Tenascin-R promotes assembly of the extracellular matrix of perineuronal nets via clustering of aggrecan

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Perineuronal nets (PNs) in the brains of tenascin-R-deficient (tn-r−/−) mice develop in temporal concordance with those of wild-type (tn-r+/+) mice. However, the histological appearance of PNs is abnormal in adult tn-r−/− mice. Here, we investigated whether similar defects are also seen in dissociated and organotypic cultures from hippocampus and forebrain of tn-r−/− mice and whether the structure of PNs could be normalized. In tn-r−/− cultures, accumulations of several extracellular matrix molecules were mostly associated with somata, whereas dendrites were sparsely covered, compared with tn-r+/+ mice. Experiments to normalize the structure of PNs in tn-r−/− organotypic slice cultures by depolarization of neurons, or by co-culturing tn-r+/+ and tn-r−/− brain slices failed to restore a normal PN phenotype. However, formation of dendritic PNs in cultures was improved by the application of tenascin-R protein and rescued by polyclonal antibodies to aggrecan and a bivalent, but not monovalent form of the lectin Wisteria floribunda agglutinin. These results show that tenascin-R and aggrecan are decisive contributors to formation and stabilization of PNs and that tenascin-R may implement these functions by clustering of aggrecan. Proposed approaches for restoration of normal PN structure are noteworthy in the context of PN abnormalities in neurological disorders, such as epilepsy, schizophrenia and addiction.

1. Introduction

Perineuronal nets (PNs) are composed of aggregates of extracellular matrix (ECM) molecules around synaptic contacts at somata, proximal dendrites and axon initial segments of distinct neuronal populations in the central nervous system. To study the function of PNs, attempts have been made to reconstitute these conspicuous ECM structures in organotypic and dissociated neuronal cell cultures [1–5].

The molecular composition of PNs in vivo and in vitro appears to be similar in regard to ECM components, such as aggrecan [6,7], brevican [3,8] and the binding sites for the lectin Wisteria floribunda agglutinin (WFA) [2,5]. In dissociated hippocampal cell cultures, we were able to demonstrate an association of three major ECM components of PNs, i.e. aggrecan, tenascin-R (TN-R) and hyaluronan [4], which mirror the composition and histological appearance of
PNs in the intact brain [9]. Formation of PNs in vivo is an experience-dependent process [10,11] and, likewise, formation of PNs in vitro requires neuronal activity [1,4]. Vice versa, in vitro experiments indicated that PNs may be involved in regulation of neuronal activity, since enzymatic alteration of PNs increased the excitability of fast-spiking interneurons [4].

The organization of PNs is distinct among neuronal cell types [7,12–14] and relative levels in expression of aggregan, TN-R and hyaluronan vary in different brain regions [15]. Similarly, variability in distribution of these components was observed for interneurons maintained in vitro [4,5]. Multiple chondroitin sulfate proteoglycans (CSPGs) bind to hyaluronan via their G1 domains and to TN-R via their lectin-like and fibronectin type III homologous domains by a glycan-independent mechanism [16]. Since native TN-R molecules are di- or trimers, they may cross-link complexes of hyaluronan and CSPGs, and thus stabilize PNs (for review, see [17]). This notion is supported by the observation of abnormally structured PNs in tn-r−/− mice [18,19]. Since abnormalities in the WFA-staining patterns of PNs are barely detectable in mice deficient in neurocan [20] and brevican [21], it is likely that aggregan contributes most prominently to formation and maintenance of the WFA-positive PN structures [5–7,22] (for review, see [23]).

Here, we used two cell culture systems to investigate the role of TN-R in the assembly of PNs. We found that in tn-r−/− organotypic and dissociated cell cultures, PNs are abnormal in their appearance. Since this abnormality is abrogated by delivery of two cross-linking reagents that induce the clustering of aggregan, we suggest that this clustering by TN-R is essential for the characteristic in vivo appearance of dendritic PNs in normal brains.

2. Results

(a) Formation of perineuronal nets in organotypic cultures from tn-r+/+ mice

The temporal course of PN appearance in murine brain organotypic cultures corresponded well with the developmental appearance described for postnatal mice [19] and rat brain organotypic cultures [1,24]. However, unlike in organotypic cultures of rat brain, chronic depolarization evoked by elevated potassium concentrations was not required for formation of PNs in organotypic cultures of mouse brain [1,5,18].

In vitro, WFA-stained PNs were first detectable in brain stem regions during the first week of culture and only in the second week did they appear in the neocortex and hippocampus. The in vivo distribution pattern of WFA-stained PNs characteristic of adult mice is clearly seen at the end of the third week (figure 1). In contrast to the staining pattern with WFA, immunoreactivity for TN-R was not restricted to PNs and was often seen with intense staining in the neuropil around TN-R-positive PNs (figure 1a,b).

In the cerebral cortex, the majority of PNs was associated with parvalbumin (PARV)-immunopositive neurons (figure 1f). Cholinergic neurons immunoreactive for choline acetyltransferase (ChAT) in the basal forebrain were devoid of PNs (figure 1f), as previously reported for different mammalian species, including human [13,25–27].

(b) Formation of perineuronal nets in organotypic slice cultures from tn-r−/− mice

The temporal course of PN appearance, the typical regional distribution pattern, as well as the neuronal cell type-specific association of PNs did not differ in organotypic cultures from forebrain obtained from tn-r−/− mice compared with tn-r+/+ mice (figure 1a,c,e,g). However, in the cultures from tn-r−/− mice the intensity of WFA-staining was faint and a granular appearance characterized the structure of PNs in cortical and subcortical regions (figure 1c,e; electronic supplementary material, figure S1b). Similar to its appearance in vivo [13,28], the axon initial segment was often clearly WFA-labelled in vitro (figure 1c; electronic supplementary material, figure S1b), whereas PNs associated with proximal dendrites appeared consistently more faintly labelled than in vivo.

(c) Formation of perineuronal nets in organotypic co-cultures from tn-r−/+ and tn-r−/− mice

Immunoreactivity for TN-R was not reconstituted in organotypic cultures from tn-r−/+ co-cultured with tn-r+/+ organotypic cultures in the same culture well, even when slices were positioned in a way that they were overlapping with each other (figure 1a), suggesting that soluble factors produced by wild-type slices, including the 160 kDa isofrom of TN-R which can be detected in the culture supernatant of tn-r+/+ slices (electronic supplementary material, figure S2), are insufficient to normalize PNs in neighbouring tn-r−/+ slices. Thus, in co-culture, the characteristic genotype-specific patterns characteristic of the in vivo phenotype of WFA-stained PNs was retained in both genotypes.

(d) Formation of perineuronal nets in the presence of elevated external potassium concentration in organotypic cultures from tn-r−/− mice

Chronic depolarization of neurons induced by elevated potassium concentrations (10 and 25 mM) in the culture medium did not influence the abnormal appearance of WFA staining of PNs in cultures from tn-r−/− mice, whereas immunoreactivity for PARV was increased after chronic stimulation with 10 mM KCl (electronic supplementary material, figure S3d) as semiquantitatively detected by measuring brightness of fluorescence of untreated versus treated cultures (data not shown). Thus, despite the known positive effects of increased neuronal activity on the formation of PNs [4], elevation in expression of ECM molecules in the absence of TN-R is not sufficient to form normally appearing PNs.

(e) Formation of perisomatic GABAergic synapses is similar in cultures from tn-r−/+ and tn-r−/− mice

The temporal course of PN formation in dissociated hippocampal cultures followed the pattern previously described (electronic supplementary material, figure S4e, [4]). The faint WFA-labelling signal of PNs and the granulated structure of perisomatic PN areas described in vivo [19] and in organotypic cultures was a characteristic feature in dissociated cell cultures (electronic supplementary material, figure S4b). The axon initial segment identified in most net-associated neurons often...
Figure 1. PNs in organotypic forebrain slice cultures from wild-type (tn-r^+/+) and TN-R-deficient (tn-r^-/-) mice. N-Acetylgalactosamine-containing ECM components were detected in cultures maintained for 21 days by WFA fluorescence labelling (green) combined with immunolabelling (red) of tenascin-R (TN-R), PARV or ChAT. (a) Co-culture of forebrain slices from wild-type and TN-R-deficient mice showing the lateral parts of hemispheres dorsally overlapping with parietal cortices. WFA-labelled PNs are seen as dot-like structures within the TN-R-immunoreactive tissue in the wild-type. Because of the low staining intensity PNs can hardly be recognized in cultures of tenascin-R-deficient mice at low magnification. The piriform cortex (PirC) is characterized by a diffuse labelling of the neuropil. (b,b') Split double-fluorescence image showing the lattice-like structure of a WFA-labelled PN (b) that is also immunoreactive for TN-R (b') in the parietal cortex. The neuropil is clearly TN-R-immunopositive. (c) WFA-stained PN showing a granular component in the mutant. (c') The corresponding image demonstrates that the PN and the surrounding neuropil are TN-R-immunonegative. (d) PARV-immunoreactive interneurons associated with PNs in the wild-type parietal cortex. (e) PARV-immunoreactivity is clearly seen in cortical PN-associated interneurons in the mutant, whereas WFA-staining of PNs appears faint. Arrowheads point to WFA-labelled axon initial segments. (f) Basal forebrain with intensely WFA-labelled PNs in the wild-type. Neurons immunoreactive for ChAT are devoid of PNs. The transition zone to the piriform cortex is at the left. (g) Basal forebrain in a tn-r^-/- slice shows well-differentiated ChAT immunoreactive neurons in the ventral pallidum (VP) but poorly developed PNs (arrowheads). One of the PNs (framed area) is shown at higher magnification in the inset. The medial part of the piriform cortex of the mutant exhibits a coarser structure when compared to that of the wild-type. Scale bars, 500 μm (a); 20 μm (b, b', c, c'); 50 μm (d, e); 200 μm (f, g).
showed a tube-like WFA-labelled structure (electronic supplementary material, figure S4b), as reported, for example, in histological sections of the superior colliculus [28].

Electronic supplementary material, figure S4b, shows that perturbation of PNs by TN-R deficiency did not abolish formation of vesicular gamma-aminobutyric acid (GABA) transporter (VGAT)-immunoreactive GABAergic synaptic contacts on somata of WFA-expressing neurons. To evaluate whether TN-R deficiency would lead to quantitative changes in the number and/or distribution of perisomatic GABAergic contacts, we sampled z-stacks of confocal images with perisomatic GABAergic synaptic terminals in cultures of tn-r<sup>−/−</sup> and tn-r<sup>+/+</sup> mice. Since synaptic contacts on the somata were distributed non-uniformly, quantification of VGAT immunoreactivity was performed separately for five different image planes of soma and for the axon initial segment (electronic supplementary material, figure S4c). No difference in distribution of GABAergic contacts could be detected between genotypes.

(f) Restoration of perineuronal nets in dissociated cell cultures of tn-r<sup>−/−</sup> mice

In the following series of experiments, we analysed whether addition of soluble TN-R protein or of molecules mimicking its activity by cross-linking aggrecan molecules could restore the structure of PNs. Figure 2a,b again demonstrate the difference between genotypes in dendritic expression of PNs described above: while in tn-r<sup>+/+</sup> neurons dendritic segments of the second order are often associated with the WFA-labelling signal and aggrecan-immunopositive PNs, in cultures from tn-r<sup>−/−</sup> mice the association of PN markers with dendrites was restricted to the most proximal dendrites. Addition of TN-R protein increased the numbers of dendritic PNs, but the difference in pattern and structural appearance between genotypes was not changed (figure 2c,f). By contrast, culturing of neurons in the presence of polyclonal antibodies to aggrecan resulted in the appearance of conspicuous WFA-positive PNs (figure 2d,f). As shown by indirect immunofluorescence, these PNs contained the antibodies to aggrecan that had been applied during the 8-day culture period. Antibodies to the ECM molecule TN-C applied in the same manner were not detectable in WFA-positive nets and did not promote formation of PNs (figure 2e,f). Despite the successful reconstitution of PNs along dendrites, PNs around cell bodies showed less prominent net-like appearance when compared with cultures from wild-type mice (electronic supplementary material, figure S5).

(g) Restoration of perineuronal nets in organotypic cultures of tn-r<sup>−/−</sup> mice

In organotypic cultures, addition of molecules that normalized PN structures induced similar effects as seen in dissociated cell cultures (figure 3). Maintenance of organotypic cultures in the presence of polyclonal antibodies to aggrecan resulted in the appearance of conspicuous WFA-positive PNs (figure 3d,g). Addition of TN-R protein tended to increase the extent of PNs along dendrites. However, this effect failed to be significant (figure 3c,g). Antibodies to TN-C did not generate normally appearing PNs (not shown). To confirm whether restoration of PNs in tn-r<sup>−/−</sup> organotypic cultures is due to cross-linking of aggrecan induced by clustering via polyclonal antibodies, another cross-linking reagent was applied. We used WFA for these experiments as it is divalent and reacts predominantly with N-acetylgalactosamines on aggrecan [5]. Furthermore, a monovalent (reduced) form WFA can be used as a negative control, since it has the same affinity for N-acetylgalactosamines on aggrecan [29] but does not cross-link aggrecan molecules. Native, but not monovalent WFA led to a clear increase in the numbers of PNs along dendrites (figure 3e–g).

3. Discussion

In this study, organotypic slice cultures and dissociated cell cultures were used to investigate the role of TN-R in the assembly of PNs. We found that in tn-r<sup>−/−</sup> organotypic and dissociated cell cultures, the localization of PNs on somata and dendrites is severely disturbed. This abnormality can, however, be ameliorated by delivery of TN-R protein, polyclonal aggrecan antibodies and native, but not monovalent, WFA which binds predominantly to glycans carried by aggrecan, to the cultures. We suggest that aggrecan antibodies and WFA substitute for TN-R by clustering of aggrecan, thus restoring the appearance of normal PNs in TN-R deficient cells to resemble those in the wild-type cells.

(a) Development of perineuronal nets in tn-r<sup>+/+</sup> versus tn-r<sup>−/−</sup> cultures

In organotypic and dissociated cultures of tn-r<sup>+/+</sup> and tn-r<sup>−/−</sup> mice, development of the basic cellular features appeared to be normally patterned. These features include the cellular characteristics, immunoreactivity for PARV and NeuN as well as the subcellular characteristics for MAP2 and a number of VGAT positive synapses. Further, the levels of the prominent ECM components of PNs, such as the CSPG aggrecan, chondroitin sulfate glycosaminoglycans and hyaluronan, resemble those seen histologically in the cerebral cortex, subcortical forebrain and brainstem of wild-type animals [19]. Co-staining with different PN markers (WFA or hyaluronan-binding protein with antibodies to aggrecan) revealed a molecular heterogeneity of PNs in distinct interneuronal subpopulations as indicated by different staining intensities of these markers, resembling those found in the cerebral cortex, subcortical forebrain and brainstem [19]. The temporal appearance in wild-type cultures of the ECM components hyaluronan, aggrecan and TN-R was similar to that of WFA-reactive glycans [19]. However, the dendrites of PARV-positive interneurons in organotypic cultures of tn-r<sup>−/−</sup> mice appeared less covered by PNs than those of tn-r<sup>+/+</sup> mice. In addition, clustering of ECM components at somata appeared less regular in organotypic cultures from tn-r<sup>−/−</sup> versus tn-r<sup>+/+</sup> mice, suggesting that TN-R may not be essential for the overall development of neurons, but to be more specific for the assembly of the characteristic PN structures.

(b) Formation of perisomatic GABAergic synapses on interneurons is not altered in cultures from tn-r<sup>−/−</sup> mice

No significant differences in the distribution of GABAergic presynaptic contacts on PN-expressing interneurons were seen between tn-r<sup>−/−</sup> and tn-r<sup>+/+</sup> hippocampal cultures. By contrast, a reduction in synaptic GABAergic coverage of
Figure 2. Appearance of PNs in dissociated hippocampal cultures from wild-type (tn-r+/+) and tenascin-R (tn-r−−)-deficient mice maintained for 15 days in vitro. (a,b) Untreated cultures from tn-r+/+ mice (a) and tn-r−− mice (b). (c–e) Cultures from tn-r−− mice treated with TN-R (c), or antibodies to aggrecan (Aggr IgG) (d) or tenascin-C (TN-C IgG) (e). The genotype and type of treatment are indicated by white letters in the left image of each row. WFA fluorescence labelling (first column) is combined with immunostaining for aggrecan (Aggr) or tenascin-C (TN-C) (second column) and NeuN (third column). An overlay is shown in the fourth column. (a,b) All immunolabelling was performed with fixed cultures. (d,e) Antibodies to aggrecan and tenascin-C were applied only to live cultures, whereas all secondary antibodies were applied after fixation. (f) Quantification of results obtained in the experiments with reconstitution of PNS in dissociated cultures (n = 15 interneurons per untreated tn-r−− cell and n = 20 for all other groups). *p < 0.05, **p < 0.001 significantly different from untreated tn-r+/+ cells, +++p < 0.001 significantly different from untreated tn-r−− cells (t-test or U-test, when normality test failed). Scale bars, 100 μm for all images.
Figure 3. Restoration of dendritic PNs in organotypic slice cultures from \( \text{tn-r}^{-/-} \) mice compared to organotypic slice cultures from \( \text{tn-r}^{+/+} \) mice. (a) Untreated organotypic slice cultures from \( \text{tn-r}^{+/+} \) mice. (b) Untreated organotypic slice cultures from \( \text{tn-r}^{-/-} \) mice. (c) Organotypic slice cultures from \( \text{tn-r}^{-/-} \) mice treated with TN-R protein. (d) Organotypic slice cultures from \( \text{tn-r}^{-/-} \) mice treated with antibodies to aggrecan (Aggr IgG). (e) Organotypic slice cultures from \( \text{tn-r}^{-/-} \) mice treated with monovalent WFA (mono WFA). (f) Organotypic slice cultures from \( \text{tn-r}^{-/-} \) mice treated with native WFA (poly WFA). (g) Quantification of results obtained in the experiments with reconstitution of PNs in organotypic cultures (\( n = 9 \) interneurons per untreated \( \text{tn-r}^{-/-} \) cell and \( n = 9 \) for all other groups). *\( p < 0.05 \), **\( p < 0.01 \) significantly different from untreated \( \text{tn-r}^{+/+} \) cells (t-test). +++\( p < 0.001 \) significantly different from untreated \( \text{tn-r}^{-/-} \) cells (t-test). Scale bar, 10 \( \mu \)m for all images.
CA1 pyramidal neurons in tn-r−/− mice [30,31] was observed, which was accompanied by a decrease in minimal perisomatic IPSC amplitudes [32]. These morphological and functional alterations were found in pyramidal cells, onto which the TN-R-associated HNK-1 carbohydrate directly impinges via postsynaptic GABA_{A} receptors [33]. However, in PNs TN-R is complexed with aggrecan and other CSPGs. Thus, it is conceivable that the accessibility of the HNK-1 carbohydrate to postsynaptic GABA_{A} receptors on fast-spiking interneurons may be sterically hindered, and hence TN-R does not affect formation of presynaptic GABAergic terminals on fast-spiking interneurons, at least in vitro.

(c) Restoration of normally appearing perineuronal nets in dissociated and organotypic cultures of tn-r−/− mice

Addition of TN-R to the culture medium of dissociated cell cultures increased the extent of dendritic PNs. Although only a partial restoration of the dendritic PN structure was achieved, this result suggests that TN-R is needed for formation of the appropriate PN architecture. A functional link between TN-R and aggrecan became tangible by exposing the cultures to polyclonal antibodies against aggrecan, resulting in apparent normalization of a WFA-positive PN pattern. We would thus like to suggest that the scaffolding function of TN-R, which can be isolated in di- or trimeric forms from brain homogenate, can at least be partially mimicked by a native lectin or an aggrecan cross-linking reagent, such as an antibody to aggrecan, which promoted the clustering of ECM components, thus leading to increased covering of dendrites by PNs in cultures from tn-r−/− mice.

In organotypic cultures of tn-r−/− mice, addition of TN-R tended to increase the extent of dendritic PNs. This tendency could not be shown to be significant, possibly due to the limited extent of diffusion of TN-R into and in the more compact tissue of organotypic cultures, as compared with dissociated cell cultures. It is also possible that the developmentally late application of TN-R to the cultures leads to an improper interaction of TN-R and aggrecan with their neuronal receptors, for instance contactin-1 [34]. However, as in dissociated cell cultures, also the organotypic cultures from tn-r−/− miceaggrecan antibodies normalize the WFA-positive PN architecture which tended to be accompanied by a more extensive coverage of dendrites with PNs. Furthermore, native WFA, which binds to glycans carried predominantly by aggrecan, also restored the structure of PNs, whereas monomeric WFA did not. Thus, clustering of ECM components appears to be an important mechanism in formation of PNs.

(d) Functional consequences of perineuronal net deficiency

A deficit in tenascin-R leads to a deficit of links interconnecting aggrecan molecules, and relatively weak interactions between hyaluronic acid and aggrecan seem to be insufficient for stabilization of hyaluronic acid–aggrecan complexes and aggregation of these molecules around dendrites. In the absence of aggrecan-based polyanionic PN aggregates between synaptic contacts, the extracellular molecules in tn-r−/− mice may lose their capacity to build a barrier for the lateral diffusion of transmembrane molecules (e.g. neurotransmitter and neurotrophin receptors, adhesion molecules, ion channels), for buffering positively charged ions (e.g. Ca^{2+}), for being neuroprotective [35,36] and for compartmentalization of cytokines (e.g. fibroblast growth factor, brain-derived neurotrophic factor; for reviews, see [37,38]). An altered diffusion of neuroactive substances in the extracellular space inevitably affects extrasynaptic transmission, synaptic efficacy and, not least, neuron–glia interactions.

Recent studies revealed abnormalities in the structure of PNs during epileptogenesis and after intake of addictive drugs [39,40]. Furthermore, there is a genetic association between schizophrenia and the enzyme B3GAT2 involved in synthesis of HNK-1, the major functional carrier of which is TN-R [41]. These considerations and the reported reduction of perisomatic inhibition of CA1 pyramidal neurons in tn-r−/− mice [32] suggest that TN-R and associated molecules are likely to play important roles in synaptic transmission by securing the fidelity of interactions of perisomatic interneurons with each other and with their neighbouring cells. The analysis of the underlying mechanisms will be the topic of further investigations. The importance of this study is the demonstration that abnormalities in PN structures can be pharmacologically abrogated using reagents that cluster, for instance aggrecan, suggesting that PNs are a promising target for therapeutic interventions.

4. Material and methods

Generation of tn-r−/− mice has been described [18]. A static culture method [42] was used for preparation of organotypic slice cultures from the forebrain as described [1,24]. Cytochemistry and immunocytochemistry in organotypic slice cultures were performed according to Brückner & Grosche [1]. Preparation and cytochemical and immunocytochemical labelling of dissociated hippocampal cell cultures and quantification of perisomatic GABAergic contacts have also been described [4].

(a) Experimental manipulations of organotypic slice cultures

To induce depolarization of neurons and to increase spontaneous activity rates, the concentration of KCl in the organotypic culture medium was raised from 2.5 mM (‘standard medium’) to 10 mM or 25 mM (‘high KCl medium’ [43]) starting from the third day of culture. After an initial period of 10 days in normal culture medium, organotypic cultures were also treated for 8 days with either recombinant human TN-R (h-rec-TN-R, 20 µg ml−1, 3865TR; R&D Systems, Minneapolis, MN, USA), or TN-R purified from chicken brain [44], rabbit polyclonal anti-aggrecan antibodies (20 µg ml−1, AB1031; Millipore, Billerica, MA, USA), rabbit polyclonal anti-mouse TN-C antibodies (20 µg ml−1, L-8258; Sigma) and monovalent WFA (20 µg ml−1, prepared under reducing conditions according to Zhou et al. [29]).

(b) Treatments of dissociated cell cultures

Dissociated cultures were prepared from 1–3 day old mice. After being maintained for 7 days in normal culture media, some dissociated cultures were treated for 8 days with one of the following reagents: the chicken homologue of murine TN-R, restrictin (20 µg ml−1, AB1031; Millipore, Billerica, MA, USA), rabbit polyclonal anti-aggrecan antibodies (20 µg ml−1, AB1031; Chemicon), rabbit polyclonal anti-tenascin-C antibodies (20 µg ml−1, KAF14).
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Data accessibility. More detailed descriptions of materials and methods are provided in the electronic supplementary materials.

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