Summary. Neurotrophins are substances that have been shown to be important in growth and remodelling phases in different types of tissue. There is no information concerning the possible occurrences of neurotrophins and their receptors in tendons. In this study, sections of both chronic painful (tendinosis) and pain-free (non-tendinosis) human Achilles tendons were immunohistochemically stained with antibodies against the neurotrophins NGF and BDNF, and their receptors TrkA, TrkB and p75. There were marked immunoreactions for NGF and BDNF in the tendon cells (tenocytes) of both tendinosis and non-tendinosis specimens. The tenocytes were also reactive for the receptor p75, but not for the receptors TrkA and TrkB. In addition, p75 immunoreactions were seen in nerve fascicles and in the walls of arterioles. This is the first study to identify neurotrophins in the tenocytes of human tendon. It is clear from this study that the local cells of tendons are sources of neurotrophins. The neurotrophins may play an important role in the tendon through their interaction with the receptor p75 in the tenocytes. These interactions may regulate trophic modulatory, and apoptotic effects. In conclusion, the observations show a new concept concerning production and function of neurotrophins, namely in the tenocytes of tendons.

Key words: Achilles tendon, Neurotrophins, Tendinopathy, p75, NGF
2007a,b) tendons have thus the capacity to produce the nerve signal substances acetylcholine and catecholamines. Furthermore, the results of a recent study suggest that these cells can produce glutamate (Scott et al., 2008). These findings revealed functions of tenocytes that were previously unidentified. One important observation was the evidence of increases in nerve signal substance production and signal substance receptors in chronic painful tendons when compared with normal tendons (Danielson et al., 2006; Bjur et al., 2008a,b). Despite these findings, the underlying mechanisms causing tendinosis and the accompanying pain remain unclear.

The functional importance that neurotrophins may have in normal and tendinosis tendons still remains to be identified. As described above, there is no information as to whether neurotrophins are produced by tenocytes or other cells in the tendon, and neither is it known if neurotrophin receptors are expressed in tendon tissue. Therefore, the aim of this study was to investigate the expression of NGF and BDNF and their related receptors (p75, TrkA and TrkB) in the Achilles tendons of patients suffering from tendinosis, comparing them to non-tendinosis Achilles tendons from healthy control subjects with no reported pain symptoms.

Materials and methods

Individuals

The present study includes biopsies from 20 subjects (9 women, 11 men), with a mean age of 45, divided into two groups. In one group, all subjects (9 men, 6 women, mean age: 43, range: 23-59) suffered from mid-portion Achilles tendinosis and had long duration of pain symptoms (mean 19 months). The pain was activity-related and located in a tender, thickened portion of the Achilles tendon, 2-6 cm cranial to the tendon insertion into the calcaneus. Tendinosis was verified by clinical examination and ultrasonography (localized widening of the tendon, irregular structure and focal hypoechoic areas) or MRI (localized widening and increased signal intensity) (c.f., Alfredson, 2005). Using colour Doppler ultrasonography the occurrence of augmented blood flow in the region of the structural changes was shown. This augmented blood flow is typical for tendinosis (Alfredson, 2005). All patients were non-smokers, and besides suffering from tendinosis, were found to be healthy individuals.

The other group consisted of 5 individuals (2 men, 3 women, mean age: 45, range: 39-47) with pain-free and normal Achilles tendons. All individuals were healthy, on no medication and were non-smokers. This group is hereafter referred to as the non-tendinosis group. The tendons in this group were examined with ultrasonography and showed no structural changes and the tendon width were normal. These tendons did not show an augmented blood flow.

The study was approved by the local Committee of Ethics at the Faculty of Medicine and Odontology, Umeå University, and by the regional Ethical Review Board in Umeå. All experiments conformed to the Declaration of Helsinki.

Sampling

In the tendinosis group, biopsies were taken during surgical treatment. Under local anaesthesia (4-5 ml pilokainhydrochloride, 10 mg/ml, Södertälje, Sweden), tendon tissue (macroscopically abnormal) was taken from the ventral part of the Achilles tendon through a longitudinal incision lateral to the tendon mid-portion. The specimens contained tendon tissue proper, and to varying degrees, outer parts of the tendon (paratendinous connective tissue). Tissue samples were taken from different depths of the tendons and were approximately 2 mm wide and 1-5 mm long. From the non-tendinosis tendons, samples of the same size were collected from the dorsal part of the tendon using a longitudinal plain incision under local anaesthesia.

Sectioning and fixation

Immediately after the samples were taken, they were fixed overnight at 4°C in a solution of 4% formaldehyde in 0.1M phosphate buffer, pH 7.0. The samples were thereafter thoroughly washed in Tyrode’s solution containing 10% sucrose, at 4°C overnight, mounted on thin cardboard in OCT embedding medium (Miles Laboratories, Naperville, IL, USA) and frozen at -80°C until sectioning. A series of 7-8 µm thick sections from both groups of samples were cut using a cryostat (Leica Microsystem CM 3000, Heidelberg, Germany). The sections were mounted on slides pre-coated with chrome-alun gelatine, dried, and processed for immunohistochemistry, or stained with haematoxylin-eosin in order to explore tissue morphology.

Immunofluorescence processing

Sections were processed by the following protocol: Initially sections to be stained for BDNF and NGF were incubated in acid potassium permanganate for 2 min, to enhance the visualization of specific reaction sites (c.f., Hansson and Forsgren, 1995), and then were washed in PBS (3x5min). These sections were then immersed for 20 min a 1% solution of detergent Triton X-100 (Kebo Lab, Stockholm) in 0.01M phosphate buffered saline (PBS), pH 7.2. Such immersion in Triton X-100 solution was the initial step for the staining of sections to be processed for TrkA, TrkB and p75. The procedures that followed after immersion in Triton X-100 were the same for all sections. These procedures included initially a rinsing in PBS (3x5 min), and thereafter incubation in 5% normal swine serum in PBS supplemented with 0.1% bovine serum albumin (BSA) for 15 min. Subsequently, the sections were incubated with the primary antibody, diluted in PBS with BSA, in a humid environment. Incubation was conducted for 60 min at 37°C. After this, the sections were washed in PBS.
(3x5min), followed by another incubation in normal swine serum and an incubation with the secondary antibody corresponding to tetramethylrhodamine isothiocyanate (TRITC)–conjugated swine antirabbit IgG (Dakopatts, Denmark) diluted 1:40, for 30 min at 37°C. After a last wash in PBS (3x5min), the sections were finally mounted in VECTASHIELD microscopy mounting medium.

Examination was carried out using a Zeiss Axioskop 2 plus microscope, equipped with epifluorescence optics and a digital Camera (Olympus DP70).

Antibodies and control stainings

The following antibodies were used; Antibodies against NGF (code: sc 548, dilution 1:200), BDNF (code: sc 546, 1:100), TrKB (code: sc-12, 1:50) TrkA (code: sc-118, 1:50), and NGFR-p75 (p75) (code: N 3908, 1:50). All 5 antibodies were raised in rabbit. The p75 antibody was obtained from Sigma (Saint Louis, USA) and the others were obtained from Santa Cruz Biotechnology, Inc (California, USA).

The antigen for p75 is a synthetic peptide corresponding to the C-terminal of rat NGFR-p75 (amino acids 407-425 with N-terminally added lysine) conjugated to keyhole limpet haemocyanin (KLH) as immunogen. This sequence is reported by the supplier to be highly conserved in humans. Staining of the p75 band by immunoblotting is reported to be specifically inhibited by the p75 amino acid 407-425.

Preabsorbtions were performed

Preabsorption showed that the BDNF and NGF immunoreactions were inhibited by preincubation of BDNF and NGF antisera with BDNF (50 g/mL antiserum: sc-546P, Santa Cruz) and NGF (50 g/mL antiserum: sc-548P, Santa Cruz), respectively.

It is reported by the supplier that the TrkA antibody reacts with TrkB but not TrkB or TrkC, and that the TrkB antibody reacts with TrkA but not TrkA or TrkC. The occurrence of specific immunoreactions were delineated by preabsorption of TrkA antiserum with blocking peptide (50 µg/mL; sc-118P, Santa Cruz) and TrKB antiserum with blocking peptide (50 µg/mL; sc-12P, Santa Cruz). For control purposes, replacement of all primary antibodies with PBS supplemented with BSA or normal serum was also performed.

Results

General morphology

In the samples of non-tendinosis tendons, the tissue consisted of bundles of well-arranged collagen fibrils, between which narrow spaces of loose connective tissue containing blood vessels occurred. The tenocytes in the tendon tissue proper were evenly arranged. They exhibited a slender appearance or were slightly spindle-shaped.

The collagen in the tendon tissue proper in the tendinosis tendons was less well arranged than that in the non-tendinosis tendons. The tenocytes in the tendinosis samples showed varying appearances, being slender, rounded/widened or wavy in appearance. Hypercellularity and hypervascularity was frequently noted. These observations are in agreement with the morphologic observations made in previous studies on Achilles tendinosis tendons (Bjur et al., 2005, 2008a).

In some specimens, parts of the paratendinous tissue were also present. In these parts, large blood vessels, nerve fascicles and an abundance of loose connective tissue were present. The nerve fascicles in the paratendinous tissue, as well as the nerve fascicles that occasionally occurred in the tendon tissue proper, were located in the proximity of blood vessels.

Verifications of the occurrence of specific immunoreactions

Stainings were initially performed for the purpose of delineating whether specific immunoreactions occurred. These included preabsorption stainings for NGF, BDNF, TrkA and TrkB (see Materials and Methods for further details). These stainings confirmed immunoreactions specific for NGF and BDNF in the tenocytes (Fig. 1a-d). Specific TrkB reactions also occurred, being observed in nerve fascicles (Fig. 1e-g). No specific immunoreaction was observed for TrkA. The occurrence of specific p75 immunoreactions was evaluated by comparing p75-processed sections with parallel control stainings.

NGF immunoreactions

NGF immunoreactions were seen in most of the tenocytes (c.f. above) in both tendinosis and non-tendinosis samples. Reactions could thus be seen in tenocytes having a slender appearance (Fig. 2a), as well as in those showing widened and wavy appearances (Fig 2b-e). The reactions were distinct, showing a punctuate appearance in most cases (Figs. 1a, 2a-d). The magnitude of reactions within the tenocytes varied, particularly in the tendinosis group (c.f. Fig. 2b, d). Furthermore, there were tenocytes in both non-tendinosis and tendinosis samples that were non-reactive.

NGF immunoreactions were also found in cells of the blood vessel walls in both non-tendinosis and tendinosis samples. The reactions showed a punctuate appearance (Fig. 2f, c.f 2g). No reactions were seen in nerve fascicles (not shown). In total, the most marked immunoreactions for NGF were seen in the tenocytes.

BDNF immunoreactions

The patterns of immunoreactions for BDNF were similar to those for NGF concerning the tenocytes. Thus, most of these were reactive, showing a punctuate appearance (Figs. 1c, 3a-d). This was seen in both non-tendinosis (Fig. 3a) and tendinosis (Figs. 1c, 3b-d).
Fig. 1. a-d. Sections of tendinosis Achilles tendons processed for NGF (a) and BDNF (c). For the purpose of verification of specific immunoreactions sections were also processed with antisera that were preincubated with the respective antigen (b, d) but otherwise processed in the same way as in sections shown in (a, c). Reactions are seen in tenocytes in (a, c) (arrows) but are not seen after preabsorption of NGF antiserum with NGF (b) nor after preabsorption of BDNF antiserum with BDNF (d) (arrows at unreactive tenocytes). Note that the tenocytes are very frequent. a, x130; b, x120; c, x 315; d, x 315. e-g. Adjacent sections of non-tendinosis tendon processed for TrkB (e) and processed with TrkB antiserum that had been preabsorbed with TrkB antigen (f). A nerve fascicle (arrow) is immunoreactive in (e) but shows no immunoreaction in (f). Star at corresponding regions of a nearby located blood vessel. In (g), parts of a section of tendinosis tendon processed for TrkB is shown. Nerve fascicles are immunoreactive (arrows). Star at blood vessel. e-g, x 200
samples. However, the level of immunoreactions appeared to be lower in non-tendinosis than in tendinosis samples, and the tenocyte reactions for BDNF were generally weaker than those for NGF. Nevertheless, there were variations in immunoreaction levels within both groups and some tenocytes of the groups were non-

**Fig. 2.** Sections of Achilles tendon tissue proper of non-tendinosis (a, and inset a) and tendinosis (b-e) tendons processed for NGF. Tenocytes display specific immunoreactions that frequently show a punctuate appearance (arrows). Tendinosis tenocytes tend to be wider and wavy (b-e) compared to those of non-tendinosis tendons (a). (F) and (g) show parts of Achilles tendinosis tendon. In (f), occurrence of NGF immunoreactions with a punctuate appearance (arrows) in cells of blood vessel wall of a tendinosis specimen is shown. The occurrence of specific reaction was verified by preabsorption of the NGF antiserum with NGF (g). Star at part of the blood vessel wall (f, g). a, x 100, inset x 315; b-g, x 315
reactive. Weak reactions were sometimes observed in the
cells of blood vessel walls and in nerve fascicles (data
not shown).

**P75 immunoreactions**

Immunohistochemical stainings for p75 clearly
revealed that the tenocytes were reactive. This was noted
in both tendinosis and non-tendinosis samples (Fig. 4a-
c). There was, however, a heterogeneity for both groups
in this respect with some tenocytes not showing
reactions.

Nerve fascicles and nerve fibres were
immunolabelled for p75 in both non-tendinosis and
tendinosis samples (Fig. 4d,e). In the nerve fascicles, it
was obvious that there were p75 reactions both in the

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**Fig. 3.** Sections of non-tendinosis (a) and tendinosis (b-d) Achilles tendons processes for BDNF. Tenocytes show immunoreactions. The occurrence of a punctuate appearance of the reactions is obvious especially in the tendinosis specimens (arrows in b-d). The tenocytes in (b-d) are furthermore wider and wavier in form than those in (a). a, x 200; b-d, x 315
perineurium and within the fascicles (Fig. 4e). Immunoreactions were also detected in the adventitia of some arterioles, i.e just outside the smooth muscle layer of the blood vessel walls (Fig. 4f).

**TrkB immunoreactions**

No specific immunoreactions for TrkB were seen in the tenocytes in non-tendinosis or tendinosis samples. On the other hand, nerve fascicles showed reactions in both types of samples (Fig. 1e,g). No specific immunoreactions were seen in blood vessel walls.

**TrkA immunoreactions**

No specific immunoreactions were seen (c.f. above)

**Discussion**

**Summary of findings**

The present study shows that there are marked immunoreactions for the neurotrophins NGF and BDNF and for the p75 receptor, but not for TrkA or TrkB, in the tenocytes of the human Achilles tendon. This is the first study to report such findings. The observations were made for both chronic painful tendinosis tendons and normal, pain-free, tendons. The findings suggest that the tendon itself is capable of producing neurotrophins, and that important neurotrophin effects occur in the tenocytes of both tendinosis and non-tendinosis tendon. It is thus obvious that tenocytes can not only produce nerve signal substances (Danielson et al., 2006, 2007a,b; Bjur et al., 2008a,b), but also neurotrophic factors. NGF and BDNF immunoreactions were also to some extent noted for cells of blood vessel walls, and BDNF, p75 and TrkB immunoreactions were noted in nerve fascicles. However, the immunoreactions in the tenocytes dominated.

**Presumable functions of the p75 receptor**

The fact that p75, but not TrkA/TrkB, immunoreactions were seen for the tenocytes, suggests that the effects of neurotrophins solely occur via the low-affinity p75 receptor. One possibility is that the p75 receptor is related to modulatory effects of tenocyte apoptosis, a phenomenon that is reported to occur in tendinosis (Lian et al., 2007). It cannot be excluded that tenocytes go into apoptosis also in normal tendons. The p75 receptor is shown to promote endothelial cell apoptosis (Caporali et al., 2008) and it is involved in hypothyroidism-enhanced
apoptosis during rat cortical development (Kumar et al., 2006). Moreover, the p75 receptor modulates apoptotic processes in the endocrine and exocrine pancreas in chronic pancreatitis (Zhu et al., 2003). It has also been suggested that the p75 receptor is important for the excitotoxic degeneration that occurs in dopaminergic neurons in Parkinson’s disease (Wang et al., 2008).

Another possibility is that neurotrophins acting via the p75 receptor have remodelling, trophic and autocrine/paracrine effects. The p75 receptor is thus presumably related to regeneration mechanisms in nerve tissue, there being an increased synthesis of p75 in nerve-associated Schwann cells after injury of the sciatic nerve (Funakoshi et al., 1993). There are several reports describing that neurotrophins can have trophic and survival-promoting effects in neurons (Levi-Montalcini, 1987; Linnarsson et al., 2000; Chu et al., 2007), and that they have effects in nerve differentiation (Ibanez, 1998). Trophic effects on chondrocytes have also been suggested (Iannone et al., 2002), as well as effects in the formation of bone tissue (Asaumi et al., 2000). The results of a previous study (Yamashiro et al., 2001), and a recent study in our group (Grimsholm et al., 2008), suggest that neurotrophins are of importance for cartilage, having autocrine/paracrine effects. The p75 receptor is markedly expressed in the articular chondrocytes of the mouse knee joint (Grimsholm et al., 2008).

Further studies should clarify if the p75 receptor interactions are primarily involved in apoptosis-modifying or trophic effects in tendons. The recent findings that the p75 receptor has anti-angiogenetic actions, as shown in vitro studies (Caporali et al., 2008), are also of interest when clarifying the role of p75 in tendons. Tendinosis is thus associated with angiogenesis (Khan et al., 1999; Mafulli et al., 2004).

Occurrence of variability in neurotrophin/p75 expressions

It is often considered that NGF levels are increased during inflammation (Gillardon et al., 1995; Friess et al., 1999). However, a decrease in NGF and neurotrophin receptor expression levels occurs in the chondrocytes lining the synovial cavity in experimentally induced arthritis in mice (Grimsholm et al., 2008). Furthermore, there is a decrease in neurotrophin immunoreaction in the nerve structures in ulcerative colitis (Johansson et al., 2007). In the present study, we noted that there were variations in the levels of neurotrophin/p75 immunoreactions between different samples, particularly within the tendinosis group. These findings, together with the findings described above, show that plasticity may occur in production levels concerning the neurotrophins and their receptors, especially in the tendinosis group. A further explanation for the variability seen in the present study is the fact that the tenocyte population in tendons is not a complete uniform cell type (Riley, 2005).

Neurotrophins can interact with other neuromodulators

An important aspect concerning neurotrophins is that they show interactive effects with other neuromodulators, including neuropeptides and cytokines. Interactions between NGF and the neuropeptide substance P (SP) (Allo et al., 1995), as well as an involvement of NGF in TNF-alpha upregulation (Manni and Aloe, 1998), are thus reported for joint inflammation. NGF production is also stimulated by cytokines (Safieh-Garabedian et al., 1995), and cytokines, including TNF-alpha, are known to stimulate BDNF secretion in human peripheral blood monocytes (Schulte-Herbrüggen et al., 2005). The levels of NGF, TNF-alpha and SP were all shown to be increased in a cystitis model in mice (Gonzalez et al., 2005). Based on these observations it is of interest to note that we recently found that tenocytes in Achilles tendons, especially in tendinosis, showed expression of SP mRNA, as well as expression of the SP-preferred receptor (neurokinin-1 receptor) at both protein and mRNA levels (Andersson et al., 2008). These findings suggest that SP has autocrine/paracrine effects in tendon tissue. It might be that a part of these effects occur via interactions of SP with neurotrophins. In the case of NGF and TNF-alpha, the existence of a crosstalk between these has been recently proposed to exist in several other tissue types (Takei and Laskey, 2008).

Interference with neurotrophin effects; treatment with platelet-rich plasma

The effects of interference with neurotrophin activity have been examined in number of experimental studies. The results suggest that blocking of NGF-effects in cases of colitis might be worthwhile (Ma et al., 2003). Nevertheless, anti-NGF treatment has also been shown to increase the severity of experimentally induced intestinal inflammation (Reinshagen et al., 2000). Results of experimental studies also favour that an augmentation of NGF-effects may be desirable. NGF is thus shown to accelerate wound healing in diabetic mice (Muangman et al., 2004), and to be important in the healing of skin ulcers (Aloe, 2004).

A recently discussed treatment model for tendinosis and tendon wounds is therapy with platelet-rich plasma. Studies on human tenocyte cultures (de Mos et al., 2008), on Achilles tendon repair in the rat (Aspenberg and Virchenco, 2004; Virchenco et al., 2006) and on the rat patellar tendon (Kajikawa et al., 2008), suggest that in vivo use of platelet-rich plasma or platelet concentrate might improve tendon repair. The mechanisms are unclear. The neurotrophin BDNF is known to be stored in human platelets, and to be released to circulate in plasma (Lommatsch et al., 2005, 2007). It is therefore possible that BDNF in platelet-rich plasma exerts effects in tendons via interaction with the p75 receptor in blood vessel walls and in tenocytes.
**Conclusions**

The present study gives evidence for the occurrence of a local production of neurotrophins in tendons, particularly in the tenocytes. These observations, in parallel with those of p75 immunoreactions in the tenocytes, are of relevance, since neurotrophins have well-established effects in relation to growth and remodelling, as well as apoptosis-modifying effects. Presumably, they have modulatory effects for the tendons, including in situations when tendinosis develops. Current thoughts concerning NGF are that this neurotrophin is involved in the modulation, and not in the initiation, of inflammatory events in joints (Manni et al., 2003), and that it has a protective role in injuries (Ma et al., 2003). Future studies should be performed to clarify the importance of neurotrophins for tendon function, and whether or not interference of neurotrophin induced effects is useful in tendon disorders.

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**References**


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Neurotrophins in the Achilles tendon


