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ORIGINAL COMMUNICATION

Reduced gene expression of netrin family members in skin and sural nerve specimens of patients with painful peripheral neuropathies

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Abstract

Objective To investigate the expression of axon guidance cues in skin and sural nerve biopsies of patients with polyneuropathies (PNP) as potential markers of nerve de- and regeneration and inflammation.

Methods We prospectively recruited 88 patients with PNP and compared data between patient subgroups and healthy controls. All patients underwent skin punch and/or sural nerve biopsy at the lower leg and proximal thigh. We characterized gene expression profiles of netrin family members as target genes involved in neuronal de- and regeneration [netrin 1, deleted in colorectal cancer (DCC), uncoordinated5H2, neogenin 1 (NEO1), netrin G1, netrin G2] using quantitative real-time PCR.

Results Gene expression of netrin 1 ($p < 0.05$ in proximal skin), DCC ($p < 0.001$ in distal skin), NEO1 ($p < 0.05$ in distal skin), netrin G1 ($p < 0.05$ in proximal and $p < 0.01$ in distal skin), and netrin G2 ($p < 0.001$ in distal skin) was lower in skin biopsies of patients with neuropathy compared to healthy controls. Gene expression of NEO1 ($p < 0.05$ in distal skin), netrin G2 ($p < 0.05$ in distal skin), and DCC ($p < 0.05$ in sural nerve) was lower in samples of patients with painful compared to painless PNP and also correlated positively with intraepidermal nerve fiber density. Skin and sural nerve gene expression of the investigated target genes did not differ between neuropathies of different etiologies.

Conclusion We show reduced cutaneous and neural axon guide expression, which may contribute to a dysregulation of nerve fiber de- and regeneration.

Keywords Polyneuropathy · Axon guides · Netrin-1 · Gene expression · Skin biopsy · Nerve biopsy

Introduction

Axonal sprouting is precisely guided by growth promoting and inhibiting cues in the extracellular environment. These guidance molecules navigate axons by regulating cytoskeletal dynamics and remodeling the cell membrane by chemical and mechanotransductive mechanisms [1, 2].

Netrin 1 is a bifunctional axon guide directing axonal outgrowth by interacting with its receptors deleted in colorectal cancer (DCC), neogenin 1 (NEO1), and uncoordinated5H2 (UNC5H2) [2–4]. The ligand–receptor interaction of netrin-1 and DCC or NEO1 initiates neurite growth [5, 6]. In contrast, netrin-1 leads to axon repulsion over short distances via the UNC5H2 receptor [4, 7]. Simultaneous expression of DCC and UNC5H2 has a repulsive effect on axonal outgrowth over long distances [8]. Netrin G1 and netrin G2, two glycosylphosphatidylinositol (GPI)-anchored membrane proteins, are also part of the netrin axon guide family [9–11]. Both function as receptors for their transmembrane ligands NGL1 and NGL2, and regulate axonal and dendritic outgrowth of central neurons as well as influence synaptic plasticity [10, 12, 13].

Previous studies mainly focused on the central nervous system (CNS) effects of netrins [14–16]. In the peripheral nervous system (PNS), data are scarce. Netrin 1, DCC, and UNC5H2 were found in rat Schwann cells promoting and inhibiting Schwann cell proliferation and migration [17–19]. While the role of netrin G1 and netrin G2 is unknown in the PNS, gene expression of one of its ligands, NGL2, was reduced in axotomized rat spinal motoneurons [20]. Besides the effects of netrin 1 and its receptors on axonal outgrowth [4, 6, 8], several studies showed that netrin 1 and NEO1 are also involved in inflammatory processes [14, 21–23].

The bifunctional properties of netrins involving nerve fiber de- and regeneration as axon guides and their influence

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on inflammation make netrin family members interesting targets in the pathophysiology of PNS diseases involving both mechanisms. One example is inflammatory and non-inflammatory peripheral neuropathies (PNP) [24, 25] that can be painful or painless.

We hypothesized that gene expression of netrin family members is altered in diagnostic skin and sural nerve biopsy specimens of patients with PNP compared to controls reflecting axon degeneration and that axon guidance cues are associated with inflammation in PNP.

Patients and methods

Study design

Between 2010 and 2012, 88 patients (19 women, 69 men) with large fiber neuropathies were prospectively recruited at the Department of Neurology, University of Würzburg. Median age was 63 years (23–84). All patients underwent a comprehensive diagnostic work-up including complete neurological examination, laboratory tests, nerve conduction studies, and skin punch and/or nerve biopsy. Patients were also asked to fill in questionnaires on pain (German versions of the Neuropathic Pain Symptom Inventory [NPSI] [26] and Graded Chronic Pain Scale [GCPS] [27]) and depression ("Allgemeine Depressionsskala" [ADS] [28]). Further, we determined The Overall Disability Sum Score (ODSS) [29] for symptom severity. The Würzburg Medical Faculty Ethics Committee approved the study (85/06) and all participants gave written informed consent before enrollment.

Inclusion criteria and diagnostic workup

Inclusion criteria were adult patients ≥ 18 years and a diagnosis of PNP. Patients were excluded if they had an infection at recruitment. The diagnosis of neuropathy was based on patients' medical report, neurological examination, and nerve conduction studies. Electrophysiological studies included the assessment of at least three motor and two sensory nerves of the upper and lower limbs as individually appropriate. Patients underwent extensive laboratory studies for differential diagnosis of neuropathies including: inflammation markers (e.g., whole blood and differential cell counts, C-reactive protein and erythrocyte sedimentation rate); renal and liver function parameters including serum electrolytes; glucose metabolism (e.g., HbA1c and oral glucose tolerance test); thyroid hormones; vitamins (e.g., folic acid, vitamin B6 and B12); autoimmune diagnostics including rheumatoid factor, anti-nuclear antibodies (ANA), antibodies to extractable nuclear antigen (ENA), anti-neutrophil cytoplasmic autoantibody (ANCA); diagnostics for paraproteinemia (e.g., serum electrophoresis and immunofixation; urine Bence-Jones proteins); and infectious diseases (e.g., serology of borreliosis and lues). Diagnostic lumbar puncture was conducted at our department if not done previously.

Differentiation of PNP subgroups and healthy control group

Based on current criteria, we differentiated nine PNP subgroups including chronic idiopathic axonal polyneuropathy (CIAP) [30] and chronic inflammatory demyelinating neuropathy (CIDP) [31]. CIDP was further differentiated into "CIDPclin" (i.e., PNP with typical clinical presentation, elevated cerebrospinal fluid protein, and demyelination in neurophysiological and histological assessment, however, not fulfilling the INCAT criteria) and "CIDPsens" (i.e., PNP with purely sensory symptoms for ≥ 2 months and otherwise like $CIDP_{clin}$ [32, 33]). Further, diabetic neuropathy (i.e., diabetes mellitus type I or II and typical clinical, laboratory, and electrophysiological findings [34]), hereditary neuropathy (i.e., PNP with positive genetic testing or typical clinical and electrophysiological findings and indicative family history), paraproteinemic neuropathies [35], progressive idiopathic axonal neuropathy (PIAN) [30], vasculitic neuropathy (i.e., systemic and non-systemic vasculitic neuropathies [36, 37]) as well as a group of PNP with definite other etiology (e.g., neuropathy due to amyloidosis, renal insufficiency or chemotherapy) called PNP of "other origin" were differentiated.

Inflammatory PNP included the following subgroups: CIDP (including "CIDPclin" and "CIDPsens"), vasculitic neuropathy, PIAN, and paraproteinemic neuropathies. Non-inflammatory PNP were: CIAP, diabetic and hereditary neuropathies. In all other cases, inflammatory PNP was assumed if sural nerve biopsy showed typical signs of inflammation which was defined as perivascular cellular infiltrates around ≥ 3 endo- or epineurial vessels containing at least three T cells and/or macrophages and/or a diffuse increase in endoneurial cell counts containing ≥ 10 T cells or macrophages in ≥ 2 sections of sural nerves immunoreacted with antibodies against CD-68 or CD-3 for routine histological assessment [38]. PNP were classified as painful if patients had a current pain intensity of ≥ 3 on a numeric rating scale ranging from 0 to 10 with 0 "no pain" and 10 "worst pain" during the last four weeks. In addition, 17 healthy adult volunteers (11 women, 6 men) were recruited from among patients' acquaintances at the Department of Neurology, University of Würzburg. The median age was 54 years (24–74). Every individual in this control group underwent skin punch biopsy at the lower leg and proximal thigh. Exclusion criteria were the presence of neuropathy (nerve conduction study of at least one sural nerve was accomplished), acute or chronic pain, diabetes, history of cancer, inflammatory processes and immunosuppressive

and/or immunomodulatory therapy as well as reduced distal intraepidermal nerve fiber density (IENFD).

Skin punch biopsy

Skin punch biopsies (5 mm; device by Stiefel GmbH, Offenbach, Germany) were taken in local anesthesia from the distal lateral calf 10 cm above the malleolus and the proximal lateral thigh [39]. One half was flash frozen in liquid nitrogen and stored at − 80 °C before further processing for gene expression analysis. The other half of each specimen was processed for the assessment of IENFD [39] following standardized rules [40].

Sural nerve biopsy

Sural nerve biopsy was performed following a standard procedure at the Department of Neurosurgery, University of Würzburg, Germany [41]. The biopsy sample was further processed for histological diagnostics at the Department of Neurology, University of Würzburg. Three millimeters was dissected, instantly shock frozen in liquid nitrogen, and stored at − 80 °C for additional gene expression analysis.

Gene expression analysis from skin and nerve biopsies

All cyclers and reagents used for quantitative real-time PCR (qRT-PCR) were purchased from Life Technologies (Carlsbad, CA, USA). Skin and nerve biopsy samples were processed following a published protocol [39]. Tissue samples were thawed on ice and immersed in TRIzol reagent® (Invitrogen, Karlsruhe, Germany) using a Micra homogenizer (ART Prozess und Labortechnik, Germany). Samples were incubated in 200 µl chloroform (laboratory production of ultimate elements) (25 °C, 3 min) and centrifuged (12000*g*, 15 min, 4 °C). After spin down, the supernatant was mixed with isopropanol (Merck KGAA, Darmstadt, Germany) (500 µl) and incubated (25 °C, 10 min). After centrifugation (12,000*g*, 10 min, 4 °C), the pellet was washed using ethanol 75% and spun down again (7500*g*, 5 min, 4 °C). Then, the specimens were air dried, the pellet was dissolved in diethylpyrocarbonate-treated water and the samples were incubated in a water bath (55 °C, 10 min) before storage at − 80 °C and further processing.

Reverse transcription PCR

All cyclers and PCR reagents were purchased from Applied Biosystems (Darmstadt, Germany). To reverse transcribe the obtained mRNA to cDNA, 500 ng of mRNA and TaqMan Reverse Transcription Reagents® were applied in a total volume of 100 µl including the following components: 10 µl 10 × PCR buffer, 6.25 µl multiscribe reverse transcriptase, 2 µl RNase inhibitor, 22 µl $MgCl_2$ and 20 µl dNTPs. The PCR cycler conditions were as follows: annealing (25 °C, 10 min), reverse transcription (48 °C, 60 min), and enzyme inactivation (95 °C, 5 min).

Quantitative real-time PCR

TaqMan Universal Master Mix® and 5 µl of cDNA were used for qRT-PCR performed in a StepOnePlus™ Cycler. Investigated target genes and respective assay-IDs of the used TaqMan primers are summarized in Table 1. 18sRNA was used as endogenous control. The qRT-PCRs contained 12.5 µl TaqMan Master Mix and 1.25 µl of the specific primer in a total volume of 25 µl. The cycler conditions were as follows: incubation (2 min, 50 °C), second incubation (95 °C, 10 min), 40 cycles (15 s, 95 °C and 1 min, 60 °C). We investigated gene expression of netrin 1 and its receptors DCC, NEO1 and UNC5H2 as well as netrin G1 and G2 in skin and netrin 1 and its receptors UNC5H2 and DCC in sural nerves (Table 1).

Evaluation of qRT-PCR results

We used the comparative ΔΔCT method (i.e., relating target gene expression with individual 18sRNA expression normalized to control group) for skin samples [42, 43]. CT values were first related to the housekeeping gene 18 s and then subtracted from each other. Higher ΔΔCT values

Table 1 Target genes investigated in skin and sural nerve specimens and TaqMan assays

Target gene	Assay-ID	Investigated in skin specimen	Investigated in sural nerve specimen
Deleted in colorectal cancer (DCC)	Hs00180437_m1	x	x
Neogenin 1 (NEO1)	Hs00933950_m1	x	
Netrin 1	Hs00924151_m1	x	x
Netrin G1	Hs01552822_m1	x	
Netrin G2	Hs00287286_m1	x	
Uncoordinated5H2 (UNC5H2)	Hs00900710_m1	x	x

(i.e., signal detection at earlier PCR cycles) indicate higher gene expression. Gene expression in the patient and control groups was compared and one calibrator sample was run on each PCR plate to ensure inter-plate comparability. To exclude genomic contamination, each plate contained a negative control specimen without cDNA template. For nerve samples, we applied the ΔCT method (i.e., relating target gene expression to individual 18sRNA expression) lacking nerve biopsies from healthy controls [44]. Lower deltaCT values (i.e., early signal detection) indicate higher gene expression. We chose an intuitive data illustration for nerve samples by plotting data as 1/ΔCT, since these reciprocal values result in higher data points for higher gene expression.

Statistical *analysis*

SPSS 21 (IBM, Ehningen, Germany) was used for data analysis. The non-normally distributed data of qRT-PCR results were assessed by applying the non-parametric Mann–Whitney *U* test and the Kruskal–Wallis test for independent groups. Results are shown as boxplots giving the median values and the upper 75% and lower 25% percentile. GraphPad Prism 8 (Graphpad Software, Inc., San Diego, CA, USA) and SPSS 21 were used for graphical illustration. Statistical significance was assumed at $p < 0.05$.

Results

Baseline description of the study cohort

Table 2 shows clinical characteristics of the study cohort and distribution of PNP subgroups. 55/88 patients (64%) had an inflammatory neuropathy. In 25/88 (28%) patients, the etiology of PNP remained obscure. 5/88 (6%) patients had received immunosuppressive or immunomodulatory treatment before inclusion. 54/88 (61%) patients had a painful neuropathy; 37/54 (69%) patients with painful PNP were on analgesic medication including the following: pregabalin ($n = 19$), non-steroidal antirheumatics ($n = 13$), gabapentin ($n = 15$), opioids ($n = 10$), amitriptyline ($n = 6$), duloxetine ($n = 2$) and venlafaxine ($n = 1$). 85/88 (97%) patients underwent skin punch biopsy (84 distal, 65 proximal) and 64/88 (72%) patients underwent sural nerve biopsy. Skin innervation was reduced in patients [median proximal IENFD 7.0/mm (0–21.5/mm); median distal IENFD 2.1/mm (0–11.9/mm)] compared to healthy controls [median proximal IENFD 10.2/mm (6.1–15.49/mm); median distal IENFD 6.8/mm (3.5–14.5/mm)].

Table 2 Clinical characteristics and diagnostic subgroups of patients

Item	Number (% of entire group)
M, F (*N*)	69, 19
Median age (range)	62 (23–84)
Median disease duration (range in years)	3 (0.06–30)
Symptoms	
Pure motor	2 (2%)
Pure sensory	16 (18%)
Sensorimotor	70 (80%)
Additive autonomic symptoms	1 (1%)
Distribution of symptoms	
Symmetric	48 (55%)
Asymmetric	40 (45%)
Predominantly distal	83 (94%)
Predominantly proximal	5 (6%)
Pain	
Painful	54 (61%)
Painless	34 (39%)
Therapy before study inclusion	
Only symptomatic	50 (57%)
Only immunomodulatory	1 (1%)
Both	5 (6%)
Diagnostic subgroups	
CIAP	5 (6%)
CIDP	22 (25%)
"CIDPclin"	6 (7%)
"CIDPsens"	4 (5%)
Diabetic neuropathy	5 (6%)
Hereditary neuropathy	5 (6%)
"other origin", inflammatory (e.g., ganglionitis)	2 (2%)
"other origin", non-inflammatory (e.g., toxic)	6 (7%)
Paraproteinemic neuropathies	3 (3%)
Progressive idiopathic axonal neuropathy (PIAN)	2 (2%)
"Unknown etiology"	25 (28%)
Vasculitic neuropathy	13 (15%)
Inflammation	
Inflammatory	55 (63%)
Non-inflammatory	33 (37%)
Electrophysiological pattern	
Axonal	43 (49%)
Demyelinating	37 (42%)
Both axonal and demyelinating	8 (9%)

CIAP chronic idiopathic axonal polyneuropathy, *CIDP* chronic inflammatory demyelinating polyneuropathy, *CIDPclin* patients with a clinical presentation typical of CIDP, but not fulfilling electrophysiological INCAT criteria, *CIDPsens* patients with pure sensory clinical presentation and otherwise like CIDP, but not fulfilling electrophysiological INCAT criteria, *F* female, *INCAT* Inflammatory Neuropathy Cause and Treatment Group, *M* male, *N* number, *PIAN* progressive idiopathic axonal neuropathy

Netrin-1, DCC, NEO1, netrin G1, and G2 gene expression is lower in skin biopsies of patients with PNP compared to healthy controls

In skin samples, patients with PNP had lower gene expression of netrin 1 (proximal $p < 0.05$; Fig. 1a, b) and its growth-promoting receptors DCC (distal $p < 0.001$; Fig. 1c, d), and NEO1 (distal $p < 0.05$; Fig. 1e, f) compared to healthy controls, while gene expression of the repellent axon guide netrin 1 receptor UNC5H2 (Fig. 1g, h) did not differ between the groups. Cutaneous gene expression of the assumed growth-promoting receptors netrin G1 (proximal $p < 0.05$, distal $p < 0.01$; Fig. 2a, b) and netrin G2 (distal $p < 0.001$; Fig. 2c, d) was lower in PNP patients compared to controls.

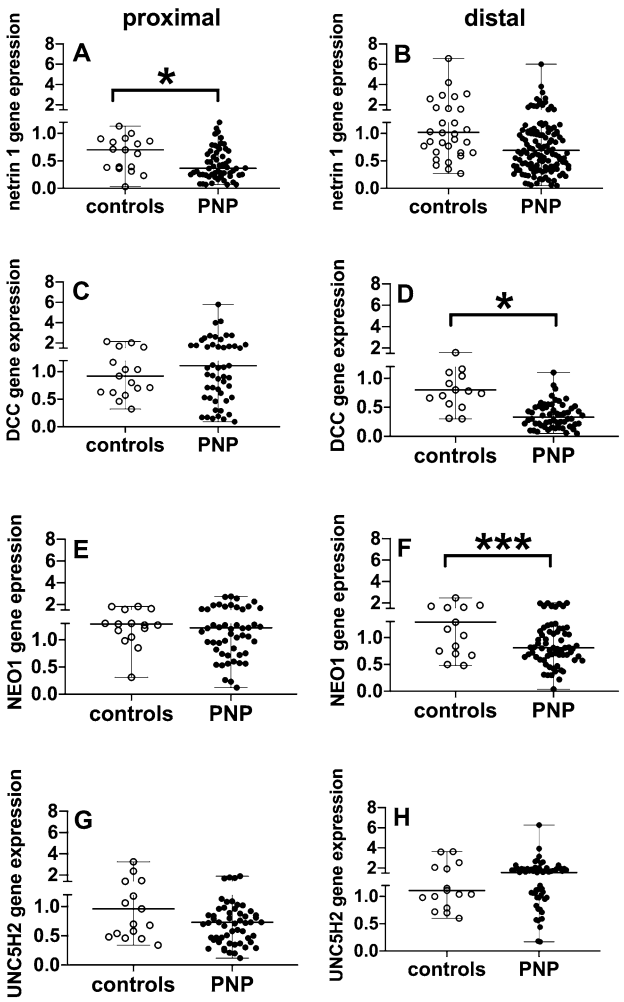

Fig. 1 Gene expression of netrin1 and its receptors in proximal and distal skin specimens. Scatter plots show ΔΔCT values of the target genes normalized to the housekeeping gene 18sRNA compared to healthy controls for netrin 1 (netrin 1; **a**, **b**) and its receptors deleted in colorectal cancer (DCC; **c**, **d**), neogenin 1 (NEO1; **e**, **f**) and uncoordinated5H2 (UNC5H2; **g**, **h**). The ΔΔCT values illustrate higher gene expression as higher scatter points. Gene expression of netrin 1 (proximal *$p < 0.05$; Fig. 1a), DCC (distal ***$p < 0.001$; Fig. 1d) and NEO1 (distal *$p < 0.05$; Fig. 1f) is lower in patients with polyneuropathies (PNP) compared to healthy controls. Number of investigated skin biopsy samples: PNP: 149 (65 proximal and 84 distal skin biopsies); healthy controls: 17

NEO1 and netrin G2 gene expression is lower in skin specimens of painful PNP and growth-promoting axon guides show a positive correlation with skin innervation

No intergroup difference was found when comparing the diagnostic subgroups for the assessed target genes in skin biopsies and also not when distinguishing inflammatory and non-inflammatory PNP (data not shown).

In distal skin biopsies, gene expression of the assumed growth-promoting receptors netrin G2 ($p < 0.05$; Fig. 3a) and NEO1 ($p < 0.05$; Fig. 3b) was lower in patients with painful PNP compared to those without pain. When correlating axon guide gene expression in skin with the individual IENFD, we found a positive correlation for DCC (distal gene expression and proximal IENFD; Spearman correlation coefficient + 0.434; $p < 0.01$; Fig. 5a) and also very weak correlation for netrin 1 (proximal gene expression and proximal IENFD; Spearman correlation coefficient + 0.285; $p < 0.05$)

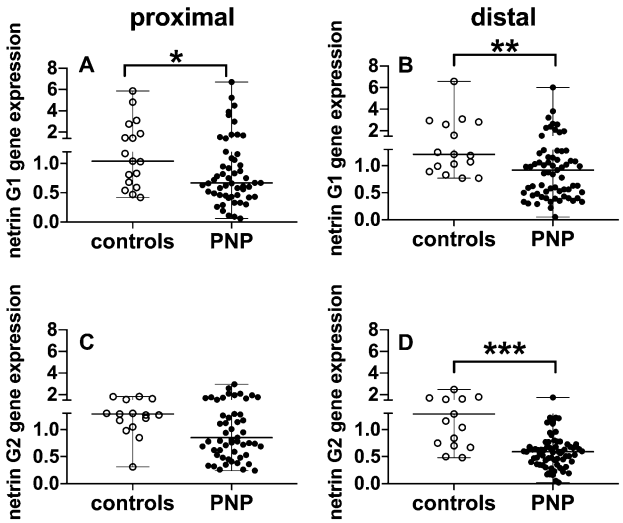

Fig. 2 Gene expression of the two membrane-linked netrin G proteins netrin G1 (netrin G1; **a**, **b**) G2 (netrin G2; **c**, **d**) in proximal and distal skin specimens. Scatter plots show ΔΔCT values of the target genes normalized to the housekeeping gene 18sRNA compared to healthy controls for netrin G1 (netrin G1; **a**, **b**) and netrin G2 (netrin G2; **c**, **d**). The ΔΔCT values illustrate higher gene expression as higher scatter points. Gene expression of netrin G1 (distal **$p < 0.01$); and proximal *$p < 0.05$; Fig. 2a, b) and netrin G2 (distal ***$p < 0.001$; Fig. 2d) was lower in patients with polyneuropathies (PNP) compared to healthy controls. Number of investigated skin biopsies: PNP: 149 (65 proximal and 84 distal skin biopsies); healthy controls: 17

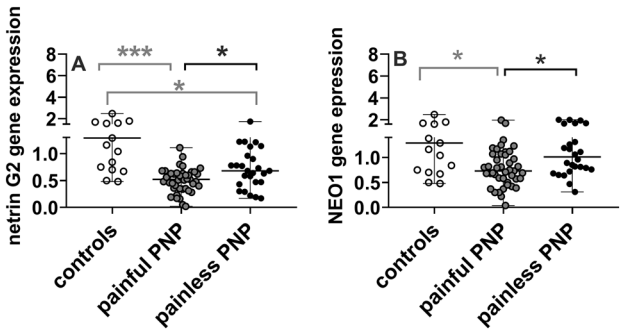

Fig. 3 Gene expression of netrin G2 (netrin G2; **a**) and neogenin 1 (NEO1; **b**) in distal skin specimens. Scatter plots show ΔΔCT values of the target genes normalized to the housekeeping gene 18sRNA compared to healthy controls for. The ΔΔCT values illustrate higher gene expression as higher scatter points. Gene expression of netrin G2 (*$p < 0.05$; **a**) and NEO1 (distal *$p < 0.05$; Fig. 3b) was lower in patients with painful polyneuropathies (PNP) compared to painless. Number of investigated skin biopsies: PNP: 149 (65 proximal and 84 distal skin biopsies); healthy controls: 17

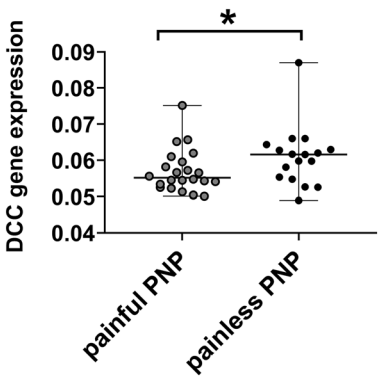

Fig. 4 Gene expression of the netrin 1 receptor deleted in colorectal cancer (DCC) in sural nerve specimens. Scatter plots show 1/ΔCT value of the target normalized to the housekeeping gene 18sRNA of DCC. The reciproval values allow illustration of low ΔCT values (i.e., high gene expression) as higher scatter points. Gene expression of DCC was lower in painful polyneuropathies (PNP) compared to painless (*$p < 0.05$). Number of investigated nerve biopsies 64

and netrin G2 (distal gene expression and distal IENFD; Spearman correlation coefficient + 0.245; $p < 0.05$). There was no difference between axonal versus demyelinating neuropathies (data not shown).

Sural nerve DCC gene expression is lower in painful compared to painless PNP

DCC gene expression in sural nerve specimens was lower in patients with painful PNP compared to painless PNP (Fig. 4). We found a weak positive correlation between DCC gene expression and proximal IENFD (Spearman correlation coefficient + 0.393; $p < 0.05$; Fig. 5b). No intergroup difference was found when comparing diagnostic subgroups including axonal and demyelinating neuropathies for the assessed target genes in sural nerve biopsies and also not when distinguishing inflammatory and non-inflammatory PNP (data not shown).

Discussion

We investigated skin and nerve gene expression profiles of axon guidance molecules in a large cohort of patients with neuropathies of different etiologies seen at one neuromuscular center. We found lower gene expression of axonal growth-promoting netrin family members in skin biopsies of patients with PNP compared to healthy controls, while there were no differences between diagnostic subgroups for skin and sural nerve biopsy specimens. We found lower gene expression of two nerve growth-enhancing receptors, NEO1 and netrin G2, in skin specimens of patients with painful compared to painless PNP and reduced gene expression of the mainly nerve growth-promoting receptor DCC in sural nerve biopsies of patients with painful compared to painless neuropathies. These findings add to the concept that neuropathies involve not only peripheral nerves, but also skin homeostasis.

We found that patients with PNP had lower gene expression of netrin 1 in proximal skin when compared to healthy controls (Fig. 1a). Whether netrin 1 has a repulsive or growth-enhancing effect on the axon depends on the composition of its receptors on the surface of the axonal growth cone [3, 45]. The expression of its receptors DCC [5] and NEO1 [4], which have an attracting effect on axonal outgrowth, was also reduced in distal skin biopsies of patients. It is possible that the simultaneous reduction of the ligand and its growth-enhancing receptors may lead to axonal degeneration and/or deficient regeneration. The UNC5H2 receptor and the simultaneous expression of DCC and UNC5H2 have a repulsive effect on axonal outgrowth. UNC5H2 gene expression was higher in the distal skin of patients with PNP compared to controls (Fig. 1h) though this effect did not reach statistical significance.

In distal and for netrin G1 also in proximal skin biopsies, we found lower gene expression of netrin G1 and G2 in patients compared to controls. For both proteins, a growth-promoting effect on axonal outgrowth was suggested [10]. Hence, reduced levels of netrin G1 and G2 may be involved in neuronal degeneration. How netrin G1 and G2 are involved in the development and regeneration of the peripheral nervous system is still unknown. Thus, the net effect of changes in the netrin system in PNP may hint toward an anti-regenerative effect.

Regarding painful and painless PNP, we found differences for the assumed growth-enhancing receptors NEO1

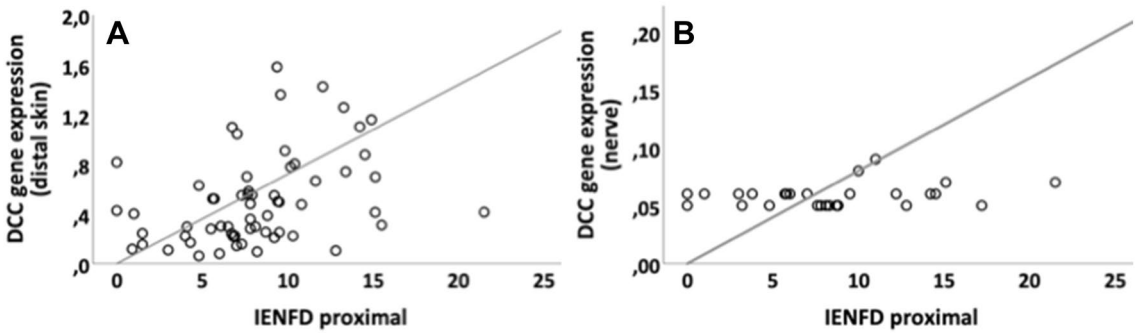

Fig. 5 Gene expression of the netrin 1 receptor deleted in colorectal cancer (DCC) in distal skin (**a**) and sural nerve specimens (**b**) correlated with proximal intraepidermal nerve fiber density (IENFD). **a** Scatter plots show ΔΔCT values of the target genes normalized to the housekeeping gene 18sRNA compared to healthy controls and IENFD. There was a positive correlation between distal DCC gene expression and proximal IENFD. Spearman correlation coefficient + 0.434 ($p < 0.01$). Number of investigated skin biopsies: PNP: 149 (65 proximal and 84 distal skin biopsies); healthy controls: 17. **b** Scatter plots show 1/ΔCT value of the target normalized to the housekeeping gene 18sRNA of DCC for sural nerve specimens and proximal IENFD. The reciprocal values allow illustration of low ΔCT values (i.e., high gene expression) as higher scatter points. There was a positive correlation between DCC gene expression and IENFD. Spearman correlation coefficient + 0.393 ($p < 0.05$). Number of investigated nerve biopsies 64

and netrin G2 in distal skin specimens. Their gene expression was lower in patients with painful PNP compared to patients with painless neuropathy. Beside NEO1's function as a growth-promoting receptor of netrin 1, its ligand repulsive guidance molecule A (RGMa) is an effective neurite growth inhibitor in the CNS. Recently, it was shown that RGMa inhibition via blocking antibodies leads to less neuropathic pain after spinal cord injury in rats and humans [46].

Although investigating a large number of nerve samples, we did not find netrin family members whose expression differed between neuropathy subgroups. However, we found lower DCC gene expression levels in sural nerve specimens of painful compared to painless neuropathies and there was a weak positive correlation between DCC gene expression in sural nerve biopsies and proximal IENFD. These results are in line with previous reports of an association between reduced DCC protein levels with mechanical allodynia in a rat postherpetic neuralgia model [47]. Thus, the netrin family ligand–receptor system may not only influence neurite outgrowth, but also initiate neuropathic pain. Our finding that cutaneous expression of two other assumed growth-promoting receptors (NEO1 and netrin G2) was lower in painful compared to painless PNP fits into this concept.

Further studies are needed to clarify which cells produce netrins. While skin consists of many highly secretory cells, in peripheral nerves Schwann cells are the main source of axon guidance molecules [17, 48]. Several studies showed that the activation of Schwann cells plays a key role in neuronal regeneration [17, 18] and an association of neuropathic pain development and peripheral nerve regeneration was discussed [49]. Besides Schwann cells, keratinocytes, fibroblasts, and cutaneous immune cells might also be involved [50–52].

One limitation of our study is the low number of skin biopsies of healthy controls that do not perfectly match with the patient group for sex and age and also the lack of healthy control nerve biopsies. This was due to the fact that we only accepted those volunteers as healthy controls who had a normal IENFD in the skin punch biopsy assessment. For ethical reasons, sural nerve biopsy is not possible in healthy volunteers. Additionally, we cannot rule out that the investigated sural nerve specimens did contain those parts with the severest pathology. Especially in inflammatory neuropathies with discontinuous inflammation, it is possible that a non- or less-inflamed part of the nerve was biopsied, which may have influenced gene expression results. Another limitation is that in spite of the large patient cohort several diagnostic subgroups contained only few patients ($n \leq 5$, see Table 2). Furthermore, we only assessed gene expression, but did not analyze protein levels due to the limited biomaterial available. Our study also cannot answer the question if alterations of netrin family members are the cause or the consequence of neuropathies; for this longitudinal and interventional studies would be needed.

Our data suggest that neuropathies are not limited to the peripheral nerves but comprise skin gene expression. We also provide evidence for a lack of growth-promoting axon guides being involved in the development of neuropathic pain which seems not to be an effect of inflammation since we did not find differences in gene expression of netrin family members in inflammatory versus non-inflammatory PNP.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflicts of interest.

Ethical approval Our study was approved by the Würzburg Medical Faculty Ethics Committee (85/06) and has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Informed consent All participants gave written informed consent before study inclusion.

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