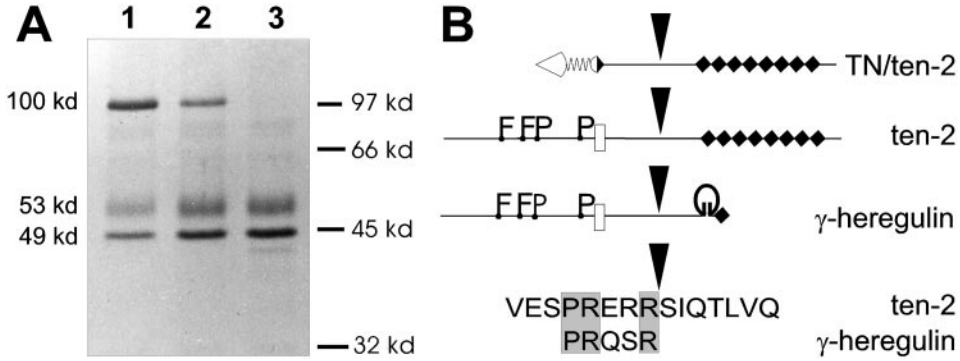


FIG. 3. Proteolytic processing of teneurin-2 HT1080 cells were transfected with a plasmid encoding the fusion protein TN/ten-2. TN/ten-2 was purified from conditioned medium by affinity purification using a tenascin-C-specific antibody. (A) Lane 1, SDS–PAGE revealed three bands in the affinity-purified fraction. The 100-kDa band corresponds to the intact TN/ten-2. The 53- and 49-kDa bands result from cleavage in teneurin-2 (ten-2) at the dibasic site. The black triangle indicates the location of the conserved proteolytic cleavage site, which corresponds to the highlighted motifs in (B). (A) Lanes 2 and 3, treatment of the affinity-purified fraction with increasing amounts of trypsin led to complete cleavage of TN/ten-2 into the two fragments. (B) Structural models: The cone and the zig-zag structure represent the tenascin-C part of the TN/ten-2 fusion protein. The black diamonds represent the EGF-like repeats of teneurin-2 (ten-2). The "F" stands for a potential calcium-binding site and the "P" stands for polyproline stretches located in the cytoplasmic domain of the teneurin-2 and γ-heregulin. The rectangular box represents the putative transmembrane domains. The loop and black diamond represent the immunoglobulin domain and the single EGF-like repeat characteristic for neuregulins.

distribution of filamentous actin and to compare it to that of teneurin-2. The actin staining showed that both the teneurin-2-negative and the teneurin-2-positive cells extended filopodia along their cell body and their neurites. However, in a vast majority of teneurin-2-expressing cells the filopodia were forming a much denser, highly branched and extensive network. This striking phenotype was never observed in any of the teneurin-2-negative cells in the same transfection experiment or in vector-transfected control cells in separate cultures (compare Figs. 4a and 4b), but was reproducible in four independent experiments in which several hundred cells were examined. To quantify these changes, 50 cells which had been transfected with either the plasmid encoding teneurin-2 or the vector alone were analyzed for the number of filamentous actin-containing filopodia emanating along their neurites. The teneurin-2-expressing cells formed an average of 7.3 filpodia/15 μm $\pm$ 1.71 (SD) and the control cells formed an average of 2.9 filopodia/15 μm $\pm$ 1.6. This represents a 2.5-fold increase in the number of filopodia in the presence of teneurin-2 along neurites. In addition, a higher density of filopodia and actin-free membrane protrusions was observed along the cell body (Fig. 4). The induction of additional filopodia seemed to be substrate dependent, as it was more pronounced when the cells were plated on laminin than on polylysine (data not shown). The dynamic interaction between the filopodia and the laminin substrate could be visualized indirectly by the fact that moving filopodia left a "footprint" of teneurin-2-stained material behind (compare actin staining to teneurin-2 signal in Fig. 4c). The teneurin-2-induced filopodia mostly formed a very fine branching network along the periphery of the cell, as seen in Figs. 4d–4f by the actin and the teneurin staining. The image in Fig. 4i represents the integration of 10 confocal planes of filopodia formed along a neurite. Visual cross-sections of this three-dimensional representation showed that the teneurin-2 staining surrounded the phalloidin-stained core, i.e., the filamentous actin (see inset in Fig. 4i). Of particular interest is the fact that a large fraction of the teneurin-2-positive cells in addition to an increased number of filopodia also developed enlarged growth cones in comparison to the nonexpressing cells (see the phase contrast image in Fig. 4g and immunostaining in Fig. 4h). To quantify growth cone sizes areas of the foremost 15 μm of neurites of the teneurin-2-expressing cells and of neurites of the vector-transfected control cells were measured. These measurements showed a marked shift toward a larger size growth cone size in teneurin-2-expressing cells. A total of 53% (20/38) of the teneurin-2-transfected cells formed a growth cone larger than 125 μm^2, whereas only 2.6% (1/39) of the control cells exceeded that size (Fig. 5). These results argue that teneurin-2 indirectly or directly influences the organization of the actin cytoskeleton in Nb2a cells leading to distinct morphological changes.

Teneurin-2 Is Predominantly Expressed in the Chicken Central Nervous System

To identify the tissues in which teneurin-2 is expressed, Northern blots (Fig. 6A) and reverse transcription PCR (Fig. 6B) were performed on RNA isolated from brain tissue and peripheral organs during development and in the adult animal. The message size detected was about 10 kb and thus much larger than expected for a protein of 831 amino acids. The large size of the message of teneurin-2 could be explained by the presence of long untranslated sequences as

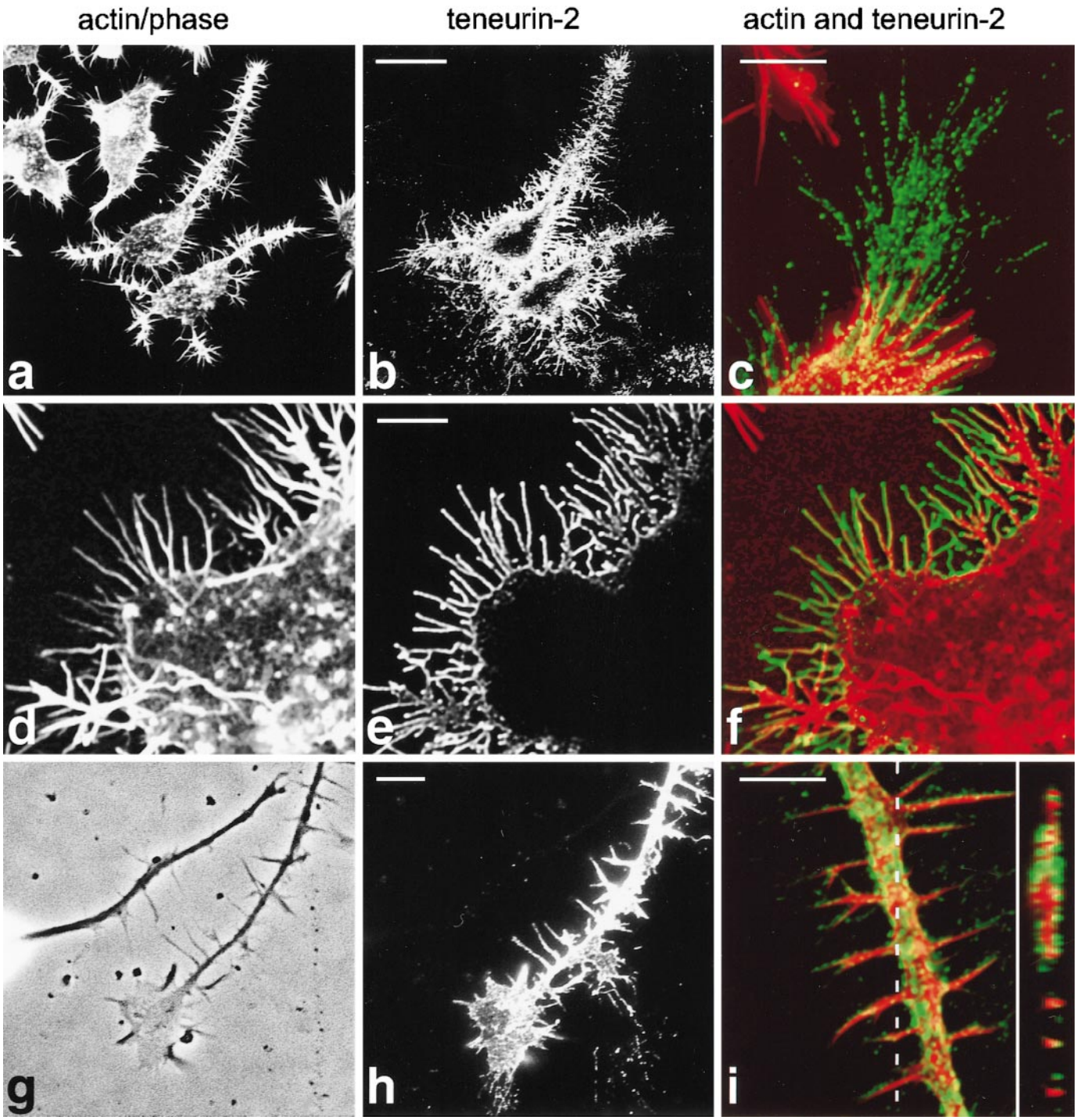


FIG. 4. Teneurin-2 induces filopodia in Nb2a cells Nb2a cells were transfected with a plasmid encoding teneurin-2 and induced to form neurites on a laminin substrate. After fixation the cells were immunostained using a polyclonal antibody directed against the extracellular part of teneurin-2 and a fluorescein-coupled secondary antibody (visible as a green signal). In parallel, cultures were incubated with phalloidin coupled to rhodamine (visible as a red signal) to reveal filamentous actin. (a–f) single optical slices; (I) the integration of 10 optical slices generated by confocal microscopy. (g and h) Conventional phase contrast and fluorescence images, respectively. The actin staining in (a) shows all cells present in the field. The corresponding field in (b) shows that the two cells having an increased number of filopodia can be identified as those expressing teneurin-2. At higher magnification in (c) one can see that the actin-positive filopodia were sometimes

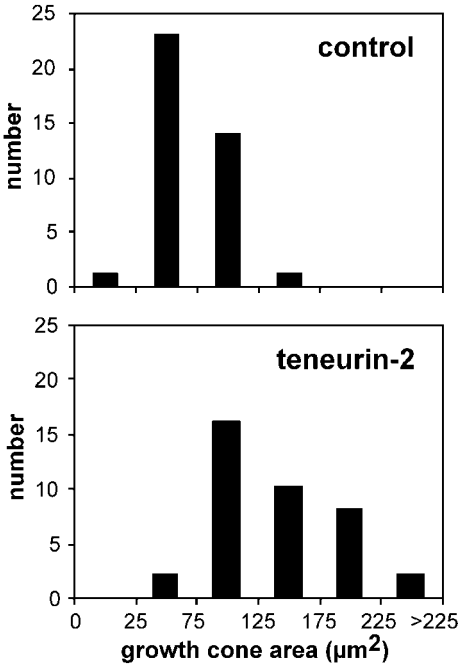


FIG. 5. Teneurin-2 induces the formation of enlarged growth cones The area of growth cones from teneurin-2 (n = 39) and vector-transfected control cells (n = 38) was measured. The results were grouped in classes (size limits are indicated on the x axis). Absolute numbers of growth cones within a certain size range are represented by the bars.

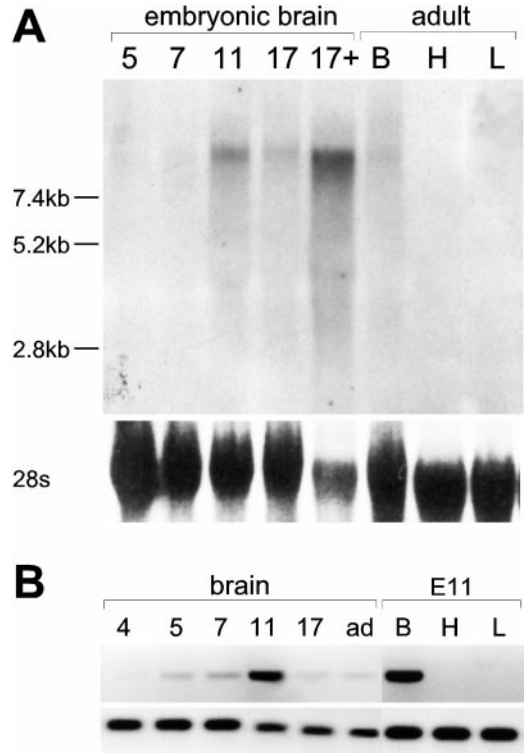


FIG. 6. Teneurin-2 mRNA is present in developing and adult chicken brain. (A) Northern blot: hybridization of total RNA and poly(A)+ mRNA (17+) isolated from different embryonic stages (days of incubation given as numbers) and adult brain (B), heart (H), and liver (L) with a teneurin-2-specific riboprobe revealed a band of about 10 kb. This signal was only present in adult brain RNA, but not in RNA from adult heart or liver. (B) Reverse transcription PCR using primers specific for teneurin-2 and glyceraldehyde 3-phosphate dehydrogenase, as a control, was performed on total RNA of different embryonic stages (days of incubation given in numbers). Teneurin-2-specific bands were visible starting from embryonic day 4 persisting in the adult (ad) brain. The strongest signal was obtained in two independent preparations of embryonic day 11 (E11) mRNA, corresponding to the strong signal seen in the Northern blot at this stage. No teneurin-2-specific band could be detected in embryonic day 11 heart (H) or liver (L).

has been shown to be the case for *Drosophila ten-a* (Baumgartner and Chiquet-Ehrismann, 1993). In agreement with this hypothesis we have obtained two independent cDNA clones of teneurin-2 containing up to 1400 bp of untranslated sequences at the 3′ end, without reaching the polyadenylation site.

By reverse transcription PCR teneurin-2 could be detected in brain starting at embryonic day 4. Northern blot and reverse transcription PCR both showed an increase in expression until embryonic day 11 and thereafter a lower relative constant level of expression persisting in the adult brain. Neither heart and liver at embryonic day 11 nor adult heart and liver contained detectable levels of teneurin-2 mRNA, as judged by Northern blots and reverse transcription PCR (Figs. 6A and 6B). The expression of teneurin-1 was also limited to the central nervous system and showed a parallel time course to that of teneurin-2 (Minet *et al.*, 1999).

retracted leaving teneurin-2 staining material attached to the laminin substrate. In (d–f) the network of filopodia protruding from the cell periphery is shown at a higher magnification. Filamentous actin was present in all of the teneurin-2 stained filopodia, forming highly branched structures. The difference in the number of filopodia and in growth cone formation between teneurin-2-expressing cells and nontransfected cells could also be visualized by phase microscopy: compare the neurite and growth cone of the immunostained cell with that of the unstained cell in (g and h). In (i) a stack of 10 individual confocal images has been integrated. The stippled line indicates the position of an oblique section taken through the integrated image. The resulting image to the right shows at high magnification that the green teneurin-2 staining surrounded the red actin core along the neurite shaft. Bar in (b), 100 μm; bars in (c, e, and i), 5 μm; and bar in (h), 10 μm.

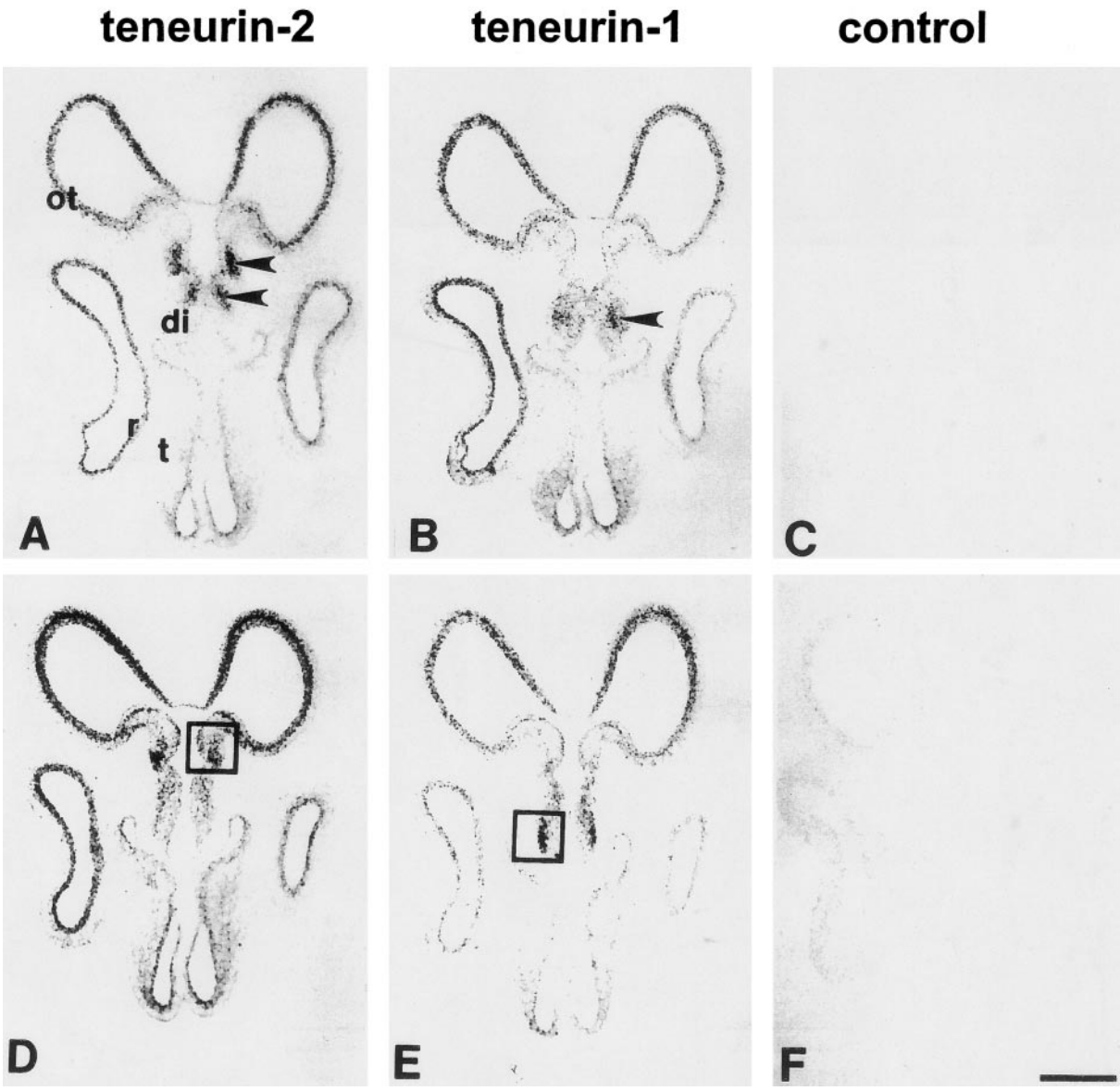


FIG. 7. *In situ* hybridization of horizontal sections through heads of E7 chicken embryos X-ray overlays of hybridizations with radioactively labeled probes to teneurin-2 (A, D), teneurin-1 (B, E), or a pUC control (C, F). Both teneurin-2 and teneurin-1 probes hybridize in the developing visual system. Signals are concentrated in the developing optic tectum (ot) and retina (r) and in specific nuclei in the diencephalon (di). There is also a faint, diffuse signal in the telencephalon (t). Different diencephalic nuclei express teneurin-2 and teneurin-1 (arrowheads). The boxes indicate the regions illustrated at higher magnification in Fig. 7. Bar, 1 mm.

Teneurin-1 and Teneurin-2 Are Expressed Widely in the Early Chicken Brain

To analyze the expression pattern of teneurin-1 and teneurin-2 in the developing brain, coronal sections through an embryonic day 7 head were hybridized with probes specific for both messages in parallel. Teneurin-1 (Figs. 7B and 7E) and teneurin-2 (Figs. 7A and 7D) are expressed in the retina, the telencephalon, the optic tectum, and the diencephalon. It is important to note that at this early stage the distribution of the two messages in the diencephalon is already complementary and confined to different nuclei. This becomes especially apparent if the two areas indicated in Figs. 7D and 7E are examined at higher magnification and compared to a counterstaining with a nuclear dye showing the general cytoarchitecture (Fig. 8). The message for teneurin-2 was mainly found in the intermediate zone of the thalamus (T), whereas the one of teneurin-1 was mostly located to the intermediate zone of the dorsal thalamus (DT) (compare Figs. 8A–8C and 8D–8F). Neither of the two messages could be detected in the cell

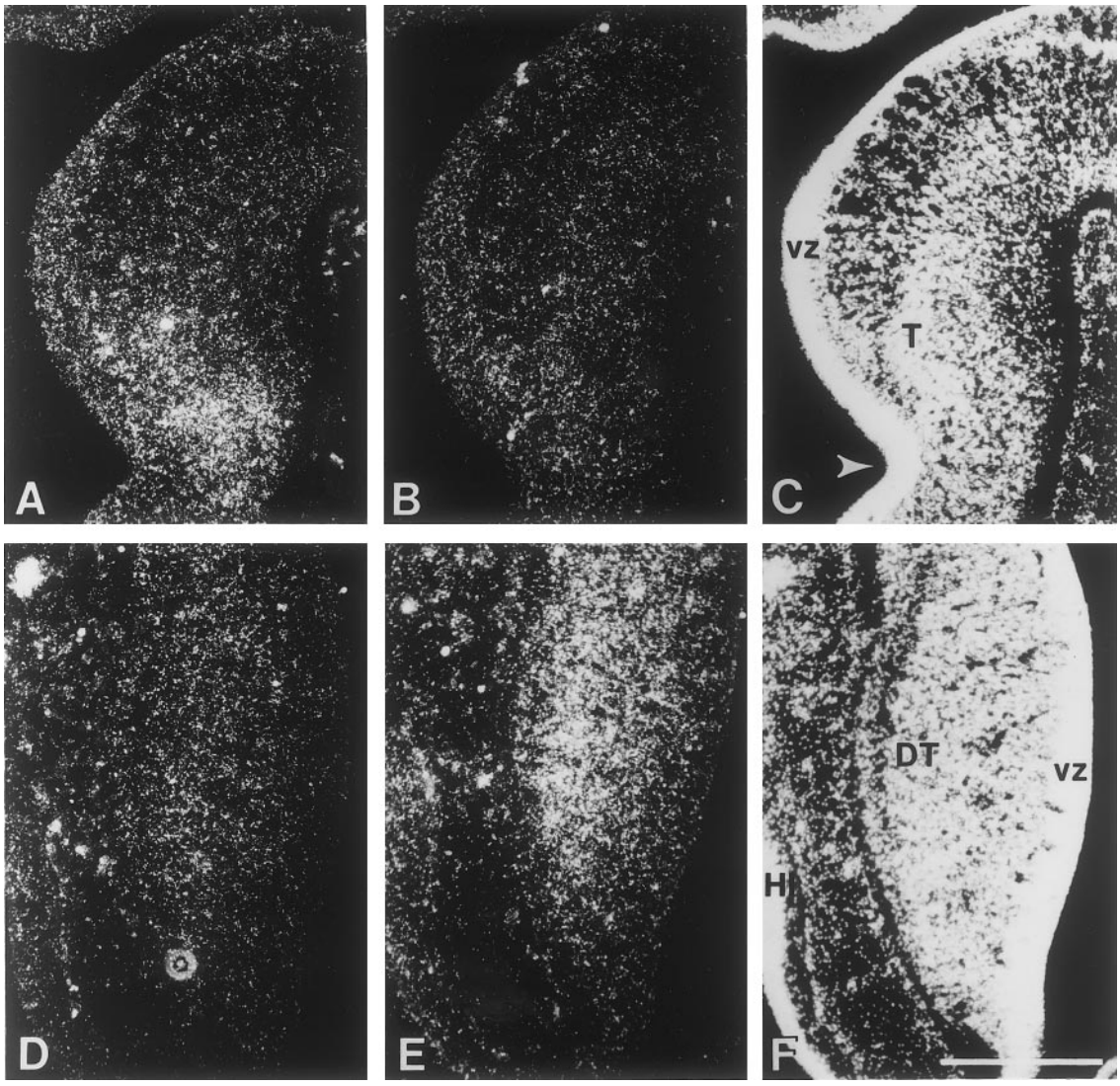
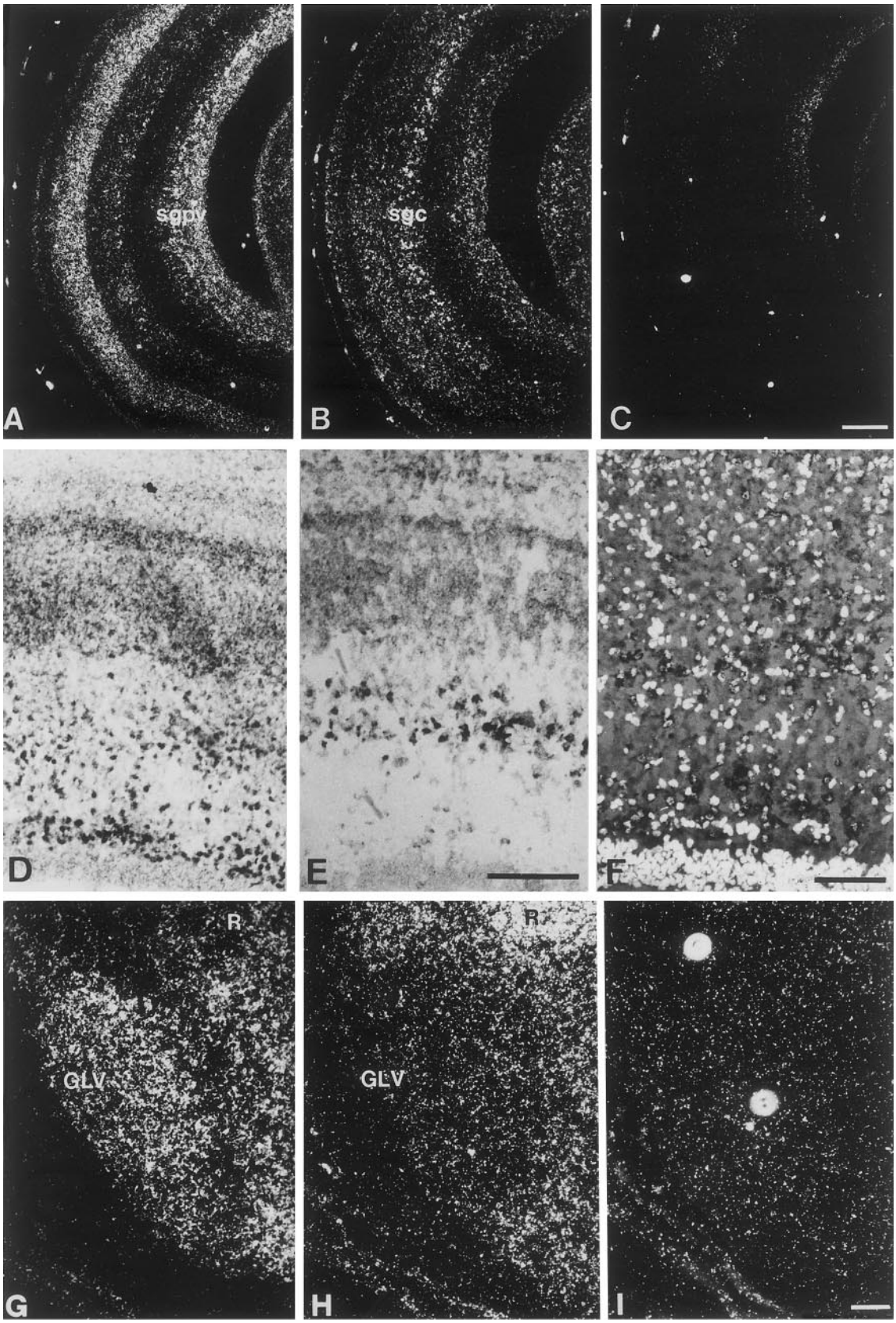


FIG. 8. *In situ* localization of teneurin-2 and teneurin-1 mRNAs in the E7 chicken diencephalon. The sections have been counterstained with a nuclear dye (C, F). Teneurin-2 mRNAs (A, D) are concentrated in the intermediate zone of the thalamus (T), whereas teneurin-1 transcripts (B, E) are concentrated in the intermediate zone of the dorsal thalamus (DT). Neither probe hybridizes over background levels in the ventricular zone (vz) or hippocampus (H). The dorsal diencephalic sulcus is indicated with an arrowhead. Bar, 250 μm.

dense ventricular zone (VZ), suggesting that the teneurins are not expressed by neuroblasts, but by postmitotic neurons about to establish new connections. The differential expression of teneurin-2 and teneurin-1 is maintained in the diencephalon and becomes more prominent as more nuclei form and the lamination of the optic tectum develops during later stages of brain development.

Chicken Teneurin-2 and Teneurin-1 Are Expressed in a Differential Fashion in the Developing Nervous System

At embryonic day 14, *in situ* analysis showed that teneurin-2 and teneurin-1 are still expressed in the optic tectum, the thalamus, and the retina. At this stage the main laminae of the optic tectum have developed and it becomes apparent that the cells which express teneurin-2 most strongly are localized in the stratum griseum periventriculare (sgpv) (Figs. 9A, 9D, and 9F), while those expressing teneurin-1 are found exclusively in the stratum griseum centrale (sgc) (Figs. 9B and 9E). There is some overlap between both cell populations expressing the different messages, as teneurin-2 seems also to be expressed by some cells in the stratum griseum centrale (Figs. 9E and 9F). This differential expression pattern is already present in the embryonic day 11 optic tectum (data not shown). The restricted distribution of the two messages suggests that both teneurin-2 and teneurin-1 are expressed by neuronal



cells specific for the different laminae. In the embryonic day 14 thalamus the complementary expression of teneurin-2 and teneurin-1 becomes even more apparent, as the cells that express them are part of different nuclei. Only teneurin-2 transcripts are present in the lateral geniculate nucleus, in contrast to the rotund nucleus where only teneurin-1 was detected (Figs. 9G and 9H).

DISCUSSION

A Novel Family of Proteins

We have shown that the teneurins constitute a highly conserved family of neuronal cell-surface proteins. The high degree of conservation between vertebrate and invertebrate protein encoding homologous sequences allowed the identification of six different members of the teneurin/ten family. Two of these seem to be species homologues encoding mouse and chicken teneurin-2, respectively. Therefore, we postulate that a family of vertebrate teneurins exists, which has evolved from an ancestral gene to two genes, *ten-a and ten-m* at the insect level to at least four genes at the vertebrate level (Oohashi *et al.*, 1999).

Teneurin-2, a Receptor and/or a Ligand?

The analysis of the amino acid sequences of vertebrate teneurin-1 and teneurin-2 suggested a structural model whereby the N-terminal half of the protein is located within the cytoplasm followed by a single transmembrane domain and an extracellular C-terminal half. This structure prediction was confirmed by the fact that if intact cells expressing recombinant teneurin-2 were stained with a polyclonal antibody raised against the C-terminal part of the protein a distinct membrane staining was visible. This and the fact that within the cytoplasmic domain of chicken teneurin-1 and -2 two potential calcium-binding sites and two putative SH3-domain-binding sites could be identified argue that the teneurins could serve as receptor proteins transmitting signals to the cell interior upon homo- or heterophilic binding of a ligand. This does not exclude, however, that they could function themselves as membrane-bound ligands. Transmembrane members of the eph family ligands play such a dual role (Bruckner *et al.*, 1997). Ten-a, teneurin-2, DOC4/Ten-m 4, and γ-heregulin all possess conserved sequences in the beginning of the extracellular part of the protein that correspond to the basic cleavage motifs recognized by members of the subtilisin family of proteases. This cleavage site was found to be active in the tenascin-C/teneurin-2 fusion protein. Furin, a member of the subtilisin family, has been shown to be a transmembrane protein. It is activated by autocatalytic cleavage and cycles between the trans-Golgi network and the cell surface (Molloy *et al.*, 1994). Furin is expressed in the central nervous system during development (Zheng *et al.*, 1994, 1997) and has been shown to modulate the activity of members of the semaphorin family of axon guidance molecules (Adams *et al.*, 1997). It is assumed that teneurin-2 becomes cleaved at the cell membrane. Therefore, either cleavage of teneurin-2 might serve to release a large part of the extracellular domain of the protein from the cell membrane, upon which it could act in addition as a soluble ligand or, alternatively, cleavage could occur after binding of a ligand and be involved in signal transduction, as is the case with notch (Nye, 1997).

Teneurin-2 Induces the Formation of Filopodia in a Neuronal Cell Line

Expression of full-length teneurin-2 in Nb2a cells, which had undergone differentiation to neuronal cells, led to a 2.5-fold increase in the number of filamentous actin-containing filopodia and a distinct shift toward a larger growth cone size. Teneurin-2 not only promoted the formation of these structures, but was also localized at their membrane surface. Filopodia and growth cones are highly dynamic structures, which are essential for the correct navigation of the outgrowing axon during development (Tessier-Lavigne and Goodman, 1996; Cook *et al.*, 1998). *In vitro,* filopodia react to subtle changes in soluble or substrate-bound signaling molecules, conferring a turning response to the entire growth cone toward an attractive

FIG. 9. Teneurin expression in the embryonic day 14 optic tectum and diencephalon. Horizontal sections of the E 14 optic tectum (A–C) were hybridized with radioactively labeled probes to teneurin-2 (A), teneurin-1 (B), or a pUC control (C). X-ray overlays of the optic tectum seen an at low magnification show that there was a differential distribution of teneurin-2 signal being more pronounced in the stratum griseum periventriculare (sgpv) (A) and the teneurin-1 signal being strongest in the stratum griseum centrale (sgc) (B). A higher magnification of horizontal sections of the E14 optic tectum stained by nonradioactive *in situ* hybridization showed that the teneurin-1 signal is indeed restricted to the large neurons of the stratum griseum centrale (E), while the teneurin-2-probe in addition to the medium-sized neurons of the stratum griseum periventriculare (sgpv) also stained some cells in the adjacent stratum album centrale and in the stratum griseum centrale (D and F). Neither probe gave a signal over background in the more superficial laminae, nor in the very cell dense periventricular layer, seen in a section hybridized with a teneurin-2 probe and counterstained with a nuclear dye (F). Bars in (E and F), 250 and 100 μm, respectively. The expression of teneurin-2 (G) and teneurin-1 (H) in horizontal sections through the E14 chicken diencephalon. A pUC control is shown in (I). At E14, the general pattern of expression first seen at E7 persists: teneurin-2 is expressed ventrally, and teneurin-1 is expressed dorsally. A specific thalamic nucleus that is clearly labeled by the teneurin-2 probe is the ventral lateral geniculate nucleus (GLV), which is not labeled by the teneurin-1 probe. In contrast, the rotund nucleus (R) is labeled intensely with the teneurin-1 probe, but not with the teneurin-2 probe. Bar in (I), 100 μm.

signal and away from a repulsive signal (Kater and Rehder, 1995; Fan and Raper, 1995; Zheng *et al.*, 1996; Song *et al.*, 1998). Growth cone motility is based on changes in cytoskeletal structures (Bentley and O'Connor, 1994). Actin and its associated proteins such as talin, vinculin (Sydor *et al.*, 1996), and myosin (Lin *et al.*, 1996) play a role during elongation and retraction of filopodia. It is possible that teneurin-2 interacts with some of the actin-binding proteins and influences thereby the reorganization of the actin cytoskeleton, the core structure of filopodia. Teneurin-2 could either increase the frequency of initiation and/or reduce the frequency retraction of filopodia; both events would lead to an overall increase in the number of filopodia. In fibroblasts the expression of the N-terminal domain of ezrin and moesin, two proteins shown to serve as links between the actin cytoskeleton and integral plasma membrane proteins, induces very similar, abundant and highly branched filopodial structures, which retract abnormally (Amieva *et al.*, 1999).

The induction of structures by teneurin-2, which are needed by neurons to sense their extracellular environment, is particularly interesting in view of the fact that chicken teneurin-1 and teneurin-2, as *Drosophila* Ten-a and Ten-m, were found to be expressed in the developing nervous system (Baumgartner and Chiquet-Ehrismann, 1993; Baumgartner *et al.*, 1994).

Do Teneurins Define the Target Specificity of Neural Circuitry?

Teneurin-1 and teneurin-2 mRNA are found to be differentially expressed by postmitotic neurons in the chicken brain from embryonic day 7 on. The differential expression pattern is maintained at later stages of development. Interestingly, in the chicken visual system the projection of neurons, expressing one type of teneurin to neurons, expressing the same type of teneurin could be demonstrated. From anatomical studies it is known that neurons of the stratum griseum centrale project to the rotund nucleus, forming the major tectofugal projection in the avian visual system (Benowitz and Karten, 1976). Both these structures contain cells expressing teneurin-1 mRNA. In an analogous manner neurons of the deep layers of the optic tectum project to the lateral geniculate nucleus (Crossland and Uchwat, 1979). Again both these parts of the visual system contained teneurin-2-positive cells. Therefore, the teneurins, as has been suggested for the cadherins in the vertebrate central nervous system (Redies, 1997; Wohrn *et al.*, 1998), could serve to define the projections of subgroups of neurons to their corresponding target cells.

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