Co-existence of Up-regulated NMDA Receptor 1 and Glutamate on Nerves, Vessels and Transformed Tenocytes in Tendinopathy

N. Schizas[1], Ø. Lian[2, 3], F. Frihagen[4], L. Engebretsen[2, 4], R. Bahr[2], P. W. Ackermann[1]

[1]Orthopedic Laboratory, Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden, [2]Oslo Sports Trauma Research Center, Department of Sports Medicine, Norwegian School of Sport Sciences, Oslo, Norway, [3]Kristiansund Hospital, Kristiansund, Norway, [4]Orthopaedic Center, Ullevål University Hospital, Oslo, Norway.

Running title: Up-regulation of NMDAR1 in Tendinopathy

Address correspondence to:

Paul W Ackermann, MD, PhD

Orthopedic Dept., Karolinska University Hospital, 171 76 Stockholm, Sweden

Telephone: +468 517 726 99

Telefax: +468 517 761 03

E-mail: paul.ackermann@ki.se

Article type: Original article

Keywords: tendon, pain, glutamate, NMDAR1, tendinopathy, tendinosis, angiogenesis, cell proliferation

Abstract

Elevated levels of the neurotransmitter glutamate and the presence of its receptor, N-methyl-d-aspartate receptor type 1 (NMDAR1) have been established in patients with tendinopathy, i.e. chronic tendon pain and degeneration. However, whether NMDAR1 is up- or downregulated in tendinopathy and co-localized with glutamate is still unexplored. We hypothesize that an alteration in tissue expression and in co-existence of NMDAR1 and glutamate occurs in tendinopathy and might play a role in nociception and possibly also progression of tendon degeneration (tendinosis).

We therefore examined tissue distribution and levels of NMDAR1 and glutamate in biopsies from patients with patellar tendinopathy (n=10) and from controls (n=8). The biopsies were single- and double-stained immunohistochemically for glutamate and NMDAR1 and assessed subjectively and semi-quantitatively.

The chronic painful tendons exhibited a significant elevation of NMDAR1 (9-fold), which was independent from the observed increase in glutamate (10-fold). This up-regulation of NMDAR1 and glutamate was found to be co-localized on nerve fibers as well as on morphologically altered tenocytes and blood vessels. None of the controls exhibited neuronal co-existence of glutamate and NMDAR1. The neuronal co-existence of glutamate and NMDAR1, observed in painful tendinosis but not in controls, suggests a regulatory role in intensified pain signalling.

Introduction

The pathogenetic mechanisms leading to tendinopathy, i.e. chronic tendon pain and tissue degeneration, are largely unknown. Thus, effective targeted therapies are lacking.

Recently, accumulating data have indicated that the peripheral nervous system, in addition to nociception, is implicated in regulating tendon tissue proliferation [Ackermann et al 2002, Ackermann et al 2003]. Thus, increased neuronal occurrence of the sensory neuropeptide, substance P, was observed during early tendon repair but also in patients with tendinopathy [Ackerman et al 2001, Schubert et al 2005, Lian et al 2006,].

Substance P has the ability to unblock the glutamate receptor, N-methyl-d-aspartate receptor type 1 (NMDAR1), permitting the ligand glutamate to activate the receptor and trigger a variety of chronic pain disorders [Besson 1999, Madden 2002]. The role of NMDAR1 in nociception has been studied extensively during the past decade. Several studies show that upregulated NMDAR1 is associated with allodynia and hyperalgesia [Besson 1999, Danbolt 2001, Cairns et al 2003, Cairns et al 2006]. The presence of NMDAR1 has recently been established in the periphery, also in tendon tissue [Scannevin and Huganir 2000, Alfredson et al 2001, Danbolt 2001, Madden 2002,]. Whether NMDAR1 is up- or downregulated in tendinopathy has so far not been explored.

Although elevated levels of glutamate have been found in tendon degeneration (tendinosis) by microdialysis, this has not been associated with nociception [Alfredson et al 2001, Alfredson and Lorentzon 2002]. Thus, the specific localization of the glutamate increase and the co-localization with its receptor NMDAR1 has yet not been clarified in tendinopathic patients. We hypothesize that NMDAR1 is up-regulated and also co-localized with glutamate in human chronic painful tendinosis. In the present study we investigated the occurrence and specific localizations of NMDAR1 and its ligand glutamate as well as their co-existence in tendinopathic biopsies and control tissue.

Materials and Methods

Patients: The study included ten patients (9 males and 1 female) and eight controls (5 males and 3 females). It was approved by the regional committee for research ethics, and the participation was voluntarily [Lian et al 2006]. The patient group included athletes from different sports with symptoms of tendinopathy. All patients met the four inclusion criteria which were: 1) Symptoms of exercise-related pain in the proximal patellar tendon or the patellar insertion and distinct tenderness to palpation [Blazina et al 1973]. 2) Clinical diagnosis of jumpers knee grade IIIB, which means that the patient has pain before and after activity and is unable to participate in sports at the same level as before [Roels et al 1978, Lian et al 2006]. 3) Thickening and signal changes on MRI corresponding to the painful area and 4) symptom duration for a minimum of three months.

All patients underwent a standardized interview and they were asked to answer the VISA questionnaire, which assesses symptoms, function, and the ability to participate in sports. It has been reported to be a reliable and valid measure of symptoms in patients with patellar tendinopathy [Visentini et al 1998]. The maximum VISA score is 100 points and the minimum is 0 points, where 100 points represent full ability to participate in sports without symptoms. The average age of the patient group was 28 years (range 19-32, n=10), the average VISA score was 37 (range 16-53, n=10) and the average duration of patellar tendon pain was 33 months (range 6-81 months, n=10).

The control group consisted of patients with tibia fractures treated with intramedullary nailing without current or previous knee pain. In both groups the minimum age was 18 years. The average age of the controls was 32 years (range 19-60, n=8). VISA scores were not collected, but all of them reported being completely symptom-free during physical activity and never having had symptoms of jumper’s knee. Subjects in both groups were excluded if they had a

history of shock wave therapy or corticosteroid/ polidocanol injections in or around the knee, knee or patellar tendon surgery, inflammatory or degenerative joint condition.

Surgical technique: The surgical technique was identical in both groups and has been described in detail in a previous study [Lian et al 2006]. A 5-cm longitudinal midline or lateral parapatellar incision was done, followed by splitting of the paratenon, and exposure of the patellar ligament. If clearly abnormal tissue was not seen macroscopically, the excision was guided by the MRI signal changes. Starting at the patellar tendon origin a wedge 10 mm wide and 20-30 mm long was removed.

Histology: Immediately after surgery the samples were transferred to Zamboni´s fixative (4% paraformaldehyde in 0.2 mol/liter Sorensen phosphate buffer, pH 7.3, containing 0.2% picric acid). They were stored there for 24-48 hours and then washed in 0.1M phosphate-buffered saline (PBS), pH 7.2, with 15% sucrose (weight/volume) and 0.1% sodium azide. This washing was performed approximately six times, until the yellow color of Zamboni was no longer seen in the solution. The samples were frozen, sectioned at 12µm and 7µm on a Leitz cryostat and the frozen sections were mounted directly on Super-Frost /Plus glass slides, three sections per slide consecutively.

Immunohistochemistry: The sections were stained using the avidin-biotin system, rinsed in PBS for five minutes and incubated in 5% normal goat or horse serum for 40 minutes. Normal horse serum was used when the NMDAR1-mouse raised primary antibody (BD Biosciences C.N. 556308) was analyzed. Afterward, one of the NMDAR1 (1/250), L-Glutamate (1/50 Chemicon international C.N. AB5018), PGP9.5 (1:10 000, UltraClone, Wellow, United Kingdom) and GAP-43 (1/5000, Boehringer Mannheim Biochemicals, Germany) primary antisera was added and the samples incubated over night in room temperature, wet atmosphere. The sections were rinsed in PBS (3x5min) and incubated with biotinylated goat anti-rabbit or horse anti-mouse secondary antibody for 40 minutes. For immunofluorescence,

the sections were rinsed in PBS (3x5 min) and incubated with Cy2- conjucated avidin (1/2000 Amersham UK).

For double staining, after completing the staining steps for the first antibody, the sections were incubated with avidin blocking solution followed by biotin blocking solution (Vector Laboratories) (15 min each). The staining for the second antibody was repeated as described for the first antibody, but with a different flourochrome, CY3 (1:5000; Amersham Int., Poole, UK).

For DAB, the sections were blocked with peroxidase (H_2O_2) 0,3% in a dark place for 30 minutes. After rinsing, the samples were incubated with ABC solution for 30 minutes and then, incubation with DAB solution (Vector Laboratories) followed for 5 minutes. After washing with water, one drop of haematoxylin was dropped on the sections for 10 seconds and then washed in water for 10 minutes. The samples were then dehydrated in 70%, 95% and 100% alcohol for 2, 5 and 5 minutes respectively and finally 5 minutes in xylene.

Control staining: To demonstrate the specificity of NMDAR1 and glutamate primary antisera, the following controls were included: (a) Pre-adsorption techniques described in detail in previous studies [Marc et al 1990, Siegel et al 1994, Billig et al 2003]. (b) Omission of either the primary antiserum, or the secondary biotinylated antibody. Additionally, control tissues (spinal cord, intestine, sciatic nerve) as suggested by the manufacturers were stained and used as positive controls.

Image Analysis

Subjective analysis: Sections from both the single- and double-staining experiments were subjectively evaluated by two independent observers. The observers were blinded for the identity of the sections analysed and the sections were analyzed in random order. Double staining was done to demonstrate the relation between NMDAR1 and PGP9.5 (mature)/ GAP-43 (regenerating) nerves, also that between NMDAR1 and glutamate.

Quantitative analysis: Semiquantitative analysis was performed by two independent observers to assess the occurrence of NMDAR1 receptors and glutamate in the vessel walls in both tendinopathic samples and controls. The observers were blinded for the identity of the sections analysed and the sections were analyzed in random order.

The following steps developed in an earlier study [Ackermann et al 2002] were applied to optimize the semiquantitative analysis: The patellar tendons were longitudinally sectioned, and the sections were numbered consecutively from the dorsal to the ventral aspect. Three sections from different levels (ventral, middle, and dorsal parts of the tendon) were chosen to represent the full thickness of the tendon. Staining was performed simultaneously for all sections to be compared. For microscopic analysis, a video camera system (DXM 1200 Nikon) was attached to the epifluorescence microscope and connected to a computer. From each section, three non overlapping images, microscopic fields (x 20 objective NMDAR1) and (x 40 objective glutamate), were taken from the tendon proper and the loose connective tissue, exhibiting the strongest vascular immunofluorescence as assessed subjectively [Ackermann et al 2002].

Thereafter, the images were stored in the computer and analyzed using Easy Analysis software (Technooptik, Skarholmen, Sweden). The software denotes and considers all positively stained antibodies beyond a defined threshold of fluorescent intensity which was set and remained unaltered for all images assessed. The results were expressed as the fraction area occupied by positive staining in relation to the total area of the vessel. The fluorescent/total area was determined in 9 images in each biopsy of the patient and control groups, respectively. In the microscopic analysis, the mean interobserver coefficient of variation was 16% and the intraobserver variation 15%. For statistical analysis, the mean fluorescent/total area was calculated for each of the biopsies from both the patient and the control groups.

The quantitative assessment of glutamate positive tenocyte density was performed by counting the number of positive cells per objective field by two independent observers. Semi-quantitative assessment of tenocyte transformation was performed according to the Bonar scale [Lian et al 2006], which assesses tenocyte morphologic characteristics (Table 1).

Statistical analysis: For statistical analysis the mean area fraction of positive ligand or receptor / area of the vessel as well as the mean number of positive cells per objective field were calculated for each sample. Non-parametric Mann-Whitney *U*-tests for independent samples were used. Correlation between the vascular occurrence of NMDAR1 and glutamate as well as between glutamate occurrence in vessels and in the tendon proper was assessed according to Pearson's correlation analysis. The statistical tests were conducted using Statistica 7.0 (StatSoft Inc. Tulsa USA). The level for significance was set at $p<0.05$.

Results

Quantitative assessments

The numbers below reflecting the occurrence of vascular NMDAR1 and glutamate are expressed in % of positive receptor or ligand area divided by the area of the vessel.

N-methyl-d-aspartate receptor type 1 (NMDAR1): Semi-quantitative assessment demonstrated an approximately 9-fold increase in vascular NMDAR1 in the painful tendons (7.6%) (p=0.027) compared to the controls (0.9%) as analyzed in the loose paratendinous connective tissue (fig 1). Vascular NMDAR1 in the tendon proper (5.9%) of the tendinopathic group exhibited similar levels as in the surrounding loose connective tissue. However, no vessels within the tendon proper could be found in the controls.

Glutamate: The quantitative analyses revealed that the glutamate occurrence in vessels and cells was elevated approximately 10-fold in the tendinopathic group. Vascular glutamate localized in the loose connective tissue (6.7%) of the painful tendons was significantly (p=0.006) elevated as compared to the controls (0.6%) (fig 1). In addition, counting disclosed an increased number (p=0.009) of glutamate positive tenocytes in tendinopathic tendons (4.8 per obj field) compared to controls (0.5 per obj field) (fig 1).

Correlation of NMDAR1 with glutamate occurrence: No correlation was found in either of the two groups (tendinopathy: r^2=0.087, p=0.41) (controls: r^2=0.047, p=0.61).

Male and female subgroup analysis: No differences between the male (5) and female (3) controls were found regarding the assessed parameters (NMDAR1, Glutamate, Glutamate positive tenocytes) (p=0.57, p=0.78, p=1 respectively). The one female tendinopathic sample did not exhibit outlier values. Quantitative analyses including only the male samples (9 tendinopathic and 5 controls) demonstrated that the results were still significant between the two groups in all the parameters assessed quantitatively (NMDAR1, Glutamate, Glutamate positive

tenocytes p=0.011, p=0.041, p=0.041). We thus considered that there were no subgroups and hence the data were analyzed together.

Combined immunofluorescence and DAB staining

Immunofluorescence studies were conducted to assess the neuronal and cellular staining. DAB staining confirmed the immunofluorescence findings and additionally showed the cellular morphology in detail.

NMDAR1: The glutamate NMDAR1 was identified in all samples analyzed (tendinopathic and controls). The combined analysis by immunofluorescence and DAB staining suggested increased NMDAR1 immunoreaction in the painful tendons, in line with the quantitative assessment. Moreover, the immunohistochemical analysis showed the localizations of increased NMDAR1 immunostaining in the tendinopathic samples, as seen in nerve fibers (fig 2,3), blood vessel walls (fig 2) and tendon cells.

The neuronal occurrence of NMDAR1, according to double staining with PGP9.5, a general nerve marker, was found to be elevated in tendinopathy in free nerve endings (fig 3) and in the adventitial layer of blood vessels (fig 2) in the tendon proper and surrounding loose connective tissue. Immunoreaction to NMDAR1 in the endothelial vessel layer was non-neuronal (not co-localized with PGP9.5). Moreover, in five of the ten tendinopathic samples, but not in the controls, pathologic granulation tissue accompanied by NMDAR1 positivity in the endothelial layer of blood vessels was observed in the tendon proper.

Double staining for GAP-43, a nerve regenerative marker, and NMDAR1 disclosed no co-localization.

Tenocytes in the tendon proper were positive for NMDAR1 in 7/10 (6/9 male, 1/1 female) samples of the tendinopathy group and 2/8 (2/5 male, 0/3 female) of the controls. All of the tendinopathy biopsies exhibiting NMDAR1 positive tenocytes also showed signs of tenocyte transformation (table 2).

Glutamate: The NMDAR1 ligand glutamate was identified in both tendinopathic and control samples. The immunohistochemical analysis demonstrated that glutamate was elevated in the painful tendons, as shown by the quantitative assessments. This increase was observed in nerve fibers (fig 4), blood vessels and tenocytes (fig 5), i.e similar to NMDAR1.

In the painful tendons double staining of PGP9.5 and glutamate demonstrated the neuronal existence of glutamate in nerve bundles and the adventitial layer of vessel walls localized in the loose connective tissue, as well as in free nerve endings within the tendon proper (fig 3,4). Notably, no neuronal occurrence of glutamate was found in the normal controls (no co-localization with PGP9.5) (fig 4).

Moreover, the analysis demonstrated an increased number, compared to the controls, of glutamate positive tenocytes in the painful tendons (fig 5), which also exhibited signs of tenocyte transformation according to the Bonar scale (table 1).

Co-localization of NMDAR1 and glutamate: A co-localization of glutamate and NMDAR1 was demonstrated in nerve fibers (fig 6) in all the painful tendinopathic samples, but not in any of the controls. A co-existence was also found in blood vessel walls exclusively observed in the tendon proper of the tendinopathy group, and on transformed tenocytes both in the tendinopathic samples and to a lesser extent in the controls. In some areas of the tendons, however, immunoreaction to NMDAR1 and glutamate was seen without co-localization.

Discussion

To our knowledge this is the first study on patients showing that painful tendinosis is associated with an increase in peripheral NMDA-type 1 glutamate receptors, which morphologically are accompanied by an increase in their ligand glutamate. The ligand-receptor co-existence in nerve endings confined to the painful tendons strongly supports the idea of a peripheral neuronal NMDAR1 activation involved in nociception.

The elevated occurrence of the ligand glutamate and/or the NMDAR1 in tendinopathic patients may be seen as a response to repetitive mechanical stimulus. Bone glutamate signalling has been reported to be activated in response to mechanical loading [Skerry 1999]. Interestingly, in our study the individual correlation analysis demonstrated that there was no correlation seen in the occurrence of glutamate and NMDAR1 neither in the controls nor in the tendinosis samples. Moreover, co-localization of NMDAR1/glutamate was identified in some areas of the tendon, while in other areas immunoreaction to NMDAR1 or glutamate was seen without co-localization. This may reflect that the regulation of glutamate and NMDAR1, respectively, occurs independently. The observation is strengthened by the fact that glutamate binds not only to NMDAR1, but also to its receptors AMPA, kainate and metabotrobic receptors [Scannevin and Huganir 2000, Nedergaard et al 2002]. Thus, tendinosis patients with elevated levels of glutamate in the tendon can be pain free [Alfredson and Lorentzon 2003]. This may be due to the observation that NMDAR1 up-regulation and also activation occur independently of glutamate presence. In this study however, double staining revealed neuronal co-localization of NMDAR1 and glutamate in painful tendinosis, which seems to reflect their interaction, presumably by NMDAR1 activation.

Our finding of increased NMDAR1 occurrence in patients with painful tendinosis is also supported by a recent study on rat supraspinatus tendon overuse likewise demonstrating NMDAR1 upregulation [Molloy et al 2006]. Moreover, the observation of an approximately

10-fold elevation of glutamate levels in the painful tendons is corroborated by microdialysis of tendinopathic patients showing approximately 5-7 times increased glutamate levels [Alfredson et al 2001, Alfredson and Lorentzon 2002]. Our study provides, additonally, morphological information on the specific neuronal, vascular and cellular localizations for this peripheral, up-regulated glutamate-NMDAR1 system.

Since NMDAR1 was not co-localized with regenerating (GAP-43) nerves, we speculate that neuronal up-regulated NMDAR1 may be involved mainly in autocrine/paracrine pain signalling. This hypothesis is supported by the finding that the controls exhibited no neuronal glutamate or neuronal glutamate/NMDAR1 co-existence. The neuronal glutamate occurrence observed exclusively in painful tendons could be explained by an interaction with the neuropeptide substance P, which has also been found to be up-regulated in tendinopathy [Ackermann et al 2001, Schubert et al 2005, Lian et al 2006], and known to unblock the NMDAR1 [Besson 1999] thereby allowing glutamate binding. Glutamate pain signalling in Aδ- and C- nerve fibers was significantly enhanced after injections of substance P in rat skin [Zhang 2006].

Although glutamate-injections may lead to pain sensitization [Cairns et al 2003, Cairns et al 2006], elevated levels of free glutamate alone do not reflect pain in tendinopathy, but rather a response to mechanical loading [Alfredson and Lorentzon 2003]. Peripheral neuronal up-regulation of the glutamate receptor NMDAR1, however, in experimental adjuvant inflammation causes significantly increased nociception [Du et al 2003]. Thus, our observation of neuronal co-existence of upregulated NMDAR1 and its ligand glutamate in painful tendinosis strongly suggests a role in pain regulation, which has also been proposed in other musculo-skeletal tissues [Besson 1999, Cairns et al 2003, Cairns et al 2006].

The up-regulated NMDAR1 in the vascular network localized in the loose connective tissue, which supplies the proper tendon, may be a potent regulator of blood flow and vascular

inflammation. An increased blood flow has been demonstrated in the knee joint on activation of the NMDAR1 [Lawand et al 2004].

The extensive glutamate-NMDAR1 system found in vessel walls of the pathologic tendon proper could also reflect a regulatory role in blood supply and additionally in neo-vascularization, since the tendon proper in healthy conditions is practically devoid of blood vessels and nerve fibers. The ingrowth of NMDAR1 positive vessels in the tendon proper was found in 50 % of the painful tendons, suggesting that these patients exhibited more advanced tendinosis, considering that angiogenesis and granulation tissue formation occur in late stages of tendinosis [Lian et al 2006, Aspenberg 2007]. In fact, increased glutamate-NMDAR1 presence was observed in the granulation tissue within the tendon proper of the tendinosis group.

Glutamate-NMDAR1 co-localization on transformed tenocytes in the tendon proper was also detected by double staining. Morphologic transformation of the cells is characterized by rounding and enlargement of both the nucleus and the cell body. This type of tenocyte transformation was observed in all tendinopathic samples exhibiting NMDAR1 positive tenocytes. Notably, increased glutamate/NMDAR1 signalling is involved in other cell type transformations such as osteoblast differentiation and also in pathways leading to cell apoptosis [Hinoi et al 2003] [Villmann et al 2007].

It must be emphasized that this cross-sectional study offered only morphological and quantitative analyses on NMDAR1 and glutamate density in painful tendinosis and control specimens without direct evidence to secure a conclusion on causal relationships. Future studies should use interventional approaches, e.g. local NMDAR1-receptor antagonists, to examine the causal role of NMDAR1 in painful tendinosis.

In conclusion, we demonstrate an up-regulation of NMDAR1 (9-fold) and glutamate (10-fold), co-existing on vascular, cellular and neuronal structures in patients with painful

tendinosis. The up-regulated NMDAR1/glutamate system may represent hyper-excitation of the cells – leading to cell proliferative effects observed as angiogenesis, tenocyte transformation, and nerve sprouting. Moreover, the neuronal co-existence of glutamate and NMDAR1 observed in painful tendinosis, but not seen in any of the controls, strongly suggests a role in pain signalling.

Perspectives

Tendinopathy entails pain and tissue proliferative alterations including cell-, nerve- and vessel ingrowth [Maffulli et al 2004, Aspenberg 2007]. Pain and proliferation are suggested to be modulated via nerve transmitters, including substance P (SP) and glutamate, both upregulated in tendinopathy [Alfredson et al 2001, Schubert et al 2005, Lian et al 2006]. Prolonged SP and glutamate stimulation may lead to excessive tissue proliferation [Lian et al 2006].

Glutamate up-regulation alone is not correlated with tendinopathic pain [Alfredsson and Lorentzon 2003] and its spatial distribution in tendinopathic tissue has been unknown. However its receptor NMDARl, activated by SP, is crucial for pain regulation [Besson 1999]. Whether NMDAR1 is up- or downregulated in tendinopathy has not been explored.

This study demonstrates that patients with tendinopathy exhibit: 1) a 9-fold up-regulation of NMDAR1, 2) 10-fold increase in glutamate 3) co-localization of NMDAR1/glutamate on nerve fibers – not in controls, 4) NMDAR1/glutamate co-existence on morphologically altered tenocytes and blood vessels.

Further studies are focused on peripheral NMDA receptor modulation in tendinopathic patients. Existing drugs e.g. NMDA receptor antagonists are tested clinically. Whether peripheral neuronal ligand-receptor pathways could be utilized to develop targeted therapies to mitigate the symptoms in tendinopathic patients warrants investigations.

Acknowledgements

This study was supported by the regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet (project nr. SLL20050313) and the Swedish National Centre for Sports Research. The Oslo Sports Trauma Research Center has been established through grants from the Norwegian Eastern Health Corporate, the Royal Norwegian Ministry of Culture, the Norwegian Olympic Committee & Confederation of Sport, and Norsk Tipping AS. Special thanks to our colleague Aglaia Maleka MD for helping us with the double blinded analysis.

References

Ackermann PW, Ahmed M, Kreicbergs A. Early nerve regeneration after Achilles tendon rupture: a prerequisite for healing? A study in the rat. J Orthop Res. 2002;20:849-856

Ackermann PW, Li J, Lundeberg T, Kreicbergs A. Neuronal plasticity in relation to nociception and healing of rat Achilles tendon. J OrthopRes. 2003;21:432-441

Ackermann PW, Renström P. Sensory neuropeptides in Achilles tendinosis: transactions of the Third Biennial Congress of the International Society of Arthroscopy, Knee Surgery and Orthopaedic Sports Medicine. Arthroscopy. 2001;17(suppl):516

Alfredson H, Forsgren S, Thorsen K, Lorentzon R. In vivo microdialysis and immunohistochemical analyses of tendon tissue demonstrated high amounts of free glutamate and glutamate NMDAR1 receptors, but no signs of inflammation, in Jumper's knee. J Orthop Res. 2001 Sep;19(5):881-6

Alfredson H, Lorentzon R. Chronic tendon pain: no signs of chemical inflammation but high concentrations of the neurotransmitter glutamate. Implications for treatment? Curr Drug Targets. 2002 Feb;3(1):43-54

Alfredson H, Lorentzon R. Intratendinous glutamate levels and eccentric training in chronic Achilles tendinosis: a prospective study using microdialysis technique. Knee Surg Sports Traumatol Arthrosc. 2003 May;11(3):196-9

Aspenberg P. Is inflammation harmless to loaded tendons? J Appl Physiol 102: 3–4, 2007

Besson JM. The neurobiology of pain. Lancet. 1999 May 8;353(9164):1610-5

Billig I, Card JP, Yates BJ. Neurochemical phenotypes of MRF neurons influencing diaphragm and rectus abdominis activity. J Appl Physiol. 2003 Jan;94(1):390.

Blazina ME, Kerlan RK, Jobe FW, Carter VS, Carlson GJ. Jumper's knee. Orthop Clin North Am. 1973;4:665-673

Cairns BE, Svensson P, Wang K, Castrillon E, Hupfeld S, Sessle BJ, Arendt-Nielsen L. Ketamine attenuates glutamate-induced mechanical sensitization of the masseter muscle in human males. Exp Brain Res. 2006 Mar;169(4):467-72.

Cairns BE, Svensson P, Wang K, Hupfeld S, Graven-Nielsen T, Sessle BJ, Berde CB, Arendt-Nielsen L. Activation of peripheral NMDA receptors contributes to human pain and rat afferent discharges evoked by injection of glutamate into the masseter muscle. J Neurophysiol. 2003 Oct;90(4):2098-105

Danbolt NC. Glutamate uptake. Progress in Neurobiology 65 (2001) 1–105

Du J, Zhou S, Coggeshall RE, Carlton SM. N-methyl-D-aspartate-induced excitation and sensitization of normal and inflamed nociceptors. Neuroscience. 2003;118(2):547-62

Hinoi E, Fujimori S, Yoneda Y. Modulation of cellular differentiation by N-methyl Daspartate receptors in osteoblasts. FASEB J. 2003 Aug;17(11):1532-4

Lawand NB, Reddig WJ, Cashin AE, Westlund KN, Willis WD. NMDA receptors and associated signaling pathways: a role in knee joint blood flow regulation. Eur J Pharmacol. 2004 Sep 19;499(1-2):155-61

Lian Ø, Dahl J, Ackermann PW, Frihagen F, Engebretsen L, Bahr R. Pronociceptive and antinociceptive neuromediators in patellar tendinopathy. Am J Sports Med. 2006 Nov;34(11):1801-8

Madden DR. The structure and function of glutamate receptor ion channels. Nat Rev Neurosci. 2002 Feb;3(2):91-101

Maffulli N, Testa V, Capasso G, Ewen SW, Sullo A, Benazzo F, King JB. Similar histopathological picture in males with Achilles and patellar tendinopathy. Med Sci Sports Exerc. 2004 Sep;36(9):1470-5.

Marc RE, Liu WL, Kalloniatis M, Raiguel SF, van Haesendonck E. Patterns of glutamate immunoreactivity in the goldfish retina. J Neurosci. 1990 Dec;10(12):4006-34

Molloy TJ, Kemp MW, Wang Y, Murrell GA. Microarray analysis of the tendinopathic rat supraspinatus tendon: glutamate signaling and its potential role in tendon degeneration. J App Phys. 2006 Dec;101(6):1702-9

Nedergaard M, Takano T, Hansen AJ. Beyond the role of glutamate as a neurotransmitter. Nat Rev Neurosci. 2002 Sep;3(9):748-55

Roels J, Martens M, Mulier JC, Burssens A. Patellar tendinitis (jumper's knee). Am J Sports Med. 1978 Nov-Dec;6(6):362-8

Scannevin RH, Huganir RL. Postsynaptic organization and regulation of excitatory synapses. Nat Rev Neurosci. 2000 Nov;1(2):133-41

Schubert TE, Weidler C, Lerch K, Hofstädter F, Straub RH. Achilles tendinosis is associated with sprouting of substance P positive nerve fibres. Ann Rheum Dis. 2005 Jul;64(7):1083- 6

Siegel SJ, Brose N, Janssen WG, Gasic GP, Jahn R, Heinemann SF, Morrison JH. Regional, cellular, and ultrastructural distribution of N-methyl-D-aspartate receptor subunit 1 in monkey hippocampus. Proc Natl Acad Sci U S A. 1994 Jan 18;91(2):564-8

Skerry TM. Identification of novel signaling pathways during functional adaptation of the skeleton to mechanical loading: the role of glutamate as a paracrine signaling agent in the skeleton. J Bone Miner Metab 17: 66-70, 1999

Villmann C, Becker CM. On the hypes and falls in neuroprotection: targeting the NMDA receptor. Neuroscientist. 2007 Dec;13(6):594-615

Visentini PJ, Khan KM, Cook JL, Kiss ZS, Harcourt PR, Wark JD. The VISA score: an index of severity of symptoms in patients with jumper's knee (patellar tendinosis). Victorian Institute of Sport Tendon Study Group. J Sci Med Sport. 1998 Jan;1(1):22-8

Zhang Q, Zhao Y, Guo Y, Cao DY, Tian YL, Yao FR, Wang HS. Electrophysiological evidence for the interaction of substance P and glutamate on Adelta and C afferent fibre activity in rat hairy skin. Clin Exp Pharmacol Physiol. 2006 Dec;33(12):1128-33

Table legends

Table 1. A semi-quantitative tendon score based on tenocyte morphology.

Table 2. Distribution of NMDAR1 positive/negative tenocytes in relation to tencocyte morphology.

Figure legends

Figure 1A-C. Quantitative assessment of the mean vessel area fraction (%) immunoreactive to NMDAR1 (A), glutamate (B) and the number of glutamate positive tenocytes (C) in the tendinopathic group, as compared to healthy controls (* $p<0.05$).

Figure 2A-D. Immunofluorescence double staining micrographs of longitudinal sections through the patellar tendon of painful tendinopathy (A,B) and healthy control (C,D) after incubation with antisera

for a general nerve marker (PGP9.5) (A,C, thin arrows) and for NMDAR1 (B,D). Thick arrows denote neuronal NMDAR1 (co-localized with PGP9.5) whereas arrow heads denote non-neuronal NMDAR1. In all tendinopathic samples vascular NMDAR1 immunostaining was found to be increased in the loose connective tissue surrounding the tendon proper (v = vessel, Bar = 25µm)

Figure 3A-D. Immunofluorescence double staining micrographs of longitudinal sections through tendinopathic patellar tendons focusing on the tendon proper, after incubation with antisera for PGP9.5 (A,C, thin arrows), NMDAR1 (B) and glutamate (D). Thick arrows denote neuronal NMDAR1 and within the tendon proper arrow-heads denote neuronal glutamate, which is totally absent in the controls. (Bar = 25µm).

Figure 4A-D. Immunofluorescence double staining micrographs of longitudinal sections through the patellar tendon focusing on the loose connective tissue of tendinopathy (A,B) and healthy control (C,D) after incubation with antisera for PGP9.5 (A,C) and glutamate (B,D). Thin arrows denote PGP9.5 positive fibers and arrow-heads denote neuronal glutamate. Neuronal localization of glutamate was not found in the controls. (Bar = 25µm).

Figure 5A-B. Immunohistochemical DAB micrographs of longitudinal sections through the patellar tendon of painful tendinopathy (A) and control (B) stained for glutamate. An increased number of glutamate positive tenocytes (arrows), exhibiting signs of tenocyte transformation, was observed in the tendon proper of the tendinopathic samples. (Bar = 25µm)

Figure 6A-B. Immunofluorescence double staining micrographs of longitudinal sections through tendinopathic patellar tendons focusing on the granulation tissue after incubation with antisera for NMDAR1 (A, arrows) and glutamate (B, arrow heads). The NMDAR1-glutamate co-localization on nerve structures was clearly observed in tendinopathic samples, but not in controls (not shown). (Bar = 25µm).