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Teneurin 2 is expressed by the neurons of the thalamofugal visual system in situ and promotes homophilic cell-cell adhesion in vitro

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SUMMARY

The transmembrane glycoprotein teneurin 2 is expressed by neurons in the developing avian thalamofugal visual system at periods that correspond with target recognition and synaptogenesis. Partial and full-length teneurin 2 constructs were expressed in cell lines in vitro. Expression of the cytoplasmic domain is required for the induction of filopodia, the transport of teneurin 2 into neurites and the co-localization of teneurin 2 with the cortical actin cytoskeleton. In addition, expression of the extracellular domain of teneurin 2 by HT1080 cells induced cell aggregation, and the extracellular domain of teneurin 2 became concentrated at sites of cell-cell contact in neuroblastoma cells. These observations indicate that the homophilic binding of teneurin 2 may play a role in the development of specific neuronal circuits in the developing visual system.

Key words: DOC4, Neurestin, Odz, Ten^{m}, Ten^{a}, Ten-m, Chicken

INTRODUCTION

Teneurins are a family of type II transmembrane proteins originally discovered in *Drosophila*. The first member was Ten-a (Baumgartner and Chiquet-Ehrismann, 1993; Minet and Chiquet-Ehrismann, 2000), which was found in a search for *Drosophila* homologues of tenascins and shares with this protein family the same type of EGF-like repeats. The second member of the teneurin family, *Drosophila* Ten-m/Odd oz (Odz), was discovered independently in two laboratories (Baumgartner et al., 1994; Levine et al., 1994). It is expressed in seven stripes during the blastoderm stage in early embryos. Later in development, teneurins are prominently expressed by specific subpopulations of neurons in *Drosophila* (Levine et al., 1997; Minet et al., 1999) as well as in mouse (Ben-Zur et al., 2000; Oohashi et al., 1999; Wang et al., 1998), rat (Otaki and Firestein, 1999a), chicken (Minet et al., 1999; Rubin et al., 1999; Tucker et al., 2000; Tucker et al., 2001) and zebrafish (Mieda et al., 1999). Although teneurin 2 shows highest expression in the nervous system, it is also expressed at other locations known to be crucial regulatory sites of morphogenesis, such as the apical ectodermal ridge and the dorsomedial lip of the somite (Tucker et al., 2001).

The domain organization of teneurins in invertebrates and vertebrates is highly conserved. All teneurins have a proline-rich cytoplasmic domain, and extracellularly contain a series of EGF-like repeats and 26 YD repeats (Minet and Chiquet-Ehrismann, 2000). The cytoplasmic domain may be involved in a signal transduction cascade, as mutational analysis showed that *ten-m/odz* is a member of the 'pair-rule' gene family and has a central role in determining the segmentation of the *Drosophila* embryo (Baumgartner et al., 1994). A highly conserved dibasic furin-like cleavage site is found between the transmembrane domain and the EGF-like repeats (Rubin et al., 1999), meaning that teneurins may be proteolytically processed in the same way as Notch (Logeat et al., 1998). The function of the EGF-like repeats is unknown, but a recombinant murine teneurin forms side-by-side dimers in vitro that appear to be linked via disulfide bridges between the EGF-like repeats (Oohashi et al., 1999). The YD repeats bind heparin (Minet et al., 1999) and are similar to those found in the *rhs* element of *E. coli* and in wall associated protein A of *Bacillus subtilis*, where they may have appeared due to horizontal gene transfer from an ancestral teneurin (Minet and Chiquet-Ehrismann, 2000).

Unlike *Drosophila*, which has two teneurin genes, vertebrates have up to four teneurin genes (Minet and Chiquet-Ehrismann, 2000; Oohashi et al., 1999). Although different nomenclatures have been developed in laboratories using different animal models, the number designation at the end of each name can be used to identify the orthologous genes: murine ten-m1 (Oohashi et al., 1999) is the same as murine odz1 (Ben-Zur et al., 2000) and corresponds to avian teneurin 1 (Rubin et al., 1999), etc. Note that the murine *Doc4* gene (Wang et al., 1998) encodes ten-m4 (Oohashi et al., 1999), and that the rat teneurin 2 ortholog has also been called neurestin (Otaki and Firestein, 1999b). In addition, at least three alternatively spliced variants

of teneurin 2 have been identified (Tucker et al., 2001), including one variant that lacks the YD repeats. The functional significance of these variations is unknown.

There is some experimental evidence that teneurins may play a role in neurite outgrowth and pathfinding. In vitro, the YD repeats of teneurin 2 support the outgrowth of neurites, and this outgrowth is abolished by heparin (Minet et al., 1999). Transfection of Nb2a neuroblastoma cells with chicken teneurin 2 expression constructs results in the formation of numerous teneurin 2-enriched filopodia and enlarged growth cones, suggesting an interaction of teneurin 2 with the cytoskeleton (Rubin et al., 1999). In situ hybridization reveals non-overlapping neuronal expression of teneurin 1 and teneurin 2 in interconnected parts of the developing diencephalon and midbrain (Rubin et al., 1999). The possibility of homophilic interactions between these teneurins is supported by the observations of Oohashi et al. (Oohashi et al., 1999) that labeled ten-m1 binds to ten-m1 on blots, and labeled ten-m1 binds to ten-m1-rich regions of tissue sections. Finally, the human teneurin 1 gene maps to the same part of the X-chromosome (Xq25) as an X-linked mental retardation syndrome characterized by sensory neuropathology (Minet et al., 1999).

We show by immunohistochemistry that teneurin 2 is expressed in specific brain regions by neurons that are known to be part of a specific circuit, namely the thalamofugal visual system of the chicken. Teneurin 2 is expressed at the time when axons find their targets. Furthermore, functional tests prove teneurin 2 to be a homophilic cell-adhesion protein fitting the hypothesis that teneurin 2 provides neurons with the capability of recognizing and forming synapses with other teneurin 2-expressing neurons.

MATERIALS AND METHODS

Immunohistochemistry and in situ hybridization

For immunohistochemistry, chicken embryos at E3, E5, E6, E7, E11, E13 and E18 were fixed in cold 4% paraformaldehyde in phosphate buffered saline (PBS) overnight (the brains and eyes of the E18 embryos were dissected from the heads after a few hours and returned to the fixative). After fixation, the tissues were rinsed in PBS, cryoprotected in 20-25% sucrose in PBS overnight, and frozen in embedding medium using dry ice. Frontal, sagittal and transverse sections were cut at 14 μm in a Leitz cryostat and collected on FisherPlus (Fisher Scientific) microscope slides. The sections were then processed for indirect fluorescence immunohistochemistry with an affinity-purified rabbit anti-chicken teneurin 2 polyclonal serum (Rubin et al., 1999) and Alexa Fluor 568-tagged goat anti-rabbit secondary antibody (Molecular Probes) using methods detailed elsewhere (Tucker et al., 2001). At least three embryos were analyzed per developmental stage and they all revealed a consistent expression pattern.

Whole-mount in situ hybridization was carried out on E10 brains using methods described elsewhere (Tucker et al., 2001; Wilkinson and Nieto, 1993). In brief, whole brains were dissected from E10 chicken heads after fixation in 4% paraformaldehyde, cut sagittally at the midline, then dehydrated in methanol and stored at –20°C. Hybridization with a digoxigenin-labeled RNA probe to teneurin 2 (ten-2L) (Tucker et al., 2001) or a sense control probe was followed by extensive rinses and incubation for 40 hours at 4°C in TBST/1% goat serum with alkaline phosphatase-tagged anti-digoxigenin (Boehringer Mannheim) diluted 1:1000. NBT/BCIP was used for the color reaction. Frozen sections (see above) through retinas at E12 and whole brains at E18 were processed similarly to localize teneurin 2 transcripts in cells.

Teneurin 2 constructs and transfection of cells

Four different teneurin 2 constructs were used in the present study. All of them are cloned in pcDNA3/Neo (Invitrogen). They are named according to the protein domains contained within their coding regions (see Fig. 3). Construct CTE has been used previously (Rubin et al., 1999) and contains the complete coding region of a short splice variant of teneurin 2 (Accession Number, AJ245711). Construct CTEY contains the complete coding region of the long form of teneurin 2 described elsewhere (Tucker et al., 2001) (Accession Number, AJ279031). Constructs TEY and TE are derived from each of the above constructs, respectively, by deletion of amino acids 1-362 of the cytoplasmic domain. The plasmids were either transfected into COS-7 or Nb2a neuroblastoma cells for transient expression studies or into HT1080 cells to isolate stably expressing clones using the transfection reagent fugene (Roche Diagnostics). After transfection, HT1080 cells were plated at low enough dilution to result in clonal growth of transfected cells. Several clones representing each construct were picked and screened for recombinant protein expression by immunofluorescence as well as by immunoblotting. Phalloidin staining and detection of the recombinantly expressed teneurin 2 proteins with the teneurin 2 antibody by immunocytochemistry was done as described previously (Rubin et al., 1999). Teneurin 2-containing cellular extracts for immunoblotting were prepared as follows. After washing the cells on 5 cm culture dishes with cold PBS, plates were frozen at –20°C. After thawing, they were extracted for 30 minutes on ice with 600 μl of hypotonic buffer [20 mM KCl, 2 mM sodium phosphate (pH 7.0), 1 mM β-mercaptoethanol]. Cells were collected by scraping with a rubber policeman and transferred into Eppendorf tubes. After spinning for 10 minutes at maximum speed the pellet was dissolved at 37°C for 20 minutes in 60 μl detergent buffer [150 mM NaCl, 50 mM Tris (pH 8), 1% NP-40, 6 M urea, 5 mM EDTA] per plate. An equal volume of SDS-PAGE sample buffer [0.2 M Tris-HCl (pH 6.8) 4% SDS, 17.4% glycerol/20% β-mercaptoethanol, 6 M urea] was added and the sample was incubated for 1 hour at 52°C. The samples were separated on SDS-PAGE and transferred to PVDF membranes. The teneurins were detected by the anti-teneurin 2 serum (Rubin et al., 1999) and the signals revealed using the ECL system (Amersham).

Aggregation assays

HT1080 cells were harvested by incubation with 0.2% EDTA, pelleted by centrifugation and resuspended at 2.5×10^5 cells/ml of Leibovitz L15 medium (Gibco) containing 1% fetal calf serum (Gibco). Cell suspensions (3 ml of each) were added to 5 cm bacterial plates (Sterilin) and incubated at 37°C on a rotary shaker at 80 rpm. Photographs were taken at 15 minutes intervals and cell aggregation was analyzed by counting single cells versus cells in double, triple or higher number aggregates. The aggregation index was calculated as N_o-$N_t/N_o\times100$, where N_o is the initial number of particles corresponding to the total number of cells and N_t is the number of remaining particles at the incubation time point t.

Cos7 cells were co-transfected with the ten-2 construct CTEY and a plasmid encoding EGFP under the control of a β-actin promoter (kindly provided by Andrew Matus, Friedrich Miescher Institute) using the transfection reagent fugene (Roche Diagnostics). The transfected cells were cultured for 24 hours before harvesting them as described for the HT1080 cells. Aggregation assays were performed as with the HT1080 cells. After 3 hours, pictures of the aggregates were taken using a Zeiss Axiophot microscope (Carl Zeiss).

RESULTS

Teneurin 2 expression coincides with target recognition and synaptogenesis

The earliest teneurin 2 expression in the central nervous system was revealed by faint immunostaining in the optic fiber layer (OFL) of the avian retina at embryonic day (E) 7 (not shown). By E11, the immunostaining was found in the inner plexiform layer (IPL) in addition to the OFL and the optic nerve (Fig. 1A,B). At E12, the staining was most intense in the IPL (Fig. 1C). The retina itself is a source of teneurin 2, as the entire retinal ganglion cell layer is strongly labeled with a digoxigenin-RNA probe at E12 (Fig. 1D). The labeled cells are neurons and not glia, as oligodendrocytes do not appear in the retina until E10, do not reach IPL until E14, and do not reach the periphery of the retina until E16 (Ono et al., 1998; Fu and Qiu, 2001). The only other significant anti-teneurin 2 immunostaining found in the central nervous system between E7 and E11 is in the optic tectum, where both the stratum opticum (SO; representing retinal ganglion cell fibers) and the large neurons of the stratum griseum periventriculare (SGP) are positive for anti-teneurin 2 (Fig. 1E,F). As in the retina, this staining precedes the appearance of oligodendroglial markers (Perez Villegas et al., 1999). In the chick, the axons of retinal ganglion cells first extend from the retina at E3 (Prada et al., 1981) and reach the optic tectum at E6 (DeLong and Coulombre, 1965), i.e. just before the first detection of teneurin 2 in the OFL. Retinal ganglion cell processes leave the SO and invade the underlying optic tectum at E9, and form synapses shortly thereafter (Thanos and Bahnhoeffer, 1987; Yamagata et al., 1995). Synapses also first appear within the IPL between E9 and E10 (Hering and Kroger, 1999). Thus, the appearance of teneurin 2 immunoreactivity in this well studied system corresponded not with initial outgrowth and pioneering, but with target recognition, fasciculation and synaptogenesis.

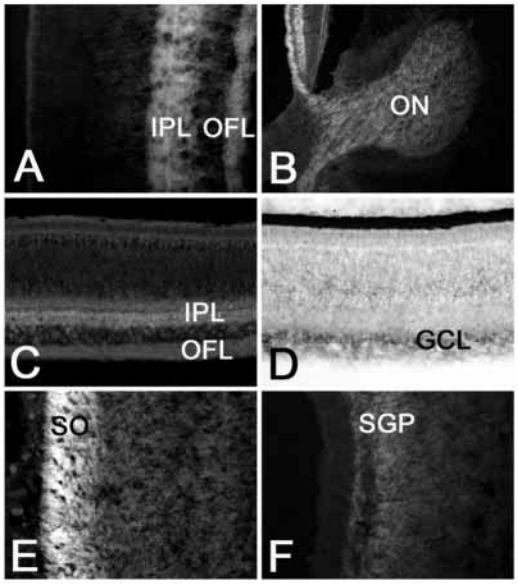

Fig. 1. Expression of teneurin 2 in the visual system. (A) Anti-teneurin 2 labels the inner plexiform layer (IPL) and optic fiber layer (OFL) of the E11 retina. (B) The staining in the OFL extends through the optic nerve head and into the optic nerve (ON). (C) At E12 anti-teneurin 2 labels the IPL and OFL, with distinctive laminae being labeled in the former. (D) An adjacent section subjected to in situ hybridization with a teneurin 2 cRNA probe reveals teneurin 2 transcripts concentrated in the retinal ganglion cell layer (GCL). Nearby sections treated with a sense control probe were unlabeled (not shown). (E) The stratum opticum (SO) of the optic tectum is stained with anti-teneurin 2 at E11. (F) The deepest layer of the optic tectum, the stratum griseum periventriculare (SGP), is also labeled with anti-teneurin 2 at E11.

Teneurin 2 is concentrated in the thalamofugal visual system

Whole-mount in situ hybridization of E10 brains revealed significant teneurin 2 expression in the avian forebrain and midbrain (Fig. 2A). In the forebrain, there was a strong hybridization reaction in the hippocampus (Hp) and hyperstriatum accessorium (HA or visual Wulst). There was also a strong signal in the dorsal thalamus. In the midbrain the optic tectum was strongly labeled. The neuronal origins of teneurin 2 were confirmed by examination of frozen sections hybridized with the teneurin 2 probe (Fig. 2B,C). Teneurin 2-positive perikarya are larger than oligodendrocytes (>20 μm versus 12-15 μm) and are absent from fiber tracts. By E13, the anti-teneurin 2 also revealed the presence of the protein in these areas (not shown).

By E18, the architecture of the avian brain resembles that of the adult, permitting identification of the specific brain regions stained with the anti-teneurin 2 serum. These regions are summarized in Table 1 as well as in Fig. 2D-O. Teneurin 2 immunoreactivity was found in the visual Wulst, as well as in dorsal thalamic nuclei and the ventral lateral geniculate nucleus (vLGN). A prominent fiber tract originating in the HA, the tractus septomesencephalicus (TSM), was also positive. In the midbrain, pretectal nuclei were labeled, and in the optic tectum the SGP was still teneurin 2 positive, although the staining was considerably fainter than at earlier stages. These teneurin 2-

Table 1. The expression of teneurin 2 in the E18 avian central nervous system

Region	Intensity*
Hyperstriatum accessorium (HA, visual Wulst)	+++
Medial hyperstriatum ventrale (HV)	+++
Tractus mesencephalicus (TSM)	++
Hippocampus (Hp)	+++
Area parahippocampus (APH)	+++
Posteromedial cortex piriformis (CPi)	+++
Nucleus septalis lateralis (SL)	++
Nucleus taeniae (Tn)	++
External granular layer, bulbus olfactorius (BO)	+
Nucleus dorsolateralis anterior thalami (DLA)	++
Nucleus triangularis (T)	++
Nucleus geniculatus lateralis (vLGN)	++
Nucleus pretectalis (PT)	++
Nucleus spiriformis (Sp)	++
Nucleus lentiformis mesenchephali (LM)	++
Stratum griseum periventriculare (SGP)	+/–
Nucleus of Edinger-Westphal (EW)	++
Nucleus isthmi, pars magnocellularis (Imc)	++
Nucleus isthmi, pars parvocellularis (Ipc)	++
Nucleus isthmo-opticus (IO)	++
Cerebellum (Cb)	+

*+++, intense signal, visible at low magnification; ++, strong signal, visible at intermediate magnification; +, weak signal, visible at high magnification; +/–, weak and sometimes absent.

positive regions are interconnected and correspond to the thalamofugal visual pathway (reviewed by Mey and Thanos, 2000): retinal ganglion cells project to the dorsal thalamus, which in turn projects to the visual Wulst. The Wulst then projects to the vLGN, pretectal nuclei (PT) and the optic tectum, which were also stained with the anti-teneurin 2. With the exception of retinal ganglion cells, which belong to both major visual circuits, the major parts of the tectofugal visual pathway (the rotund nucleus and the ectostriatum) were not positive for teneurin 2 expression. However, the triangular nucleus (T), which receives efferents from the stratum griseum centrale of the optic tectum (Hellmann and Güntürkün, 2001), was teneurin 2 positive. Note that in addition to all parts of the thalamofugal visual pathway, teneurin 2 was associated with the nucleus of Edinger-Westphal (EW), a mesencephalic nucleus composed of preganglionic parasympathetic motoneurons that innervate the smooth muscle of the ciliary body and pupil. There was also a strong signal in the nucleus isthmo-opticus (IO), which projects to the teneurin 2-positive retina, and in the mesencephalic lentiform nuclei (LM), which receive fibers from the retina (Ehrlich and Mark, 1984).

Although most of the teneurin 2 in situ hybridization and immunohistochemical signals in the avian CNS were associated with the thalamofugal and minor visual pathways, there was also a strong signal in the Hp and one of its targets, the lateral septal nucleus (SL). There was also a faint signal in the cerebellum at E18 and in the external granular layer of the olfactory bulb. This latter observation is consistent with the cloning of the rat teneurin 2 ortholog neurestin from an olfactory bulb library by others (Otaki and Firestein, 1999b).

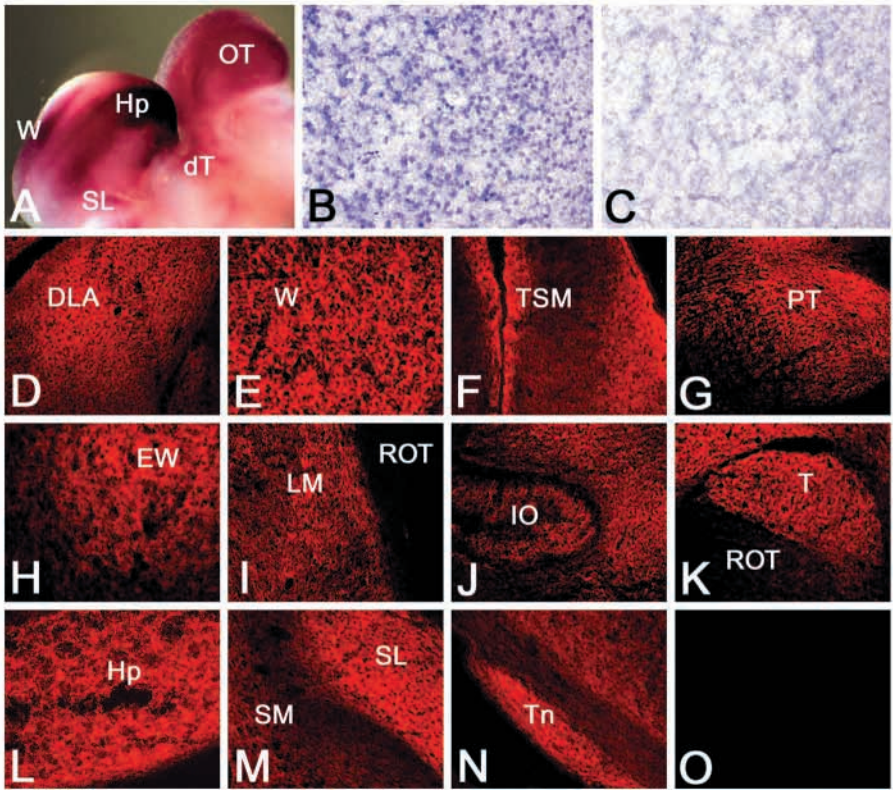

Fig. 2. Expression of teneurin 2 in the avian central nervous system. (A) Whole-mount in situ hybridization with a teneurin 2 probe at E10. The whole brain has been cut at the midline and the medial surface is being viewed. In the forebrain (left and center), the visual Wulst (W), hippocampus (Hp), lateral septal nucleus (SL) and nuclei in the dorsal thalamus (dT) are strongly labeled. In the midbrain (right), teneurin 2 transcripts are seen in the optic tectum (OT). (B) Sections through the developing Hp followed by in situ hybridization show teneurin 2 transcripts in large neurons. (C) Sections through a brain incubated with a control probe show the low level of background. (D,E) At E18, anti-teneurin 2 labels the anterior dorsolateral thalamic nucleus (DLA) and the primary target of its efferent projections, the visual Wulst (W). (F-H) The mesencephalic tract (TSM), which is formed largely from Wulst efferent fibers, and targets of the Wulst like the pretectal nucleus (PT) are also stained with anti-teneurin 2 at E18. The nucleus of Edinger-Westphal (EW), which receives input from the pretectal nucleus, expresses teneurin 2 as well. (I,J) In addition to the thalamofugal visual pathway, another non-tectal target of retinal projections [the mesencephalic lentiform nucleus (LM)] and a source of projections back to the retina [the isthmo-optic nucleus (IO)] are positive for teneurin 2 at E18. (K) The triangular nucleus (T), which rests like a cap on the rotund nucleus (ROT), expresses teneurin 2. The ROT, which is part of the tectofugal visual pathway, does not. (L-N) A major site of teneurin 2 expression at E18 is the Hp and one of the principal targets of its efferent projections, the SL. The nucleus taeniae (Tn), which projects to the teneurin 2-positive parahippocampus, is also a site of teneurin 2 expression. (O) Sections incubated with preimmune serum are unlabeled.

In vitro expression of teneurin 2 constructs

The timing and distribution of teneurin 2 expression in the developing nervous system implied a possible function of teneurin 2 in neurite fasciculation and/or neuron target recognition. We decided to test this hypothesis on the cellular level by expressing teneurin 2 recombinantly in cell cultures. First we expressed four different membrane-anchored teneurin 2 constructs in COS-7 cells: CTEY (for cytoplasmic, transmembrane, EGF-like and YD-repeats), containing the cytoplasmic domain as well as the entire long form of the extracellular domain; TEY, the same with a deleted cytoplasmic domain; CTE, corresponding to a short splice variant of teneurin 2 (Rubin et al., 1999); or TE, the same but without a cytoplasmic domain (Fig. 3A). Cell extracts were prepared 24 hours after transfection and analyzed by SDS-PAGE and immunoblotting with anti-teneurin 2 serum. Each construct resulted in the presence of a major band of roughly the expected size. The proteins without the cytoplasmic tails (TEY and TE) always had an M_r of ~40×10^3, which is smaller than the corresponding proteins with these domains present (CTEY and CTE) (Fig. 3B). Immunostaining of the transfected cells without permeabilization prior to antiserum incubation revealed the presence of the extracellular domains of teneurin 2 on the cell surface. Interestingly, the morphology of the cells expressing the constructs including the cytoplasmic domains was very different from the ones without the cytoplasmic

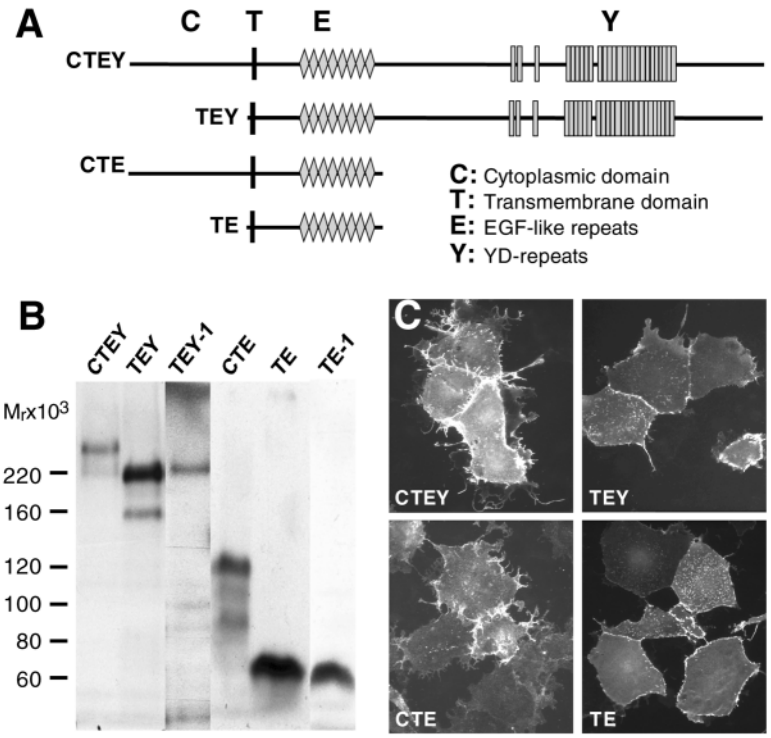

Fig. 3. Expression of teneurin 2 constructs. (A) Models of the four teneurin 2 proteins encoded by the constructs used for transfection. (B) Immunoblots with anti-teneurin 2 of cell extracts of COS-7 cells transfected with constructs CTEY, TEY, CTE and TE as indicated. Lanes TEY1 and TE1 represent immunoblots with anti-teneurin 2 of cell extracts of stable cell clones of HT1080 cells selected after transfection with TEY or TE, respectively. (C) Immunostaining of COS-7 cells with anti-teneurin 2 after transfection with the constructs indicated reveals cell surface expression. Note the numerous filopodia of cells transfected with the constructs containing the cytoplasmic domains (CTEY and CTE).

domain (Fig. 3C). Whereas CTEY and CTE induced prominent filopodia in the transfected cells, TEY and TE was present in cells with smooth surfaces. This implies an interaction of the teneurin 2 cytoplasmic domain with yet-to-be identified cytoskeletal components.

Expression of the extracellular domain of teneurin 2 leads to cell aggregation

Next we tried to isolate stable cell lines expressing these four types of teneurin 2 proteins. HT1080 cells were transfected and replated at high enough dilution to allow clonal growth of the transfected cells. We selected several clones of two constructs, TEY and TE, that lack the cytoplasmic domain (Fig. 3B; Fig. 4). However, several attempts to obtain clones from constructs CTEY or CTE (containing the cytoplasmic domain) were unsuccessful. It appears that the presence of the cytoplasmic domain is not tolerated very well by the cells and interferes with their ability to survive or proliferate. A comparison between the morphology of the parental cell line and examples of several clones are shown in Fig. 4. We consistently noticed that all clones expressing the long form of the extracellular domain (TEY1, TEY2, TEY3) showed a much flatter morphology than either the parental cells (HT1080) or the cells expressing the short teneurin extracellular domain (TE1) as can be seen both on the phase contrast pictures (Fig. 4A), as well as after phalloidin staining of the actin cytoskeleton (Fig. 4B). Despite their flat morphology the clones, TEY1, TEY2 and TEY3 did

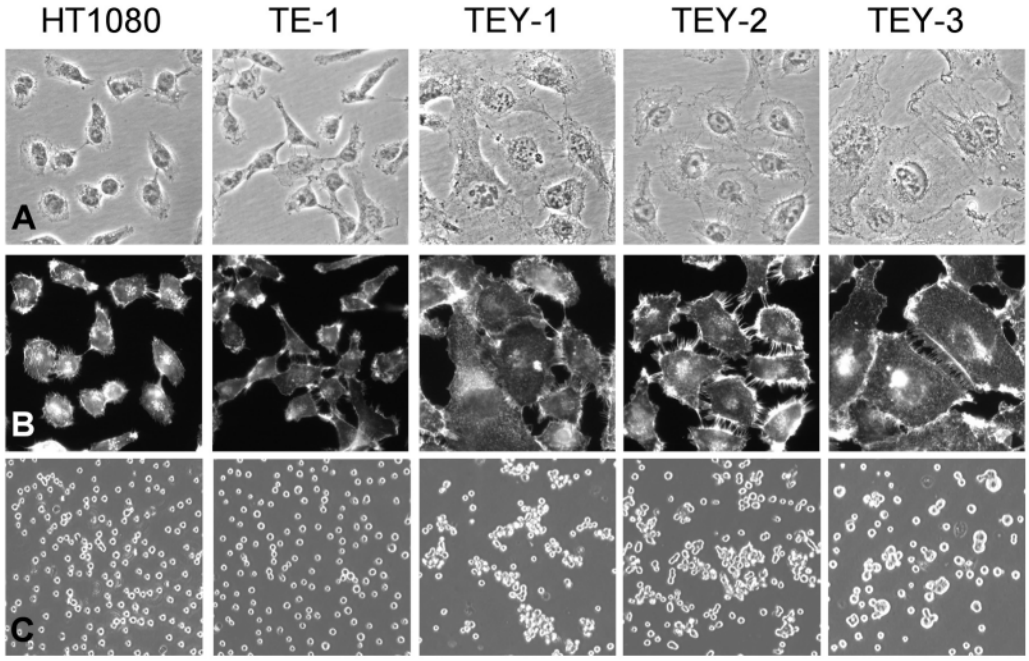

Fig. 4. The long form of the extracellular domain of teneurin 2 induces morphological changes and aggregation of transfected HT1080 cells. (A) Phase-contrast pictures of parental HT1080 cells and cell clones transfected with the membrane-anchored short extracellular domain (TE1) or cell clones expressing the long extracellular domain (TEY1, TEY2 and TEY3). Note that TEY-transfected cell clones show increased cell spreading and more cell-cell contacts. (B) Staining of the actin cytoskeleton by RITC-phalloidin of the cells shown in A reveals mostly cortical actin staining and no increased stress fiber formation even in the highly spread cells. (C) Phase-contrast pictures of cells dissociated by EDTA treatment and incubated in suspension on a rotary shaker for 1 hour. All clones expressing the long extracellular domain of teneurin 2 aggregated, whereas the parental cells and the cells expressing the short form of teneurin 2 remained as single cells.

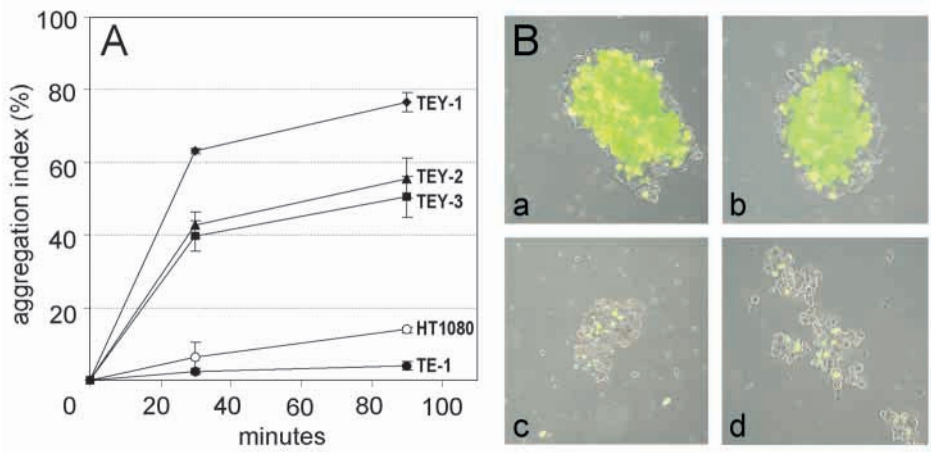

Fig. 5. Time course and quantitative measurement of aggregation. (A) All clones expressing the long extracellular domain of teneurin 2 aggregate rapidly reaching aggregation indices of over 40% within 30 minutes. HT1080 cells and cells expressing the short form of teneurin 2 do not aggregate over the 90-minute incubation period. This experiment was repeated five times with the same qualitative results. (B) Photographs of typical aggregates formed by Cos-7 cells after transfection of the full length teneurin 2 construct CTEY together with an EGFP construct. Green cells formed tight aggregates excluding non-transfected cells in their center (a,b), whereas the aggregates of non-transfected cells were looser and smaller and only contained occasional green cells (c,d).

not contain a more pronounced actin cytoskeleton than the parental cells and all cells revealed mostly cortical actin staining.

In addition to their flat phenotype, we noticed that the cell clones TEY1, TEY2 and TEY3 grew in epithelial cell-like patches suggestive of increased cell-cell adhesion. By contrast, the parental cells and clone TE1 grew as dispersed fibroblast-like cells. Therefore, we investigated whether or not these clones showed increased cell-cell adhesion in an aggregation assay of cells in suspension. The cells were harvested by EDTA treatment, centrifuged and taken up in medium as single cell suspensions. The suspended cells were placed in petri dishes and incubated at 37°C on a rotary shaker. Photographs of the cells after one hour of incubation are shown in Fig. 4C. Although the parental HT1080 cells as well as the TE-1 cells remained as single cells, all clones expressing the long teneurin 2 extracellular domain (TEY1, TEY2, TEY3) aggregated into clumps of cells. Clearly, the presence of the extracellular domain C-terminal to the EGF repeats of teneurin 2 on these cells resulted in their aggregation. Quantitative comparison of the cell aggregation showed that after 30 minutes of incubation all TEY cell lines had already approached maximal aggregation values, whereas the HT1080 and TE-1 cells did not aggregate even after 90 minutes of incubation (Fig. 5A).

These experiments suggested that teneurin 2 expression may lead to homophilic cell-cell adhesion. To exclude the possibility that the teneurin 2 causes cell aggregation by interaction with an endogenous heterophilic ligand present on the cells, we studied the aggregation of a mixed population of Cos-7 cells. We transiently transfected Cos-7 cells with the teneurin 2 construct CTEY, together with a GFP plasmid to visualize the transfected cells. This resulted in a mixed culture of green, transfected cells and pale non-transfected cells. After 1 day in culture, aggregation assays were performed and photographs of the resulting aggregates are shown in Fig.5B. We found that the aggregates consisted either of mostly green or mostly pale cells. The green aggregates tended to be bigger and tighter, whereas the pale cells formed looser aggregates.

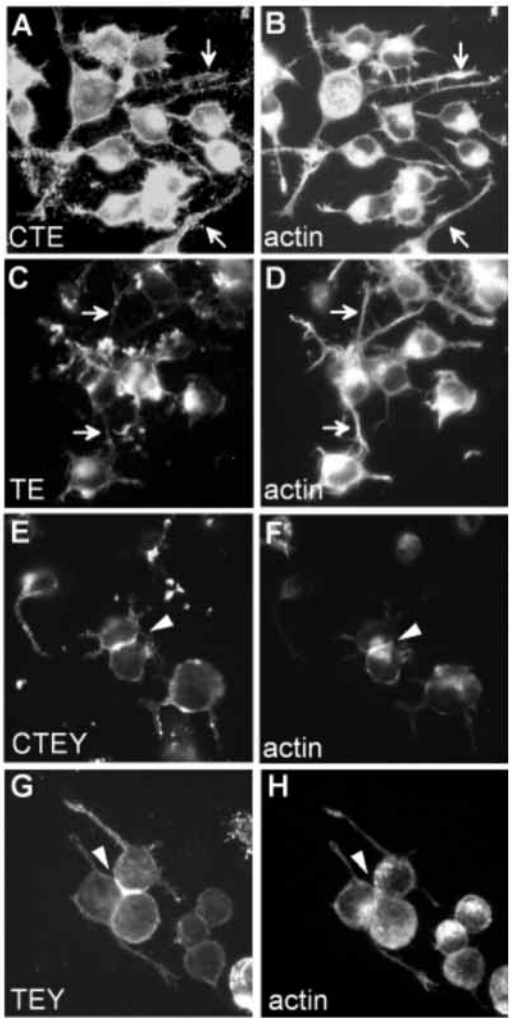

Fig. 6. Expression of teneurin 2 constructs in Nb2a neuroblastoma cells. The following constructs were transiently expressed in Nb2a cells. CTE (A,B), TE (C,D), CTEY (E,F) and TEY (G,H). In the left panel (A,C,E,G), the staining pattern of the transfected teneurin 2 as revealed by anti-teneurin 2 staining is shown and the right panel reveals the actin cytoskeleton of the same cells by phalloidin staining (B,D,F,H). In each pair of pictures, arrows or arrowheads indicate sites of teneurin 2 expression that do (A,B,E,F) or do not (C,D,G,H) overlap with the actin staining.

We conclude that the green teneurin 2-expressing cells preferentially aggregated with teneurin 2-expressing cells and not with non-transfected cells supporting the hypothesis of a homophilic interaction between teneurin 2-positive cells.

The cytoplasmic domain of teneurin 2 is required for transport into neurites

We further investigated the effect of the expression of the teneurin 2 constructs on Nb2a neuroblastoma cells. As previously reported (Rubin et al., 1999), expression of CTE constructs in Nb2a cells led to the induction of filopodia and enlarged growth cones (Fig. 6A,B). This effect was not seen after transfection of the TE construct (lacking the cytoplasmic domain). In addition, the TE protein mainly localized to the plasma membrane of cell bodies and not to neurites (Fig. 6C,D). This suggests that the cytoplasmic domain is required for the translocation of teneurin protein to neurites and growth cones in addition to the induction of filopodia. The transfection of the longer constructs CTEY and TEY resulted in quite different teneurin 2 expression patterns. Both of these proteins were expressed on the cell surfaces and were heavily enriched in cell-cell contact areas, giving further support for the promotion of homophilic interactions between these neuronal cells (Fig. 6E-H). Interestingly, the CTEY expression led to the accumulation of actin to these teneurin-rich cell-cell contacts as revealed by phalloidin staining (Fig. 6F), which was not the case for the TEY construct (Fig. 6H). This provides evidence for an interaction between teneurin 2 and the actin cytoskeleton through its cytoplasmic domain.

DISCUSSION

Neuronal cell bodies in the vertebrate nervous system are organized in discrete nuclei and laminae that can be histologically distinguished. Each lamina consists of specific types of neurons, which in turn arborize and form synapses with neurons of other common subsets of laminae in other brain regions. This specificity of synaptic connections is so striking that it appears to be a major determinant of the defined connectivity in the central nervous system (for a review, see Sanes and Yamagata, 1999). In the 1960s, Roger Wolcott Sperry proposed the ‘chemoaffinity hypothesis’ where neurons recognize their synaptic partners through interactions between molecular ‘specifiers’ displayed on neuronal surfaces (reviewed by Sperry, 1963). The molecular basis underlying this morphological specification is just beginning to be understood.

The visual system is commonly used as a model to test Sperry’s hypothesis. In the avian visual system there are two principal circuits: the tectofugal pathway and the thalamofugal pathway (Fig. 7A). In the tectofugal pathway, retinal ganglion cells project to the optic tectum where they synapse in an ordered fashion to create a map of the visual field. Neurons within the optic tectum, in turn, project to the rotund nucleus of the diencephalon, which then projects to the ectostriatum of the telencephalon. This pathway is responsible for color discrimination, brightness, acuity and other crucial visual functions (for reviews, see Mey and Thanos, 2000; Shimizu and Bowers, 1999). The retina also sends processes to visual centers in the dorsal thalamus. These in turn project to the visual Wulst, which then projects back to the mesencephalon, including the optic tectum. This latter circuit is the thalamofugal pathway, which is believed to play a role in modulating the tectofugal pathway and in the detection of movements (reviewed by Mey and Thanos, 2000).

We show that the complex spatial and temporal pattern of teneurin 2 expression largely coincides with the development of the thalamofugal visual pathway (Fig. 7B). At early stages of development this was seen by teneurin 2 expression in the retina and dorsal thalamus. At later stages, the visual Wulst and its targets were major sites of teneurin 2 expression. Interestingly, we showed previously that teneurin 1, another member of the same protein family, was expressed in the retina, optic tectum and rotund nucleus at E14 (Rubin et al., 1999). Thus, teneurin 1 was expressed by interconnected neurons in the tectofugal visual pathway, and teneurin 2 was expressed by interconnected neurons in the thalamofugal pathway (Fig. 7). These observations led us to hypothesize that homophilic binding of teneurins may assist in the formation of appropriate synapses and fasciculation in the developing visual system. We tested this hypothesis in vitro by examining the morphology and behavior of cells transfected with full-length and partial sequences encoding teneurin 2. We found that teneurin 2 expressing HT1080 cells aggregated with each other. Furthermore, in transiently transfected Nb2a cells teneurin 2 accumulated at cell-cell contact sites. In the presence of the cytoplasmic domain of teneurin 2, F-actin became concentrated at these cell-cell contact sites as well. This is intriguing as F-actin was shown to be required for the

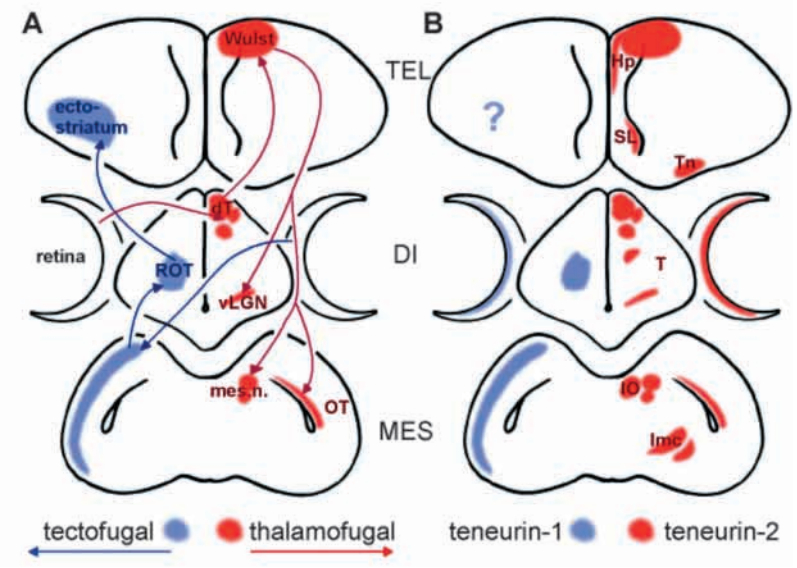

Fig. 7. The primary visual pathways of the chick and patterns of teneurin expression. (A) There are two primary visual circuits in birds, the tectofugal pathway and the thalamofugal pathway. (B) Teneurin 1 is expressed by neurons in at least two of the three major parts of the tectofugal pathway (expression in the telencephalon has not been examined). Teneurin 2 is expressed by the neurons of the thalamofugal visual pathway. See text for details and abbreviations.

development and stabilization of young synapsis in cultured hippocampal neurons (Zhang and Benson, 2001). It will be interesting to analyze whether other synapse-specific proteins are attracted to these sites and which cellular proteins can interact with the cytoplasmic domain of teneurin 2.

Although the principal sites of teneurin 2 expression were parts of the thalamofugal visual pathway, teneurin 2 was found in a few other parts of the embryonic CNS as well. The most prominent of these were the expression seen in Hp, the SN and nucleus taenia. Although not considered part of the thalamofugal visual pathway, these interconnected regions are speculated to play a role in spatial memory and pattern recognition (Colombo et al., 2001). Another interesting site of expression is the IO, which sends processes back along the teneurin 2-positive optic tract to the retina (Von Bartheld and Johnson, 2001). Both the Hp and the IO project to regions that also expressed teneurin 2, supporting the hypothesis that teneurin 2/teneurin 2 interactions may play a role in the development of appropriate synapses. Finally, two other regions that were positive for teneurin 2 expression at E18, the olfactory bulb [see also the observations made by Otaki and Firestein (Otaki and Firestein, 1999a; Otaki and Firestein, 1999b)] and cerebellum, are connected to the teneurin 2-positive regions of the developing visual system. The latter receives fibers from the visual Wulst (Wild and Williams, 2000), and the former receives fibers from a part of the hypothalamus that: (1) is a target of retinal projections (Ehrlich and Mark, 1984) and (2) also projects to the visual Wulst and Hp (Ehrlich and Mark, 1984).

Other protein-protein interaction systems have been shown to insure proper wiring of the brain. A prominent example was discovered through the cloning of the mutated gene in the reeler mouse. In this mouse line, neurons fail to reach their correct locations in the developing brain, leading to a disruption of the laminar organization of the cerebellar and cerebral cortices. The affected gene product, reelin, is a large secreted extracellular matrix protein (D'Arcangelo et al., 1995). There is evidence for several types of reelin receptors present on neurons, namely protocadherins (Senzaki et al., 1999), the VLDL-receptor and ApoE receptor 2 (Hiesberger et al., 1999), and integrin $\alpha 3\beta 1$ (Dulabon et al., 2000), all of which are though to be part of a reelin signaling pathway. Reelin and its signaling pathway through disabled 1 are also important in the development of the visual system, as mice deficient in reelin or disabled 1 show disturbed patterns of synaptic connections in the retina (Rice et al., 2001). The exact mechanism of reelin action is still unclear and a direct effect on axon growth has recently been questioned (Jossin and Goffinet, 2001). At least part of its action could be due to its recently discovered role as a serine protease that is able to degrade the integrin ligands fibronectin and laminin (Quattrocchi et al., 2002).

Protocadherins (Wu and Maniatis, 1999) and the classical cadherins are also well recognized candidates for cell-surface recognition proteins delineating and determining neural circuits. Experimental evidence suggests that cadherins contribute to central nervous system regionalization, morphogenesis and fiber tract formation, probably be conferring homotypic adhesiveness between neurons (for reviews, see Shapiro and Colman, 1999; Ranscht, 2000; Redies, 2000; Hamada and Yagi, 2001). Genetic evidence in *Drosophila* clearly demonstrates a role for N-cadherin in target finding in the visual system (Lee et al., 2001; Chiba, 2001). In humans, mutations in specific protocadherin genes cause sensorineural deafness and vestibular dysfunction as well as visual impairment due to retinitis pigmentosa (Bolz et al., 2001; Bork et al., 2001; Ahmed et al., 2001; Petit, 2001). In addition to the cadherins, protein-protein interactions between ephrins and Eph-recepters (Braisted et al., 1997; Castellani et al., 1998) or neuroligin and neurexin are also important factors determining synaptogenesis in the central nervous system (Cantallops and Cline, 2000).

Using specific constructs, we have begun to dissect the domains of teneurin 2 to understand the functions of its different domains. The cytoplasmic domain is responsible for transportation of teneurin 2 into cellular processes and its association with the actin cytoskeleton. This domain is also responsible for the induction of filopodia and may also play a role in regulating cell growth and proliferation. Future studies should be directed to identifying the binding partners of this domain. Others have suggested that the EGF-like repeats are involved in the side-by-side dimerization of teneurins (Oohashi et al., 1999), and our results demonstrate that these domains alone do not play a role in the homophilic interactions that lead to the aggregation of cells in vitro. This activity depends instead on the part of the molecule distal to the EGF-like repeats. This part of teneurin 2 also plays a role in regulating cell spreading in vitro. Thus, through a combination of morphological and experimental methods, we have shown that teneurin 2 is expressed at the right time and place, and has the appropriate properties in vitro to be a molecular 'specifier' that regulates the development of appropriate synapses in the avian visual system.

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