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Type III intermediate filament peripherin inhibits neuritogenesis in type II spiral ganglion neurons *in vitro*

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Abstract

Peripherin, a type III intermediate filament protein, forms part of the cytoskeleton in a subset of neurons, most of which have peripheral fibre projections. Studies suggest a role for peripherin in axon outgrowth and regeneration, but evidence for this in sensory and brain tissues is limited. The exclusive expression of peripherin in a sub-population of primary auditory neurons, the type II spiral ganglion neurons (SGN) prompted our investigation of the effect of peripherin gene deletion (pphKO) on these neurons. We used confocal immunofluorescence to examine the establishment of the innervation of the cochlear outer hair cells by the type II SGN neurites *in vivo* and *in vitro*, in wildtype (WT) and pphKO mice, in the first postnatal week. The distribution of the type II SGN nerve fibres was normal in pphKO cochleae. However, using P1 spiral ganglion explants under culture conditions where the majority of neurites were derived from type II SGN, pphKO resulted in increased numbers of neurites/explant compared WT controls. Type II SGN neurites from pphKO explants extended ~ double the distance of WT neurites, and had reduced complexity based on greater distance between turning points. Addition of brain-derived neurotrophic factor (BDNF) to the culture media increased neurite number in WT and KO explants ~30-fold, but did not affect neurite length or distance between turning. These results indicate that peripherin may interact with other cytoskeletal elements to regulate outgrowth of the peripheral neurites of type II SGN, distinguishing these neurons from the type I SGN innervating the inner hair cells.

Keywords

cochlea; spiral ganglion neuron; unmyelinated; neurite outgrowth; neuritogenesis; BDNF

Introduction

Several types of intermediate filaments (IF) contribute to the development and maintenance of the neuronal cytoskeleton. These include nestin, internexin and the neurofilament

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proteins; neurofilament – heavy (NF-H), - medium (NF-M) and - light (NF-L). These proteins associate with a range of cytoskeletal elements to influence neurogenesis, axon and dendrite growth and survival [15,19,22]. The function of peripherin, a type III IF, remains controversial [17]. It is expressed in a subset of sensory, motor and autonomic neuronal populations, the majority of which have at least some part of their axon in the peripheral nervous system [1,7,9,24,25,34] and studies suggest a role in axon outgrowth and regeneration based on spatial and temporal expression patterns [7,34] and functional studies in rat pheochromocytoma (PC12) cells [13]. However, peripherin null mice do not exhibit an overt phenotype and, with the exception of the reduction of non-peptidergic nociceptive afferent fibres in the spinal cord, axon development proceeded normally for the majority of peripherin-expressing peripheral neurons [18]. Furthermore, over-expression of peripherin in transgenic mice, leads to late onset motor neuron disease which has a similar pathology to that commonly observed in degenerating motor neurons of amyotrophic lateral sclerosis (ALS) patients [2,3].

Overall there is a paucity of information regarding the role of peripherin in neurons in animal models. To address this, we have studied the contribution of peripherin to the establishment of the afferent innervation of the mouse cochlea. In this model, peripherin is specifically expressed by the type II spiral ganglion neurons (type II SGN), after embryonic day 18 [14]. These neurons exhibit a distinctive innervation pattern and extend unmyelinated peripheral axons that turn basally after crossing the tunnel of Corti to form three parallel tracts of outer spiral bundle fibres (OSB) beneath the rows of outer hair cells (OHC), innervating multiple OHC in an *en passant* fashion [31]. While there is transient innervation of OHC by separate fascicles of type I fibres, this is resolved by postnatal day (P) 7 [14], where multiple type I SGN (peripherin – negative) neurites selectively innervate individual IHC forming the inner spiral plexus [6,26,29]. Thus the neonatal mouse cochlear model provides the opportunity to evaluate the function of peripherin in an identifiable sub-class of neurons, the type II SGN and their neurites, which form the OSB.

The mechanisms that drive the refinement of afferent innervation in the cochlea are poorly understood, although changes in neurotrophin signalling, specifically Brain-Derived Neurotrophic Factor (BDNF), have been implicated [29,36]. Our model, the spiral ganglion explant, enabled investigation of the hypothesis that peripherin expression confers specific properties to axon outgrowth from type II SGN during the developmental period when these neurites extend along the rows of OHC. We examined the effect of peripherin gene deletion (peripherin knockout mouse) on the morphology of type II innervation of the cochlea *in vivo* as well as on neurite outgrowth in SGN explants *in vitro*.

Methods

Animals

All procedures in the study were approved by the University of Auckland Animal Ethics Committee or by the University of New South Wales Animal Ethics Committee. Cochlear tissue was obtained from P1 and P7, C57/BL6 strain (wildtype) and 129/BL6 strain (peripherin null - pphKO) mice [18].

Fixed tissue preparation

P1 and P7 mice were killed by decapitation and the cochleae were isolated and fixed in 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) at 4°C (overnight). The cochleae were then placed in 10% sucrose for 4 hours, 30% sucrose for 2 days and a 1:1 solution of 30% sucrose and Tissue-Tek OCT (Miles, Diagnostics Division, Elkhart, IN, USA) for one hour. Cochleae were subsequently mounted in OCT, snap frozen on dry ice

and cryosectioned (CM1900 cryostat microtome, Leica, Germany) at 50 μ m into 0.1 M PBS. Floating sections were processed for immunofluorescence.

Explant cultures

Cochleae were immediately removed from C57/BL6 (WT) and 129/BL6 (pphKO) P1 mice and placed in Dulbecco's PBS (138 mM NaCl; 8.06 mM Na_2HPO_4 ; 2.67 mM KCl; 1.47 mM KH_2PO_4 ; 0.9 mM CaCl_2 ; 0.49 mM MgCl_2). The apical turn spiral ganglion was isolated (hair-cell containing organ of Corti and spiral limbus removed) and transferred onto a poly-D-lysine - coated glass coverslip (50 μ g/ml, BD Biosciences, San Diego). Coverslips were transferred to wells on a 24 well plate containing 200 μ l neuron maintenance medium: DMEM (Gibco), 10% Fetal Bovine Serum (FBS) (Gibco), 10 μ l/ml N2 supplement (Invitrogen) 25 mM Hepes (Sigma-Aldrich), 300 units/ml penicillin (Sigma-Aldrich), 6 mg/mL glucose (Sigma-Aldrich). This control maintenance media, which included FBS, provided a baseline level of support for the neuronal cultures. Media for half of the explants also contained 100 ng/ml BDNF (Promokine, Heidelberg, Germany). Explants were cultured at 37°C in a humidified incubator with 5% CO_2 for 48 hours then fixed for 20 mins in 4% PFA and processed for immunofluorescence.

Immunofluorescence

Rabbit polyclonal (PRB-435P) and mouse monoclonal (MMS – 435P) antisera against class III β -tubulin (Covance, Emeryville, CA, USA) were used for the immunolabelling of all SGN and their peripheral processes. The peripherin polyclonal rabbit antiserum (PII/SE411, a gift from Dr. Annie Wolff, Division de Biochimie, Universite Pierre et Marie Curie, Paris) was used for selective labelling of type II SGN in WT tissue. Cross sections and cultured tissue were incubated in blocking/permeabilising solution (10% normal goat serum [NGS, Vector Laboratories, Burlingame, CA, USA] and 2.5% Triton X-100 in PBS) for 2-3 hours at room temperature and then overnight at room temperature in primary antibody solution containing β -tubulin antisera (1:500 dilution) and peripherin antisera (1:1000 dilution) with 5% NGS and 0.25% Triton X-100 in 0.1 M PBS. The following day, sections were washed in PBS, then incubated for 2 hours at room temperature in secondary antibody solution containing Alexa-488 conjugated goat anti-rabbit IgG (1:500); Alexa-488 conjugated goat anti-mouse; IgG (1:500) and Alexa-647 conjugated goat anti-rabbit (1:200) (All Invitrogen) with 5% NGS and 0.25% Triton X-100 in 0.1 M PBS. Following washing with PBS, tissue was mounted on glass slides in Vectastain (Vector Laboratories, Burlingame, CA, USA) and stored at 4°C.

Image acquisition and quantitative analysis

Images were acquired by confocal microscopy (Olympus FV1000, Japan and Zeiss LSM710, Germany), saved as TIFF files and processed using Adobe Photoshop CS2 (Adobe Systems, San Jose, California, USA) and Image J (NIH, USA) software; levels were adjusted to remove low levels of background fluorescence, whilst ensuring that no neurite detail was lost. NeuronJ (an ImageJ plugin) was used to trace and measure neurite lengths and count neurites. SigmaPlot 11 (Systat Software, Germany) was used for all graphing and statistical analyses.

Results

Comparison of afferent innervation in WT and pph-KO cochleae

The possible influence of loss of peripherin expression on the development of the cochlear afferent innervation was examined by immunofluorescence of cryosectioned mouse cochlea at P1 and P7. This spans the period of afferent innervation and synaptic consolidation just

prior to the onset of hearing [14]. β -tubulin labelling resolved the soma and peripheral neurites of the type I and type II SGN and type II SGN were specifically labelled in the WT using a peripherin antiserum (Fig. 1A). Afferent innervation of the pphKO mice exhibited no morphological variation from the WT based on β -tubulin labelling of the OSB in both P1 (compare Fig. 1B and C) and P7 (compare Fig. 1D and E) tissue. At P7, the OSB exclusively represent type II SGN innervation of the OHC [14]. These data indicate that peripherin expression is not obligatory for establishment of type II innervation of the OHC.

Functional significance of peripherin in neuritogenesis

To investigate the putative role of peripherin in cochlear neuritogenesis, we established a P1 mouse spiral ganglion explant model, roughly corresponding to the onset of type II SGN innervation of the OHC. After 48 hours culture in maintenance medium, 75% of neurites were immunolabelled for both β -tubulin and peripherin (Fig. 2A, B). This pattern of double labelling indicates that the majority of the neurons in these preparations were type II SGN. PphKO explants extended significantly more neurites (15 ± 3 neurites/explants ($n = 11$) vs 4 ± 1 for WT ($n = 7$); unpaired Student's t-test, $P < 0.05$) in the maintenance media (Fig. 2C, D and Fig. 3A). This indicates that peripherin inhibits type II SGN neuritogenesis. This effect was also apparent with regard to the length of the neurites and the distance between turning (neurite complexity) (Fig. 2C, D and Fig 3B, C). The pphKO explants in maintenance media had neurite lengths approximately double that in the WT controls ($601 \pm 24 \mu\text{m}$ pphKO ($n = 11$) vs $343 \pm 29 \mu\text{m}$ WT ($n = 7$); unpaired t-test, $P < 0.001$). Average distance between turns was $171 \pm 13 \text{ m}$ in the pph-KO vs $124 \pm 29 \mu\text{m}$ in the WT (unpaired t-test, $P < 0.05$).

Media supplemented with BDNF was used to promote neuritogenesis to enable evaluation of peripherin activity on larger numbers of neurites (Fig. 2E – H). BDNF produced a 30-fold increase in neuritogenesis for both WT and pphKO explants (Fig. 2E - H and Fig. 3A). Type II SGN neurites maintained their dominant representation under these culture conditions (63% of neurites in WT explants were double labelled for peripherin and β -tubulin) (Fig. 2E, F). However, the pphKO explants in BDNF did not exhibit a greater BDNF-induced increase in neurite numbers compared with the WT-BDNF explants, with the result that the number of neurites/explant observed for the two genotypes was statistically similar after BDNF treatment (109 ± 18 and 141 ± 21 neurites/explant respectively; $P > 0.05$) (Fig. 3A). This suggests that the modest increase in neurite number that we observed in the pph-KO explants in maintenance media was masked by the very large increase in neuritogenesis produced by BDNF for both genotypes. While BDNF caused a small increase in neurite length for both genotypes, this was not significant ($p > 0.05$), however, a large increase in neurite length produced by KO of peripherin was still evident. With BDNF, pphKO neurite length was $670 \pm 41 \mu\text{m}$ ($n = 11$ explants) compared to $475 \pm 54 \mu\text{m}$ for the WT+BDNF neurites ($n = 7$ explants) (unpaired t-test, $P < 0.05$) (Fig. 2 F, H and Fig. 3B). BDNF also had no effect on the distance between turns (Fig 3C), and the increased distance to turning in the pphKO seen in maintenance media, was replicated in the pphKO+BDNF explant group ($180 \pm 13 \text{ m}$; $n = 11$ explants) compared with the WT+BDNF control ($122 \pm 6 \text{ m}$; $n = 7$ explants) (unpaired t-test, $P < 0.01$) (Fig. 3C).

Discussion

Previous examination of the peripherin null mouse used in this study found no effect of peripherin deletion on the development of the dorsal roots, retina, gut and the majority of the ventral roots and spinal cord and only a small sub-population of unmyelinated nociceptive dorsal root ganglion afferent neurons were lost [18]. In parallel with these observations, we found no discernable effect of peripherin gene deletion on the distribution of the type II SGN dendrites in the OSB *in vivo*. This lack of effect may be related to redundancy with

other IFs. A recent study showed that the loss of the small diameter nociceptive fibres observed in pphKO mice likely arises from the lack of expression of other IF proteins, namely NF-M and NF-H [4]. The expression of these neurofilament subunits by type II SGN [11,28] therefore likely compensates for the loss of peripherin expression. However, it should be noted that in sections we were not able to evaluate the lengths of type II SGN dendrites in the pphKO cochlea.

We subsequently investigated the effect of peripherin knockout on neurite outgrowth from type II SGN in apical turn explant cultures containing the most recently differentiated SGN in the cochlea [30]. While recent studies in rats indicate that peripherin is up-regulated in type I SGN following explant culture of P5 SGN [16], it seems that this up-regulation is species specific. Thorough examination of mouse SGN following organotypic and explant culture has shown that peripherin is not up-regulated in type I SGN [23,27]. While we show a large number of SGN express peripherin in our P1 explants, this likely reflects the relatively larger representation of type II SGN near birth [14] as the number of peripherin immunolabelled neurons in P7 SGN explants reflects the mature representation of type II SGN in the cochlea (M. Barclay, A. F. Ryan and G. D. Housley, unpublished observations).

Our *in vitro* data indicates that peripherin expression in type II SGN reduces the possible extent of neurite extension. Previously the effects of loss of functional peripherin protein on neuritogenesis were examined by knockdown of peripherin expression in PC12 cells [13] and by the expression of a disrupted copy of the peripherin gene in 'disruptor mice', where the portion critical for IF assembly was removed [4]. Loss of functional peripherin expression gave rise to a loss of the outgrowth and maintenance of neurites in differentiated PC12 cells and a loss of sprouting of nociceptive sensory fibres in 'disruptor mice' following denervation and likely reflects the lack of NF-H and NF-M expression in these cells. Our results provide novel insight to the potential effects of peripherin on axon outgrowth from neurons that do express these neurofilament proteins.

Axon extension requires polymerisation of actin microfilaments and microtubules at the growth cone [21]. The early onset of peripherin expression in the developing embryo and its localisation near the growth cone indicates that peripherin stabilises the extending axon whereas the later onset of expression (and proximal localisation) of neurofilament proteins (e.g. NF-H) provides the stability that is necessary for the maintenance of neurites [8,32,33,35]. Our data indicates that this initial axon stabilisation by peripherin may hinder neurite extension, while the later recruitment of neurofilament proteins stabilises and maintains the structure of these small diameter, unmyelinated fibres. While studies have demonstrated a linkage between peripherin subunits and actin microfilaments [5,20] and crosstalk between peripherin and microtubules and actin microfilaments [12], the effect that IF polymerisation and cross linking has on the assembly rate of microtubules and actin has yet to be elucidated.

Conclusion

Loss of the type III IF peripherin in sensory neurons, the type II SGN, leads to increased neuritogenesis, longer neurites and changes in neurite growth patterns *in vitro*. This suggests that during the establishment of the innervation of the cochlear OHC by the type II SGN, peripherin may interact with other cytoskeletal elements to regulate spatiotemporal outgrowth that distinguishes these afferent fibres from the majority of (type I) neurons that innervate the IHC.

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Figure 1.

Confocal immunofluorescence of neonatal mouse cochlear sections shows type II spiral ganglion neuron (SGN) innervation of the outer hair cells (OHC) via the outer spiral bundles (osb / arrowheads). β -tubulin antisera (green) immunolabels all SGN and their peripheral fibres in wildtype (WT; **A, B, D**) and KO (**C, E**) tissue from P1 (**A, B, C**) and P7 (**D, E**) mice. Peripherin immunostaining (red) distinguishes type II SGN soma and neurites in WT tissue (**A, B, D**). The outer spiral bundles are preserved in the peripherin knockout tissue (**C, E**). IHC, inner hair cells; Rm, Reissner's membrane; SM, scala media; ST, scala tympani; SV, scala vestibule. Scale bars: 25 μ m.

Figure 2.

Confocal immunofluorescence (β -tubulin (β -tub - green) and peripherin (pph - red)) of wildtype (WT) P1 spiral ganglion explants cultured for 48 hours in control maintenance media (WT-BDNF(-)) (**A, B**) or media supplemented with 100ng/ml BDNF (WT-BDNF(+)) (**E, F**) indicates that the majority of neurites were derived from type II SGN. Explants from peripherin knockout mice cultured in maintenance media (KO-BDNF(-)) (**C, D**) and with BDNF supplemented media (KO-BDNF(+)) (**G, H**) had longer neurites and greater growth between turning (**C, D and G, H**) than the respective WT controls. Peripherin knockout explants under maintenance media culture had greater numbers of neurites than the WT-BDNF(-) controls. B, D, F and H are magnified images of regions of A, C, E and G respectively. Scale bars: A, C, E and G = 250 μ m; B, D, F and H = 100 μ m.

Figure 3.

Quantitative analysis of neurite outgrowth following 48 hour culture of P1 wildtype (WT) and peripherin knockout (KO) apical turn spiral ganglion explants. **A.** Number neurites per explant. **B.** Total neurite length. **C.** Distance neurite travels before turning. The significant increase in neurite number and length shown for the peripherin KO explants reflects the influence of this type III intermediate filament on neuritogenesis. Supplementing the maintenance media with BDNF (BDNF(+)) did not significantly increase neurite length or distance between turns. Data represented as Mean and SE, unpaired t-tests, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.