

The role of glutamate in rotator cuff tendinopathy

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Abstract

The role of glutamate in rotator cuff tendinopathy

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Thesis questions:

1. Is the glutaminergic system altered in rotator cuff tendinopathy?
2. Is the glutaminergic system altered by common treatments?
3. Are glutaminergic changes related to pain symptoms?
4. What are the effects of glutamate and glutamate receptor modulation on tendon derived cells?

Summary answers:

1. The glutaminergic system is altered in rotator cuff tendinopathy
2. Changes within this system are seen after common treatments
3. Specific glutaminergic changes are associated with the resolution of pain following shoulder surgery but do not predict the severity of pain symptoms
4. Glutamate has significant effects on tendon derived cells

What is known: It is known that extracellular glutamate concentrations are increased in both Achilles and patellar tendinopathy. It has also been previously shown that the glutamate receptors NMDAR1 and mGluR5 are upregulated in patellar tendinopathy.

What this thesis adds: This thesis has shown for the first time that glutamate and NMDAR1 are increased in rotator cuff tendinopathy. Increases in cell proliferation, vascularity and HIF1 α are seen after surgical rotator cuff repair and these features are not seen after glucocorticoid injection. There are significant differences between painful and pain-free rotator cuff tendons in terms of glutamate receptor expression (KA1, mGluR7 and mGluR2) and inflammatory cell numbers (CD45 and CD206). Exposure to 1.875mM glutamate for 72 hours results in reduced cell viability, decreased collagen (COL1A1 and COL3A1) and increased aggrecan gene expression; NMDAR antagonism with MK-801 attenuates the deleterious effect on cell viability but had no effect on the changes in matrix gene expression.

Bias, confounding and other reasons for caution: The observational histological work was limited by the control tissue. Some control tissue was not age matched, while some of the pain-free control tendons were post-surgical intervention. Confounding factors include tendon structure, length of symptoms and previous treatments. Caution must be applied when discussing the *in vivo* implications of the *in vitro* work.

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Abbreviations

ADP	Adenosine Diphosphate
AMN082	N,N'-dibenzhydrylethane-1,2-diamine dihydrochloride
AMPA	α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor
ATP	Adenosine Triphosphate
C-A	Coracoacromial
CAL	Coracoacromial Ligament
CI	Confidence Interval
DAPI	4',6-diamidino-2-phenylindole
DMEM-F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
FBS	Foetal Bovine Serum
FTT	Full Thickness Tear
GAP43	Growth Associated Protein 43
GCI	Glucocorticoid Injection
GFP	Green Fluorescent Protein
GHJ	Glenohumeral Joint
HIF-1 α	Hypoxia Inducible Factor 1 α
HTM	High Threshold Mechanoreceptor
IQR	Inter-Quartile Range
iGluR	ionotropic glutamate receptor
KAR	Kainate receptor
LHBT	Long Head of Biceps Tendon
LDH	Lactate Dehydrogenase
LTM	Low Threshold Mechanoreceptor
mGluR	metabotropic glutamate receptor
MK-801	Dizocilpine
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NGF	Nerve Growth Factor
NMDA	N-Methyl-D-Aspartate
OSS	Oxford Shoulder Score

Abbreviations continued

PCR	Polymerase Chain Reaction
PGP9.5	Protein Gene Related Product 9.5
PROMS	Patient Reported Outcome Measures
PTT	Partial Thickness Tear
P53	Tumour protein p53
P75	Low-affinity Nerve Growth Factor receptor
qRT-PCR	Quantitative Reverse Transcription PCR
RCR	Rotator Cuff Repair
RCT	Rotator Cuff Tendinopathy
SAB	Subacromial bursa
SAD	Subacromial decompression
SD	Standard Deviation
SSCN	Suprascapular nerve
TBP	TATA Binding Protein
TrkA	Tyrosine Kinase A
VAS	Visual Analogue Scale
VEGF	Vascular Endothelial Growth Factor
WDR	Wide Dynamic Range

Chapter 1 Introduction and literature review

1.1 Background and burden

Musculoskeletal pain is a very common and costly health care problem affecting a third of the population^[1]. Shoulder pain is the 3rd most common cause of musculoskeletal pain in the community and approximately 1% of adults consult a general practitioner with new shoulder pain annually^[1]. The majority of shoulder pain is associated with an abnormality of the rotator cuff tendons^[2]. Tendinopathy is a generic term used to describe pathology in a tendon and the term 'rotator cuff tendinopathy' (RCT) may be used to describe pathology specifically related to the rotator cuff tendons. 'Rotator cuff tendinopathy' (RCT) covers a whole spectrum of disorders from an intact tendon without tearing to massive rotator cuff tendon tears^[3].

The cost of managing shoulder pain in the United States is estimated to be around \$3billion^[4], accounting for over 4 million medical appointments per annum^[5]. Operative treatment is generally reserved for when conservative non-operative treatment has failed^[2]. However recent trends in the United Kingdom demonstrate a rapid increase in the rate at which surgery is being undertaken for RCT^[5]. This is concerning given the lack of high quality evidence to demonstrate the effectiveness of these surgical treatments above placebo or physiotherapy^[6]. In this context of an increasingly ageing population and the increasing disease burden of RCT, it is vital to better understand the disease process and how it may be affected by common treatments.

1.2 The Normal Shoulder

The scapula, the clavicle and the humerus are the bones that make up the human shoulder. The shoulder is linked to the axial skeleton by the clavicle, whose proximal end is linked to the sternum by the sternoclavicular joint and whose distal end is anchored to the scapula by the acromioclavicular joint. The movement of the shoulder joint is largely dependent on the glenohumeral and scapulothoracic articulations. The shoulder is an extremely mobile joint and this fact in combination with the mobility of the elbow means that the hand can be positioned anywhere within the visual working space. The glenohumeral joint is a synovial joint between the glenoid fossa of the scapula and the head of the humerus. The shallowness of the glenohumeral joint means both capsuloligamentous structures and the musculotendinous rotator cuff is extremely important in joint stability. The rotator cuff tendons have the important role of transmitting force between muscle and bone in order to enable shoulder movement and to prevent the humeral head from subluxing superiorly on abduction.

1.2.1 Anatomy of the Rotator Cuff

The rotator cuff is a key musculotendinous unit around the shoulder joint and is made up of supraspinatus, infraspinatus, teres minor and subscapularis. The anatomy of the rotator cuff is summarised in Table 1.1. Not only is the rotator cuff important in contributing to movement, but as previously mentioned it has a key role in stabilising the glenohumeral joint. The subacromial bursa (SAB) is located between the rotator cuff and the bony under surface of the acromion; it is of particular importance in terms of providing minimal friction during shoulder abduction. 'Bursitis' (an inflammation of the subacromial bursa) has been recurrently proposed as a cause of shoulder symptoms.

Table 1.1 Summary of rotator cuff structure and function

Rotator cuff muscle	Origin	Insertion	Function	Innervation
Supraspinatus	Supraspinous fossa	Superior and middle facets of greater tuberosity	Abducts arm	Suprascapular nerve
Infraspinatus	Infraspinous fossa	Posterior facet of greater tuberosity	External rotates arm	Suprascapular nerve
Teres Minor	Middle of lateral border of scapula	Inferior facet of greater tuberosity	Externally rotates arm	Axillary nerve
Subscapularis	Subscapular fossa	Lesser tuberosity	Internal rotates arm	Upper and lower subscapular nerves

The rotator cuff insertion onto the humeral tuberosities is broad, continuous, multilayered and interwoven^[7]. The supraspinatus and infraspinatus tendons fuse approximately 1.5cm proximal to their insertions.

1.2.2 Microstructure of the Rotator Cuff Tendons

The rotator cuff tendons consist of three general zones: the musculotendinous junction, the main tendon substance and the osseotendinous junction (the so called 'enthesis'). The main tendon substance consists of collagen bundles, cells and ground substance^[8 9]. Tropocollagen molecules spontaneously form collagen fibrils, collagen fibrils then aggregate to form fibres, while groups of fibres form fascicles, and fascicles are surrounded by a layer of loose connective tissue called the 'epitenon' as is demonstrated in Figure 1-1.

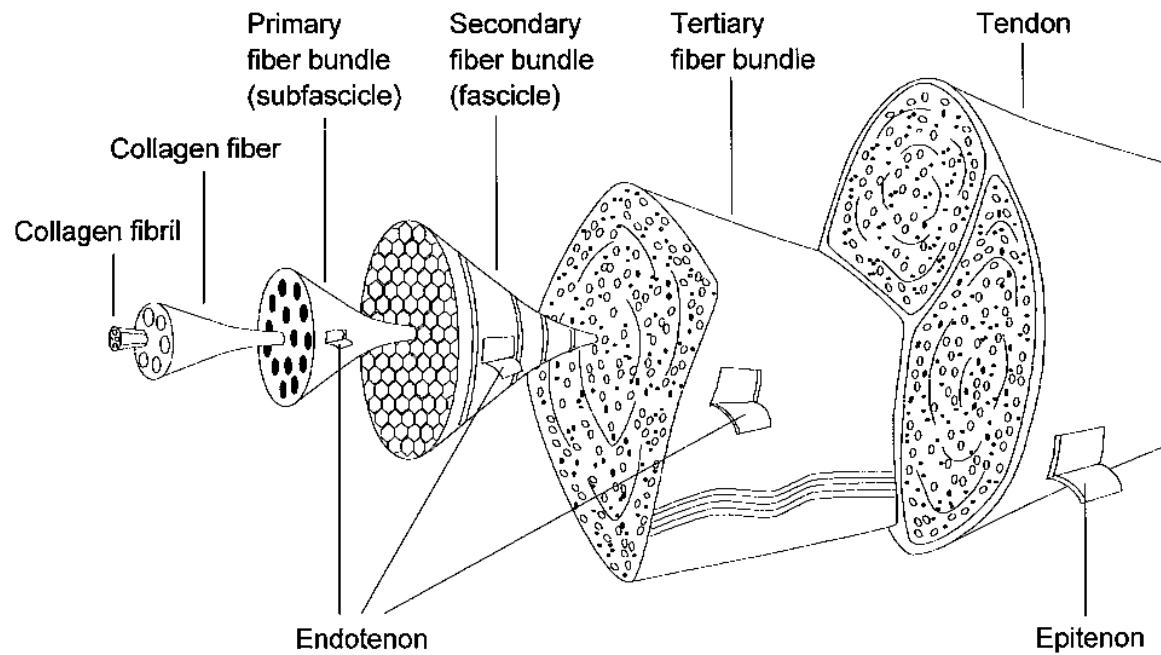


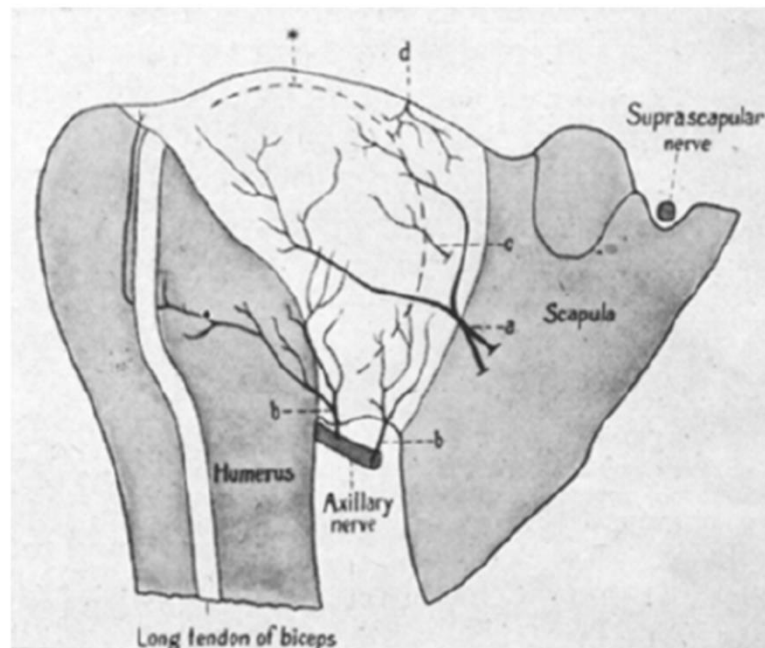
Figure 1-1 The structure of collagen (reproduced with permission^[9])

The vast majority of the collagen in the main tendon substance is type I and this provides tendon with its tensile strength. Under light microscopy normal tendon consists of well-defined parallel and slightly wavy collagen bundles. The ground substance is rich in proteoglycans and highly viscous. The anatomy of the supraspinatus tendon's insertion is of key relevance in terms of its varied extracellular matrix composition^[7] and has been categorised into four transition zones at the so called 'enthesis'. The first zone is proper tendon made up of largely type I collagen and small amounts of decorin (a small leucine rich proteoglycan). The second zone is fibrocartilage and consists of largely types II and III collagen, with small amounts of types I, IX and X collagen. The third zone is mineralised fibrocartilage and consists of type II collagen, with significant amounts of type X collagen and aggrecan (a proteoglycan). The fourth zone is bone and is largely type I collagen with a high mineral content. This effective bone-tendon attachment is achieved through a functional grading in mineral content and collagen fibre orientation. The supraspinatus tendon enthesis is a highly specialised

heterogeneous structure that is subjected to both tensile and compressive forces; this appears important in both the development of and propagation of cuff tears. The resident fibroblasts are frequently referred to as 'tenocytes' and these cells are crucial in maintaining the structural integrity of the extracellular matrix (ECM). In normal tendon there is a fairly even and sparse distribution of these tenocytes with thin wavy nuclei between the collagen bundles. The subacromial bursa is lined by a synovial membrane containing both type A and type B synoviocytes^[10]. Type A synoviocytes are derived from blood-borne mononuclear cells and are typically thought of as resident macrophages. Type B synoviocytes have a greater proliferative capacity and are capable of secreted ECM components such as collagen and fibronectin, unlike type A synoviocytes.

1.2.3 Neural anatomy

The nerves that contribute to the innervation of the anterior shoulder joint are the subscapular (C5/6), axillary (C5/6) and the lateral pectoral (C5/6) (Figure 1-2)^[11-13].

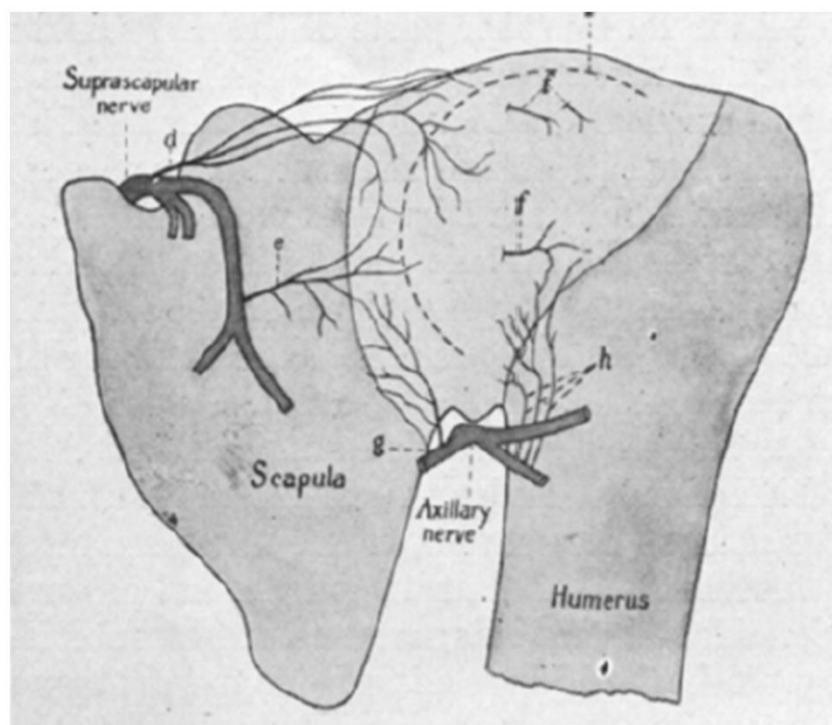
Figure 1-2 - The neural anatomy of the shoulder joint (anterior view)(reproduced with permission^[11])

The subscapular nerves arise high from the posterior cord of the brachial plexus and supply a small portion of the anterior joint. The axillary nerve is the last nerve of the posterior cord of the brachial plexus, before the latter becomes the radial nerve. As the axillary nerve courses across the subscapularis muscle, it releases its first articular branch as it runs across the antero-inferior capsule. The axillary nerve divides into two main branches at the lower aspect of subscapularis, the medial and lateral branches; the medial branch supplies the scapular side of the antero-inferior capsule, while the lateral branch supplies the humeral side of the antero-inferior capsule. The muscular branch, which innervates teres minor also issues a small articular branch at the level of the insertion of the long head of triceps. The lateral pectoral nerve arises from the lateral cord of the brachial plexus, passes superficial to the axillary vessels and pierces the

clavipectoral fascia to reach the deep surface of pectoralis major. A small articular branch arises just before it pierces the fascia, this gives off branches to the coracoacromial (C-A) and coracohumeral (C-H) ligaments on its way laterally. Between these ligaments it then divides into two main branches; one passes beneath the C-A ligament to supply the subacromial bursa, the other passes across the C-A ligament to supply the anterior portion of the acromioclavicular joint.

The nerves that contribute to the innervation of the posterior joint are the suprascapular nerve (SSCN) (C5/6) and axillary nerve (C5/6) (Figure 1-3).

Figure 1-3 - The neural anatomy of the shoulder (posterior view)(reproduced with permission^[11])



The SSCN arises from the upper trunk of the brachial plexus. A large superior articular branch separates from the main nerve at an average of 4.5cm proximal to the transverse scapular ligament^[13], together these nerves pass underneath the transverse scapular ligament and enter the suprascapular notch. After entering the notch, the SSCN turns

laterally and releases a small branch to the coracoclavicular ligaments. The main articular branch then advances laterally between the coracoid and supraspinatus, it divides into two terminal branches at this point; one descends to innervate the C-H ligament and the adjacent capsule, the other splits into several small branches which innervate the subacromial bursa and the posterior portion of the acromioclavicular joint capsule. The main SSCN passes into the suprascapular fossa where it release a large muscular branch supplying supraspinatus; at the level of the scapular spine a large inferior articular branch separates and travels obliquely to the posterior capsule. This inferior articular branch releases several branches, which deviate upward and downward to terminate where the tendon of infraspinatus merges with the posterior capsule. The suprascapular nerve terminates by supplying infraspinatus. The contribution of the axillary nerve has been previously described. The sympathetic innervation of the shoulder joint is from the cervical ganglia (superior, middle and lower) via the grey rami communicantes.

1.2.4 Vascular Anatomy

Historically the vascular supply to the rotator cuff has been a subject of some controversy. Codman was the first to describe the so called 'watershed' area within the rotator cuff tendons located in the distal 10mm near the insertion into the great tuberosity^[14 15]. Rothman and Clarke^[7] described a consistent bloody supply to the rotator cuff from the suprascapular, anterior circumflex and posterior circumflex humeral arteries in all cases. The contributions of the thoracoacromial, supra-humeral and subscapular arteries were more variable. Chansky and Ianotti^[16] demonstrated that major arterial supply to the rotator cuff is from the ascending branch of the anterior circumflex humeral artery, the acromial branch of the thoracoacromial artery, as well as the suprascapular and posterior humeral circumflex arteries; the anterior humeral

circumflex artery and the suprascapular artery supplies the anterior portion of the rotator cuff, while the posterior humeral circumflex artery supplies the posterior portion.

1.2.5 Biomechanics

Human evolution has seen significant changes in both shoulder morphology and rotator cuff function^[17]. The glenoid has shifted from a vertical orientation towards a far more horizontal one, a significant factor in this may be the greater throwing power enabled by the more horizontal glenoid^[18-19]. Consequently, a key function of the rotator cuff appears to be stabilizing the humeral head in the glenoid during abduction, and this is achieved by the supraspinatus counteracting the upwards pull of the middle deltoid^[20]. The load on supraspinatus during abduction is dependent on the 'critical shoulder angle'^[21], an angle representing the laterality of the lateral acromion relative to the glenoid, and patients with larger critical shoulder angles are at greater risk of developing degenerative rotator cuff tears^[21].

1.3 Pathology of Rotator Cuff Tendinopathy

Tendon homeostasis and its failure in degenerative disease is a complex process which involves the interplay between a variety of cells, matrix components, enzymes, cytokines, growth factors and proteins. Supraspinatus tendon tears are the most common type of rotator cuff tear and they are most frequently found near to the tendon's bony insertion; as supraspinatus tendon tears become larger they are more likely to involve infraspinatus due to their common insertion.

Historically a scientific understanding of tendinopathy has relied on work carried out on cadavers or biopsy specimens removed at the time of tendon surgery. Early pioneering work by Neer^[22] and Codman^[14] on RCT utilized open surgical biopsy

techniques to more fully understand this disorder. Nevertheless, despite advances, the study of cadaveric and surgical biopsy specimens each has limitations. Cadaveric tissue lacks a clinical history to accurately phenotype the individual from whom the tissue was obtained. Furthermore, with this method there is the potential for tissue changes to occur *post mortem* before laboratory analysis has occurred. A serious limitation with the use of surgical tissue is that specimens represent a relatively end stage of the disease process, in which mechanical failure of the tendon has already occurred in the way of a full thickness tendon tear. Due to the limitations of these techniques, novel, minimally invasive biopsy techniques have been developed to sample tendon at multiple stages throughout the evolution of a tendinopathy.

These minimally invasive biopsy techniques are a significant reason for recent progress in our understanding of tendinopathy and have been developed, and well described, in both Achilles and patellar tendinopathy^[23-29]. In contrast, for the shoulder, sampling of intact, tendinopathic supraspinatus tendon has been limited to very few studies. In an early study by Tillander et al^[30] large 4x4mm biopsies were taken from the mid portion of the supraspinatus tendon during subacromial decompression surgery. Unfortunately, the location of these biopsies does not represent the common site of failure in the supraspinatus and such large biopsies are not ethically feasible to take in studies today. Tuoheti et al^[31] obtained tissue from the insertion region of the supraspinatus tendon. However, the study was limited to five tissue samples and was carried out during open subacromial decompression surgery, an operation rarely performed now due to the widespread use of arthroscopic techniques.

Furthermore, control tissue used in RCT studies has also been limited to the subscapularis or cadaveric tendon. Subscapularis tendon is different in its anatomy, function and pathology relative to supraspinatus and thus does not represent a faithful

control.^[30 32-37]. Finally, similar to the work by Tilander et al with open biopsies, efforts to sample tissue from the supraspinatus tendon for controls has only been undertaken using large biopsies that are no longer ethically justifiable^[30]. As a direct result of the difficulty in accessing well phenotyped tissue samples from the spectrum of rotator cuff tendinopathy, there is a distinct lack of tissue studies investigating earlier stages of the condition. Regrettably, this is precisely the stage of disease when patients present with symptomatic shoulders with intact or only partially torn tendons.

The following sections summarise the findings of a systematic review that I carried out as part of the background work for my thesis^[38]. This systematic review used the PRISMA-Statement (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) as a guide in the development of the study protocol and the report; the inclusion criteria and methods of analysis were specified in advance and are documented in the full review^[38].

1.3.1 Histological changes

The histology of RCT reveals a characteristic pattern of changes to the cells, the collagen and the overall tendon structure. The rounding of tendon cell nuclei has been well described by a number of studies^[39-41]. Generally increased overall cellularity and a less homogeneous cellular distribution are seen in RCT^[39 42 43]. An increased number of inflammatory cells have been demonstrated in both the tendon proper and the adjacent bursal tissue^[36 40 44 45]. There is increased matrix disorganization and increased overall tendon degeneration present in RCT^[41 42]. The collagen shows disorientation, disorganisation and fibre separation^[41]. Fibrocartilagenous change and fatty infiltration have been seen at edges of rotator cuff tears in multiple studies^[46-49]. A wide variety of morphological terms have been used to describe the histological appearance of RCT including ‘fibroid degeneration’, ‘calcification’, ‘chondroid metaplasia/change’,

‘mucoid/myxoid degeneration’, ‘hyaline degeneration’ and ‘hypoxic degeneration’^[38]. These different pathologies may coexist, and their prevalence may vary depending on factors such as disease stage and the nature of a patient’s disease aetiology. The topic of vascularity in RCT is one of some contention^[15]. Although some studies have demonstrated focal areas of increased vascularity^[49 50], others have shown no change in overall vascularity^[51]; this points to the importance of objectively investigating the vascularity of normal and diseased rotator cuff tendons. Of note studies which have described increased vascularity have generally relied upon rather subjective measurement techniques which do not analyse the whole of a tendon specimen objectively and systematically.

The change to vascularity in RCT is a subject of significant controversy. A systematic review by Hegedus et al^[15] revealed highly conflicting results. Of note studies which have described increased vascularity have generally relied upon rather subjective measurement techniques which do not analyse the whole of a tendon specimen objectively and systematically^[42 55]. The presence of a critical zone in terms of reduced vascularity close to the insertion of supraspinatus has been repeatedly demonstrated by histological studies^[52 53]. Reduced vascularity has been demonstrated at the edges of rotator cuff tears^[51] and vascularity has been shown to decrease more in relatively end stage disease^[34]. Reduced vascularity in disease versus control has been robustly supported by some *in vivo* work^[38]. Innervation appears reduced in rotator cuff tears when compared to control tendon; studies by Xu et al and Tomita et al have both shown reduced nerve density in tears versus control^[37 54]. Small nerves are difficult to reliably identify in small tissue sections and many so called ‘nerve markers’ are expressed by other cells types such as endothelial cells and synoviocytes^[55]. It is arguably more

reliable to use larger tendon specimens which can generally only be obtained from cadavers.

1.3.2 Molecular changes

Broadly the molecular changes in the matrix, matrix enzymes, cytokines and growth factors reflect the recognized histological changes in terms of showing a general state of failing homeostasis involving matrix catabolism and increased inflammatory mediators^[56-57]. Increases in both type II and type III collagen have been demonstrated in RCT^[46-58-60], indicative of the fibrous type scar formation present in disease. The overall collagen content is reduced^[59-60] and there is an increased deposition of glycosaminoglycans (GAGs) such as chondroitin sulphate, dermatan sulphate and hyaluronan^[32-59]. Broadly catabolic matrix enzymes such as certain matrix metalloproteinases (MMPs) are increased and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) are decreased^[38]. The pro-inflammatory enzyme cyclooxygenase-2 (COX-2) has been shown to be increased^[44-61], while several pro-inflammatory cytokines such as Interleukin-1 β and Interleukin-6 have been shown to be increased in RCT^[10-44-61-62]. Markers of apoptosis, such as Caspase 3 and 8, are increased in RCT^[63-64], while substances associated with increased cellular stress such as the heat shock proteins 27 and 70 are elevated^[63-64].

1.4 Epidemiology of Rotator Cuff Tendinopathy

Age is the strongest risk factor for RCT with shoulder pain becoming the most common musculoskeletal problem in patients aged over 65^[65-66]. The incidence of rotator cuff tears increases dramatically from just over 10% in the sixth decade of life to 50% in

the ninth decade^[67]. Other risk factors for rotator cuff tears include smoking, hypercholesterolemia, and a family history^[68]. Baumgarten et al^[69] found a history of smoking to significantly increase the risk of developing a rotator cuff tear. Abboud and Kim^[70] found that patients with rotator cuff tears were more likely to have hypercholesterolemia than those in a control group. The work of Harvie et al and Gwilym et al^[71] has demonstrated a significant genetic component in both the development and progression of rotator cuff tears; siblings of patients with a previously diagnosed cuff tear were almost 3 times as likely to develop new tears as controls. Bodin et al^[66] showed an association between several biomechanical factors, including high-perceived physical exertion and arm abduction, and clinically diagnosed RCT.

Shoulder pain has been associated with metabolic, biomechanical and psychosocial risk factors. A cross-sectional study by Bodin et al^[66] found that 'shoulder pain' was associated with low levels of workplace supervisor support in both men and women. An automatic work pace was associated with shoulder pain only in men, while high perceived physical exertion and exposure to cold temperatures were associated with 'shoulder pain' only in women. A large cross-sectional study by Rechartt et al^[72] demonstrated several metabolic associations with RCT and shoulder pain. Smoking, waist circumference and waist-to-hip ratio were all associated with shoulder pain in both males and females. Increased waist circumference and type I diabetes were both associated with RCT in men.

1.5 Aetiology of Rotator Cuff Tendinopathy

The pathogenesis of cuff disease is multifactorial and results from a combination of intrinsic, extrinsic and environmental factors^[73]. The natural history and molecular pathophysiology of cuff disease is far from being fully understood. Historically the idea of mechanisms both intrinsic and extrinsic to the tendon have been researched and argued. Codman initially proposed in 1934^[14] that degeneration within the tendon was the 'intrinsic' primary cause of cuff tears. The 'extrinsic' theory relating to tendon damage secondary to attrition by surrounding structures was popularised by Neer in 1972^[22] and the term 'impingement' was coined. Broadly the modern consensus recognises both the role of intrinsic and extrinsic factors, but sees the intrinsic factors are being more dominant overall in RCT^[74]. It is probable that different patients have different disease phenotypes with different intrinsic and extrinsic factors playing variable roles. Intrinsic tendinopathy is defined as tendon pathology that originates from within the tendon. Physical reasons for intrinsic failure included overuse and overload; obviously cumulative use increases with age and this is consistent with age being the primary risk factor for RCT. The mechanism of overuse has been well demonstrated in an animal model by Soslowky et al^[75]. Tendon cells progressively fail to meet the homeostatic demands of maintaining the structural integrity of the rotator cuff and this is the key reason for intrinsic tendon failure. Other factors such as vascularity and inflammation have also been implicated. The bottom line remains that a single aetiological factor is unlikely to cause RCT; the degenerative structural failure that may result in tendon rupture is a result of a complex network of associated factors, with age being of primary importance.

1.6 Pain and the Shoulder

1.6.1 Classifying Rotator Cuff Structure

Numerous classification systems for rotator cuff tears have been described in the literature; radiological findings and surgical findings are the two most widely used methods of analyzing the rotator cuff's structure. The classification system may describe the topography of the tear (partial thickness versus full thickness, size, location, geometry) or other structures such as the muscle condition, humeral head position or glenohumeral joint condition. The reliability of various classification systems is highly variable. Intra-observer agreement is generally high for discriminating between full thickness and partial thickness tears; however the reliability of grading partial thickness tears is invariably poor^[76]. The number of tendons involved in the tear is also a reasonably reliable measure^[76]. For the purposes of this work the classification system of full thickness tears of Post, Silver and Singh^[77] has been used. This measures the tear in its longest diameter, a small tear is defined as less than 1cm, a medium-sized tear is from 1cm to 3 cm; a large tear is from 3cm to 5cm, while a massive tear measures more than 5cm in diameter, usually with retraction of the torn cuff edge. For practical purposes small and medium sized tears generally only involve supraspinatus, while large and massive tears extend beyond supraspinatus into infraspinatus.

1.6.2 Measurement of Pain

It is important to adequately assess the severity of the pain, as well as the impact of the pain upon the patient's shoulder function and quality of life; in this way any response to treatment can be more objectively measured and quantified. The visual analogue scale (VAS) is one of many measures that can be used to assess pain^[78], while there are numerous shoulder specific scoring systems^[79] and quality of life measures^[80]. The

Oxford Shoulder Score is a well validated patient reported outcome measure which contains questions related to both shoulder function and shoulder pain^[81]. There are also more specific scoring systems which can help identify and quantify the neuropathic element of a patient's pain such as the painDETECT questionnaire^[82].

1.6.3 Pain Related to Diagnosis and Management

The traditional triad of history, examination and investigations are used to guide the clinician towards a reasoned management plan for the patient's pain. It is also important to focus on features of the history and examination which hint at a greater degree of central sensitisation being present. Shoulder pain frequently radiates below the shoulder, however a history of the pain radiating down the arm to below the elbow and the presence of 'light touch' hyperalgesia around the shoulder are both features of central sensitisation^[83]. Significant symptoms in the absence of radiological abnormality should ring alarm bells; in these cases treating an assumed peripheral pathology surgically may result in disaster as the majority of the pathology is in the higher pain processing systems.

The lack of reliable diagnostic tests for many shoulder pathologies^[84] including 'impingement syndrome'^[85 86] reflects how subjective and variable clinical tests may be. This can be explained by mechanosensitivity changing as a result of any form of peri-articular inflammation with the majority of nociceptive fibres (C and δ) showing increased mechanosensitivity; indeed a large proportion of high-threshold mechanoreceptors (HTMs) are sensitised such that they respond not just to high-intensity stimuli but also to movements in the normal working range. This has been confirmed in the shoulder by Yamashita et al ^[87]who showed that inflammation peripherally sensitised both low and high threshold mechanoreceptors in a rabbit model. The classical inflammatory mediators such as bradykinin, the prostaglandins and

serotonin excite joint afferents and sensitise them to mechanical stimulation. Spinal cord neurons may be activated by noxious movements (NS – nociceptive specific) or they may respond with increasing intensity when the stimulus is increased from the innocuous to the noxious range (WDR – wide dynamic range). Both WDR and NS spinal cord neurons show enhanced responses to noxious stimuli applied in the inflamed joint, and NS neurons show a reduction in their mechanical activation threshold so that they now become excited by innocuous stimuli.

This means that any cause of inflammation in or around the shoulder may give rise to the so called ‘irritable’ shoulder joint. Therefore clinically, it may be difficult to discriminate between rotator cuff tendinopathy and calcific tendinitis or inflammatory joint disease for example; there is a clear lack of clarity with regard to whether common diagnostic tests used in clinical examination are useful in differentially diagnosing pathologies of the shoulder^[84 88]. Consequently there has been the modern trend away from these bedside clinical tests towards radiological imaging modalities such as Ultrasound and Magnetic Resonance Imaging (MRI).

1.6.4 Pain and Structure

Despite the high number of asymptomatic patients with rotator cuff tears^[89 90], there is still a very strong relationship between pain symptoms and rotator cuff integrity. Rotator cuff tendinopathy (RCT) is the commonest ‘cause’ of shoulder pain and accounts for about three quarters of the patients presenting. Patients with rotator cuff tears are far more likely to have symptoms than those without^[68 71], while previously asymptomatic patients are far more likely to develop symptoms as their tear size increase^[91 92]. While a recent study by Koike et al^[93] demonstrated that patients with symptomatic rotator cuff tears had significantly higher radioisotope uptake in the proximal humerus than patients with asymptomatic tears. It is unlikely that any one specific factor which precisely

determines symptom development will ever be identified, pain is complex and many factors may contribute to its development. The glenohumeral joint kinematics change as tear size increases but this abnormality is equally prevalent in both symptomatic and asymptomatic patients^[94].

1.6.5 Treatments

The treatment of shoulder pain depends largely on the clinical diagnosis reached and it is beyond the scope of this thesis to deal with the specifics of all shoulder pathologies.

The placebo effect

An important obstacle to measuring the effect of any treatment on pain is the significance of the placebo effect, which is ‘the patient response to inactive or sham treatments’. Placebo treatments have powerful analgesic effects and even placebo-controlled randomised trials may result in the efficacy of a treatment being misinterpreted^[95]. The Descending Pain Modulatory System (DPMS) is crucially important to placebo analgesia^[96]. A variety of factors including patient expectation, emotions such as anxiety and mood all have significant effects on the complex neurobiological process that lies behind placebo analgesia^[97-98]. Different types of neuropathic pain are affected in different ways by the placebo effect^[99]. While different treatments may have hugely differing strengths of placebo effect depending on multiple complex patient and treatment factors, it has been argued that the placebo effect of sham surgery is likely to be particularly strong^[100]. Consequently the interpretation of treatment effects in shoulder pain is a highly contentious and controversial area. Maximising the placebo effect is useful for any clinician treating shoulder pain; ensuring that the patient has confidence in the treatment plan and allaying any anxieties are examples of ways in which this can be done.

Pharmacotherapy

The World Health Organisation's analgesia ladder is frequently used to guide the treatment of pain and has been modified since its introduction in 1986^[101]. Simple agents such as Paracetamol and the non-steroidal anti-inflammatory drugs (NSAIDs) are generally first line. Paracetamol has both peripheral and central effects in inhibiting prostaglandin synthesis and activating the descending serotonergic system^[102]. The NSAIDs inhibit the cyclooxygenases (COX-1 and COX-2) to varying degrees resulting in both peripheral and central analgesic effects. Peripherally they inhibit the prostaglandin induced nociceptor sensitisation that occurs via TRPV1 and sodium channel activation; centrally their effects are thought to be partly due to the inhibition of the prostaglandin mediated glycinergic neurotransmission.

Weak and strong opiates can then be added to the simpler agents in a step wise fashion. The mu, delta, kappa and ORL1 opioid receptors have been found in humans^[103]. The coupling of these receptors to potassium and sodium channels is believed to be the main mechanism by which endogenous and exogenous opioids produce analgesia. At a spinal level the most important mechanism of action is the presynaptic opioid receptor agonism which leads to a decrease in nociceptive afferent transmitter release; postsynaptic and interneuron opioid receptor agonism attenuates spinal cord outputs. Important sites of supraspinal opioid action are the PAG and RVM. Other drugs including certain anti-depressants and anti-convulsants are particularly important in managing neuropathic pain^[104]. Table 1.3 summarises the classes of drug that may be in the management of acute and chronic shoulder pain. The decision making process in analgesic pharmacotherapy involves the consideration of several complex factors including the nature and the chronicity of the pain; the evolving specifics relating to chronic neuropathic pain are a huge topic in their own merit^[104 105].

Table 1.2 - Summary of the analgesics that may be used in the management of shoulder pain

Class of drug	Examples	Simplified mode of action
NSAIDs	Paracetamol, Diclofenac	Cyclooxygenase inhibition
Weak Opiates	Codeine Phosphate, Tramadol	Peripheral and central opioid receptor agonism
Strong Opiates	Oral morphine solutions	Peripheral and central opioid receptor agonism
Tri-cyclic Antidepressants (TCAs)	Amitryptiline, Clomipramine, Imipramine	blockade of serotonin and norepinephrine re-uptake in the CNS
Selective serotonin re-uptake inhibitors (SSRIs)	Fluoxetine, Citalopram	blockade of serotonin re-uptake in the CNS
Conventional anti-convulsants	Carbamazepine	Blockade of voltage-gated sodium channels
Other anti-convulsants	Gabapentin, Pregabalin	GABA agonism?/calcium channel inhibition?

Injections and nerve blocks

A number of different injections sites have been used in treating shoulder pain including the glenohumeral joint, the subacromial space and several nerve blocks. Peripheral injections may be diagnostic, prognostic or therapeutic; they may consist of local anaesthetic with or without steroid. Local anaesthetics are sodium channel blockers and directly block the nerve conduction. The pain-relieving mechanisms of steroids are complex, not only do they have an anti-inflammatory effects, but they also appear to reduce nociceptor sensitivity and central sensitisation^[106].

The benefits of steroid injections have been demonstrated in both frozen shoulder and in cuff tendinopathy^[107 108]. However the Cochrane review of the use of steroid injections for shoulder pain in rotator cuff tendinopathy concluded that injections may be beneficial, but that their effect may be small and not well-maintained^[109]. This conflicting evidence reflects small trial sample sizes, variable methodological quality and a general heterogeneity.

Nerve blocks are widely used in managing intraoperative and postoperative pain in shoulder surgery; several nerve blocks or combinations of blocks are commonly used including the interscalene, suprascapular and axillary nerve blocks. The suprascapular nerve block has been shown to more effective than subacromial infiltration or placebo after shoulder surgery^[110]. The suprascapular nerve block has also been shown to reduce pain in cases of frozen shoulder one month following injection^[111] and in cases of general chronic shoulder pain of multiple aetiologies^[112]. Pulsed radiofrequency of the suprascapular nerve has also been reported as an effective treatment of chronic shoulder pain^[113].

Acupuncture

It is hypothesised that acupuncture relieves pain by activating A δ and possibly C fibres via the mechanical stimulation of needles. Some trials have shown a benefit over placebo in treating shoulder pain^[114], however the benefit could be attributed to the greater placebo effect of actual acupuncture over sham acupuncture. The Cochrane review regarding acupuncture for shoulder pain concluded that there was insufficient evidence to support or refute it as a treatment^[115].

Physiotherapy and activity modification

The proposed analgesic mechanisms of physiotherapy include rotator cuff muscle strengthening, augmenting scapulothoracic movement, increasing proprioceptive feedback and stretching tight structures. The exact nature of what shoulder physiotherapy consists of varies widely in the literature; for example some regimes are patient led, others more therapist-led, while some are particularly aggressive in terms of provoking pain symptoms. This has been emphasised by the Cochrane review which

stated that the small sample sizes, variable methodological quality and heterogeneity in terms of population studied, physiotherapy intervention employed and length of follow up of randomised controlled trials of physiotherapy interventions results in little overall evidence to guide treatment^[116]. However there is evidence to support the use of some interventions in specific cases^[117 118], while physiotherapy has compared favourably with surgery in some trials^[119]. In this particular example the mechanisms behind the pain, the role of capsular receptors and the reason for pain improving with stretching remain unclear. Physiotherapy and activity modification frequently overlap, for example if pain is felt to be related to overuse then a reduction in activity levels may be followed by a supervised structured programme of rehabilitation. In some instances the individual may wish to accept that certain activities are no longer possible or that living with a certain level of pain is acceptable, this decision is often made having discussed all available treatment options with their physiotherapist, doctor or surgeon.

Surgery

Many of the surgical treatments of shoulder pain involve attempting to switch off the peripheral environmental triggers of pain by attempting to 'fix' the underlying 'causal' pathological process. Defining a causal pathological process is not always straightforward and this has been emphasised by the confusion surrounding the diagnosis and management of 'impingement syndrome'^[85 86]. There remains a lack of high quality evidence showing that surgery is effective in RCT for example^[6 120]; the heterogeneous nature of patient groups and treatment methods make it hard to draw firm conclusions^[121]. There is emerging evidence that certain surgical treatments such as rotator cuff repair result in significant sustained benefits in terms of pain and function^[122], but these results have not been confirmed by randomised trials^[6]. Certainly

more large, high quality randomised controlled trials are needed to answer these questions surrounding the effectiveness of surgery in specific shoulder pathologies.

The role of central sensitisation in shoulder pain has recently been explored in relation to 'impingement syndrome' and outcome after subacromial decompression (SAD)^[83]. Patients with higher levels of punctate hyperalgesia and/or referred pain (features demonstrative of central sensitisation) did significantly worse in terms of post-operative outcome. Central sensitisation has also been demonstrated in patients with hip and knee osteoarthritis^[123 124]. Gwilym et al^[123] demonstrated increased activity in the periaqueductal grey (PAG) with stimulation of the patient's skin in the areas of their referred arthritic pain. Joint arthroplasty can reverse pain-related changes in the thalamus in patients with hip and knee osteoarthritis, in association with their reduced pain and increased function following surgery^[125]. The normalisation of hyperaesthesia following knee joint arthroplasty has been demonstrated by Graven-Nielsen et al^[126], implying that the central pain processes are maintained by peripheral input.

1.7. Glutamate

Glutamate (L-glutamic acid) is a vital amino acid and is part of the 'glutamate family' of amino acids^[127]. This family comprises approximately 25% of dietary amino acid intake and consists of glutamine, the most abundant plasma amino acid, as well as proline, histidine, arginine and ornithine. Glutamine is of key importance in the growth of cells *in vitro* and is an essential metabolic precursor in nucleotide, glucose and amino sugar biosynthesis, glutathione homeostasis, protein synthesis and a source of oxidative energy^[127]. Glutamate is the most abundant intracellular amino acid, concentrations

varying from between 2 to 20mM, while glutamine is the most abundant extracellularly *in vivo* with a concentration of approximately 0.7mM. Glutamate cannot readily diffuse across cell membranes but there are a number of amino acid transporters capable of transporting glutamate across the cell membrane. Glutamate may be co-transported into cells with Na⁺ or H⁺ ions or counter-transported with K⁺ or OH⁻ ions^[128]. Generally the process of transporting glutamate into cells consumes energy, for example the co-transport into cells with Na⁺ ions is powered by the ionic gradient driven by the ATP consuming Na⁺/K⁺ exchanger. The presence of the Na⁺ dependent uptake of glutamate by cultured fibroblasts has been confirmed experimentally^[129].

1.7.1. Metabolic role

Glutamate appears to be at a vital crossroads between amino acid metabolism and carbohydrate metabolism. Glutamate can either donate its amino group for new amino acid synthesis which involves the process of 'transamination' or can lose its amino group as NH₄⁺ via deamination to 2-oxoglutarate^[127].

Figure 1-4 - Summary of metabolic pathways involving glutamate

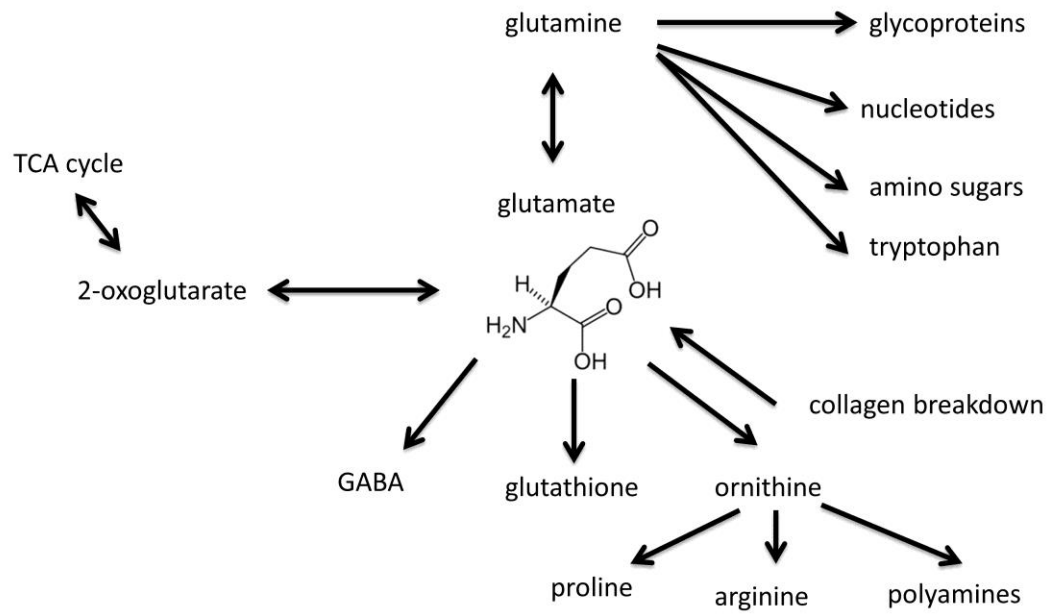


Figure 1-4 summarises some of the key metabolic pathways in which glutamate is involved. The production of glutamate from glutamine is catalyzed by the glutaminase enzymes, while the conversion of glutamate to 2-oxoglutarate is catalyzed by glutamate dehydrogenase. The tricarboxylic acid (TCA) cycle is vital in the aerobic generation of ATP in all living cells. Of note glutamate is one of the most abundant amino acids in collagen, making up approximately 11% of collagen's total amino acid content^[130]. Other amino acid constituents of collagen such as proline, ornithine and arginine may be broken down into or synthesized from glutamate^[130].

Fibroblasts are highly metabolically active cells, even when in a so called 'quiescent' state, using both glucose and glutamine-derived carbons to generate energy^[131 132]; of note glutamine, and not glucose, seems to serve as the major anaplerotic precursor in proliferating fibroblasts^[133]. Glucose and glutamine utilisation are reciprocally regulated by human fibroblasts in culture^[132]. The uptake of glutamate appears important in fibroblast growth in the absence of glutamine^[132]. Human fibroblasts are also capable of metabolizing glutamine to both pyruvate and lactate^[133].

Bone-resorbing osteoclasts have been shown to secrete glutamate via the vesicular glutamate transporter 1 (VGLUT1)^[128] and catabolism is a recurrent feature in RCT. The macrophage appears to have an important role in regulating extracellular glutamate concentration in the brain and express excitatory amino acid transporters (EEATs) which serve to transport glutamate into cells^[134]. It is known that cellular metabolic function declines with age and the reduction in proliferative capacity of cells that occurs with age is often referred to as 'cellular senescence' ^[135]. Decreased glutamate uptake by fibroblasts has been observed in human neurodegenerative disease and correlated with disease severity^[136].

It has been shown that glutamate increases cell proliferation in rat retinal epithelial cells *in vitro*^[137]. The increase in cell proliferation was blocked by the NMDAR inhibitor MK-801 and an increase in basic Fibroblast Growth Factor (bFGF) was seen after glutamate administration; these results led the authors to suggest that the glutamate-induced cell proliferation was as a result of NMDAR activation and an increase in bFGF levels. Hinoi et al have demonstrated that glutamate increases cell proliferation in rat synovial fibroblasts^[138], interestingly this effect was only seen in the fibroblasts of arthritic rats and not the fibroblasts of control animals.

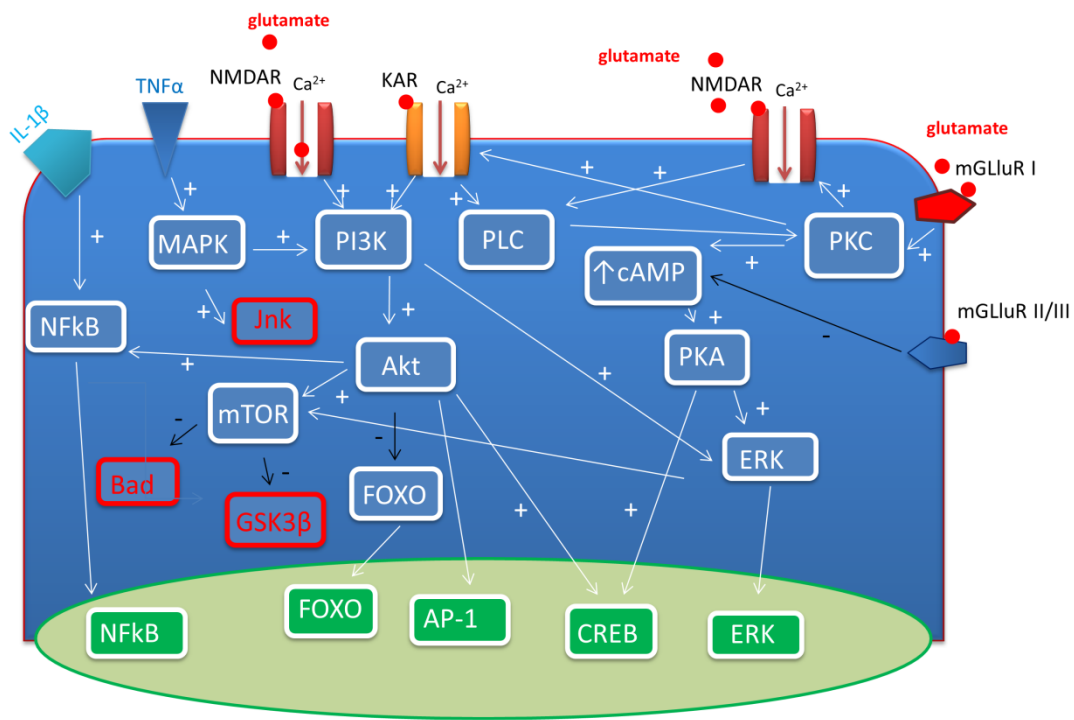
1.7.2. Glutamate receptors and excitotoxicity

Glutamate receptors can be broadly broken down into two major types: ionotropic, which are glutamate-gated ion channels (iGlu), and metabotropic, which are G-protein coupled receptors that modulate signal transduction cascades (mGlu)^[139-140]. The ionotropic receptors include Kainate (KA) receptors, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and N-Methyl-D-Aspartate (NMDA) receptors^[141]. On a basis of their effect on signal transduction mechanisms, agonist pharmacology and sequence homologies metabotropic receptors have been classified into three subgroups, termed Group I (mGluR1 and 5), Group II (mGluR2 and 3) and Group 3 (mGluR 4,6,7 and 8)^[142].

Group I receptor activation leads to the formation of inositol 1,4,5-triphosphate and diacylglycerol, whereas receptors from Group II and III induce a decrease in cAMP levels. It is known that Group 1 metabotropic receptors (mGluR1/5) increase NMDAR activity, while Group 2 (mGluR2/3) and Group 3 receptors (mGluR4/6/7/8) decrease NMDAR activity^[139]. These ionotropic and metabotropic receptors have vital roles within the central nervous system (CNS), however they are also widely distributed in peripheral tissues and their peripheral roles has historically been rather under-

investigated^[77]. Figure 1-5 summarises some of the intracellular signalling pathways related to these key glutaminergic receptors.

Figure 1-5 Diagram summarising some of the possible intracellular signalling pathways relating to key glutamate receptors



The majority of work relating to glutamate has been carried out regarding its role in central nervous system as a key excitatory neurotransmitter. Glutamate is also a key factor in the mechanism of hypoxic-ischaemic neuronal cell death^[143]. Glutamate neurotoxicity may be largely mediated by a toxic influx of extracellular calcium and this may be blocked by the antagonism of the NMDA subtype of glutamate receptor ^[143]. The NMDA-receptor (NMDAR) activated ion channel is a major route by which glutamate induces the toxic calcium influx as NMDA antagonists attenuate these toxic effects^[141]. Extracellular glutamate is increased by the mitochondrial glutaminase following

neuronal cell death^[144]. Hypoxia inducible factor 1 (HIF-1) and neurotrophic factors have both been shown to protect against the glutamate-induced excitotoxicity^[145 146].

The role of the NMDA receptor is complex and dichotomous^[147]. It can be protective against both apoptosis and excitotoxicity^[147] with the activation of the PI3K (phosphoinositide-3-kinase) -Akt kinase pathway and the suppression of pro-death genes appearing implicated in these effects. The pro-survival role of NMDAR is demonstrated by the fact that its blockade *in vivo* can promote oxidative damage and neuronal cell death. However at the other end of the toxicity curve excessive NMDAR activity promotes free radical production and oxidative stress *in vitro and in vivo*^[141]. Notably Akt deactivation occurs in multiple models of cell death including NMDA excitotoxicity^[148].

1.7.1. Glutamate and pain

There is extensive evidence demonstrating the key role of glutamate and its receptors in both peripheral and central sensitisation as described in earlier chapters. Glutamate has been shown to induce pain and hyperalgesia when injected around human tendon tissue^[149]. Glutamate injection causes pain, pain sensitisation and mechanical allodynia when injected into the human masseter muscle^[150]. Glutamate has also been shown to cause peripheral and central sensitisation when injected into the rat temporomandibular joint^[151 152]. Increased glutamate levels have also been correlated with pain intensity in the musculoskeletal condition trapezius myalgia^[153 154]. The involvement of ionotropic glutamate receptors in central sensitisation has been repeatedly demonstrated^[155 156]. Peripheral AMPA and KA glutamate receptors have also been implicated in the pathogenesis of painful human osteoarthritis^[157], and an attenuation of NMDA receptor phosphorylation is associated with reduced experimental osteoarthritis and nociception in a rat model^[157]. An up-regulation of group I metabotropic receptors have been shown to have an important role in pain behaviour in a rat model of knee inflammation^[158]. The antagonism of group II and III metabotropic receptors has been demonstrated to reduce nociceptive behaviour in a rat model of inflammatory pain, while the group III receptor mGluR7 is down regulated following peripheral nerve injury in the rat^[159]. Peripheral neuronal NMDA, AMPA and KA receptors are increased in a rat model of inflammatory pain^[160], while the activation of presynaptic NMDA receptors enhances glutamate release in the dorsal horn in a rat model of neuropathic pain^[161]. Overall the evidence points to a pivotal role for glutamate and the dynamic balance of the activation of its many receptors in pain sensitivity in human musculoskeletal disease^[139].

1.7.2. Glutamate in tendon healing and tendinopathy

The metabolic activity of a healing tendon after acute injury is hugely increased^[162] and fibroblasts are metabolically adapted for survival in this hypoxic environment^[163]. The elevated lactate metabolite present in the acutely traumatised tendon is also a potent stimulant for collagen synthesis^[164]. The upregulation of metabolites including glutamate demonstrated after Achilles tendon repair in humans^[165] is consistent with this increased metabolic activity and this appears likely to be important in the increased cell proliferation present in healing tendon. The metabolic activity in early tendon repair can in fact be enhanced by intermittent pneumatic compression, and healing tendons demonstrated a significantly higher level of extracellular glutamate to be present^[165]. Glutamate signalling in fibroblasts appears to have an important role in both a rat model of Achilles healing^[166] and in a rat model of RCT^[167]. Metabolic changes have been demonstrated in Achilles tendinopathy, with an increased degree of anaerobic metabolism being present alongside a reduction in aerobic activity^[168]. A reduced level of aerobic metabolic activity has been seen close to the edges of rotator cuff tears in humans^[169].

The finding of raised extracellular glutamate levels and an upregulated glutaminergic system in human tendinopathy has been well documented in recent years^[170 171]. Control levels of extracellular glutamate have been shown to be around 50µM, while in tendinopathy levels are typically well above 100µM. Schizas et al demonstrated that NMDAR1 and glutamate were co-localised on the nerves, blood vessels and transformed tenocytes of patients with patellar tendinopathy^[172]; there were far greater levels of glutamate and NMDAR1 in patients with tendinopathy versus control. Further detailed investigation into the specific glutaminergic receptors involved in patellar tendinopathy was carried out by Schizas et al using immunohistochemistry^[173].

The finding of increased NMDAR1 was repeated, while an increased immunodensity of phospho-NMDAR1 and mGluR5 were found in tendinopathy versus control. Of note there was no difference in mGluR6 and mGluR7 receptors as a group when compared between control and tendinopathy patients. Again the NMDAR1 immunoreactivity was only seen within the tendon proper in transformed tenocytes in patients with tendinopathy and not in controls; mGluR5 was also predominantly seen on the morphologically altered tenocytes. Scott et al investigated whether the vesicular glutamate transporters VGLUT1 and VGLUT2 were present in Achilles and patellar tendinopathy specimens^[174]. It was confirmed using immunohistochemistry and in situ hybridisation that VGLUT2 expression was increased in tendinopathy and that VGLUT1 was not expressed in tendon. Based on these results Scott et al hypothesised that the increased extracellular glutamate seen in tendinopathy may be related to altered glutamate signalling involving VGLUT2.

1.8.Pain processing

1.8.1.The peripheral receptors

There are numerous types of peripheral sensory receptors present in the human musculoskeletal system^[175]. They may be classified based on their function (as mechanoreceptors, thermoreceptors or nociceptors) or morphology (free nerve endings or different types of encapsulated receptors)^[175]. The different types of receptor can then be further subclassified based on the presence of certain chemical markers. There are significant overlaps between different functional classes of receptor, for example between the mechanoreceptor and nociceptor, and this has the potential to cause significant confusion. For this reason it is absolutely vital to understand that there is no universally accepted receptor classification system and that receptors are best seen as a continuum, in which the artificial boundaries between different receptor types are somewhat arbitrary and fluid. Despite this, it is still worthwhile considering how sensory receptors may be classified in more detail.

Sensory receptors are supplied by afferent nerves of varying size, degrees of myelination and conduction velocity. On this basis sensory nerves can be simply divided into three main groups (conduction velocity given in brackets): thick diameter myelinated Group II or A β fibres (>20m/sec), small diameter myelinated Group III or A δ fibres (2.5 to 20m/sec) and unmyelinated Group IV or C fibres (<2.5m/sec)^[176]. Receptors are sometimes referred to simply by the characteristics of the nerve that innervates them, for example 'A δ nerve endings'. This description makes no reference to a receptor's other specific characteristics as outlined above and described in more detail below.

Receptors that respond preferentially to noxious stimuli and that have a high threshold to the adequate stimulus are termed 'nociceptors'^[177]. Nociceptors may respond to multiple energy forms such as thermal, mechanical and chemical stimuli. Nociceptors can be comprehensively subclassified based on four criteria: myelination of nerve supply (unmyelinated C fibres versus myelinated A δ fibres), modalities of stimulus that evoke a response, response characteristics and distinctive chemical markers^[178]. On a molecular basis, nociceptors can be either peptidergic or non-peptidergic based on whether peptides are expressed in their dorsal root ganglion cells^[178]. The peptides expressed include Substance P (SP), calcitonin gene-related peptide (CGRP) and somatostatin. The sub classification of nociceptors is complex; furthermore they have a degree of functional and chemical plasticity which ensures that their threshold and responsiveness, as well as the efficacy of their synaptic contacts, are affected by activity, inflammation, and axonal injury^[178].

The second form of receptors is the mechanoreceptor which conveys information about the mechanical stimuli to the central nervous system. The main innocuous-touch receptors include (skin stimulus is in brackets): Meissner corpuscles (dynamic deformation), Merkel cell-neurite complexes (indentation depth), Pacinian/Lamellar corpuscles (vibration), Ruffini receptors/endings/corpuscles (stretch) and free nerve endings (touch). The term 'proprioceptor' is used to describe when a mechanoreceptor's role is that of responding to the mechanical variables associated with muscles and joints^[179]. Proprioceptors include muscle spindles, Golgi tendon organs and Ruffini type receptors.

Mechanoreceptors can be classified as low-threshold mechanoreceptors (LTMs), so called because they respond to innocuous mechanical forces, or high-threshold mechanoreceptors (HTMs), so called because they are excited only by greater mechanical

forces. As with nociceptors, different types of mechanoreceptor are associated with specific afferent nerve types. Undoubtedly there is a significant degree of cross over between the properties of both nociceptors and mechanoreceptors, demonstrated by the fact that HTMs can also be described as nociceptors^[180 181]. Certainly creating any absolute division between the two is somewhat artificial and potentially misleading, especially given the plasticity of receptor characteristics.

Generally mechanoreceptors supplied by A β nerve endings are LTMs, while those supplied by A δ nerve endings may be either HTMs or LTMs. HTMs supplied by A δ nerve endings and the majority of C-fibre nerve endings may be termed nociceptors. Schaible and Grubb^[176] have studied the mechanosensitivity of the different fibre types (A β , A δ and C) supplying the cat knee joint. They found that most A β nerve endings were activated by innocuous stimuli and hence could be termed LTMs. A much higher percentage of A δ and C fibre nerve endings were insensitive to innocuous stimuli (50% and 70% respectively) and could be classified as HTMs. Many LTMs fire in the innocuous range but have a far stronger response in the noxious range, demonstrating the dynamic complexity of their function.

A typical joint nerve is innervated by thick diameter myelinated A β , small diameter myelinated A δ and a high proportion (circa 80%) of unmyelinated C fibres. C fibres are further subdivided into either sensory afferents or sympathetic efferents, with about 50% of each^[176]. Muscle nerves consist of axons from motor neurons, sensory neurons and postganglionic sympathetic neurons. Each of these nerve types may end in a number of different 'receptors' as described above. Freeman and Wyke^[182] classified the different types of articular nerve endings based on their pioneering work in the knee joints of cats in 1967, these being summarised in **Table 1.3**. They classified the different endings as types I, II, III and IV on a morphological and functional basis. Some of the eponymous

terms used to describe receptors of different types are included in Table 1, for example 'Ruffini ending' and 'Golgi-Mazzoni body'. Other authors have since modified the eponymous terms mentioned in Freeman and Wyke's classification system^[183] (Table 1.3).

Table 1.3 - the Freeman and Wyke classification of joint nerve endings

Type	Morphology	Location	Diameter of parent fibre (micrometre)	Principle functional characteristics	Eponymous or descriptive terms
I	Globular or ovoid corpuscles, fine capsule, in clusters of 3-6	Fibrous capsule	5-8 (small myelinated)	Mechanoreceptor (low threshold, slowly adapting)	Ruffini ending, Golgi-Mazzoni ending, Meissner corpuscle,
II	Cylindrical or conical corpuscles, thick capsule, in groups of 2-3	Fibrous capsule	8-12 (medium myelinated)	Mechanoreceptor (low threshold, rapidly adapting)	Pacinian corpuscle, Golgi-Mazzoni body, Meissner corpuscle
III	Fusiform corpuscles	Ligaments/tendons	13-17 (large myelinated)	Mechanoreceptor (high threshold, very slowly adapting)	Golgi ending, Golgi-Mazzoni corpuscle
IV	Unmyelinated nerve endings	multiple	<2 (unmyelinated)	Pain receptors	

Articular A β fibres terminate as corpuscular endings of the Ruffini-, Golgi- and Pacini-type in the fibrous capsule, articular ligaments, menisci and adjacent periosteum^[184]. While articular A δ and C fibres terminate as non-corpuscular or free nerve endings in the fibrous capsule, adipose tissue, ligaments, menisci, periosteum and synovium. The cartilage is not innervated. In muscle, thick myelinated afferents terminate as organised endings such as muscle spindles and tendon organs, while A δ and C fibres terminate as free nerve endings. The receptor types and innervation specifically relating to the shoulder will be explored in greater detail the next section.

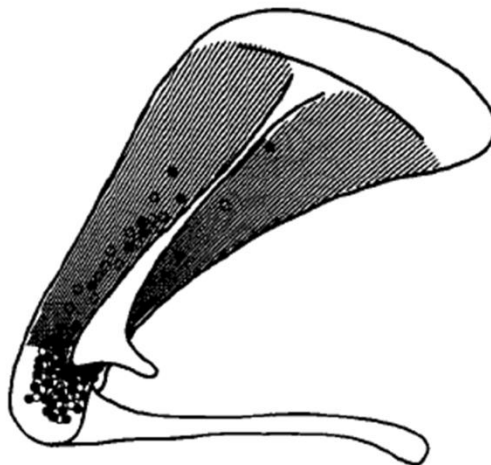
1.8.2.Shoulder Nociception

The peripheral receptors play a pivotal role in the way in which tissue pathology may generate pain. Therefore understanding both the location of receptors and the types of receptor present in the shoulder are of key importance in diagnosing and treating shoulder pain.

The rotator cuff muscles and tendons

Minaki et al^[185] investigated the innervation of the rabbit rotator cuff electrophysiologically and demonstrated a high density of nociceptors to be present around the rotator cuff's humeral insertion. Supraspinatus was particularly densely innervated with both mechanoreceptors and nociceptors as shown in Figure 1-6 which shows the locations of receptive fields identified in the posterior shoulder.

Figure 1-6- The receptive fields present in the rabbit rotator cuff.
Filled circles are units with mechanical thresholds greater than 7 g, and open circles are units with mechanical thresholds less than 7 g.



In a histological study in rats Backenkohler et al^[186] showed that Golgi tendon organs were found within tendons where they merge into muscle and in the tendinous insertion near the joint capsule; it was also shown that the muscle spindles tended to accumulate near the musculotendinous junction. Some lamellated corpuscles were found in the connective tissue of muscle septa and tendons, in contrast Ruffini corpuscles were not.

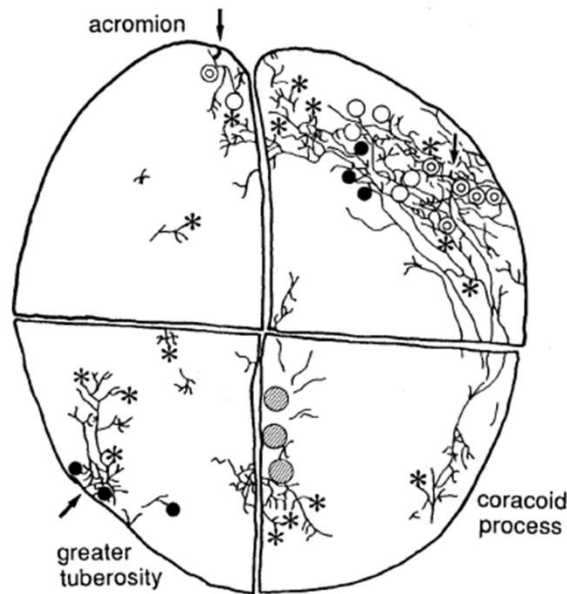
The subacromial bursa (SAB)

The SAB is an important structure that has been implicated in the pain of patients with rotator cuff tendinopathy and 'impingement syndrome'. Human tissue from the SAB is readily available because of its frequent removal as part of 'subacromial decompression' (SAD) surgery. Numerous studies have shown that the SAB is richly innervated^[187-189]. Soifer et al^[188] examined cadaveric shoulder tissue using IHC and found that neural elements to be present in SAB, cuff tendon, biceps tendon and tendon sheath; the nerve supply to the SAB was significantly denser than that to the other tissues. Tomita et al^[189] examined tissue from the SAB of patients with varying degrees of cuff tendinopathy. They found that the SAB was innervated by large numbers of free nerve endings and mechanoreceptors (encapsulated corpuscles); they also found that there was a strong correlation between the density of neural elements at rest and shoulder pain at rest, those with higher densities of neural elements being more likely to experience pain at rest.

Ide et al^[187] investigated the innervation of the SAB in great detail using IHC and their results are shown in Figure 1-7. A large number of C and A δ fibres were found to innervate the SAB and they were shown to be immunoreactive to SP and CGRP, confirming their nociceptive role.

Figure 1-7 - A schematic drawing of the major nerve fibre bundles and the distribution of sensory nerve endings in the SAB (reproduced with permission^[187])

Double circles indicate Pacinian corpuscles. Filled circles indicate Ruffini endings. Asterisks indicate free nerve ending plexus. Hatched areas indicate areas where cluster of Golgi-Mazzoni corpuscle-like receptors exists. White circles indicate type II unclassified receptors.



A variety of mechanoreceptors were innervated by larger A β fibres and these fibres were not immunoreactive to substance P. The Pacinian corpuscles were localised in the roof of the coracoacromial (C-A) arch side of the bursa, while the Ruffini endings were located at both the greater tuberosity and C-A side of the bursa; other unclassified mechanoreceptors were found in the bursa, some of which resembled Golgi-Mazzoni corpuscles. An intriguing study by Gotoh et al^[190] showed that patients with higher levels of SP in the SAB had greater levels of pain as measured by VAS, and that substance P levels were higher in non-perforated cuff tears versus perforated cuff tears.

The long head of biceps tendon (LHBT)

The LHBT is often implicated in shoulder pain and a degenerate LHBT frequently coexists with rotator cuff tendinopathy. Alpantaki et al^[191] analysed four cadaveric LHBT in humans using IHC. An extensive neural network was demonstrated along the tendon, with the densest innervation being present proximally and decreasing distally. This innervation was positive for SP and CGRP, suggesting the presence of thinly myelinated and unmyelinated sensory neurons; it was also positive for tyrosine hydroxylase, indicating the presence of post-ganglionic sympathetic fibres. Singaraju et al^[192] compared the neurohistology of the LHBT in patients undergoing surgery for pain related to degeneration with that of cadaveric specimens, none of which had evidence of cuff or LHBT degeneration. No difference was found in CGRP and SP staining between the two groups but there was a trend towards increased inflammation and vascularity in the surgical specimens that was not statistically significant. These results led the authors to conclude that neither the LHBT nor its sheath was the sole cause of anterior shoulder pain. Hashimoto et al^[193] showed that, in the central portion of the long head of biceps (LHB) tendon, relatively large nerve fibres inserted from the shoulder capsule. Tosounidis et al^[194] demonstrated, by staining for the S-100 protein and neuropeptide Y, that cells with neural differentiation within the LHBT were increased in acute injury and hypothesised that this provided a mechanism for LHBT related pain. The function of the LHB tendon and its role in glenohumeral kinematics presently remain only partially understood^[195]; likewise its role in shoulder pain remains controversial and incompletely understood.

The Coracoacromial ligament (CAL)

Konttinen et al^[196] demonstrated the CAL to be aneural but the adjacent connective tissue and fat to be richly innervated; some of these nerves stained positively for Neuropeptide Y (sympathetic marker) and none for SP/CGRP. The rich innervation of

the connective tissue adjacent to the CAL is described above (Figure 1.4). The aneural nature of the CAL parenchyma has been subsequently confirmed by Tamai et al^[197]. Tamai et al^[197] also showed that the superficial periligamentous bursal tissue over the CAL was richly innervated with nociceptive fibres in patients with rotator cuff disease and not in controls; they hypothesised that this may be one of the causes of pain in this group of patients.

The glenohumeral joint

Two studies using human cadavers have analysed the neurohistology of the shoulder joint. Guanche et al^[198] analysed three cadaveric human shoulders using gold chloride staining and light microscopy. They found that the superior, middle and inferior glenohumeral ligaments all contained mechanoreceptors (Golgi tendon organs, Ruffini's endings and Pacinian corpuscles) and free nerve endings. Only free nerve endings were identified in the biceps tendon and glenoid labral tissue. Hashimoto et al^[193] analysed 26 shoulder joints from 13 cadavers using immunohistochemistry (IHC) and light microscopy. A small number of free nerve endings were identified in the innermost layer of the joint capsule. Many nerve fibres of large diameter were found in both the anteroinferior and posteriosuperior portions of the outermost layers of capsule (stratum fibrosum), while nerve fibres of various sizes were more numerous in posteriosuperior and anteroinferior portions of the boundary zone between the labrum and capsule in all layers.

Animal studies investigating the innervation of the shoulder joint have been relatively consistent in their findings. Backenkohler et al^[186] showed the Ruffini corpuscles were exclusively found in the ventral aspect of the joint capsule, while most lamellated corpuscles were located in the axillary portion of the joint capsule. Solomonow et al^[199]

examined the distribution of neural elements in the glenohumeral capsule of cats. Again the greatest density of neural elements was found in the inferior aspect of the capsule; nerve fibres, free nerve endings, Golgi tendon organs, Ruffini's endings and Pacinian corpuscles were found in all areas of the capsule. Tarumoto et al^[200] studied the glenohumeral capsule in monkeys and again confirmed this dense inferior capsular innervation, particularly with Ruffini-like endings. They also found a high density of smaller peptidergic fibres that were presumed to be nociceptive, in the posterior half of the capsule; a small number of nociceptive fibres were found to innervate the marginal labrum. Overall an increased density of mechanoreceptors in certain areas of the shoulder joint region coincides with zones where sensory control is most important^[201]. The presence of corpuscular mechanoreceptors in the joint capsule and glenoid labrum shows their important role in inducing protective reflex actions in phases of extreme or abnormal movement^[201 202].

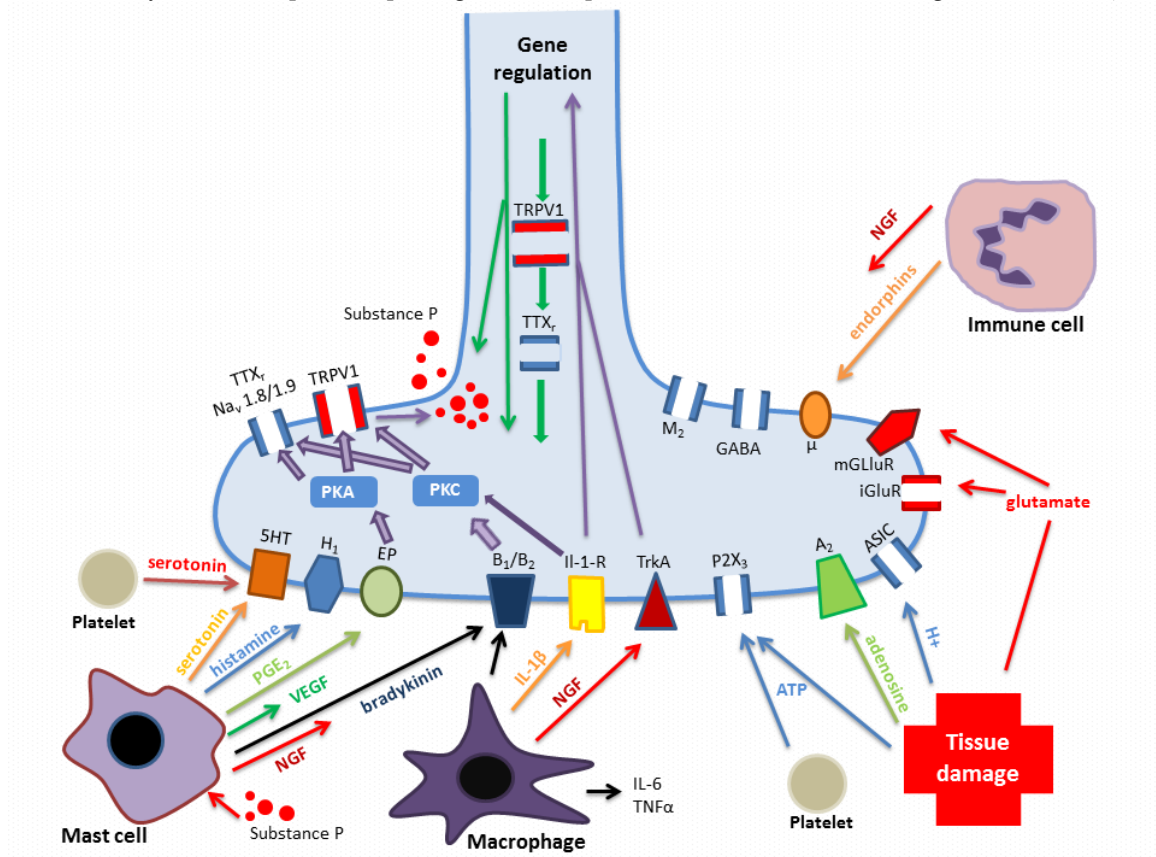
1.8.3. Peripheral pain processing

Tissue injury involves a variety of inflammatory mediators being released by damaged cells including bradykinin, histamine, 5-hydroxytryptamine (5-HT), ATP, nitric oxide (NO) and certain ions (K^+ , H^+). The activation of the arachidonic acid pathway leads to the production of prostaglandins, thromboxanes and leukotrienes. Cytokines, including the Interleukins (ILs) and $TNF\alpha$, and neurotrophins, such as nerve growth factor (NGF), are also released and are intimately involved in the facilitation of inflammation^[203]. Other substances such as excitatory amino acids (glutamate) and opioids (endothelin-1) have also been implicated in the acute inflammatory response^[204 205]. Some of these agents may directly activate nociceptors, while others bring about the recruitment of other cells which then release further facilitatory agents^[206]. This local process resulting

in the increased responsiveness of nociceptive neurons to their normal input and/or the recruitment of a response to normally sub-threshold inputs is termed 'peripheral sensitisation'. Figure 1-8 summarises some of the key mechanisms involved.

Figure 1-8 - Peripheral sensitisation and inflammation at the peripheral nerve terminal

(ASIC, acid sensing ion channel; iGluR, ionotropic glutamate receptor; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; mGluR, metabotropic glutamate receptor; NGF, nerve growth factor; PGE₂, prostaglandin E₂; PKA, protein kinase A; PKC, protein kinase C; TNF- α , tumour necrosis factor α ; TrkA, tyrosine kinase receptor A; TTXr, tetrodotoxin-resistant sodium channel; μ , mu-opioid receptor; M₂, muscarinic receptor; 5-HT₃, serotonin; H₁, histamine receptor; B₁/B₂, bradykinin receptors; A₂, adenosine receptor; GABAR, γ -aminobutyric acid receptor; EP, prostaglandin receptor; VEGF, vascular endothelial growth factor^[207]).



NGF and the Transient Receptor Potential cation channel subfamily V member 1 (TRPV1) receptor have a symbiotic relationship when it comes to inflammation and nociceptor sensitisation. The cytokines produced in inflamed tissue result in an increase in NGF production^[208]. NGF stimulates the release of histamine and serotonin by mast cells, and also sensitises nociceptors, possibly altering the properties of A δ fibres such that a greater proportion become nociceptive. The TRPV1 receptor is present in a

subpopulation of primary afferent fibres and is activated by capsaicin, heat and protons. The TRPV1 receptor is synthesised in the cell body of the afferent fibre, and is transported to both the peripheral and central terminals, where it contributes to the sensitivity of nociceptive afferents. Inflammation results in NGF production peripherally which then binds to the tyrosine kinase receptor type 1 (TrkA) receptor on the nociceptor terminals, NGF is then transported to the cell body where it leads to an up regulation of TRPV1 transcription and consequently increased nociceptor sensitivity^[208 209]. NGF and other inflammatory mediators also sensitise TRPV1 through a diverse array of secondary messenger pathways. Many other receptors including cholinergic receptors, GABA receptors and somatostatin receptors are also thought to be involved in peripheral nociceptor sensitivity.

A large number of inflammatory mediators have been specifically implicated in shoulder pain and rotator cuff disease^[56 61 190 210 211]. While some chemical mediators directly activate nociceptors, most lead to changes in the sensory neuron itself rather than directly activating it. These changes may be early post-translational or delayed transcription dependent. Examples of the former are changes in the TRPV1 receptor or in voltage gated ion channels resulting from the phosphorylation of membrane bound proteins. Examples of the latter include the NGF-induced increase in TRV1 channel production and the calcium induced activation of intracellular transcription factors.

1.8.4. Peripheral sensitisation

Primary hyperalgesia is defined as hyperalgesia, or hypersensitivity, at the site of injury (the primary zone)^[212]. Several mediators may lead to the sensitisation of a nociceptor and therefore play a role in primary hyperalgesia^[213]. Primary hyperalgesia relates to both heat and mechanical stimuli^[214]. The hyperalgesia to heat stimuli that occurs at the site of injury is thought to be due to the sensitisation of primary afferent nociceptors, ie

peripheral sensitisation. However the hyperalgesia to mechanical stimuli at the site of injury results from not only peripheral sensitisation, but probably from central sensitisation as well^[215]. Different types of mechanical hyperalgesia have been described: one type is hyperalgesia to light touch or 'allodynia', another is 'punctate' hyperalgesia and a third is termed 'pressure' or 'impact' hyperalgesia^[216]. It must be remembered that the acute presence of both allodynia and 'punctate' hyperalgesia at the site of injury may be demonstrative of a completely normal pain response, in response to acute injury e.g. sunburnt skin. A lowered threshold to stimulation, an increased response to supra-threshold stimuli and the expansion of receptive fields are all ways which the nociceptor sensitisation of primary hyperalgesia may be manifest^[217].

1.8.5. Central sensitisation

Secondary hyperalgesia is defined as hyperalgesia outside the original zone of injury and relates to the sensory response to mechanical stimuli only^[214]. The localised activation of nociceptors leads to a 'flare response' outside the zone of initial injury through the spreading chemical activation of adjacent nociceptors. However La Motte et al^[218] demonstrated that proximal nerve blocks prevent the development of secondary hyperalgesia, indicating that higher pathways must be involved and that the flare response cannot fully account for secondary hyperalgesia. The flare response and secondary hyperalgesia have also been shown to be distinct entities for several reasons: the zone of secondary hyperalgesia is generally larger than the 'flare', they can exist independently, and secondary hyperalgesia does not migrate across the body's midline whereas flare does^[218]. Peripheral sensitisation cannot adequately account for secondary hyperalgesia; hence the central nervous system must have a key role to play in this phenomenon^[213 219]. It is clear that the peripheral signal for pain is not purely driven by nociceptors; under pathological circumstances other receptors types, which are normally

associated with the sensation of touch, acquire the ability to elicit pain^[219 220]. This applies to neuropathic pain as well as to acute secondary hyperalgesia.

The term 'central sensitisation' is used to describe the phenomenon whereby central pain-signalling neurons become hypersensitive to the input of LTMs^[220]. 'Central sensitisation' may also be more generally described as 'an amplification of neural signalling within the CNS that elicits pain hypersensitivity'^[156]. Secondary hyperalgesia (and therefore central sensitisation) is seen in many clinical situations including the acutely inflamed joint^[221], the arthritic knee^[126] and the painful shoulder^[83].

Two distinct forms of secondary hyperalgesia have been observed: punctuate hyperalgesia and light touch hyperalgesia (allodynia). Punctate hyperalgesia is thought to be different to light touch hyperalgesia for several reasons: the area of punctuate hyperalgesia is larger than that of light touch hyperalgesia and light touch hyperalgesia post capsaicin is significantly shorter lived than punctate hyperalgesia. Light touch hyperalgesia^[218] and punctate hyperalgesia^[219] both appear to be mediated by LTMs. Ziegler et al^[222] used an intradermal capsaicin model to show that punctuate hyperalgesia was mediated by smaller myelinated fibres than light touch hyperalgesia. Certainly the central sensitisation is induced by unmyelinated C-fibre nociceptors, but the exact higher mechanisms have yet to be fully determined^[223]; it is likely that the sensitisation of nociceptive neurons in the dorsal horn of the spinal cord are involved, although a novel pre-synaptic model has been proposed by Cervero^[224]. The way in which the facilitated pathways (myelinated A-fibres) are separate from the facilitating pathways (unmyelinated C-fibres) in this process is called a 'heterosynaptic' form of facilitation^[215]. Heterosynaptic facilitation is distinct to 'homosynaptic' facilitation, an example of the latter being 'wind up' i.e. the temporal summation to punctuate stimuli in the zone of capsaicin injection. In 'homosynaptic' facilitation the events responsible

for triggering synaptic strengthening occur at the same synapse that is being strengthened. In 'heterosynaptic' facilitation synaptic strengthening between a presynaptic and a postsynaptic cell may occur as a result of the firing of a third neuron, a modulatory interneuron, whose terminals end on and regulate the strength of the specific synapse^[225].

1.8.6. The Spinal Cord and Brain

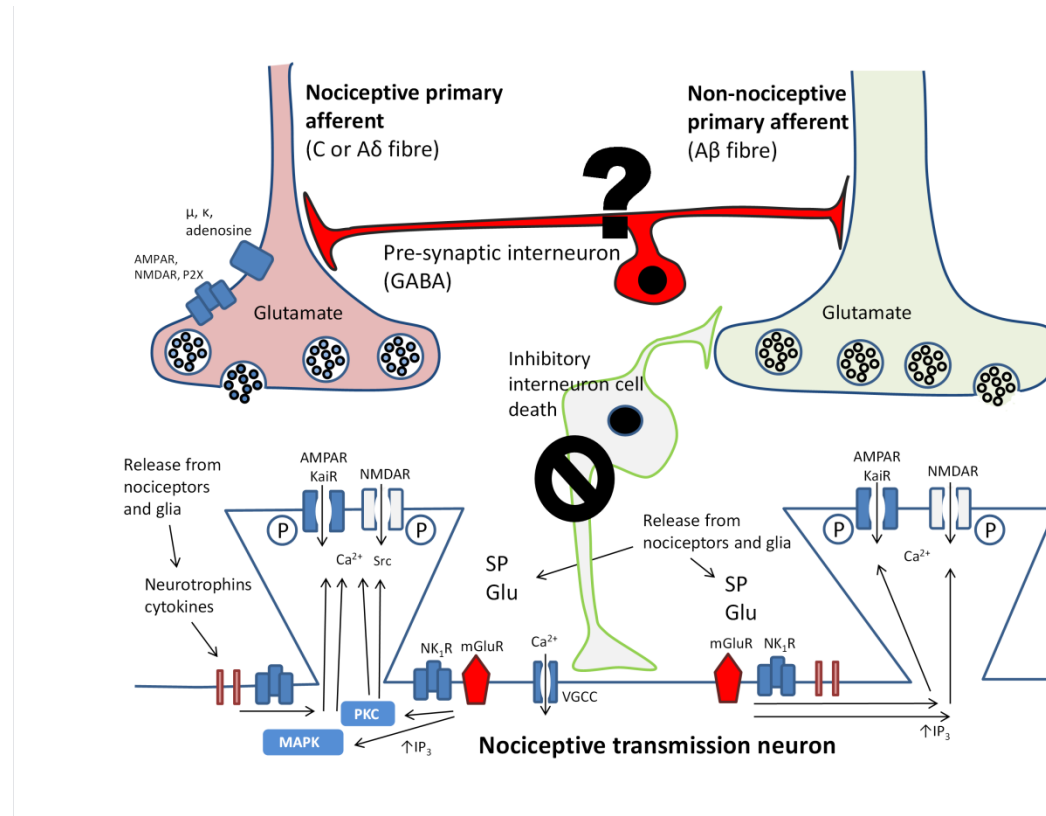
The spinal dorsal horn receives inputs from a wide variety of primary afferent fibres including those from nociceptors and mechanoreceptors^[223]. Primary afferents generally use glutamate as their principal neurotransmitter and form excitatory (glutamnergic) synapses with the neurons in the dorsal horn^[223]. The primary afferent fibres may synapse with two broad types of neuron: projection cells which travel in rostral parts of the spinal cord to the higher brain centres and interneurons which remain in the spinal cord and contribute to local neuronal circuits. Interneurons can be either inhibitory, using GABA/glycine as their neurotransmitter, or excitatory (glutamnergic)^[226]. The intrathecal administration of drugs that antagonise GABA/glycine receptors can cause allodynia, suggesting that one key function of inhibitory interneurons is to suppress the activity of tactile afferents so that they do not normally elicit pain. The dorsal horn also receives an important descending input from higher brain centres, these systems are serotonergic (5-HT) and norepinephrnergic (NA), with serotonin (5-HT₃) receptors and α_2 adrenoceptors being found in the superficial dorsal horn^[226]. Stimulation of these descending inhibitory circuits produces analgesia; the mechanism behind this appears to be related to inhibitory actions on excitatory interneurons and projection neurons. These descending systems may be called the Descending Pain Modulatory System (DPMS).

Examples of the synaptic plasticity of the dorsal horn include the activity dependent plasticity of 'wind-up', the 'heterosynaptic' central sensitisation as previously described,

the 'homosynaptic' increases in response seen in long term potentiation and transcription-dependent central sensitisation^[203]. Wind-up's summative increase in dorsal horn neuron output is related to the SP and glutamate induced change in dorsal horn post synaptic sensitivity^[227]. Heterosynaptic central sensitisation is partly related to transmitters and mediators released from primary afferents and glial cells, these then act at a distance on dorsal horn neurons to produce long-lasting heterosynaptic potentiation of fast synaptic transmission: the two general mechanisms implicated in this are the post-translational processing of ion channels/receptors/regulatory proteins and the cell surface expression/trafficking of ion channels^[220]. Long term potentiation is the homosynaptic increase in response to primary afferent inputs and involves NMDA/AMPA receptor activated pathways^[139 228]. Transcription-dependent central sensitisation involves multiple signalling pathways in the sensory and dorsal horn neurons^[228]. Figure 1-9 summarises our current understanding of some of the mechanisms behind central sensitisation in the spinal cord.

Figure 1-9 - Mechanisms underlying central sensitisation in the spinal cord

(AMPA, AMPA receptor; NMDAR, NMDA receptor; KaiR, kainite receptor; P2X, purinoreceptor; Src, Src tyrosine kinase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; NK1R, neurokinin-1 receptor; mGluR, metabotropic glutamate receptor; SP, substance P; Glu, glutamate; P, phosphate residue; VGCC, voltage-gated calcium channel^[207])



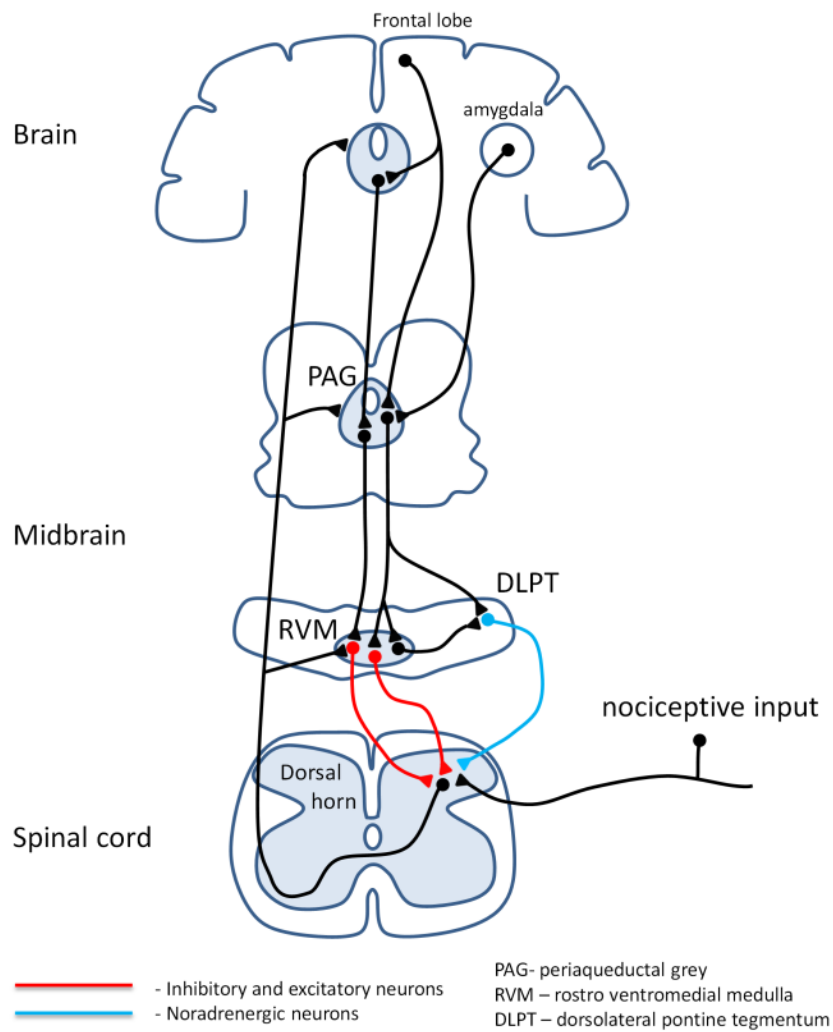
Nociceptive information is transmitted from the spinal cord to the brain via several different pathways^[203]. These ascending pathways include direct projections to the thalamus (spinothalamic tract), direct projections to homeostatic control regions (spinomedullary and spinobulbar) and projections to the hypothalamus/ventral forebrain (spinohypothalamic). The periaqueductal grey (PAG) and reticular formation are important parts of the spinobulbar system. The thalamus is a key structure in pain processing and has ascending projections to multiple areas including the primary and secondary somatosensory cortices, the anterior insular cortex and the cingulate cortex^[229]. The subjective experience of pain is highly complex and involves other regions such as the amygdala, prefrontal cortex, cerebellum and basal ganglia. Key modulatory circuits involving the rostral ventromedial medulla (RVM) and PAG exert

bidirectional control over dorsal horn nociceptive transmission^[229]. This network receives numerous direct and indirect inputs from pain pathways that include the amygdala, the anterior cingulate cortex and the anterior insula; providing a mechanism for the way in which emotion may affect pain perception. The RVM has separate descending anti-nociceptive and nociceptive outputs to the dorsal horn. Figure 1-10 summarises some of the key pain modulating circuitry.

Figure 1-10 - Pain modulating systems

(PAG, periaqueductal grey, RVM, rostroventromedial medulla, DLPT, dorsolateral pontine tegmentum^[207])

Pain modulating systems



1.9.Scope and Aims of Thesis

To my knowledge the role of glutamate and its many receptors has never previously been investigated in human RCT. Therefore this thesis aims to investigate the role of the peripheral glutaminergic system in human RCT.

Broadly the pathogenesis of RCT is incompletely understood and developing the scientific knowledge in this regard is vital for several reasons. The frequent clinical mismatch between structural shoulder tendon failure and the pain symptoms needs to be better understood, so that patients with RCT can be better managed in the future.

It is plausible that a better understanding of the role of the peripheral glutaminergic system in RCT will lead to a better understanding of this common clinical mismatch. Not only may this result in a better understanding of the natural history of RCT, but it may also lead to a better rationales for treating patients with RCT.

There is a paucity of effective treatments for patients with RCT; a better understanding of disease pathogenesis relating to the peripheral glutaminergic system has the potential of identifying novel therapeutic targets which may be the subject of future research.

In this context the specific aims of this thesis are as follows:

- The first aim is to describe the peripheral glutaminergic phenotype at different stages of the disease process in patients with rotator cuff tendinopathy (RCT), including before and after common treatments such as steroid injection and rotator cuff repair. This aim will be addressed in Chapters 3 and 4.
- The second aim is to describe how the peripheral glutaminergic phenotype relates to the severity of pain symptoms as measured by the Oxford Shoulder Score in patients with RCT. This aim will be addressed in Chapter 3.

- The third aim is to determine whether there are significant differences between painful and pain-free rotator cuff tendons. This aim will be addressed in Chapter 5.
- The fourth aim is to explore the effects of glutamate on human tendon derived cells *in vitro* and to determine whether the pharmacological modulation of specific glutaminergic receptors can affect these cellular changes. This aim will be addressed in Chapter 6.

Chapter 2 Methods

2.1 Sampling

2.1.1 Rotator cuff tears

Tendon tissue was obtained as part of the United Kingdom Rotator Cuff Trial (UKUFF) (REC Ref 10/H0402/24) and was collected with informed donor consent in full compliance with National and Institutional ethical requirements, the United Kingdom Human Tissue Act, and the Declaration of Helsinki. The rotator cuff tendon was excised as part of debridement before tendon repair. The rotator cuff tear sizes were classified by the operating surgeon according to the system used by Post, Silver and Singh^[230].

2.1.2 Biopsy technique

A percutaneous ultrasound guided core biopsy technique was used to acquire the supraspinatus tendon tissue samples in several of the patient groups. This validated technique has been developed at our institution and is described in more detail elsewhere ^[231]. All longitudinal tendon biopsies were taken at a consistent location approximately 5–10 mm posterior to the rotator interval under ultrasound guidance. A single biopsy was obtained at each time point with the approximate size of each biopsy being 2 mm by 2 mm by 10mm. In the different patient cohorts biopsies were taken at specific time points before and after treatment. There were no local or systemic complications as a result of the biopsy procedure.

2.1.3 Tendon for *in vitro* work

Tendon tissue was obtained from the Oxford Musculoskeletal BioBank (Oxford REC C 09/H0606/11) and was collected with informed donor consent in full compliance with

National and Institutional ethical requirements, the United Kingdom Human Tissue Act, and the Declaration of Helsinki. Tendon derived cells were isolated by explant culture of both rotator cuff tendon excised as part of debridement before tendon repair and hamstring tendon excess to requirements for autograft repair for anterior cruciate ligament rupture.

2.2 Histology and Immunohistochemical procedures

2.2.1 Sectioning and Fixation

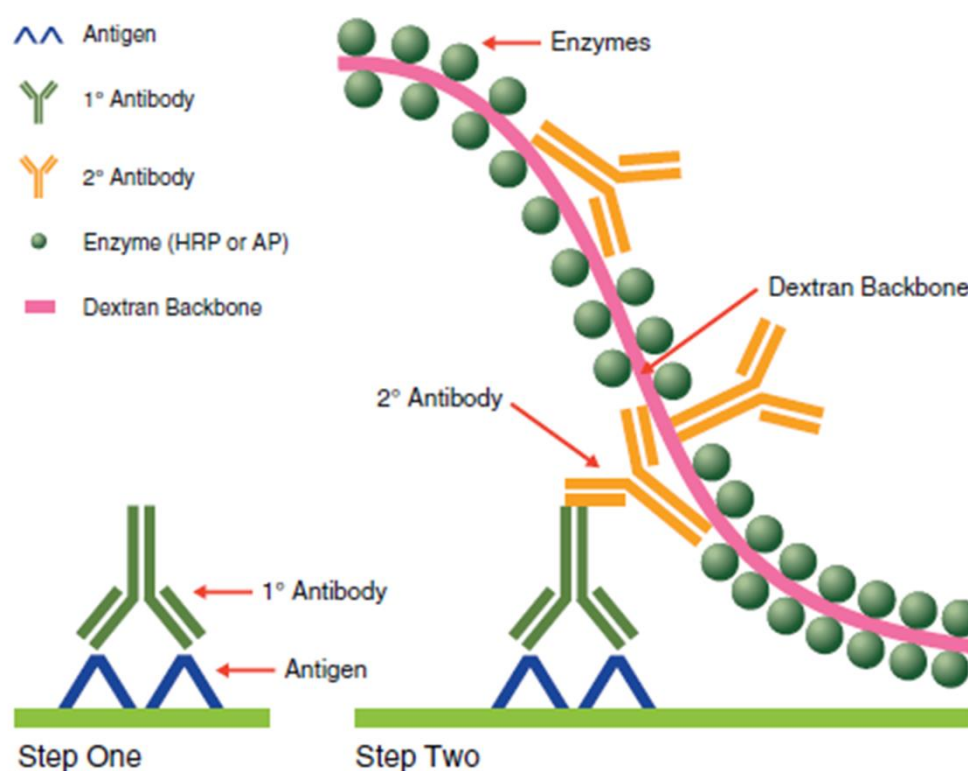
All fresh samples were immediately fixed in 10% buffered formalin and allowed sufficient time for the fixative to penetrate the tissue. Upon full fixation the tissue samples were processed using a Shandon Pathcentre Tissue Processor (Thermo Scientific, Cheshire, U.K.) and embedded in paraffin wax. Tissue was sectioned at 4µm using a rotary RM-2135 microtome (Leica Microsystems Ltd, Bucks, U.K.), and collected onto adhesive glass slides.

2.2.2 Histology and Immunohistochemistry

A Haematoxylin and Eosin (H&E) stain was performed on samples from every patient to assess the general structural condition of the tissue samples and to confirm they were tendon. Prior to antibody staining, tissue sections were taken through deparaffinisation and target retrieval steps using an automated PT Link (Dako, Denmark). All antibody staining was performed using an Autostainer Link 48 (Dako, Denmark). All immunohistochemical staining protocols used a peroxidase-blocking reagent and the EnVision™ FLEX (Dako, Denmark) detection system which detects primary mouse and rabbit antibodies and the reaction is visualized by EnVision™ FLEX DAB+ Chromogen. Haematoxylin was used as a counterstain. The Dako EnVision™ FLEX /HRP detection

reagent consists of a dextran backbone to which a large number of peroxidase (HRP) molecules and secondary antibody molecules have been coupled. The chemistry of the coupling reaction permits the binding of up to 100 HRP molecules and up to 20 antibody molecules per backbone (Figure 2-1).

Figure 2-1 Diagram demonstrating the principle of the EnVision™ FLEX detection system (taken with permission from Dako education guide)



The EnVision™ FLEX+ Rabbit or EnVision™ FLEX+ Mouse linkers were used for the signal amplification of primary rabbit antibodies or primary mouse antibodies, based on the results of preliminary antibody testing; these linkers are buffered solution containing stabilising protein and an antimicrobial agent.

Each antibody was optimised using a series of steps depending on the quality of staining observed. First the antibody was trialled at the manufacturer's recommended

concentration with and without the appropriate linker. If there was no staining present then the concentration of antibody was increased sequentially until adequate staining was present. Likewise if there was excessive non-specific staining then the antibody's concentration was sequentially diluted until staining was deemed to be optimal. The full staining protocols are detailed in Chapter 0. Sections were incubated with the primary antibodies for 60 minutes unless otherwise stated.

Details of antibodies used and their working concentrations are detailed in

Table 2.1.

Table 2.1 Details of antibodies used

Antibody	Raised in	Linker	Dilution	Make	Reference	Concentration
α 2a adrenergic receptor	rabbit	1/1000	rabbit	Abcam	Ab85570	1mg/ml
AMPA	rabbit	none	1/200	Abcam	Ab31232	0.6mg/ml
CD34	mouse	mouse	1/50	Dako	M7165	12mg/L
Caspase-3	rabbit	None	1/200	Abcam	Ab13847	0.6mg/ml
CD45	mouse	mouse	1/50	Dako	M0701	
CD68	mouse	mouse	1/50	Dako	M0814	
CD206	mouse	Mouse	1/200	Abcam	Ab117644	0.5mg/ml
GAP43	rabbit	rabbit	1/500	Abcam	Ab12274	Whole Antiserum
Glutamate	rabbit	rabbit	1/100	Millipore	AB5018	ND
HIF-1 α	mouse	mouse	1/250	R&D	MAB1935	0.5mg/ml
KA1	rabbit	rabbit	1/4000	Abcam	Ab67402	1mg/ml
LDH	rabbit	rabbit	1/250	Abcam	Ab134187	ND
mGluR1	mouse	mouse	1/250	R&D	MAB4836	0.5mg/ml
mGluR2	rabbit	rabbit	1/350	abcam	Ab118447	1mg/ml
mGluR3	sheep	rabbit	1/200	R&D	AF4668	0.2mg/ml
mGluR4	sheep	rabbit	1/100	R&D	AF7387	0.2mg/ml
mGluR5	rabbit	rabbit	1/200	Abcam	Ab53090	1mg/ml
mGluR7	rabbit	none	1/150	Abcam	Ab53705	1mg/ml
mGluR8	mouse	mouse	1/150	R&D	MAB5277	0.5mg/ml
Neurokinin-1	rabbit	rabbit	1/100	Neuromics	RA20053	1mg/ml
NGF	rabbit	rabbit	1/250	Abcam	Ab6199	1mg/ml
NMDAR1	mouse	mouse	1/140	BD	556308	0.5mg/ml
P53	mouse	mouse	1/50	Invitrogen	AHO0152	200 μ g/ml
P75	mouse	none	1/400	Abcam	Ab8874	Whole Antiserum
PCNA	mouse	none	1/200	DAKO	M0879	327mg/L
PGP9.5	rabbit	rabbit	1/200	Thermo	PA1-21097	0.2mg/ml
Substance P	rabbit	rabbit	1/500	Millipore	AB1566	ND
TrkA	goat	rabbit	1/100	R&D	AF175	0.1mg/ml
Tyrosine Hydroxylase	sheep	rabbit	1/250	R&D	AF7566	0.2mg/ml
VEGF	goat	rabbit	1/200	R&D	AB-293-NA	1mg/ml
Controls						
Immunoglobulins	rabbit	both	1/2000	Dako	X0903	20mg/ml
IgG1	mouse	both	1/600	Dako	X0931	6.2mg/ml

Immunoglobulins	goat	both	1/40	Dako	F0250	0.4mg/ml
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Human brain tissue sections (Neuromics, Minnesota, USA) were used as positive controls for each neuronal antibody staining run. Isotype control staining was also performed. Every tissue sample had one tissue section stained per antibody. The staining for each antibody was carried out simultaneously on the same run for all the sections.

2.2.3 Immunofluorescence to determine cell types expressing neuromodulators

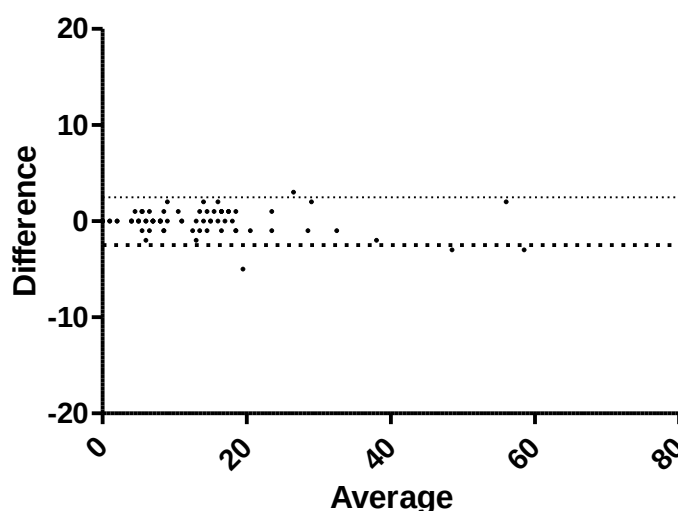
After antigen retrieval described in A1.1, tissues were blocked in 5% normal goat serum (Sigma G9023) in phosphate buffered saline (PBS) for 60 minutes in a humid chamber at room temperature. Sections were subsequently incubated with the primary antibodies diluted in 5% normal goat serum in PBS for 2 hours. Antibody combinations included dual labelling with macrophage marker CD206 with nociceptive markers NMDAR, PGP9.5 or glutamate at the same concentrations shown in

Table 2.1. After washing in PBS Tween, slides were incubated in the secondary antibodies diluted 1:200 in 5% normal equine serum (Sigma) in PBS for 2 hours. Alexa Fluor secondary antibodies were purchased from Life Technologies and goat anti-mouse IgG₁ was purchased from Southern Biotech. After washing with PBST, sections were incubated in 2 μ M POPO-1 nuclear counterstain (Life Technologies) diluted in PBS containing 0.05% Saponin (Sigma) for 20 minutes. Tissue auto fluorescence was quenched with a solution of 0.1% Sudan Black B (Appllichem) in 70% ethanol for 10 minutes^[232]. After washing, slides were mounted using fluorescent mounting medium (VectaShield) and stored at 4°C until image acquisition.

2.2.4 Image Analysis and Statistics

The assessment of the microscopic appearance of the slides stained for Haemotoxylin/Eosin was undertaken using ImageJ® (public domain software, National Institutes of Health, Bethesda, MD, USA: <http://rsb.info.nih.gov/ij/>). The nuclei count program was validated against the counts of two blinded observers using Bland Altman plots and the calculation of the Intraclass Correlation Coefficient (ICC - 0.971)(Figure 2-2).

Figure 2-2 - Bland Altman plot of manual counts: difference versus average



Note lines above and below $y=0$ represent $\pm 2SD$

The assessment of the microscopic appearance of the slides immunostained with DAB was undertaken by a single blinded investigator. Nikon inverted microscope (Nikon, Tokyo, Japan) using Axiovision software (Zeiss, Cambridge, UK) was used to capture 20 images using a systematic approach starting at one corner of the tissue moving systematically in a horizontal-vertical-horizontal manner until the tissue section was

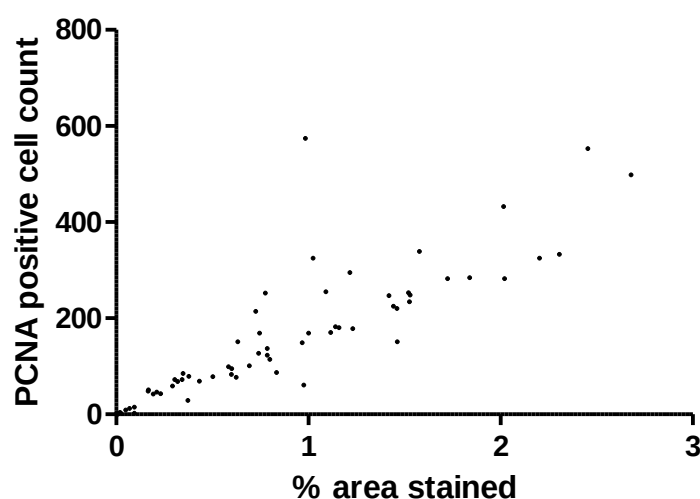
exhausted using 100X magnification with oil immersion. A minimum of 10 images were captured for each tissue section. Each antibody set was imaged using the same exact light intensity at the same sitting. The images were then analysed using an ImageJ® algorithm in order to quantify the percentage (%) of DAB staining present in each image. For each antibody stain DAB threshold levels were manually determined and kept constant for each antibody dataset to ensure that results were comparable. This mode of image analysis using colour convolution has been well described in several other studies [233-235] which have shown that measure of percentage area stained correlates well with the subjective grading systems used by experienced histopathologists [236 237]. The percentage area stained was adjusted for cell number using the validated cell counting program for antibodies which resulted in significant staining of the resident fibroblast population. Positive vessel counts were carried out manually. Statistical analysis was carried using SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) and GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Statistical significance was set at a level of $p < 0.05$ unless otherwise stated.

2.2.5 Staining quantification metrics: reliability and validity

Inter-observer and intra-observer reliability for this method have been tested using the Intraclass Correlation Coefficient (ICC). These compared the intra-rater and inter-rater observations. The ICCs relating to inter-rater measurement were between 0.724 and 0.909 for three full patient sets (minimum 70 patients). The intra-observer ICC was 0.830 after one month. We compared the quantitative analysis results with blinded subjective analysis as to the presence (1) or absence (0) of DAB staining as the use of subjective grading is a well validated current method of assessing staining^[238]. Significant differences were found between groups ($p < 0.0001$) for all antibody datasets analysed,

confirming the criterion validity of the method. The quantitative results for CD34 were also correlated against the results of the well validated method of manual vessel counts yielding Spearman correlation coefficients of 0.853 and 0.839. The quantitative results for PCNA (Proliferating Cell Nuclear Antigen), p53, CD45 and NMDAR1 revealed a strong correlation between % area stained per cell and % positive cells as counted manually (Spearman Rhos of 0.943, 0.967, 0.857 and 0.812 respectively). Figure 2.2 demonstrates the correlation between the total PCNA positive cell count and the mean % area stained (Spearman Rho 0.943, $p=2.5 \times 10^{-34}$).

Figure 2-3 Plot of total PCNA positive cells versus % area stained



2.3 *In vitro* Cell Culture

2.3.1 Cell culture protocols

Tendon derived cells were cultured in DMEM-F12 containing 10% FBS (Gibco, Life technologies) at 37°C and 5% CO₂ (this solution contains 0.05mM glutamic acid). Cells were fed regularly every 2-3 days. In experiments involving glutamate and/or drug treatment, cells were passaged at 80% confluence and used up until passage 2. Cells were plated at a density of 40-50,000 cells per ml.

2.3.2 Solutions preparation and treatment

Glutamate free DMEM-F12 media was custom made by Life technologies (ThermoFisher Scientific, USA). L-Glutamic acid (product number G5667; Sigma Aldrich, St Louis, USA) was dissolved in DMEM-F12 and the pH was normalised to the pH of standard media by titrating NaOH (1M) solution. 1M NaCl was added to the control solution to ensure an equivalent osmolality to the 30mM glutamate solution. The pH of all solutions was checked to be the same as standard media (7.25-7.35) using a calibrated SevenEasy pH meter (Mettler Toledo, Leicester, UK) and pH Fix indicator strips (Fisher Scientific, Loughborough, UK). MK 801 maleate (ab120027; Abcam, United Kingdom) was dissolved in PBS, while NBQX (ab120045; Abcam, United Kingdom) was dissolved in DMSO. The different concentrations of solutions were then made up using PBS or DMSO and DMEM-F12 so that each solution contained an equivalent volume of solvent. In the low glutamate concentration experiments, cells were treated with glutamate free DMEM-F12 media, standard DMEM-F12 (0.05mM glutamate) and 1.875mM glutamate in DMEM-F12. In the high glutamate concentration experiments, cells were then treated with either vehicle control or glutamate (0.1875mM to 50mM). In the experiments

involving pharmacological treatment, cells were pre-treated with either MK-801/AMN082/NBQX (10 μ M to 200 μ M) or vehicle control (phosphate buffered saline (PBS) or DMSO (dimethyl sulfoxide) dissolved in identical amount of DMEM-F12); this was done 2hrs before glutamate exposure in experiments involving glutamate.

2.3.3 Determination of cell viability

Cell viability was determined using Alamar Blue, an indicator dye that detects respiring cells by sensing electron transport chain activity (BioSource, Camarillo, CA). Cells were cultured in 96-well plates in 10% FBS/DMEM-F12 with treatments as appropriate for individual experiments for 24 hrs. Cells were plated at a density of 4 to 5,000 cells per well. At both a zero time point and two hours before the end of the culture period, Alamar Blue (1:11 vol/vol) was added to each well. At the zero time point and at the end of the culture period, fluorescence was measured using a Spectramax Gemini XS plate reader (Molecular Devices, Sunnyvale, CA). All viability experiments were conducted in quadruplicate.

2.4 Immunocytochemistry

Tendon derived cells were grown on 1-cm diameter cover glasses for 24 hours and plated at a density of approximately 10,000 cells per well. The staining protocols are detailed in Appendix 2: Laboratory protocols. Nikon inverted microscope (Nikon, Tokyo, Japan) using Axiovision software (Zeiss, Cambridge, UK) was used to capture images systematically using 20X magnification within 24 hours of staining using the DAPI, GFP and Texas Red channels. The assessment of the staining intensity was undertaken using ImageJ®. The ImageJ® plugin 'measure RGB' was used to measure the mean pixel intensity to quantify the nuclear staining density. Optical density has been

shown to be a reliable measure of the amount of immunohistochemical staining present^[237].

2.5 RNA extraction and cDNA synthesis

For basal gene expression (untreated cells) RNA extraction was then carried out when passage 2 cells were at 80% confluence 1 day after feeding using the TRIzol method. All gene expression relating to glutamate and drug experiments were carried out using the Cells-to-cDNA method.

2.5.1 TRIzol method

Cells were treated with TRIzol® Reagent and the samples were stored at -80°C. RNA was then extracted using the Direct-zol™ RNA MiniPrep kit (Zymo Research, United States) according to the manufacturer's instructions. This protocol included in-column DNase I digestion. The quality of the RNA was then tested using the NanoDrop 1000 spectrophotometer prior to the samples being stored at -80°C. The ratio of absorbance of 260nm and 280nm was used to determine the purity with all samples achieving a minimum of 1.80. cDNA was synthesized using the transcriptor first strand cDNA synthesis kit (Roche, UK) and was then stored at -20°C until further use.

2.5.2 Cells-to-cDNA method

Treated cells (glutamate or drug experiments) were harvested into 30 µl of Cells-to-cDNA II cell lysis buffer (Applied Biosystems) and subjected to heat inactivation of endogenous RNases followed by DNase I treatment according to the manufacturer's instructions. The samples were then stored at -20°C. For reverse transcription, DNase I-treated lysates (8 µl) were transferred to a fresh 96-well PCR plate and combined with 2.5 mM dNTP (Bioline) and 200 ng random primers (Invitrogen) at 70°C for 5 minutes.

Reverse transcription was carried out in a total volume of 20 μ l using 100 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen), according to the manufacturer's instructions, in the presence of 40 units of RNasin (Promega), followed by the addition of 30 μ l of nuclease-free water. cDNA was then stored at -20°C until further use.

2.6 Quantitative RT-PCR

Table 2.2 - Details of primers used

Primer	Company		5' to 3' sequence (or Qiagen code if QuantiTect)	Annealing temperature (°C)	Amplicon size (bp)
Aggrecan	Qiagen		QT00001365		62
Beta actin	Qiagen		QT00095431		146
CASP3	Qiagen		QT00023947		147
CD45	Qiagen		QT00028791		108
CD55	Qiagen		QT00099190		136
COL1A1	Qiagen		QT00037793		118
COL3A1	Qiagen		QT00058233		95
Decorin	Qiagen		QT00032459		87
GAPDH	Qiagen		QT00079247		95
GRIK1	Primer Design	Fwd Rev	CCTCTCTCCTTCTTCGTTAGTCT TCTCCCTCATTTCGTCCTTTGG	57	99
GRIN1	Primer Design	Fwd Rev	GCCATCCTAGTTAGCCATCCA ACGGGTATGCGGTAGAAGC	57	89
GRM1	Primer Design	Fwd Rev	GCTGGCATCTTCTTGGTTATG GTCACTAAAGCAGAGTAGCACATC	58	125
GRM2	Qiagen		QT00020356		133
GRM5	Primer Design	Fwd Rev	GTGACCTGAACCCATTGC ATGATGAAGACTACAGTAACAAAACAG	56	82
GRM7	Qiagen		QT00069727		133
IL1B	Qiagen		QT00021385		117
IL6	Qiagen		QT00083720		
LDHA	Primer Design	Fwd Rev	GGAATGAATGTTGCTGGTGTCT GGATGTGTAGCCTTTGAGTTTGA	57	141
MRC1 (CD206)	Primer Design	Fwd Rev	TGGGTTCTCTCTGTTTCC CAACATTTCTGAACAATCCTATCCA	56	114
mTOR	Primer Design	Fwd Rev	CTGATGCTGGACCGTCTGA TCTTGTTAGTCTAAATGGAATCTTCTC	56	114
PCNA	Primer Design	Fwd Rev	GCCGAAACAGCTAGACTTT TCGTTGATGAGGTCCTTGAGT	57	140
TBP1	Qiagen		QT00000721		132
TNF1	Qiagen		QT00029162		98
UCLH1	Qiagen		QT00092666		110
VEGF	Primer Design	Fwd Rev	CCAGGAAAGACTGATACAGAACG GGTTTCTGGATTAAAGGACTGTTC	57	93

Real-time quantitative PCR were performed using the ViiA™ Real Time PCR system (Fisher Scientific, Loughborough, UK), a Fast SYBR® Green PCR kit (Life technologies, UK) and QuantiTect Primer Assays (QIAGEN, Hilden, Germany), as well as validated assays designed by Primerdesign (Southampton, UK). Primer details and sequences are listed in Table 2.2 - Details of primers used are detailed in Table 2.2. Samples without

reverse transcriptase served as negative controls in real-time PCR to exclude genomic DNA contamination. No-template control was included to indicate there was no contamination in real-time PCR reagents. Samples were run in triplicate with a coefficient of variation between duplicates of <1.0 cycle, and the average value was used for analysis. A total of 250ng of cDNA was used in each reaction whose volume was in total 20 μ L. A total of 5 μ L of cDNA was used within the total reaction volume of 20 μ L. Standard curves were created for all genes measured and a set threshold of 0.15 was used for all calculations of relative gene expression. Relative quantification is expressed as $2^{\Delta C_t}$, where ΔC_t is $C_t(\text{target gene}) - C_t(\text{TBP})$. Two housekeeping genes were used (TBP and β -actin) and results were consistent using both.

Chapter 3 The Neurohistology of Rotator Cuff Tears

3.1 Abstract

3.1.1 Background

Pain related to rotator cuff tendinopathy is a common clinical problem, but little is known regarding the origin and cause of pain from the tendon substance. The aim of this study was to describe the peripheral neuronal phenotype in painful rotator cuff tears and to determine correlations between tissue change and patient symptomatology.

3.1.2 Methods

Tendon samples of the supraspinatus were taken from patients undergoing surgery to repair a rotator cuff tendon tear. Patients were classified as having small/medium or large/massive tears. Control tissue was obtained from patients undergoing surgery for posttraumatic shoulder instability. Immunohistochemical techniques were performed using antibodies to known nociceptive and neuronal markers as well as general tissue structural markers.

3.1.3 Results

A significant increase in the expression of glutamate was seen in tendon tears. There was no correlation between tissue changes and patient symptomatology. There were differences in the expression of metabotropic and ionotropic glutamate receptors. Expression changes were also observed for markers of the sensory and autonomic systems; however, no differences were found in neurotrophins.

3.1.4 Conclusion

This study shows that glutamate is significantly more abundant and that the relative expression of several glutaminergic receptors are significantly altered in painful supraspinatus tears. The precise mechanisms behind these changes are unclear. Further work is needed to determine the specific role that glutaminergic system plays in disease pathogenesis and how this relates to pain symptomatology.

3.2 Introduction

The presence of glutamate in tendon disease was originally determined by Alfredson et al^[239] who noticed an increase in glutamate in patients with painful chronic Achilles tendinopathy. This has been confirmed in more detail in human patellar tendinopathy and rat tendinopathic models^[167 173] with significant increases in glutamate expression measured by microarray and immunohistochemistry, respectively. No previous study has investigated the role of glutamate and its receptors in human rotator cuff tendinopathy.

3.3 Aims and Hypotheses

The primary aim of this study was to describe changes in tendon histology, focusing on the glutaminergic system, which occur in painful full-thickness supraspinatus tendon tears in the rotator cuff requiring surgery. I hypothesised that torn tendon would have a different neurohistological phenotype when compared to healthy control tendon.

The secondary aim was to determine if histological and immunohistochemical features are correlated with the severity patient symptomatology. The null hypothesis was that there would be no correlation between tendon histology and symptom severity.

3.4 Methods

3.4.1 Tissue cohort

This study utilized a cohort of patients participating in the United Kingdom Rotator Cuff Trial (UKUFF), funded by the National Institute for Health Research Health Technology Assessment Programme in the United Kingdom (project no. 05/47/02). This was a

multicentre randomized controlled trial designed to investigate the clinical effectiveness and cost-effectiveness of open versus arthroscopic surgical repair for supraspinatus tendon tears in the rotator cuff. The tissue study protocol was approved by the multicentre research and ethics committee (REC reference 10/H0402/24) and all local National Health Service research and development committees. Patients were recruited from orthopaedic clinics and had symptomatic degenerative full-thickness supraspinatus tears diagnosed by ultrasound or magnetic resonance imaging and confirmed at surgery. The rotator cuff tear sizes were classified by the operating surgeon according to the system used by Post, Silver and Singh^[230]. Before surgery, each patient completed the Oxford Shoulder Score (OSS) and Shoulder Pain and Disability Index (SPADI), both of which are well validated and widely used clinical outcome measures. Control supraspinatus tendon specimens were obtained from patients who underwent shoulder surgery for posttraumatic instability, and who had intact non-degenerative supraspinatus on ultrasound and confirmed at surgery. Ethical approval for this study was granted by the local research ethics committee (Oxfordshire REC B reference 09/H0605/111).

The exclusion criteria for all patients in this study included previous shoulder surgery, dual shoulder pathological lesions, significant problems in the other shoulder, rheumatoid arthritis or systemic inflammatory disease, significant osteoarthritis problems, and significant neck problems.

3.4.2 Sampling, sectioning and fixation

The lead surgeon took all tissue samples from the edge of the supraspinatus tendon tear during surgery in patients undergoing arthroscopic or open tendon repair. This is a commonly performed surgical procedure in which significant tissue is removed as part of the repair. The percutaneous ultrasound guided core biopsy technique that has been

described in the Chapter 2 in obtaining the control group biopsies. There were no local or systemic complications as a result of the biopsy procedure. The sectioning and fixation methods have been described in Chapter 2.

3.4.3 Histology and Immunohistochemical procedures

The histological and immunohistochemical procedures have been described in Chapter 2. Details of antibodies used and their working concentrations, as well as all staining protocols, can be found in Appendix 2: Laboratory protocols. Bonar scores were calculated as described previously^[240].

3.4.4 Image analysis and statistics

Image analysis and the validation of these techniques have been described in Chapter 2. Statistical analysis was carried using SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) and GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Statistical significance was set at a level of $p < 0.05$ unless otherwise stated. Histograms for all data sets were analysed. All the data was non-parametric in distribution. Unpaired t-tests were used to test for the difference between groups that were parametric in distribution, while Mann-Whitney U test was used for testing for differences between groups that were non-parametric in distribution. Spearman correlation coefficients were calculated to test the relationships between tissue markers and the OSS/ SPADI values.

3.5 Results

3.5.1 Metrics: reliability and validity

This has been described in Chapter 2.

3.5.2 Demographics

Patients in the rotator cuff cohort had a mean age of 61years (range, 50-78 years) and comprised 39 men and 25 women (Table 3.1). The median duration of symptoms ranged from 7 months to 2 years. There was no significant difference in either symptom duration or the level of preoperative symptoms between small/medium and large/massive tears (Table 3.1) as measured by the OSS and SPADI. No correlation was found between the OSS or SPADI values with any of the immunohistochemistry antibody staining. The control patients were free from pain at the time of surgery. There were 14 male and 2 female patients, with a mean age of 23 years (range, 17-29 years).

Table 3.1 Summary of Study Demographics and Patient Outcomes Quantified by Baseline Questionnaires

	Control (No Tear)	Tear (All Tears)	Small/Medium Tears	Large/Massive Tears
No. of patients	16	64	32	32
Age, mean +/- SD, years	23.1 +/- 3.8	61.4 +/- 6.6	60.4 +/- 6.6	62.5 +/- 6.4
Patient sex, male/female, n	14/2	39/25	15/17	24/8
Median length of symptoms, months	N/A	14 (9-24)	13 (9-25)	16 (7-24)
Mean tear size, cm	N/A	3.25 (1.125-5.75)	1.25 (1-2.4)	5.5 (4-6)
Median OSS score	N/A	24 (17-30)	25 (17-31)	24 (17-30)
Median OSS pain element score	N/A	5 (2-6)	2 (2-6)	2 (2-7)
Median SPADI pain score	N/A	38 (31-45)	38 (31-45)	39 (28-47)

Values in parentheses are interquartile ranges (25th-75th percentile). N/A, not available; OSS, Oxford Shoulder Score (scale of 0-48 [pain element scale of 0-16], with 0 = most painful); SD, standard deviation; SPADI, Shoulder Pain and Disability Index (scale of 0-50, with 50 = most painful).

3.5.3 Basic histology and cell viability

Histopathological observations using H&E staining of controls showed a healthy tendon structure (Figure 3-1). Stained tissue from tendon tears showed rounded tendon cell

nuclei with a high degree of collagen fibre separation and loss of architecture. A significant increase in the number of haematoxylin-positive nuclei was seen in tear tissue (n=64) compared with controls (n=16), and there were significantly more nuclei present in large/massive tears compared with small/medium tears ($p=0.012$). The mean standard deviation of the nuclei count was significantly higher in the tissue from tendon tears than control ($p=0.01$), demonstrating the more irregular structure in disease. A change in tendon cell size was also confirmed by a statistically significant increase in the cell nuclear area in tendon tear tissue compared with controls ($p=0.02$) (Table 3.2). The number of proliferative cells significantly decreased ($p=0.004$) (Figure 3.1) in all tear sizes compared with controls; however, there were no changes in the percentage of cells expressing the tumour suppressor protein p53 (Table 3.2). There was a significant decrease in vascularity (mean vessel count) in torn tendons compared with controls ($p<0.0001$) (Table 3.2 and Figure 3-1) using CD34, but no change in vascular endothelial growth factor (VEGF) was determined. Hypoxia-inducible factor 1a (HIF1a) was increased in large/massive tears compared with small/medium tears (Table 3.2).

Figure 3-1 Haematoxylin and Eosin (A-D) and Immunohistochemical (E-L) staining of human rotator cuff tendon. Photomicrographs showing the staining in control and tear groups for H&Es (A-D), CD34 (E-H) and PCNA (I-L). Scale bar: 20 μ m.

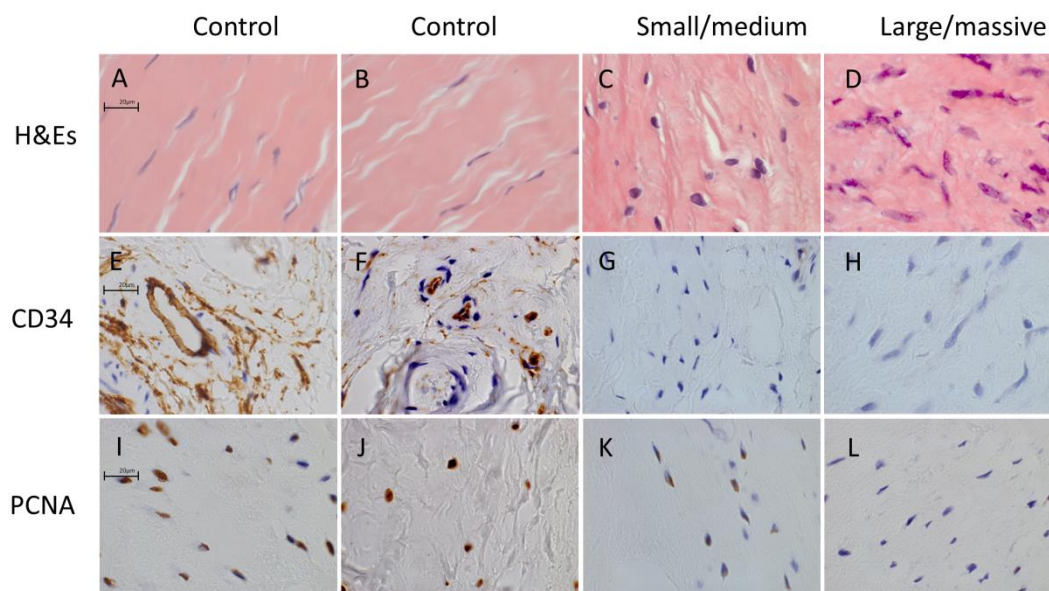


Table 3.2 Data representing the basic histological characteristics and quantified immunohistochemistry of tissue viability

	Control (No Tear)	Tear (All Tears)	Small/Medium Tears	Large/Massive Tears
Median vessel count	2.21 (1.34-3.13)**	0.31 (0.0-0.86)**	0.004 (0.0-0.37)	0.06 (0.0-0.29)
Median nuclei count	8.90 (6.95-14.2)**	12.97 (8.66-19.55)**	11.28 (6.77-16.71) †	16.29 (11.53-23.87) †
Median standard deviation of nuclei count	4.58 (3.90-8.02)**	7.85 (5.13-12.97)**	7.50 (4.37-13.05)	8.12 (5.84-13.22)
Median cell nuclear area, %	0.09 (0.07-0.1)*	0.11 (0.08-0.15)*	0.10 (0.08-0.14)	0.13 (0.08-0.16)
Bonar score	2 (1-2) **	10 (8.25-10) **	9 (8-10)	10 (9-11)
Proliferation, % of PCNA-positive cells	78 (64-100)**	41 (18-71)**	29 (2-56)	55 (19-70)
p53, % of p53-positive cells	28 (20-36)	26 (9.7-71)	24 (5-55)	26 (13-58)
HIF1a, median % of area stained ^{pc}	0.05 (0.019-0.075)	0.027 (0.007-0.097)	0.018 (0.003-0.046)†	0.045 (0.01-0.12)†
VEGF, median % of area stained ^{pc}	0.018 (0.004-0.26)	0.06 (0.02-0.13)	0.08 (0.03-0.16)	0.04 (0.01-0.12)

SD (standard deviation) and IQR (interquartile range) (p25-p75) are shown, unless otherwise stated

*- p <0.05 ** - p<0.01 (between control and tear groups), † p <0.05 †† - p<0.01 (between small/medium and large/massive tear groups), ^{pc} – per cell

3.5.4 Neurohistological results

The neurohistological results are summarised in Table 3.3, Figure 3-2 and Figure 3-3. Additional Figures demonstrating the immunohistochemical staining are included in Appendix 3 - Additional Figures. There were significant differences in the expression of glutamate and some of its related receptors between controls and torn tendons. There was a significant increase (p =0.0002) in glutamate staining in all tears compared with controls; however, there were no differences between the tear size groups. The staining of the ionotropic glutamate receptor, NMDA receptor 1 (NMDAR1), was strongly increased in all tear sizes compared with the controls with no tears (p=0.0008). Glutamate staining was of both the cells within tendon and vascular structures. NMDAR1 staining was of some cells residing within tendon and vascular structures.

NMDAR2 did not appear to be expressed in any tendon samples (data not shown). The staining of mGluR7 was dramatically reduced ($p<0.0001$) in all tears versus control. The mGluR7 staining was largely related to endothelial cells.

Staining of the Tachykinin receptor, neurokinin-1 (NK-1) was significantly lower in disease compared with controls ($p=0.007$). No significant changes were seen in the expression of selected neurotrophins and their receptors. Of note the staining of NGF, NK-1 and RAMP1 was virtually exclusively of vascular structures and not of resident fibroblasts. Staining of tyrosine hydroxylase (TH) was significantly reduced in all tears versus control ($p=0.019$). Image analysis determined a significant difference in the percentage of staining of the general nerve marker protein gene product 9.5 (PGP9.5) between control and all tears ($p=0.015$). PGP9.5 staining was of vascular structures and a subpopulation of cells within the tendon; the increase in PGP9.5 staining in controls was likely related to the greater vascularity of this tissue.

3.5.5 Correlation between symptoms and histological markers

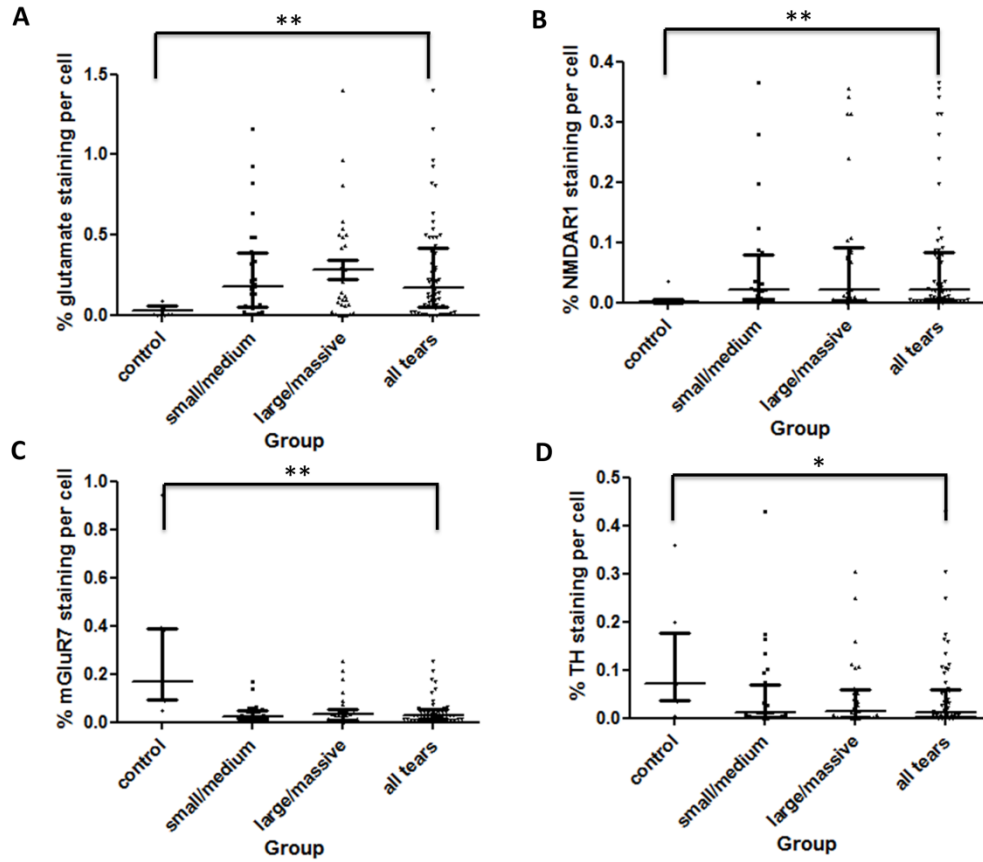
There was a significant correlation between the OSS and the SPADI pain score (Spearman $r = -0.822$, $p<0.0001$). There were no significant correlations between either the OSS or the SPADI pain score (Spearman $R>0.4$) and any of the histological markers. Patients were divided into two groups (pain positive and pain negative) based on the median OSS. No tissue marker was significantly different in the pain positive versus the pain negative groups. There was no significant differences in OSS or the SPADI pain score when patients were divided into two groups based on sex (male and female) and age (above mean and below mean).

Table 3.3 Neuronal quantitative results in format p50 (p25-p75), % area stained unless otherwise stated (pc – denotes % area stained has been adjusted per cell)

Neuronal Group	Control (No Tear)	Tear (All Tears)	Small/Medium	Large/Massive
Glutamate ^{pc}	0.002 (0.001-0.06) **	0.17 (0.05-0.42) **	0.18 (0.05-0.39)	0.14 (0.06-0.48)
NMDAR1 ^{pc}	0.001(0.0001-0.005) **	0.02 (0.004-0.08) **	0.02 (0.004-0.08)	0.02 (0.004-0.09)
mGluR1 ^{pc}	0.08 (0.003-0.36)	0.13 (0.04-0.22)	0.16 (0.024-0.25)	0.14 (0.04-0.24)
mGluR2 ^{pc}	0.08 (0.04-0.27)	0.14 (0.07-0.24)	0.21 (0.11-0.39)	0.13 (0.06-0.18)
mGluR3 ^{pc}	0.0004 (0.0001-0.02)	0.006 (0.001-0.02)	0.006 (0.001-0.03)	0.006 (0.001-0.01)
mGluR4 ^{pc}	0.004 (0.002-0.09)	0.02 (0.004-0.07)	0.04 (0.005-0.09)	0.01 (0.003-0.05)
mGluR5 ^{pc}	0.08 (0.06-0.12)	0.06 (0.01-0.14)	0.08 (0.02-0.17)	0.04 (0.01-0.12)
mGluR7 ^{pc}	0.17 (0.09-0.39) **	0.03 (0.01-0.05) **	0.02 (0.03-0.05)	0.03 (0.03-0.05)
mGluR8 ^{pc}	0.83 (0.32-0.96)*	0.1 (0.01-0.51)*	0.05 (0.01-0.42)	0.24 (0.03-0.67)
NGF	0.17 (0.01-0.94)	0.22 (0.03-0.86)	0.30 (0.03-0.75)	0.14 (0.02-1.2)
NK-1	0.26 (0.05-0.59) **	0.03 (0.01-0.08)**	0.02 (0.01-0.04)	0.04 (0.018-0.24)
NPY ^{pc}	0.1 (0.003-0.29)	0.01 (0.002-0.05)	0.004 (0.002-0.03)	0.02 (0.003-0.06)
PGP9.5 ^{pc}	0.11 (0.08-0.33)*	0.06 (0.02-0.15)*	0.04 (0.01-0.10)	0.09 (0.025-0.16)
P75 ^{pc}	0.10 (0.05-0.17)	0.06 (0.02-0.13)	0.06 (0.02-0.13)	0.04 (0.01-0.19)
RAMP	0.44 (0.24-1.0)	0.23 (0.07-0.99)	0.16 (0.02-0.41) †	0.38 (0.08-2.4) †
TH ^{pc}	0.07 (0.04-0.18)*	0.01 (0.001-0.06)*	0.01 (0.001-0.07)	0.01 (0.001-0.06)
TrkA	1.0 (0.62-1.5)	1.3 (0.37-3.22)	1.21 (0.40-2.65)	1.44 (0.37-3.70)

*- p <0.05 ** - p<0.01 (between control and tear groups), †- p <0.05 †† - p<0.01 (between small/medium and large/massive tear groups)

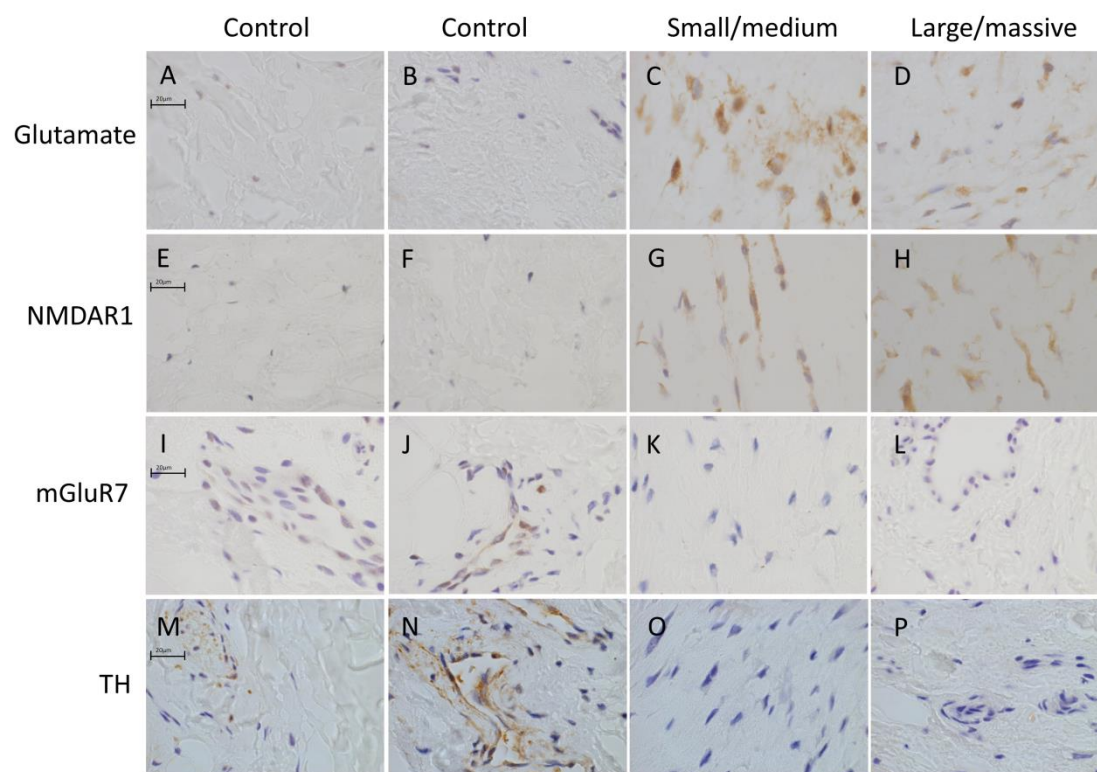
Figure 3-2 Plots of the percentage of 3,3'-diaminobenzidine (DAB) staining for individual neuronal antibodies



(A) glutamate – mean % area stained normalised to cell number (B) NMDAR1 – mean % area stained normalised to cell number (C) mGluR7 – mean % area stained normalised to cell number (D) Tyrosine Hydroxylase % staining normalised to cell number. Statistically significant differences are indicated by * - $p < 0.05$ ** - $p < 0.01$

Figure 3-3 Immunohistochemical staining of (A-D) glutamate, (E-H) NMDAR1, (I-L) mGluR7 and (M-P) TH in the human rotator cuff tendon.

Photomicrographs showing the immunohistochemical staining of different mediators in the rotator cuff tendon in control and tear groups. Scale bar: 20 μ m.



3.6 Discussion

This study describes significant changes relating to both basic histology and the glutaminergic system in RCT. The key findings relating to basic histology of reduced vascularity and reduced cell proliferation are consistent with deteriorating tendon viability being present in disease. The most significant changes to the glutaminergic system in disease were increased amount of glutamate and NMDAR1 expression alongside a reduction in mGluR7.

The first study to recognize the presence of glutamate in tendinopathy used a microdialysis technique in chronic painful Achilles tendinopathy^[239]. Since then, numerous studies have confirmed that glutamate is up-regulated in various painful tendinopathies^[170]. This study is the first to confirm that similar changes are present in human RCT. This study did not find any correlation with the severity of patient pain symptoms. This is not unsurprising as previous studies have failed to detect this link^[241]. Previous studies of tendon structure has been shown to be a powerful predictor of the presence of pain^[91], but not of its severity.

Glutamate staining was distinctly expressed in resident cells within the tendon. The release of glutamate from tendon cells was first hypothesized by Scott et al^[174] who detected vesicular glutamate transporter expression in cells localized in tendon tissue in lower limb tendinopathies. This was later detected by Schizas et al^[172]. Because of the mechanical and structural differences between tendon locations, it cannot be assumed that cell behaviour is uniform in both upper and lower limb tendinopathies. This study has also shown significant staining of certain ionotropic and metabotropic receptors on tendon cells residing in damaged rotator cuff tissue, for example NMDAR1 and mGluR1. Other receptors such as mGluR7 and NK-1 were predominantly expressed by endothelial cells and not significantly by other cells.

It is important to remember that this study has several limitations. One weakness of this study is the lack of age-matched controls. These are ethically very difficult to obtain. A strength of this study is that fresh control tissue from the supraspinatus tendon was obtained, which is significantly better tissue match than the subscapularis tendon. All the control patients were pain free at the time of surgery. The design of this study means that the mechanisms and meaning of the histological changes cannot be delineated; they have simply been described as associations.

3.7 Conclusions

This study shows that glutamate is significantly more abundant and that the relative expression of several glutaminergic receptors are significantly altered in painful supraspinatus tears. The precise mechanisms behind these changes are unclear. Further work is needed to determine the specific role that glutaminergic system plays in disease pathogenesis and how this relates to pain symptomatology.

Chapter 4 The Neurohistological Effects of Glucocorticoid Injection and Surgical Repair on the Rotator Cuff Tendon

4.1 Abstract

4.1.1 Background

Glucocorticoid injection and surgical rotator cuff repair are two widely used treatments for rotator cuff tendinopathy. Little is known about the way in which medical and surgical treatments affect the human rotator cuff tendon *in vivo*. We assessed the histological and immunohistochemical effects of these common treatments on the rotator cuff tendon.

4.1.2 Methods

Supraspinatus tendon biopsies were taken before and after treatment from twelve patients undergoing glucocorticoid injection and eight patients undergoing surgical rotator cuff repair. All patients were symptomatic and none of the patients undergoing local glucocorticoid injection had full thickness tears of the rotator cuff. The tendon tissue was then analysed using histological techniques and immunohistochemistry.

4.1.3 Results

There was a significant increase in nuclei count and vascularity after rotator cuff repair and not after glucocorticoid injection (both $p=0.008$). HIF-1 α and cell proliferation were only increased after rotator cuff repair (both $p=0.03$) and not GCI. The ionotropic NMDAR1 glutamate receptor was only increased after GCI and not rotator cuff repair ($p=0.016$). An increase in glutamate was seen in both groups following treatment (both

$p=0.04$), while an increase in the receptor mGluR7 was only seen after rotator cuff repair ($p=0.016$).

4.1.4 Conclusion

The increases in cell proliferation, vascularity and HIF1 α after surgical rotator cuff repair appear consistent with a proliferative healing response, and these features are not seen after glucocorticoid injection. The increase in the glutamate receptor NMDAR1 after glucocorticoid injection raises concerns about the potential deleterious effects that may result from this common treatment.

4.2 Introduction

Current treatments for rotator cuff tendinopathy include non-operative measures such as rest, analgesia, physiotherapy, and glucocorticoid injections^[2]. In the UK over 500 000 intra-articular glucocorticoid injections are administered per year in the primary care setting alone^[242]. Emerging evidence suggest that glucocorticoid injections may result in poorer outcomes in terms of pain and symptom recurrence in lateral epicondylitis^[243]; however there is a paucity of evidence as regards the long term outcomes following glucocorticoid injection in rotator cuff tendinopathy^[244]. There is good evidence demonstrating that glucocorticoid injections are beneficial in the short term in treating pain related to rotator cuff tendinopathy^[244], but very little is known about the way in which local glucocorticoid may affect the rotator cuff tendons *in vivo*. The vast majority of our knowledge concerning the effects of glucocorticoid on tendon is based on *in vitro* and animal *in vivo* work^[245]. Of note only one previous study has evaluated the *in vivo* effects of glucocorticoid on human tendon and this was in the Achilles^[245].

Surgical rotator cuff repair is increasingly performed in patients with symptomatic rotator cuff tendon tears^[2]. Metabolic changes involving the glutaminergic system have been demonstrated in both humans and animal models of tendon repair^[162 165 167]. Increased fibroblast cellularity and blood vessel in-growth have been demonstrated in animal models of rotator cuff repair^[246-249], while it has also been shown that bone marrow derived cells from the footprint infiltrate into the site of tendon repair^[250]. However no study has ever analysed the histological effects of surgical rotator cuff repair in humans.

4.3 Aims and Hypotheses

The primary aim was to describe the histological and immunohistochemical effects of these common treatments on the rotator cuff tendon. The secondary aim of the study was to assess whether there were any significant histological differences between the glucocorticoid group (tendinopathy) and the surgical rotator cuff repair group (full thickness tendon tears).

4.4 Methods

4.4.1 Tissue Cohort

Supraspinatus tendon biopsies were taken from eight patients undergoing rotator cuff repair and from twelve patients undergoing subacromial glucocorticoid injection for rotator cuff tendinopathy. All patients were referred to a specialist upper limb out patient service and were aged between 35 and 65 (mean age = 53 years and standard

deviation +/- 8 years). All patients in the rotator cuff repair group had symptomatic degenerative full thickness supraspinatus tears, diagnosed by ultrasound and confirmed at surgery. Tear size was based on the accepted classification of Post, Silver and Singh [230]. All patients in the glucocorticoid injection group were diagnosed with rotator cuff tendinopathy by the clinical evaluation of the senior consultant surgeon (AC), and treatment was with glucocorticoid injection. All patients had subacromial impingement pain, 'painful arc' and positive Hawkins' and Jobe's tests. Structural integrity of the rotator cuff was assessed by ultrasound in all patients. A patient completed questionnaire for the Oxford Shoulder Score (OSS) [81], a well validated and widely used clinical outcome measure, was undertaken at both the week 0 and week 7 time point in both treatment groups. Ethical approval for this study was granted by the local research ethics committee (Oxfordshire REC B, ref: 09/H0605/111) and full informed consent (according to the Declaration of Helsinki) was obtained from all patients.

Patients were excluded if there was a history of previous shoulder surgery, any other significant shoulder pathology not involving the rotator cuff (osteoarthritis, frozen shoulder, instability, or previous fracture of the humerus/clavicle/scapula), more than 3 previous glucocorticoid injections, a glucocorticoid injection within 6 weeks of the treatment intervention and systemic steroid use. The diagnosis of other significant shoulder pathology was made on the basis of clinical history, clinical examination and radiological findings by the treating consultant surgeon (AC).

Glucocorticoid injection consisted of one ultrasound-guided injection of 40 mg Depo-Medrone and 4 ml of 2% lignocaine into the subacromial bursa performed by one trained surgeon in the out-patient department using aseptic precautions. Rotator cuff repair was

performed by a single consultant surgeon (AC) using an arthroscopically assisted mini-open approach.

4.4.2 Sampling, sectioning and fixation

The percutaneous ultrasound guided core biopsy technique that has been described in the Chapter 2 was used. In the glucocorticoid injection group biopsies were taken immediately prior to and 7 weeks following glucocorticoid injection. In the rotator cuff repair group biopsies were taken from the newly created footprint at the time of surgery and 7 weeks following rotator cuff repair. All tendon repairs were intact at the 7 week time point as assessed ultrasonographically. There were no local or systemic complications as a result of the biopsy procedure. The sectioning and fixation methods have been described in Chapter 2.

4.4.3 Histology and Immunohistochemical procedures

The histological and immunohistochemical procedures have been described in Chapter 2. Details of antibodies used and their working concentrations, as well as all staining protocols, can be found in Appendix 2: Laboratory protocols.

4.4.4 Image analysis and statistics

Image analysis and the validation of these techniques have been described in Chapter 2. Statistical analysis was carried using SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) and GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Statistical significance was set at a level of $p < 0.05$ unless otherwise stated. Histograms for all data sets were analysed and Kolmogorov-Smirnov normality tests carried out. The data for nuclei count, vessel count, mean cell nuclear

area and the standard deviation of nuclei count was parametric in distribution and passed the normality test. All other data was non-parametric in distribution. As regards the paired samples the Wilcoxon signed-rank test was used for testing for differences between the groups that were non-parametric in distribution, while paired t-tests were used for testing for differences between the groups that were parametric in distribution. Unpaired t-tests were used to test the difference between the unpaired samples that were parametric in distribution, while Mann-Whitney U test was used for testing for differences between unpaired samples that were non-parametric in distribution.

4.5 Results

4.5.1 Metrics: reliability and validity

This has been described in Chapter 2.

4.5.2 Demographics

The mean age of the rotator cuff repair group was 58 years (standard deviation +/- 6) and the mean age of the glucocorticoid injection group was 49 years (standard deviation +/- 5). The length of functional and pain symptoms ranged from 8 months to 10 years (Table 4.1). In terms of sporting activity one of the glucocorticoid injection group was a professional tennis coach, while five others in the glucocorticoid injection group had a sporting pastime (golf, horse riding, kayaking, swimming and darts). One patient in the rotator cuff repair group had a sporting pastime (squash). Prior to surgery, and 7 weeks follow-up, each patient completed a questionnaire for the Oxford Shoulder Score (OSS) [81], a well validated and widely used clinical outcome measure. There were no

significant changes in OSS after rotator cuff repair or glucocorticoid injection compared to baseline. The demographics of the study cohort are summarised in Table 4.1.

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Table 4.1 Data representing the demographics of the patient cohorts, quantification of the baseline questionnaires and tear size as determined at the time of surgery

	Rotator cuff repair (RCR) group	Glucocorticoid injection (GCI) group
Number of patients	8	12
Mean age in years (SD)	58 (+/- 6.7)	49 (+/- 7.1)
Sex	5 males, 3 females	6 males, 6 females
Median length of symptoms (months) (range)	12 (10 to 120)	20 (8 to 120)
Rotator cuff integrity	5 small/medium tears, 3 large/massive tears	1 partial thickness tear, 11 intact
Median number of previous GCIs (range)	2 (0 to 3)	1 (0 to 3)
Median Oxford Shoulder Score at time zero	28 (18 to 32.5)	29 (22.75 to 35.75)
Median Oxford Shoulder Score at seven weeks	28 (15 to 34.5)	31 (22 to 34)
Median change in Oxford Shoulder Score	-2 (-6.75 to 9.75)	0 (-5 to 8)

SD (standard deviation) and IQR (interquartile range) (p25-p75) are shown, unless otherwise stated

4.5.3 Basic histology and cell viability

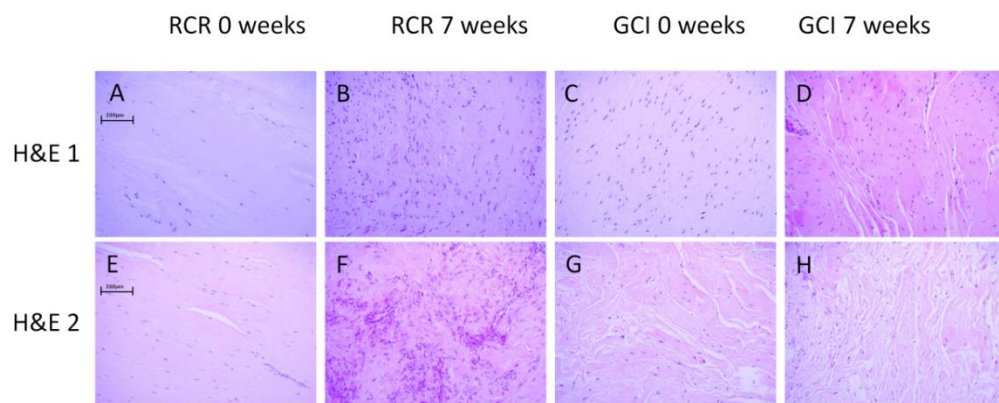
There was a significant increase in nuclei count, the standard deviation of nuclei count and vascularity (mean vessel count) after rotator cuff repair and not after glucocorticoid injection ($p=0.008$, 0.002 and 0.008 respectively). Hypoxia Inducible Factor-1 α (HIF-1 α) and cell proliferation were only increased after rotator cuff repair and not after glucocorticoid injection ($p=0.03$ for both results) (Figure 4-2 and Figure 4-3). Protein 53 (p53) was significantly increased after both treatments ($p=0.03$ for both). In terms of differences between the groups at baseline 0 weeks, vascularity (mean vessel count) was significantly reduced in the rotator cuff repair group versus the glucocorticoid injection group ($p=0.03$). Figure 4-2 depicts the quantified protein expression changes seen relating to PCNA and HIF1 α (Hypoxia Inducible Factor 1 alpha). Table 4.2 demonstrates the basic histological characteristics and quantified immunohistochemistry of tissue

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viability. Figure 4-1 and Figure 4-3 show photomicrographs relating to basic histology (H&Es) and cell viability (PCNA and HIF1 α).

Figure 4-1 Haematoxylin and Eosin (A-H) staining of human rotator cuff tendon before and after treatment.

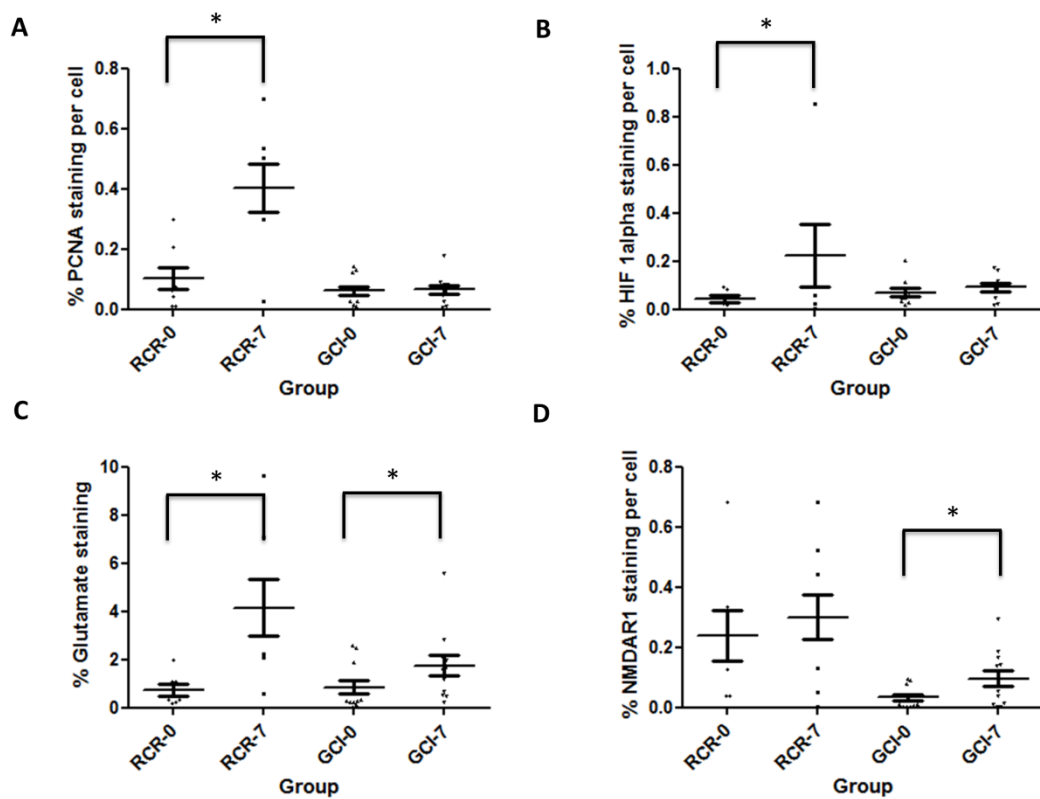
Figure 1



Photomicrographs showing the staining in the rotator cuff tendon in the rotator cuff repair group at week 0 (A, E) at week 7 (B, F), and glucocorticoid injection group at week 0 (C, G) and at week 7 (D, H) for two patients. Scale bar: 100 μ m.

The Neurohistological Effects of Glucocorticoid Injection and Surgical Repair on the Rotator Cuff Tendon

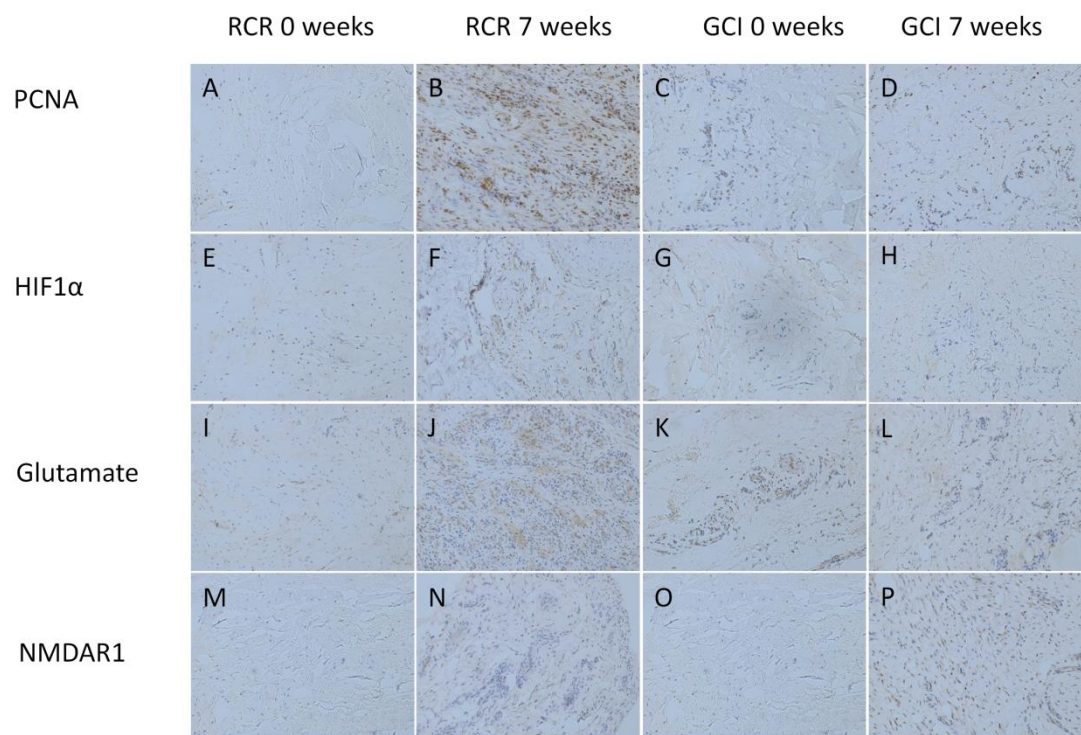
Figure 4-2 The effects of rotator cuff repair and glucocorticoid injection on the quantified immunohistochemistry of tissue viability and of the glutaminergic system



(A) cell proliferation – mean % area stained PCNA per cell (B) HIF1 α – mean % area stained HIF1 α per cell (C) Glutamate % staining (D) NMDAR1 % staining per cell. Statistically significant differences are indicated by *- $p < 0.05$ (between 0 and 7 week groups)

The Neurohistological Effects of Glucocorticoid Injection and Surgical Repair on the Rotator Cuff Tendon

Figure 4-3 Immunohistochemical staining of (A-D) PCNA, (E-H) HIF-1 α , (I-L) glutamate and (M-P) NMDAR1 in the human rotator cuff tendon before and after treatment.



Photomicrographs showing the immunohistochemical staining of different mediators in the rotator cuff tendon in the RCR at week 0 (A, E, I, M) at week 7 (B, F, J, N), and glucocorticoid injection at week 0 (C, G, K, O) and at week 7 (D, H, L, P). Scale bar: 100 μ m.

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Table 4.2 – Data representing the basic histological characteristics and quantified immunohistochemistry of tissue viability

	RCR 0 weeks	RCR 7 weeks	GCI 0 weeks	GCI 7 weeks
Mean vessel count	0.17 (+/- 0.17)**†	1.11 (+/- 0.40)**	0.33 (+/- 0.08)†	0.46 (+/- 0.19)
Mean nuclei count	9.2 (+/- 6.56)**	36.5 (+/- 15.80)**	14.2 (+/- 4.02)	19.7 (+/- 12.44)
Mean SD of nuclei count	7.9 (+/- 5.41)**	21.1 (+/- 6.61)**	11.2 (+/- 4.39)	13.2 (+/- 6.78)
Mean cell nuclear area (%)	0.12 (+/- 0.03)	0.11 (+/- 0.03)	0.11 (+/- 0.05)	0.10 +/- (0.03)
Proliferation (% PCNA staining per cell)	0.07 (0.01-0.21)*	0.42 (0.23-0.58)*	0.05 (0.02-0.12)	0.06 (0.03-0.08)
p53 (% staining per cell)	0.01 (0.001-0.02)*	0.22 (0.09-0.22)*	0.03 (0.01-0.1)*	0.08 (0.02-0.23)*
HIF1alpha (% staining per cell)	0.03 (0.01-0.09)*	0.11 (0.02-0.40)*	0.05 (0.03-0.09)	0.09 (0.04-0.13)
VEGF (% staining per cell)	0.41 (0.12-0.74)†	0.22 (0.12-1.17)	0.08 (0.04-0.14)†	0.13 (0.03-0.30)

SD (standard deviation) and IQR (interquartile range) (p25-p75) are shown, unless otherwise stated

*- p <0.05 ** - p<0.01 (between 0 and 7 week groups)

†- p <0.05 (between RCR 0 weeks and GCI 0 weeks)

4.5.4 Neurohistological results

An increase in glutamate (p=0.04 both groups) and the receptor metabotropic Glutamate Receptor 1 (mGluR1)(p=0.016) for rotator cuff repair and p=0.04 for glucocorticoid injection) was seen in both groups following treatment. The N-methyl-D-aspartate receptor 1 (NMDAR1) glutamate receptor was increased after glucocorticoid injection and not after rotator cuff repair (p=0.016) (Figure 4-2 and Figure 4-3). An increase in the receptor mGluR7 was only seen after rotator cuff repair (p=0.016). The Substance P receptor (Neurokinin Receptor 1 (NK-1)) and the Nerve Growth Factor (NGF) receptors (P75 low affinity NGF Receptor (p75) and Tyrosine Receptor Kinase A (TrkA)) were all significantly increased after rotator cuff repair and not after glucocorticoid injection (p=0.03, 0.014 and 0.03 respectively). The neural marker Protein Gene Product 9.5 (PGP 9.5) was also significantly increased after rotator cuff repair and not after glucocorticoid

injection ($p=0.008$). NMDAR1, mGluR3 and mGluR4 were all significantly increased in the week 0 rotator cuff repair group versus the week 0 glucocorticoid injection group ($p=0.006$, $p=0.005$ and $p=0.005$ respectively). In descriptive terms, a large number of the markers were predominantly expressed by cells within the tendon (NMDAR1 and mGluRs). The neurotrophin NGF and the neuropeptide substance P and their receptors (TrkA, p75 and NK-1) were predominantly expressed around blood vessels and not by the resident fibroblasts. The markers for new nerve growth and general nerves (GAP43 and PGP9.5 respectively) were largely expressed around vascular structures, but also to some degree by the resident fibroblasts. The numerical results relating to the different neuronal antibody groups are detailed in Table 4.3. Figure 4-3 shows photomicrographs relating to NMDAR1 and glutamate. There were no obvious differences between men and women in terms of both baseline histology and their histological response to treatment.

Table 4.3 –Neuronal quantitative results in format p50 (p25-p75), % area stained unless otherwise stated (pc – denotes % area stained has been adjusted per cell)

Group				
	RCR 0 weeks	RCR 7 weeks	GCI 0 weeks	GCI 7 weeks
	p50 (p25-p75)	p50 (p25-p75)	p50 (p25-p75)	p50 (p25-p75)
Glutamnergic system				
Glutamate	0.35 (0.23-1.1)*	3.4 (2.1-7.1)*	0.31 (0.22-1.7)*	1.6 (0.56-2.2)*
NMDAR1 ^{pc}	0.23 (0.1-0.42) ^{††}	0.26 (0.07-0.42)	0.02 (0.004-0.07) ^{††}	0.07 (0.01-0.16)*
mGluR1 ^{pc}	0.16 (0.06-0.57)*	0.83 (0.13-1.47)*	0.12 (0.05-0.36)*	0.39 (0.15-0.55)*
mGluR2 ^{pc}	0.22 (0.18-0.48)	0.32 (0.07-0.37)	0.20 (0.12-0.38)	0.22 (0.08-0.34)
mGluR3 ^{pc}	0.09 (0.04-0.11) ^{††}	0.13 (0.01-0.38)	0.02 (0.01-0.03) ^{††}	0.05 (0.02-0.09)
mGluR4 ^{pc}	0.19 (0.12-0.40) ^{††}	0.32 (0.08-0.52)	0.03 (0.01-0.11) ^{††}	0.09 (0.03-0.12)
mGluR5 ^{pc}	0.04 (0.01-0.06)	0.03 (0.02-0.06)	0.01 (0.01-0.04)	0.02 (0.02-0.05)
mGluR7 ^{pc}	0.01 (0.002-0.02)*	0.04 (0.02-0.10)*	0.002 (0.001-0.006)	0.02 (0.007-0.03)
mGluR8 ^{pc}	0.02 (0.01-0.06)	0.02 (0.004-0.05)	0.01 (0.007-0.02)	0.01 (0.004-0.05)
Sensory nervous system and neurotrophins				
NK-1	0.84 (0.48-2.7)*	3.6 (1.8-6.8)*	1.7 (0.94-3.2)	2.2 (1.6-3.2)
P75	6.4 (5.5-10.4)*	15.3 (11.8-20.9)*	4.6 (0.06-11.8)	5.5 (0.37-14.8)
NGF	0.22 (0.15-0.88)	0.9 (0.57-1.8)	0.21 (0.05-0.41)	0.29 (0.10-0.60)
TrkA	0.91 (0.28-1.9) [†]	6.4 (2.7-9.0)*	4.7 (2.7-5.5) [†]	2.9 (2.5-4.7)
Neural markers				
PGP9.5 ^{pc}	0.10 (0.03-0.17) ^{††}	0.52 (0.30-0.67) ^{††}	0.06 (0.04-0.15)	0.09 (0.07-0.14)
GAP43 ^{pc}	0.06 (0.02-0.15)	0.14 (0.05-0.20)	0.04 (0.01-0.13)	0.02 (0.01-0.13)

*- p <0.05 ** - p<0.01 (between 0 and 7 week groups), †- p <0.05 †† - p<0.01 (between RCR 0 weeks and GCI 0 weeks)

4.6 Discussion

This study describes the several histological and immunohistochemical changes involving the glutamnergic system that occur in the supraspinatus tendon of patients undergoing rotator cuff repair or glucocorticoid injection. The most important finding is that NMDAR1 was significantly increased in the glucocorticoid injection group and not in the rotator cuff repair group. This is in context of the pro-proliferative and pro-angiogenic changes that were seen after rotator cuff repair and not after glucocorticoid injection. The increase in glutamate, alongside the increase in cell proliferation and vascularity, seen after rotator cuff repair in our study is consistent with previous animal

studies^[162 167]. It is interesting that despite the increase in glutamate there is no significant increase in NMDAR1 after rotator cuff repair.

The clinical evidence for the use of glucocorticoid in shoulder pain is not convincing, many trials have shown only short term benefits with no long term gains^[109 244]. Emerging evidence points to poorer long term outcomes associated with glucocorticoid injection in the treatment of tendinopathy^[243]. The mechanisms of action of glucocorticoids are multiple, highly complex and incompletely understood^[251]; one important pathway involves the activation of specific cytoplasmic glucocorticoid receptors which then migrate to the cell nucleus to affect gene transcription. Generally glucocorticoids are thought to be anti-inflammatory but the reality may not be so simple^[252]. Our understanding of the tendon tissue effects of glucocorticoid injection relies almost exclusively upon *in vitro* and animal *in vivo* work. Several studies have shown that glucocorticoid is anti-proliferative, reduces type I collagen formation and has cytotoxic effects on tendon cells^[245]. The effects of glucocorticoid on the mechanical properties of tendon are conflicting^[245]. Only two previous studies have analysed the effects of glucocorticoid injection upon *in vivo* human tendon. The study by Lee and Ling ^[253] on the Achilles demonstrated reduced collagen organisation and increased collagen necrosis following glucocorticoid injection. While the more recent study by Poulsen et al^[135] showed that glucocorticoid injection induced fibroblast senescence in human rotator cuff tendon. *In vitro* studies have demonstrated that apoptosis and oxidative stress are increased in rotator cuff tendinopathy^[56].

Glutamate is a vital amino acid which has been implicated in many cellular processes including aerobic and anaerobic cell metabolism, collagen synthesis, neurotransmission, and excitotoxic cell death^[127 140 143]. The finding of raised glutamate levels and an

upregulated glutaminergic system in human tendinopathy has been well documented in recent years^[170 171]; the upregulation of NMDAR1 in painful tendinopathy has also been demonstrated^[170]. The underlying mechanism behind these glutaminergic changes does however remain unclear. It is likely that glutaminergic homeostasis is important in tendon, involving a complex balance between the variety of both inotropic and metabotropic receptors. Our findings of increased NMDAR1 in the rotator cuff repair group week 0 versus the glucocorticoid injection group week 0 is consistent with this, representing the glutaminergic changes occurring in the increased structural tendon failure of the rotator cuff repair group.

The role of the NMDA receptor is complex and dichotomous^[254]. It can be protective against both apoptosis and excitotoxicity^[255] with the activation of the PI3K (phosphoinositide-3-kinase) -Akt kinase pathway and the suppression of pro-death genes appearing implicated in these effects. The pro-survival role of NMDAR is demonstrated by the fact that its blockade *in vivo* can promote oxidative damage and neuronal cell death. However at the other end of the toxicity curve excessive NMDAR activity promotes free radical production and oxidative stress *in vitro and in vivo*^[255]. It is known that Group 1 metabotropic receptors (mGluR1/5) increase NMDAR activity, while Group 2 (mGluR2/3) and Group 3 receptors (mGluR4/6/7/8) decrease NMDAR activity ^[139]. Thus the complex interplay between the different components of the glutaminergic system is pivotal in determining the overall tissue response. It may be speculated that the increase in NMDAR1 seen after glucocorticoid injection is a pro-survival response to increased levels of cell stress. It may also be that an element of the increase in NMDAR1 after glucocorticoid injection is as a result of an increase in

inflammatory cells within tendon, a finding that has noted in previous animal studies^[245].

Generally speaking operative treatment is reserved for when non-operative measures have failed^[2]. There are a variety of operative strategies available including subacromial decompression, rotator cuff repair and several types of shoulder arthroplasty. Both patient and surgeon factors play an important role in deciding upon an appropriate treatment strategy for the individual. Rotator cuff repair is a highly successful procedure in terms of clinical outcomes but re-rupture rates remain high^[256 257]. Several studies have shown that the clinical outcome is significantly poorer if the rotator cuff repair fails^[256 258]. Tendon healing occurs with sequential inflammatory, proliferative and remodelling phases^[259]. Fibroblast proliferation, angiogenesis and nerve in-growth are all important in the healing process^[260]; this is consistent with our findings of increased vascularity and PGP 9.5 expression after rotator cuff repair. The immunopositive reactions of some fibroblasts to PGP 9.5 is of interest and may relate to a subpopulation of type B synoviocyte-like cells that has been repeatedly seen in the horse^[261]; of note PGP9.5-immunopositivity has previously been demonstrated in the human fibroblasts^[262]. The increase in the NK-1 and TrkA receptors, seen specifically around vascular structures, points towards the importance of both substance P and NGF in the process of neurovascular ingrowth^[263]. The p75 receptor was strongly expressed by both vascular structures and tendon cells; this is consistent with the work of Bagge et al^[264] which demonstrated the presence of tendon cells immunopositive to the p75 receptor in the human Achilles tendon.

The metabolic activity of a healing tendon after acute injury is hugely increased^[162] and fibroblasts are metabolically adapted for survival in this hypoxic environment^[163]. The

elevated lactate metabolite present in the acutely traumatised tendon is also a potent stimulant for collagen synthesis ^[164]. The upregulation of metabolites including glutamate demonstrated after Achilles tendon repair in humans ^[165] is consistent with this increased metabolic activity and this appears likely to be important in the increased cell proliferation present in healing tendon. The relative quantified tissue changes are generally greater after rotator cuff repair than glucocorticoid injection. This is unsurprising given the relative extent of the two treatments, with rotator cuff repair involving major mechanical change following the surgical reconstruction of the supraspinatus footprint.

No previous study has analysed the histological effects of any treatment on the rotator cuff and only one study has analysed the effect of glucocorticoid injection on *in vivo* human tendon (the Achilles) ^[253]. One of this study's great strengths is therefore its novelty. The use of paired tendon samples from these two treatment groups has increased the power of the study by enabling the comparison of the effects of glucocorticoid injection with those of the tendon healing response after rotator cuff repair. The tissue analysis has been undertaken in as objective a manner as possible by the use of automated measurement and the strict blinding of observers. The number of patients in our study is adequate to determine tissue differences, as we have demonstrated. However the study is not designed to test the effects of glucocorticoid injection and rotator cuff repair on patients' clinical outcomes. The lack of clinical improvement at 7 weeks in both treatment groups is likely to be an expected finding, as the benefits of glucocorticoid injection are typically short lasting, while the benefits of rotator cuff repair take longer to become clinically apparent.

A recognised weakness with immunohistochemistry is its semi-quantitative nature. Despite trying to make all the methods of semi-quantitative analysis as objective and unbiased as possible, the results are still semi-quantitative and must be interpreted as such. It should be noted that it has not been possible to describe all the exact details regarding the locations of the various immunohistochemical reaction patterns. Only 3 of the twelve patients in the glucocorticoid injection treatment group were truly steroid 'naïve' (i.e. had received no previous glucocorticoid injection). This reflects how widespread the use of glucocorticoid injection is in the UK, with the vast majority of patients in the UK being treated with glucocorticoid injection before referral to secondary care [242]. The tissue samples were taken at a single time point 7 weeks following treatment and therefore the persistence of these observations is unknown.

4.7 Conclusions

Increases in cell proliferation, vascularity and HIF1 α were seen after surgical rotator cuff repair and these features are not seen after glucocorticoid injection. The increase in the glutamate receptor NMDAR1 after glucocorticoid injection raises concerns about the potentially deleterious effects that may result from this common treatment.

Chapter 5 Tendon Neurohistology from Painful and Pain-free Tendons

5.1 Abstract

5.1.1 Background

The relationship between tendon structure and pain symptoms is imperfect and incompletely understood. The aim of this study was to determine whether there were neurohistological differences between painful and pain-free rotator cuff tendons.

5.1.2 Methods

Supraspinatus tendon specimens were obtained using an ultrasound guided biopsy technique from 9 patients with painful RCT resistant to conservative management (painful group) and 9 pain-free patients at over 6 years (median 6 years 11 months) following SAD (pain-free group). Pain symptoms were measured using the validated Oxford Shoulder Score (OSS). Structural tendon integrity was assessed ultrasonographically. The tendon tissue was analysed using basic histological techniques (Haematoxylin and Eosin, and Alcian Blue) and Immunohistochemistry. Image analysis was performed by two blinded observers using Image-J to quantify the amount of 3,3'-diaminobenzidine staining present

5.1.3 Results

The groups were similar in terms of age, sex and structural tendon abnormality. The painful group consisted of 7 males and 2 females, the pain-free group of 6 males and 3 females. The mean age of the painful group was 51 years and that of the pain-free group was 52 years. The median OSS in the painful group was 32 (range 23 to 36) and this was

significantly lower ($p=0.0002$) than the median OSS in the pain-free group (all 48). There were two partial thickness tears in both groups and no full thickness tears. The modified Bonar scores in the painful and the pain-free groups were comparable. There were no significant differences between groups in terms of cellularity, vascularity, proliferation and hypoxia inducible-factor 1 α expression.

The leucocyte count (% of CD45 positive cells) and macrophage count (% of CD206 positive cells) were increased in the painful group versus pain-free ($p=0.038$ and 0.0004 respectively). The expression of metabotropic glutamate receptor 7 (mGluR7) was reduced in the painful group versus pain-free ($p=0.002$), while the expression of the Kainate 1 receptor was increased in the painful group ($p=0.003$). PGP 9.5 (a nerve marker) and Lactate Dehydrogenase (LDH) expression were increased in the painful group versus pain-free ($p=0.008$ and 0.007 respectively). There were no significant differences in glutamate, the ionotropic glutamate receptor (NMDAR1) and the metabotropic glutamate receptors (mGluR1 and 2) between groups.

5.1.4 Conclusions

This study has shown that specific characteristics of tendon neurohistology are associated with the presence of shoulder pain. This provides strong evidence that the rotator cuff tendon is of key importance in the symptomatology of RCT. The mechanism behind these tendon differences is unclear. These findings are novel and improve our understanding of pain in RCT, and may help provide novel therapeutic targets.

5.2 Introduction

The likelihood of developing pain is strongly linked with changes to gross tendon structure^[91]. However in common with other musculoskeletal diseases, there is a poor association between the severity of symptoms and structural change^[241]. A significant limitation with previous research on rotator cuff tendinopathy has been the adequacy of control tissue. Out of 59 studies that have previously analysed the rotator cuff tendon, 26 have used no control tissue, 12 have used subscapularis and only 21 studies have used supraspinatus^[38]. Of the studies which have managed to obtain supraspinatus, 17 of these have been cadaveric tendon, 3 have used traumatised tendon and 1 has used the supraspinatus of patients undergoing surgery for shoulder instability. No previous study has managed to obtain supraspinatus tendon from pain-free tendinopathic patients.

There is a relative paucity of evidence in the literature relating peripheral histological changes to patient symptomatology^[170]. Tomita et al^[54] showed that an increased neural density in the subacromial bursa correlated with the presence of shoulder pain at rest. Gotoh et al^[190] demonstrated that substance P levels in the subacromial bursa appeared to correlate with pain levels as measured with a visual analogue score. A significant limitation with these two studies is the differences in gross structure that may explain the observed relationships with pain. The same limitation is relevant to much work carried out on other tendinopathies such as the Achilles and patellar, with controls invariably having normal gross tendon structure as opposed to the abnormal gross tendon structure seen in disease^[170].

5.3 Aims and Hypotheses

The aim of this study was to investigate whether patients with shoulder pain had significant different tendon neurohistology to pain-free patients. I hypothesized that there would be significant neurohistological differences between painful and pain-free tendons.

5.4 Methods

5.4.1 Tissue Cohort

Supraspinatus tendon biopsies were taken from nine patients undergoing subacromial decompression surgery (painful group) and from nine patients over 5 years after undergoing subacromial decompression surgery (pain-free group). All patients were referred to a specialist upper limb out patient service and were aged between 38 and 65 (mean age = 52 years and standard deviation +/- 8 years). All patients were diagnosed with rotator cuff tendinopathy by the clinical evaluation of a senior consultant surgeon (AC/JR), and treatment was subacromial decompression surgery. All patients in the painful group had subacromial impingement pain, 'painful arc' and positive Hawkins' and Jobe's tests. Structural integrity of the rotator cuff was assessed by ultrasound in all patients. A patient completed questionnaire for the Oxford Shoulder Score (OSS) ^[81], a well validated and widely used clinical outcome measure, was undertaken in both treatment groups. Ethical approval for this study was granted by the local research ethics committee (Oxfordshire REC B, ref: 09/H0605/111 and Oxfordshire REC B ref:12/SC/0028) and full informed consent (according to the Declaration of Helsinki) was obtained from all patients.

All patients had failed conservative management which included a minimum of one GCI, and a course of physiotherapy or a home exercise program. Patients were excluded if there was a history of previous shoulder surgery other than the subacromial decompression, a full thickness rotator cuff tear as determined on ultrasound, any other significant shoulder pathology not involving the rotator cuff (osteoarthritis, frozen shoulder, instability, or previous fracture of the humerus/clavicle/scapula), more than 3 previous glucocorticoid injections, a glucocorticoid injection within 6 weeks of the treatment intervention and systemic steroid use. The diagnosis of other significant shoulder pathology was made on the basis of clinical history, clinical examination and radiological findings by the treating consultant surgeon (AC/JR). Subacromial decompression surgery was performed by two consultant surgeons (AC/JR).

5.4.2 Sampling, sectioning and fixation

The percutaneous ultrasound guided core biopsy technique that has been described in the Chapter 2 was used. In the painful group biopsies were taken immediately prior to surgery. In the pain-free group biopsies were taken over five years following surgery (median of six years and eight months). There were no local or systemic complications as a result of the biopsy procedure. The sectioning and fixation methods have been described in Chapter 2.

5.4.3 Histology and Immunohistochemical procedures

The histological and immunohistochemical procedures, including the immunofluorescent dual labelling, have been described in Chapter 2. Details of antibodies used and their working concentrations, as well as all staining protocols, can be found in Appendix 2: Laboratory protocols.

5.4.4 Image analysis and statistics

Image analysis and the validation of these techniques have been described in Chapter 2. A modified version of the Bonar Score^[240] was developed for use in this study as outlined in Table 5.1. Cell morphology, collagen arrangement and ground substance were scored identically to the original scoring system. However new grading scores were used for cellularity and vascularity based on the work described in Chapters 3 and 4. As regards the dual labelling images were acquired on a Zeiss LSM 710 confocal microscope using a 40x oil immersion objective. POPO-1, Alexa Fluor 488, Alexa Fluor 568, and Alexa Fluor 633 flurophores were excited using the 405nm, 488nm, 561nm, and 633nm lasers respectively. Averaging was set to 2 and the pinhole was set to approximately 1 Airy unit. Two-dimensional image reconstructions were created using ZEN 2009 (Zeiss).

Table 5.1 – Modified Bonar scoring system

Modified Bonar Score for the rotator cuff	Grade 0	Grade 1	Grade 2	Grade 3
Cell morphology (four fields of view 100X)	Inconspicuous elongated spindle shaped nuclei with no obvious cytoplasm	Increased roundness: nucleus becomes more ovoid to round in shape without conspicuous cytoplasm	Increased roundness and size: the nucleus is round, slightly enlarged and a small amount of cytoplasm is visible	Nucleus is round. , large with abundant cytoplasm and lacuna formation (chondroid change)
Collagen arrangement (one field of view 100X)	Collagen arranged in tightly cohesive well demarcated bundles with a smooth dense bright homogenous polarization pattern with normal crimping	Diminished fibre polarization; separation of individual fibre bundles but with maintenance of overall bundle architecture Non homogeneous polarization	Bundle changes; separation and loss of demarcation of fibre bundles, giving rise to expansion of the tissue overall and clear loss of normal polarization pattern	Marked separation of fibre bundles with complete loss of architecture
Cellularity (mean and standard deviation of 100X images of whole section)	Mean cell count <12 and standard deviation of count <7	Either mean ≥ 12 and < 16, and/or standard deviation of count ≥ 7 and < 10	Either mean ≥ 16 and < 20, and/or standard deviation of count ≥ 10 and < 13	Either mean ≥ 20 , and/or standard deviation ≥ 13
Vascularity (mean vessel count of 100X images of whole substance)	>1.2	< 1.2 and ≥ 0.8	< 0.8 and ≥ 0.4	< 0.4
Ground substance (one field of view 100X)	Not stainable ground substance	Stainable mucin between bundles but bundles still discrete	Stainable mucin within bundles with loss of clear demarcation of bundles	Abundant mucin throughout the section with inconspicuous collagen staining

Statistical analysis was carried using SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) and GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Statistical significance was set at a level of $p < 0.05$ unless otherwise stated. Histograms for all data sets were analysed and Kolmogorov-Smirnov normality tests carried out. Data for nuclei count, vessel count, the % CD206 positive cells and VEGF % area stained was parametric in distribution and passed the normality test. All other data was non-parametric in distribution. The Mann-Whitney U test was used for testing for differences between the groups that were non-parametric in distribution, while unpaired t-tests were used for testing for differences between the groups that were non-parametric

in distribution. The Benjamini-Hochberg procedure was applied to correct for multiple testing and these results are demonstrated in Table 5.5.

5.5 Results

5.5.1 Metrics: reliability and validity

This has been described in Chapter 2.

5.5.2 Demographics

The mean age of the painful group was 51 years (standard deviation ± 8) and the mean age of the pain-free group was 52 years (standard deviation ± 8). There were 7 intact tendons and 2 partial thickness tears in both groups. The median length of symptoms before subacromial decompression surgery was 22 months in the pain-free group and 12 months in the painful group. The median number of glucocorticoid injections was one in the pain-free group (range 1 to 3) and one in the painful group (range 1 to 4). Both groups of patients completed a questionnaire for the Oxford Shoulder Score (OSS)^[81], a well validated and widely used clinical outcome measure; the median OSS was 32 in the painful group (range 23 to 36) and all OSSs in the pain-free group were 48. The demographics of the study cohort are summarised in Table 5.2.

Table 5.2 Data representing the demographics of the pain-free and painful groups, quantification of the baseline questionnaires and tendon structure

	Pain-free group	Painful group	P value
Number	9	9	-
Age (mean)	52 (+/- 7.8)	51 (+/- 8.2)	0.72
Sex	6M 3F	7M 2F	1.0
Structure	7 intact + 2PTTs	7 intact + 2PTTs	1.0
Median length of symptoms pre SAD (months)	22 (6-48)	12 (12-18)	0.43
Median no of steroid injections (range)	1 (1-3)	1 (1-4)	0.92
Oxford Shoulder Score (range)	48 (48)	32 (23-36)	0.0002**

SD (standard deviation) and IQR (interquartile range) (p25-p75) are shown, unless otherwise stated

*- p <0.05 ** - p<0.01 (between groups)

5.5.3 Basic Histology and Cell Viability

There were no significant differences between groups in terms of cellularity, mean vessel count, modified Bonar scores, proliferation and HIF1 α . The results are summarised in Table 5.3. Figure 5-1 and Figure 5-2 demonstrate the photomicrographs relating to basic histology and cell viability. There was significantly more VEGF staining in the pain-free group than in the painful group. A significant proportion of the VEGF staining was of blood vessels. There was significantly more lactate dehydrogenase staining in the painful group than in the pain-free group; this staining related to resident fibroblasts and vascular structures. There were significantly higher proportions of CD45-positive and CD206-positive cells in the painful group versus the pain-free group. CD45 is a pan-leucocyte marker while CD206 is a macrophage marker.

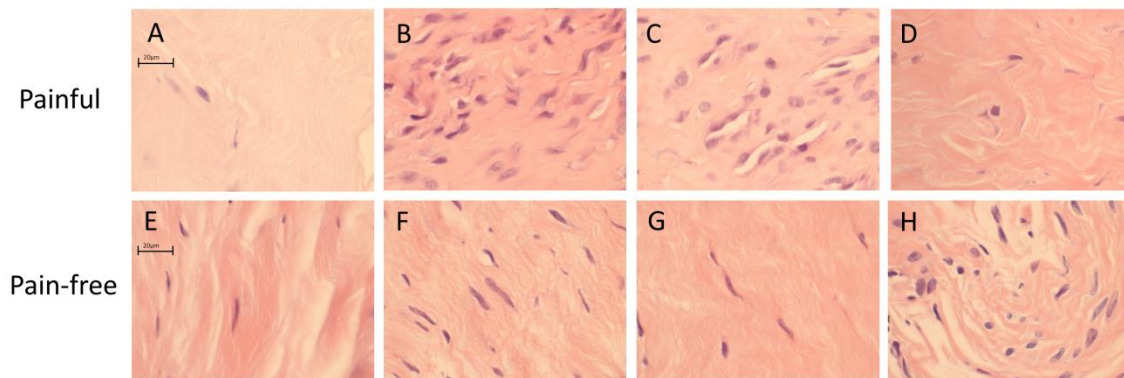
Table 5.3 Data representing the basic histological characteristics and quantified immunohistochemistry of tissue viability

	Pain-free Group	Painful Group	P value
Mean vessel count	1.09 (+/- 0.63)	0.80 (+/- 0.65)	0.47
Mean nuclei count	11.6 (+/- 4.1)	8.0 (+/- 12.5)	0.57
Median modified Bonar score	7.0 (6.5-8.0)	8.0 (6.0-10.0)	0.59
Proliferation (% PCNA staining ^{pc})	0.04 (0.03-0.06)	0.04 (0.02-0.12)	0.86
HIF1alpha ^{pc}	0.01 (0.01-0.04)	0.02 (0.01-0.04)	0.73
VEGF ^{pc}	0.27 (+/- 0.18)	0.06 (+/- 0.05)	0.004**†
CD45 (% cells positive)	6.8 (4.7-10)	12.4 (8.1-16.8)	0.038*
CD206 (% cells positive)	13.2 (+/- 6.2)	26.8 (+/- 6.5)	0.0004**†
Lactate Dehydrogenase ^{pc}	0.001 (0.0004-0.002)	0.015 (0.01-0.04)	0.007**†

SD (standard deviation) and IQR (interquartile range) (p25-p75) are shown, unless otherwise stated (*- p <0.05 ** - p<0.01 (between groups) ^{pc} – per cell)

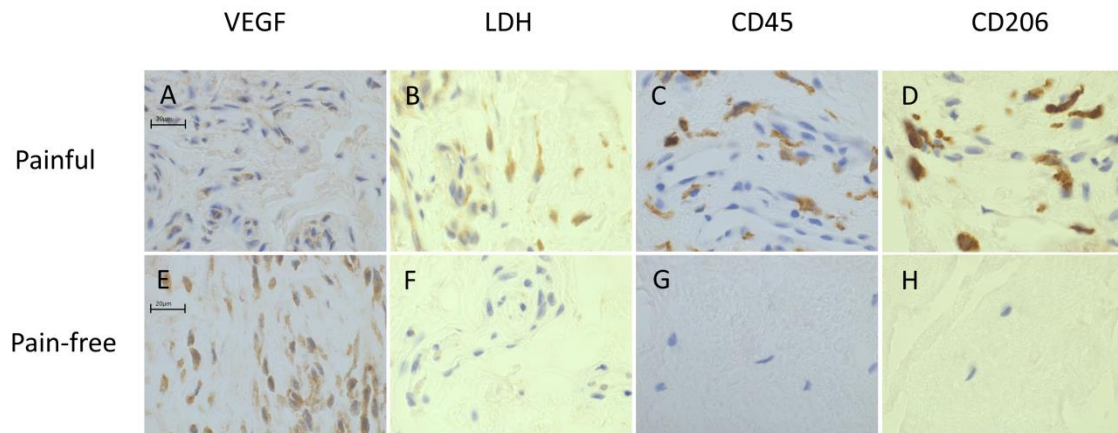
† -denotes significant result after application of Benjamini-Hochberg procedure

Figure 5-1 - Haematoxylin and Eosin (A-H) staining of painful and pain-free human rotator cuff tendon



Photomicrographs showing the staining in the rotator cuff tendon in the painful (A, B, C, D) and pain-free (E, F, G, H) groups. Scale bar: 20 µm.

Figure 5-2 Immunohistochemical staining of VEGF (A, E), LDH (B, F), CD45 (C, G) and CD206 (D, H) in the painful and pain-free groups



Photomicrographs show the immunohistochemical staining in the rotator cuff tendon in the painful and pain-free groups. Scale bar: 20 μ m.

5.5.4 Neurohistological results

These are summarised in Table 5.4 and depicted in Figure 5-3. There was minimal glutamate staining in either group. There was significantly more PGP 9.5 staining in the painful group versus the pain-free. PGP 9.5 was expressed by both cells within the tendon and endothelial cells. The mGluR7 staining was significantly increased in the pain-free group versus the painful and this staining was predominantly of endothelial cells. Both mGluR2 and KA1 staining was increased in the painful group versus the pain-free. There was staining for these glutaminergic receptors in cells within the tendon and vascular structures.

Table 5.4 –Neuronal quantitative results in format p50 (p25-p75), % area stained unless otherwise stated (pc – denotes % area stained has been adjusted per cell)

	Pain-free Group	Painful Group	P value
Glutamate ^{pc}	0.003 (0.001-0.03)	0.004 (0.002-0.007)	0.86
NMDAR1 ^{pc}	0.013 (0.006-0.015)	0.025 (0.002-0.037)	0.61
PGP 9.5	0.87 (0.35-1.86)	3.75 (1.36-4.97)	0.0079**†
mGluR1 ^{pc}	0.43 (0.24-0.58)	0.36 (0.24-0.42)	0.55
mGluR2 ^{pc}	0.0019 (0.008-0.066)	0.064 (0.058-0.096)	0.05*
mGluR7 ^{pc}	0.18 (0.12-0.64)	0.005 (0.003-0.034)	0.0019**†
KA1	0.85 (1.31-2.62)	4.55 (2.37-7.15)	0.0028**†

*- p <0.05 ** - p<0.01 (between groups) pc – per cell

† -denotes significant result after application of Benjamini-Hochberg procedure

Figure 5-3 Immunohistochemical staining of mGluR2 (A, E), mGluR7 (B, F), PGP 9.5 (C, G) and KA1 (D, H) in the painful and pain-free groups

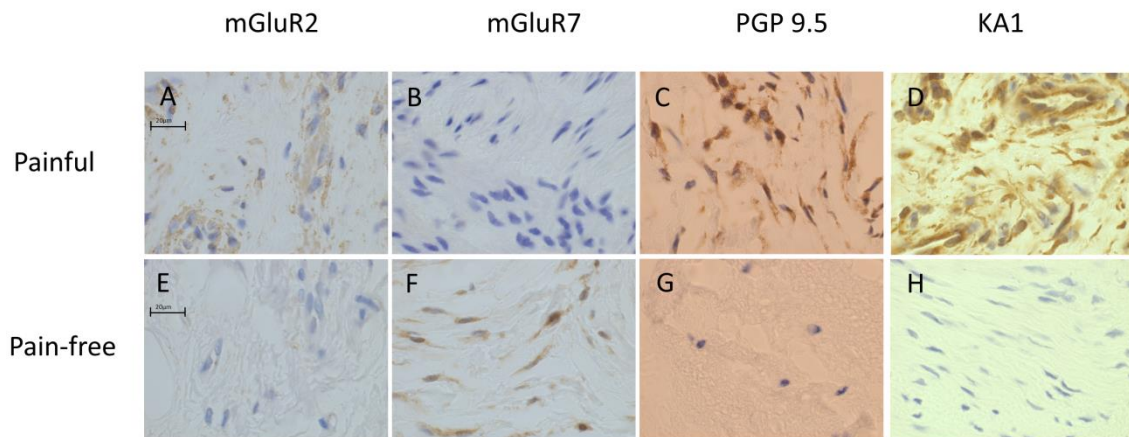
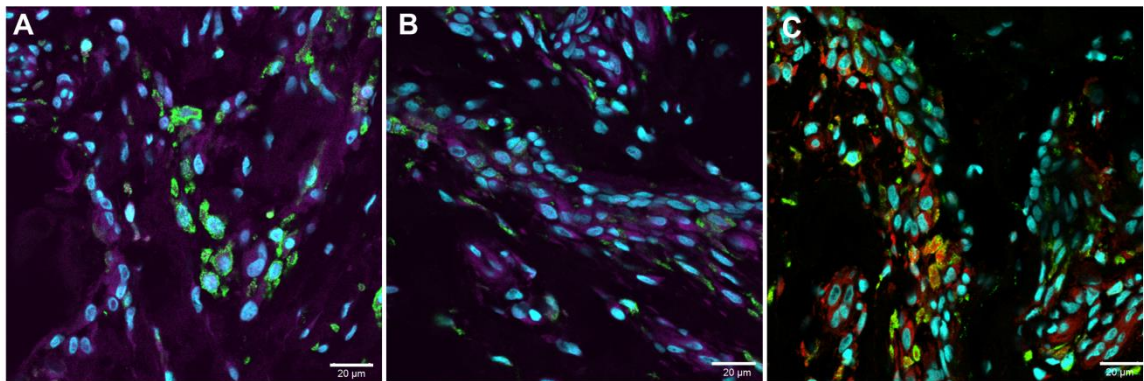


Table 5.5 – P values sorted according to the Benjamini-Hochberg procedure

Sorted P values	d x i/n	Is $P \leq d x i/n$?	Tissue marker
0.0004	0.003	yes	CD206
0.0019	0.006	yes	mGluR7
0.0028	0.009	yes	KA1
0.004	0.013	yes	VEGF
0.007	0.016	yes	LDH
0.0079	0.019	yes	PGP9.5
0.038	0.022	no	CD45

Dual labelling immunofluorescence showed NMDAR1 co-localised with CD206 positive cells, whereas PGP9.5 and glutamate were predominately expressed by non-macrophages.

Figure 5-4 - Representative immunofluorescent images of torn human supraspinatus to show cell types expressing nociceptive neuromodulators



Panel shows dual antibody labelling for CD206 (green) with (A) glutamate (violet), (B) PGP9.5 (violet) and (C) NMDAR1 (red). Cyan represents POPO-1 nuclear counterstain. NMDAR1 co-localised with CD206 positive cells, whereas PGP9.5 and glutamate were predominantly expressed by resident tendon cells. Scale bar: 20µm

5.6 Discussion

This study's main finding is that there are significant histological differences between painful and pain-free rotator cuff tendons. These findings relate to the cell type present as demonstrated by the increased proportion of 'inflammatory' cells seen in painful tendons. While the significant differences in the glutaminergic system related to the KA1 and mGluR7 receptors which were most frequently expressed by endothelial cells.

These significant differences were present despite there being no significant differences between groups in terms of both tendon macrostructure as measured by ultrasonography and basic tendon histology. There is little evidence in the literature relating histological changes in tissue to symptoms ^[170]. Tomita et al^[54] showed that an increased neural density in the subacromial bursa correlated with the presence of shoulder pain at rest. Gotoh et al^[190] demonstrated that substance P levels in the subacromial bursa appeared to correlate with pain levels as measured with a visual analogue score. A significant limitation with these two studies is the differences in gross structure that may explain the observed relationships with pain. The same limitation is relevant to much work carried out on other tendinopathies such as the Achilles and patellar, with controls invariably having normal gross tendon structure as opposed to the abnormal gross tendon structure seen in disease^[170]. An interesting recent study by Koike et al^[93] demonstrated that patients with symptomatic rotator cuff tears showed higher radioisotope uptake on bone scintigraphy compared with asymptomatic tears; this suggests that higher metabolic activity is present in painful cuff tears and this may well be associated with inflammatory tissue changes.

The role of inflammation in tendinopathy has long been a subject of significant controversy. Several studies have conclusively demonstrated that more inflammatory

cells are present in tendinopathy than in healthy control tissue^[36 38 265 266]. Increased levels of inflammatory cytokines such as Interleukin-1 β have been repeatedly demonstrated in tendinopathy^[38 267]. Certainly this study's findings point towards an association between inflammatory cells and pain symptomatology. The reason for this association is unclear and it appears that the role of inflammation in tendinopathy is poorly understood. Type A synoviocytes or 'fibrocytes' arise from monocyte precursors and are consequently CD45 positive^[268]. It is likely that type A synoviocytes make up a proportion of the CD45 and CD206 positive cells seen in this study but it is also very possible that a significant proportion of these cells migrate into tendon via the bloodstream. The increase in PGP 9.5 in the painful group may relate to a subpopulation of type B synoviocyte-like cells^[57]. The co-localisation of NMDAR1 and CD206 suggests that the glutamate receptors are predominantly located on the type A synoviocyte cell population; while the absence of NMDAR1 and PGP9.5 co-localisation suggests that this is not the case for the type B synoviocytes. The cell markers expressed by 'fibrocytes' and other tissue fibroblasts are extremely fluid and complex^[268 269]; while emerging evidence points to the potentially key role of the 'fibrocyte' in chronic inflammatory disorders^[269]. Certainly future research needs to determine whether the inflammation present in tendinopathy lies towards the 'reactive' or 'causal' side of the fence in terms of disease pathogenesis.

Interestingly mGluR2 has been shown to be an endogenous inhibitor of inflammatory pain in mice^[270], while peripheral mGluR2 agonism has analgesic properties in rats^[271]. The Kainate receptor has been implicated in the pathogenesis of osteoarthritis and KAR antagonism has been shown to be therapeutic in slowing down disease progression in animal models^[157]. It should be noted that endothelial cell staining accounted for the vast majority of the histological change relating to mGluR7 and this is the likely reason

for the absence of mGluR7 expression in explant cells (fibroblasts likely massively outnumber endothelial cells *in vitro*). Increased mGluR7 staining seen in tendon tears versus healthy control is also largely related to endothelial cells and not the tenocytes^[77]; this result does not appear to be as a result of the potential confounding factor vascularity, which was not significantly different between the groups. Little is known about the role of mGluR7 in endothelial cell function but mGluR7 is known to be expressed by rat endothelial cells^[272], while NMDA receptors are expressed by human endothelial cells^[273]. NMDA receptor antagonism in human synoviocytes and chondrocytes has been shown to be significantly anti-proliferative ^[274 275]; this is consistent with the receptor's pro-survival role being exploited in the more hostile environment of the painful tendon^[147]. NMDAR activity is decreased by mGluR7, inferring that the greater expression of mGluR7 in pain-free tendons may be representative of a less stressful environment for the endothelial cells.

The inability to gather tendon-matched and age-matched control tissue from live donors has been a significant limiting factor in many previous studies^[38]. Only a minority of studies have managed to use supraspinatus as a control and of these studies the vast majority have used cadaveric supraspinatus tendon. Only 4 studies have used supraspinatus from a live donor as a control; three of these used the supraspinatus of patients who had sustained significant shoulder trauma and one used supraspinatus from patients undergoing surgery for shoulder instability^[38]. Therefore a great strength of this study is the fact that tendon-matched and age-matched control tissue from live donors with tendinopathy has been used. The cause of these tendon differences cannot be determined given the study's cross-sectional design. The pain-free group have all had previous subacromial decompression surgery prior to becoming pain-free. This

means that the tendon differences between groups may be as a result of a true surgical treatment effect, the placebo effect or the natural history of the disease.

5.7 Conclusions

This study has shown that specific characteristics of tendon neurohistology are associated with the presence of shoulder pain. This provides strong evidence that the rotator cuff tendon is of key importance in the symptomatology of RCT. These findings are novel and improve our understanding of pain in RCT, and may help provide novel therapeutic targets.

Chapter 6 The *In Vitro* Effects of Glutamate on Human Tendon-Derived Cells

6.1 Abstract

6.1.1 Background

Little is known about the *in vitro* effects of glutamate and glutamate receptor modulation on human tendon derived cells. The primary objective was to assess the effect of glutamate exposure on human tendon-derived cells in terms of viability, protein and gene expression. The second objective was to assess whether glutamate receptor antagonism would affect the response of tendon derived cells to glutamate exposure.

6.1.2 Methods

Human tendon-derived cells were obtained from supraspinatus tendon tissue obtained during rotator cuff repair (tendon tear derived cells) and from healthy hamstring tendon tissue (control cells). The *in vitro* impact of glutamate exposure and glutamate receptor modulation was measured using the Alamar blue cell viability assay, immunocytochemistry and quantitative real-time PCR. Glutamate receptors were modulated using the NMDAR antagonist MK-801, the KAR/AMPA antagonist NBQX and the mGluR7 agonist AMN082.

6.1.3 Results

Tendon tear derived cell viability was unaffected after 24 hours of 1.875mM glutamate exposure, while control cell viability was increased. 24 hours of 1.875mM glutamate exposure reduced Type 1 alpha 1 collagen (COL1A1) and Type 3 alpha 1 collagen (COL3A1) gene expression, but increased Aggrecan gene expression in both cell groups.

Cell viability was significantly reduced after 72 hours of 1.875mM glutamate in both cell groups; this deleterious effect was attenuated by NMDAR antagonism with 10 μ M MK-801. The changes in COL1A1, COL3A1 and AggreCAN gene expression after 72 hours of 1.875mM glutamate exposure were not attenuated by 10 μ M MK-801.

6.1.4 Conclusions

The study findings provide evidence that glutamate contributes to the pathogenesis of tendinopathy. NMDAR antagonism attenuates the deleterious effect of 1.875mM glutamate on cell viability but does not affect the related changes to matrix gene expression.

6.2 Introduction

Despite the well described glutaminergic changes in tendinopathy, including an increase in extracellular glutamate concentration and the up-regulation of NMDA receptors^[172 276] little is known about the mechanisms underlying these changes. There is a paucity of *in vitro* work relating to the effects of glutamate on tendon derived cells. It is not known at what level glutamate may become toxic to cells and how the modulation of various glutamate receptors may influence them.

The harmful effects of glutamate on *in vitro* neuronal cells was first described by Choi et al^[143] who described the 'excitotoxic' effects of 500µM glutamate which were attenuated by NMDA receptor antagonism. These findings have since been replicated by other studies and glutamate induced neuronal cell death is a well described phenomenon^[140]. The underlying molecular mechanisms involve the glutamate induced activation of NMDA receptors which then leads to an influx of calcium into cells, and consequent activation of catabolic enzymes and cell death^[147]. The role of pH also appears important with cytosolic pH being decreased after glutamate exposure^[277], while the pH of the applied glutamate solution appears important in terms of determining the relative toxicity of the cellular effects^[277]. Glutamate has been shown to have pro-proliferative effects on retinal pigment epithelial cells *in vitro*^[137] using up a 1mM concentration. However in synoviocytes NMDA receptor antagonism has been shown to be significantly anti-proliferative^[275]. Similar anti-proliferative effects of NMDA receptor antagonism have been demonstrated in human chondrocytes^[274]. Glutamate receptor modulation has been linked to changes in inflammatory cytokine production^[274 278] in both human synoviocytes and chondrocytes.

6.3 Aims and Hypotheses

The overall aim of this study was to investigate the effects of glutamate on tendon-derived cells *in vitro* and how modulation of specific glutamate receptors would affect these responses.

Specifically the first aim was to determine whether there were differences in basal gene expression and cell viability between tendon tear derived cells and healthy control cells. The second aim was to determine if glutamate reduced cell viability at 24 hours. The third aim was to determine the effects of relatively small changes in glutamate concentrations on cell viability and gene expression. The fourth aim was to determine whether NMDAR antagonism would alter the responses of cells to particular concentrations of glutamate. It was also an aim to compare and contrast the effects of glutamate on tendon tear derived and control cells.

6.4 Methods

6.4.1 Cell explant and analysis

Tendon tissue was obtained from the Oxford Musculoskeletal BioBank (Oxford REC C 09/H0606/11) and was collected with informed donor consent in full compliance with National and Institutional ethical requirements, the United Kingdom Human Tissue Act, and the Declaration of Helsinki. Human tendon-derived cells were isolated by the explant culture of both rotator cuff tendon excised as part of debridement before tendon repair and hamstring tendon excess to requirements for autograft repair for anterior cruciate ligament rupture as previously described^[279]. The rotator cuff tendon was taken from 4 male and 3 female patients with symptomatic rotator cuff tears (mean age 50 +/- 11). The hamstring tendon was harvested from healthy male and female (mean age 27

+/- 9). Tendon derived cells were cultured in DMEM-F12 containing 10% FBS (Gibco, Life technologies) at 37°C and 5% CO₂. Cells were fed regularly every 2-3 days.

6.4.2 Cell Viability, Immunocytochemistry and Gene Expression

These methods have been described in detail in Chapter 2.

6.4.3 Statistics

Statistical analysis was carried using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Histograms for all data sets were analysed. Data was parametric in distribution unless otherwise stated. Results are expressed as mean $\pm$ SD unless otherwise stated. Unpaired t-tests and Mann Whitney U-tests were used to test for differences between two groups for parametric and non-parametric data respectively. Wilcoxon matched-pairs signed rank test was used to test for differences between multiple groups. The data for all cell viability experiments was normalised relative to the control. Statistical significance was set at a level of $p < 0.05$. Spearman correlation coefficients were calculated to test the relationships between cell surface marker expression (CD206 for macrophages and PGP9.5 for type B synoviocytes), cytokine expression and glutamate receptor expression in the tendon tear derived cells.

6.5 Results

6.5.1 Demographic data

The demographic data is detailed in Table 6.1. The control patient group was considerably younger than the disease patient group. The sex breakdown was similar in both groups.

Table 6.1 – Data representing the demographics of the patients from whom the control and tear derived cells were explanted

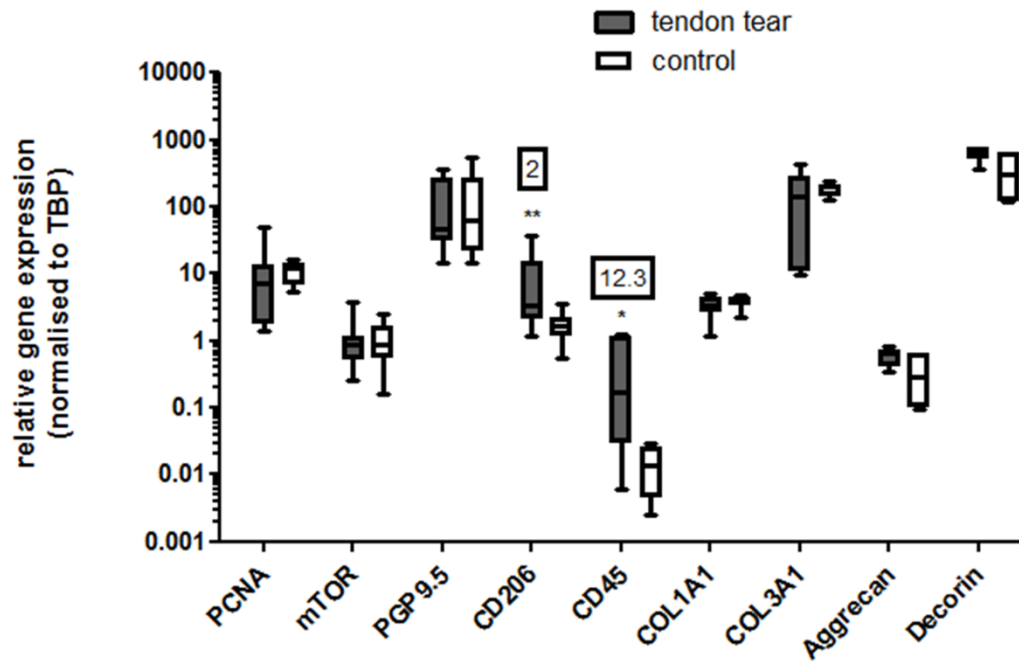
	Control	Disease
Type	Hamstring	Supraspinatus
Tendon structure	Healthy	Torn
Number	5	7
Age (mean)	27 (+/- 9)	50 (+/- 11)
Sex	3F 2M	4M 3F

6.5.2 Basal gene expression

Neuro-inflammatory genes are upregulated in cells derived from rotator cuff tendon tears versus control cells (Figure 6-1 and The boxes represent median +/- interquartile range, while the whiskers represent range. Numbers in outlined boxes represent fold change in gene expression. Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

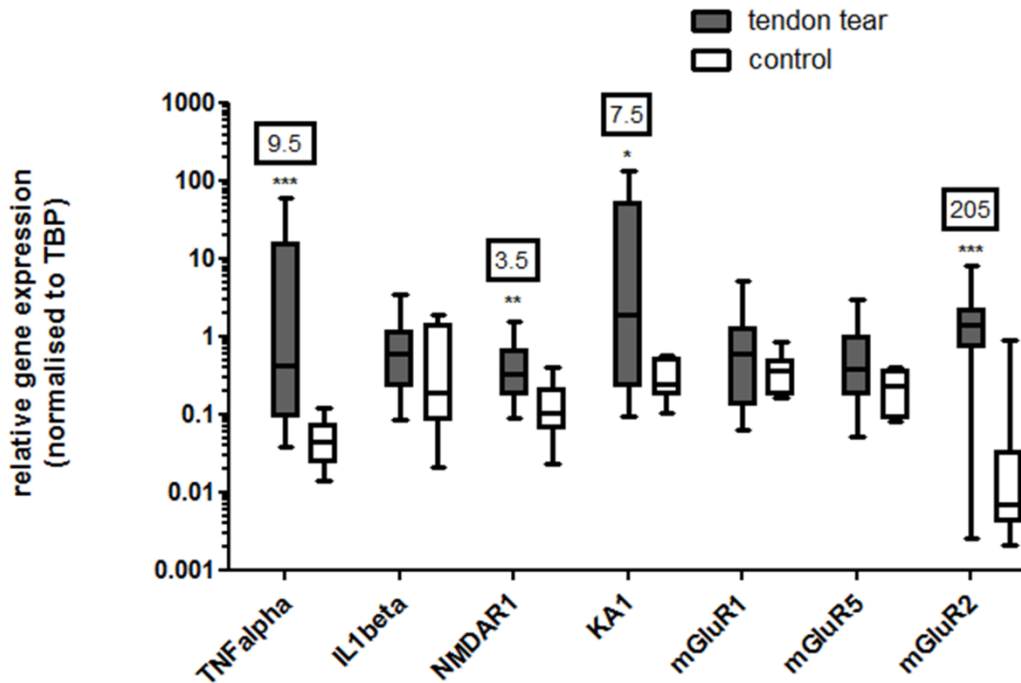
Figure 6-2). The relative expression of the cell surface markers CD45 (12.3 fold, $p = 0.03$) and CD206 (2 fold, $p = 0.004$) were significantly increased in tear derived cells. There was no significant difference in matrix gene expression between groups (COL1A1, COL3A1, aggrecan and decorin). The expression of the TNF α (9.5 fold, $p = 0.0009$), NMDAR1 (3.5 fold, $p = 0.003$), KA1 (7.5 fold, $p = 0.049$) and mGluR2 (205 fold, $p = 0.0006$) genes was significantly increased in tendon tear derived cells. mGluR7 gene expression was not detected in either disease or control cells. The gene expression of the metabotropic glutamate receptors mGluR1 and mGluR5 was not different between groups, although there was a trend towards increased expression of mGluR5 in the tendon tear derived cells ($p = 0.07$). There was no difference in PGP9.5 (type B synoviocyte marker), PCNA (cell proliferation) and mTOR (mammalian target of rapamycin) gene expression between groups.

Figure 6-1 - Relative gene expression in control and tendon tear derived cells relating to proliferation, cell surface markers and the extracellular matrix



The boxes represent median +/- interquartile range, while the whiskers represent range. Numbers in outlined boxes represent fold change in gene expression. Statistical significance denoted by *p<0.05, **p<0.01, ***p<0.001.

Figure 6-2 - Relative gene expression in control and tendon tear derived cells relating to cytokines and glutaminergic receptors



The boxes represent median +/- interquartile range, while the whiskers represent range. Numbers in outlined boxes represent fold change in gene expression. Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

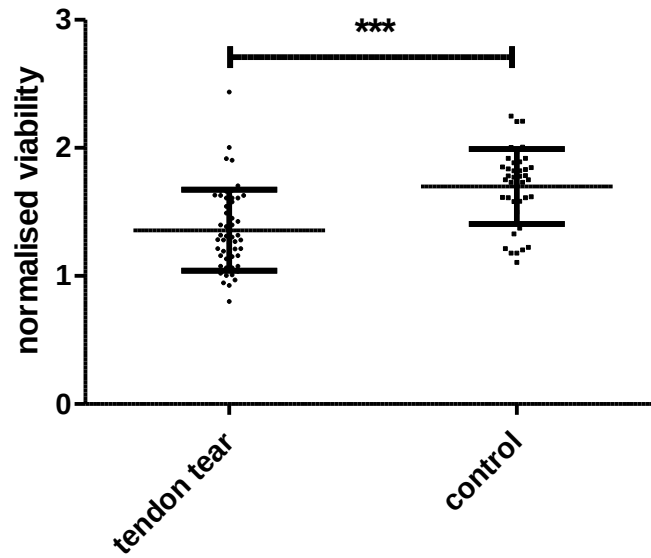
6.5.3 Correlations within gene expression data

There were strong correlations between CD206 expression and glutamate receptor expression; KA1 ($r=0.96$, $p<0.0001$), NMDAR1 ($r=0.76$, $p=0.002$), mGluR1 ($r=0.83$, $p=0.0002$), mGluR2 ($r=0.95$, $p=0.0002$) and mGluR5 ($r=0.95$, $p<0.0001$). There was no strong ($r>0.4$) or significant correlation between PGP9.5 expression and glutamate receptor expression. There was a strong correlation between CD206 expression and TNF α expression ($r=0.97$, $p<0.0001$), while there was no strong or significant correlation between PGP9.5 expression and TNF α expression.

6.5.4 Untreated cell viability

Cell viability was significantly higher in control cells than disease cells ($p < 0.0001$) (Figure 6-3).

Figure 6-3 - 24hr cell viability in tendon tear derived versus control cells

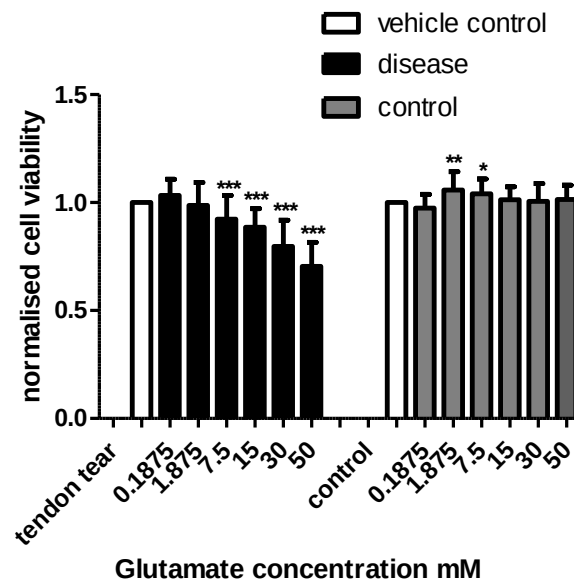


The boxes represent mean $\pm$ standard deviation. Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

6.5.5 Effect of high glutamate concentrations on cell viability and protein expression

Glutamate reduced cell viability in tendon tear derived cells but not in control cells (Figure 6-4). A significant reduction in cell viability was apparent at glutamate concentrations of 7.5mM and above. While no reduction in cell viability was observed in controls cells, a significant increase in viability was seen at concentrations of 1.875mM and 7.5mM.

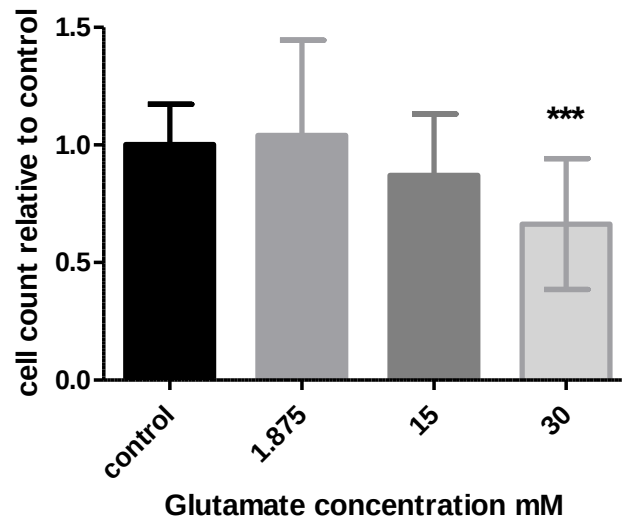
Figure 6-4 - 24 hours of glutamate exposure reduced cell viability in disease cells in a dose dependent manner but there was no significant deleterious effect on viability in controls cells



The bars represent mean $\pm$ standard deviation. All results were normalised with respect to the control. Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle control. $n=7$ disease and $n=5$ control

The number of cells was manually counted to validate the results of the Alamar Blue assay and this data is represented in Figure 6-5.

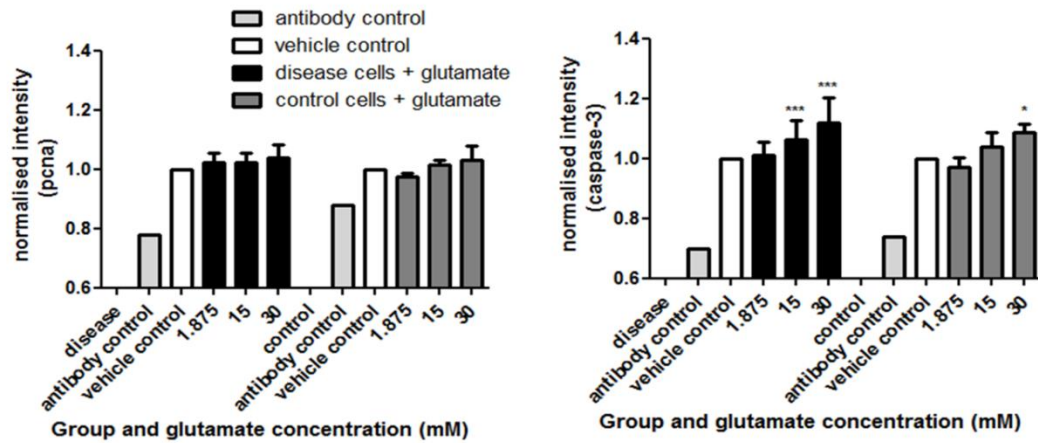
Figure 6-5 – Bar graph depicting the manual cell counts after 24 hours of glutamate exposure in disease cells



The bars represent mean $\pm$ standard deviation. All results were normalised with respect to the control. Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle control. $n = 4$

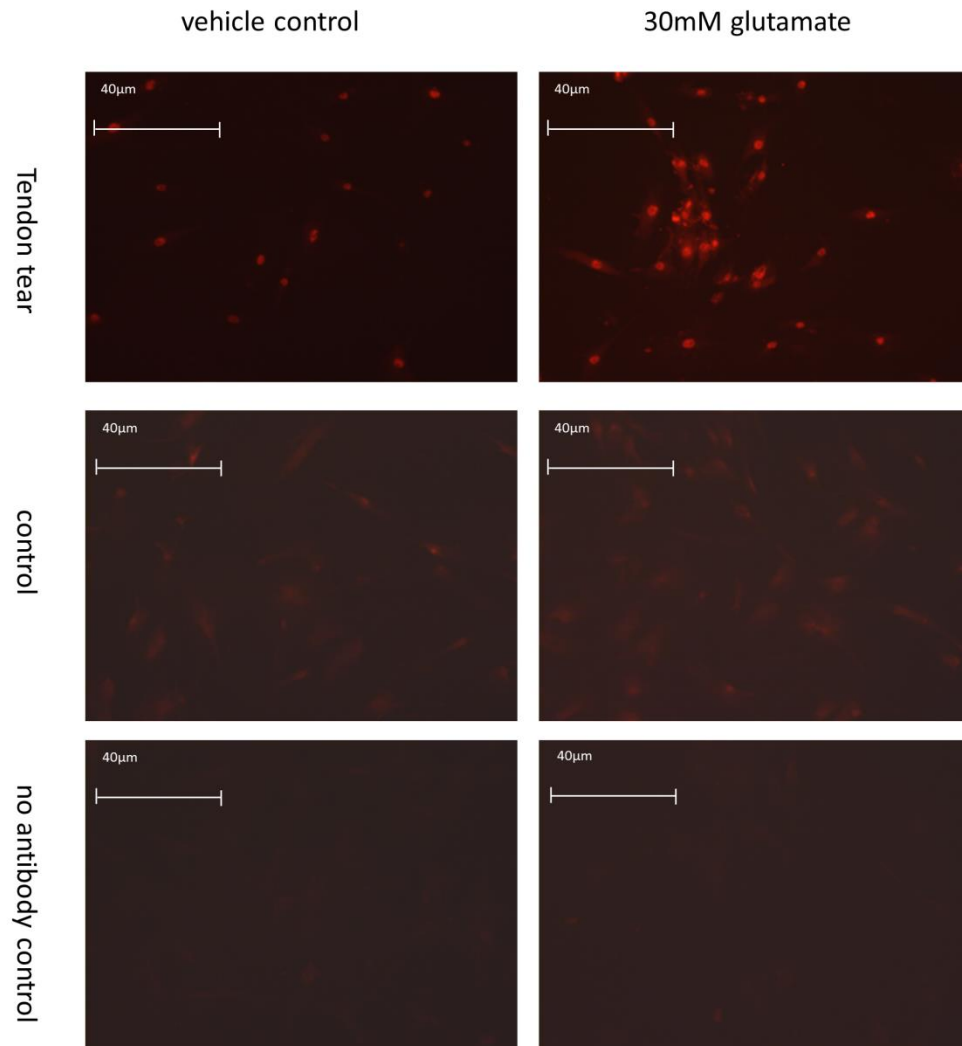
There was a significant increase in activated caspase-3 expression in both tendon tear derived and control cells, of note this was more significant in the tendon tear derived cells (Figure 6-6 and Figure 6-7). There were no significant changes in PCNA expression.

Figure 6-6 -The quantified changes in protein expression after 24 hours of glutamate exposure



The boxes represent mean $\pm$ standard deviation. All results were normalised with respect to the control. Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle control. $n = 5$ disease and $n = 4$ control

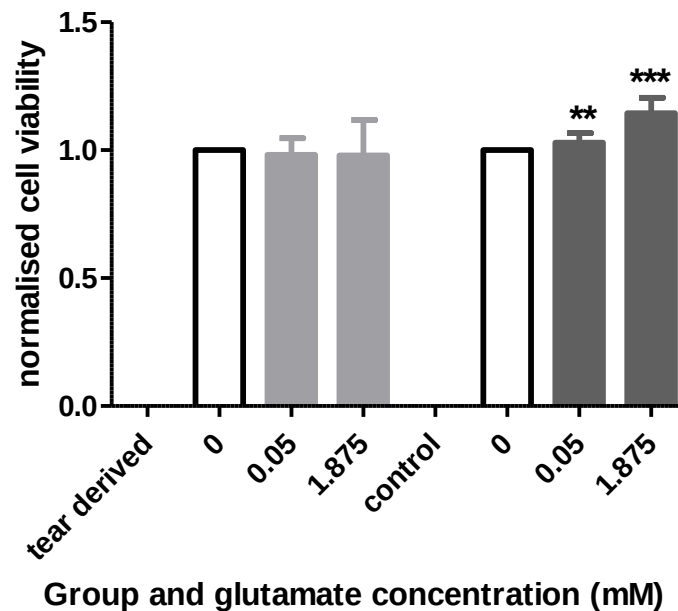
Figure 6-7 – Photomicrographs depict the immunocytochemical staining of activated caspase-3 in disease cells (top), control cells (middle) and antibody free controls (bottom). Scale bars: 40µm.



6.5.6 Effect of lower glutamate concentrations on cell viability and gene expression

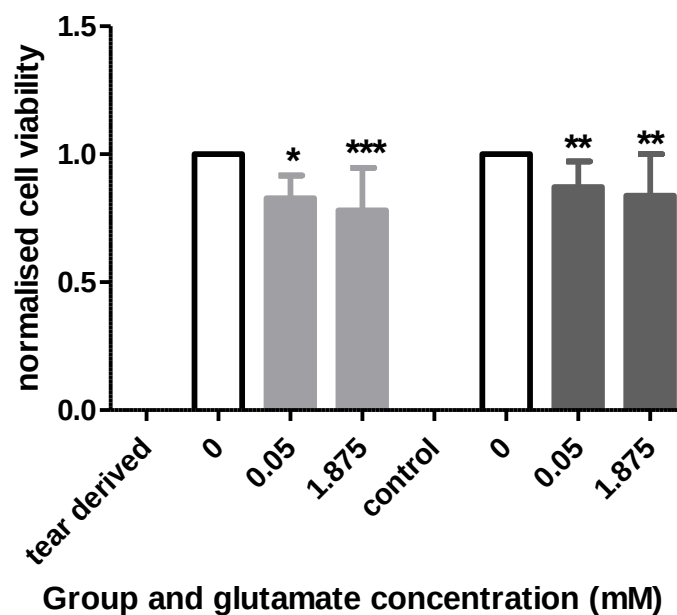
Cell viability at 24 hours was similar in tendon tear derived cells in all three solutions; however cell viability was significantly increased in 0.05mM and 1.875mM glutamate versus the glutamate free control solution (Figure 6-8). After 72 hours of exposure cell viability was reduced in 0.05mM and 1.875mM glutamate solutions versus glutamate free control in both cell groups (Figure 6-9).

Figure 6-8 - Bar graph depicting cell viability after 24 hours of exposure to different concentrations of glutamate



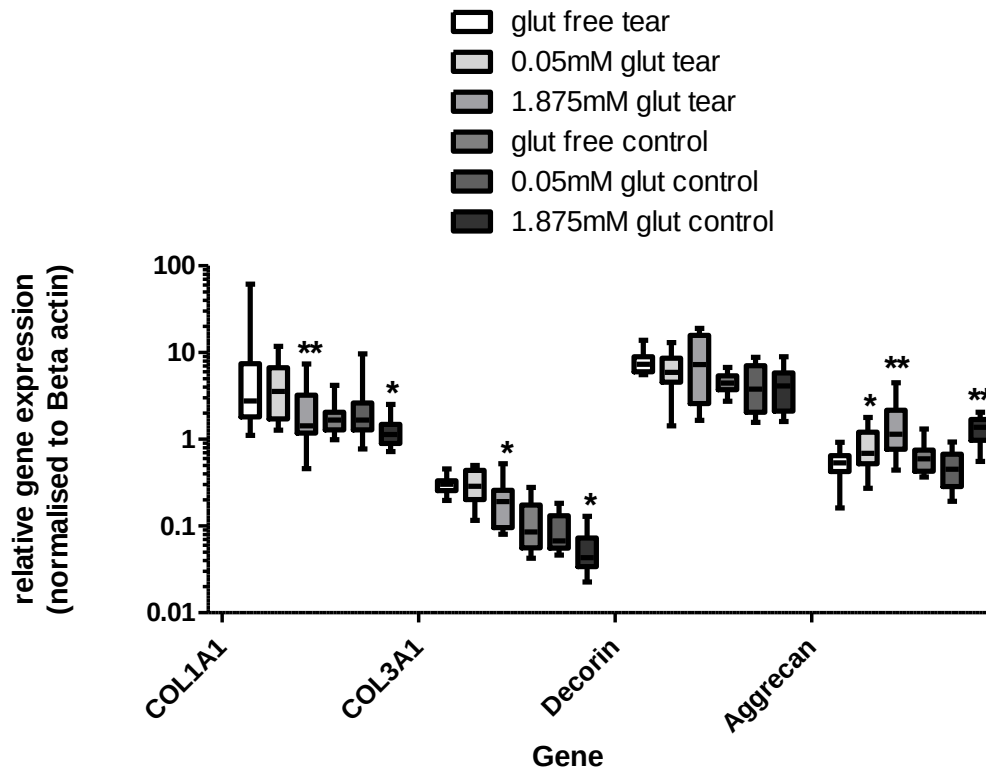
The boxes represent mean $\pm$ standard deviation. All results were normalised with respect to the control. Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus glutamate free control. $n=4$ disease and $n=4$ control

Figure 6-9 - Bar graph depicting cell viability after 72 hours of exposure to different concentrations of glutamate



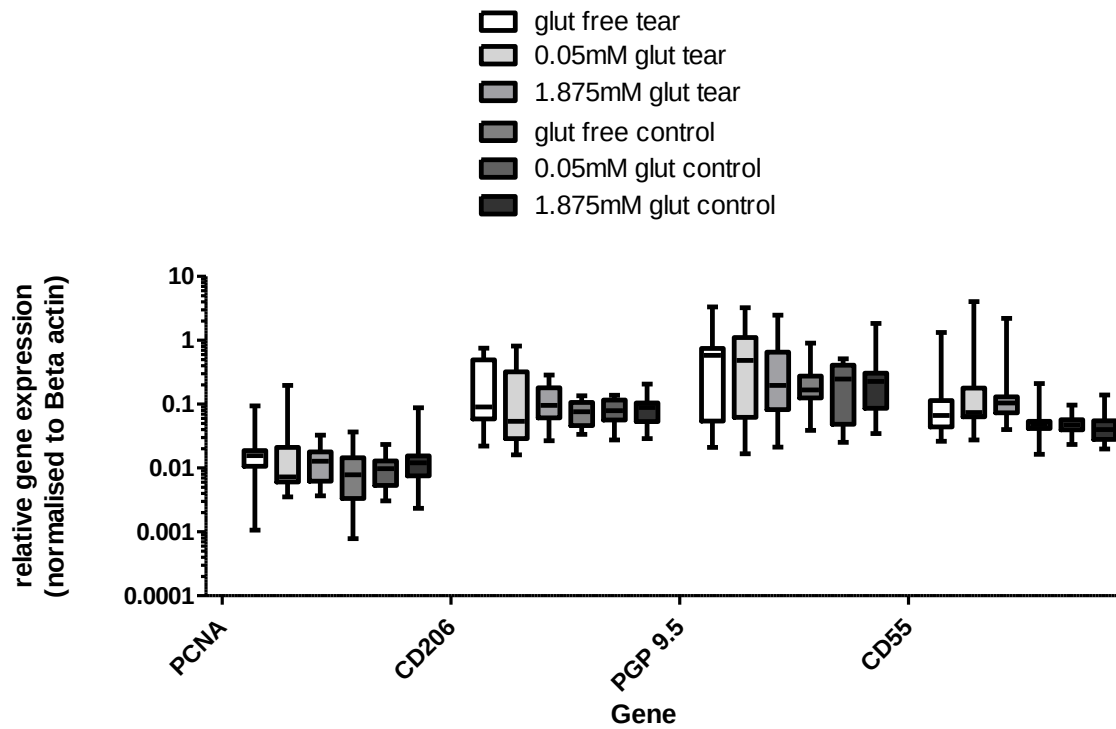
The boxes represent mean $\pm$ standard deviation. All results were normalised with respect to the control. Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus glutamate free control. $n=4$ disease and $n=4$ control

Figure 6-10 – Box and whiskers plot demonstrating the changes in matrix gene expression after 24 hours of glutamate exposure in tendon tear derived and control cells



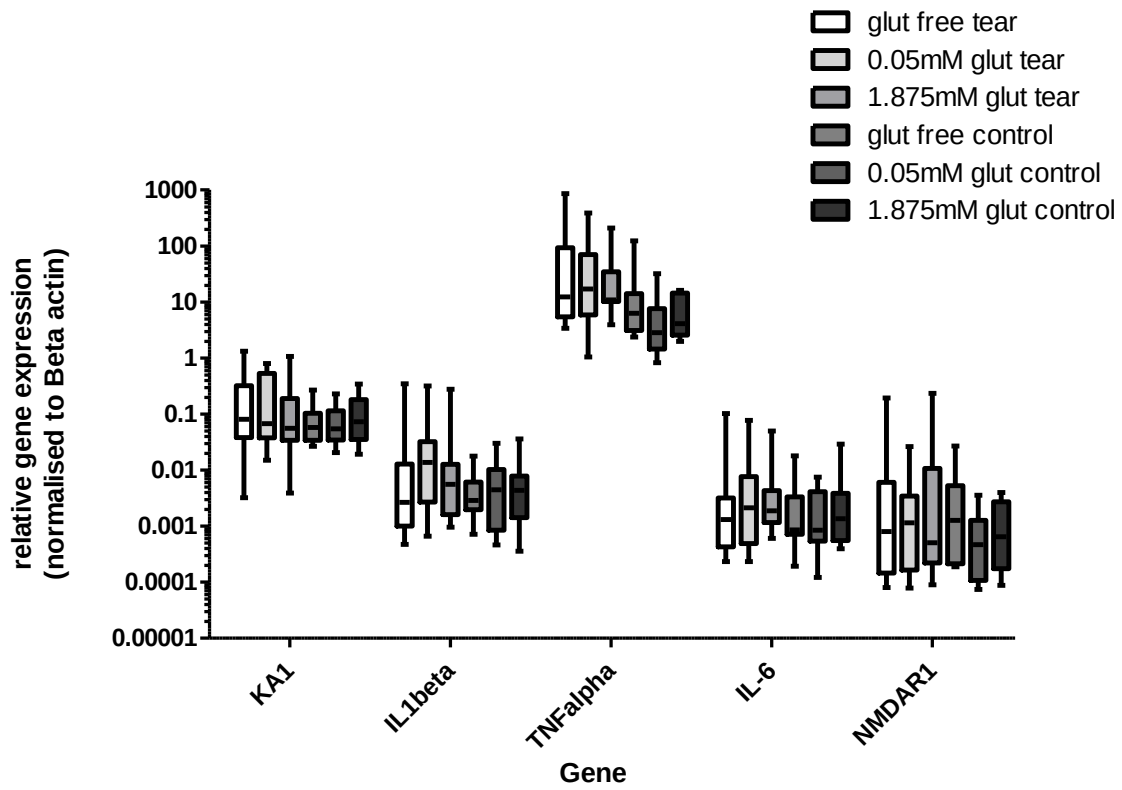
The boxes represent median +/- interquartile range, while the whiskers represent range. Numbers in outlined boxes represent fold change in gene expression. Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus glutamate free control. $n=4$ disease and $n=4$ control

Figure 6-11 - Box and whiskers plot demonstrating the changes in cell marker gene expression after 24 hours of glutamate exposure in tendon tear derived and control cells



The boxes represent median +/- interquartile range, while the whiskers represent range. Numbers in outlined boxes represent fold change in gene expression. Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus glutamate free control. $n = 4$ disease and $n = 4$ control

Figure 6-12 - Box and whiskers plot demonstrating the changes in cytokine and glutamate receptor gene expression after 24 hours of glutamate exposure in tendon tear derived and control cells

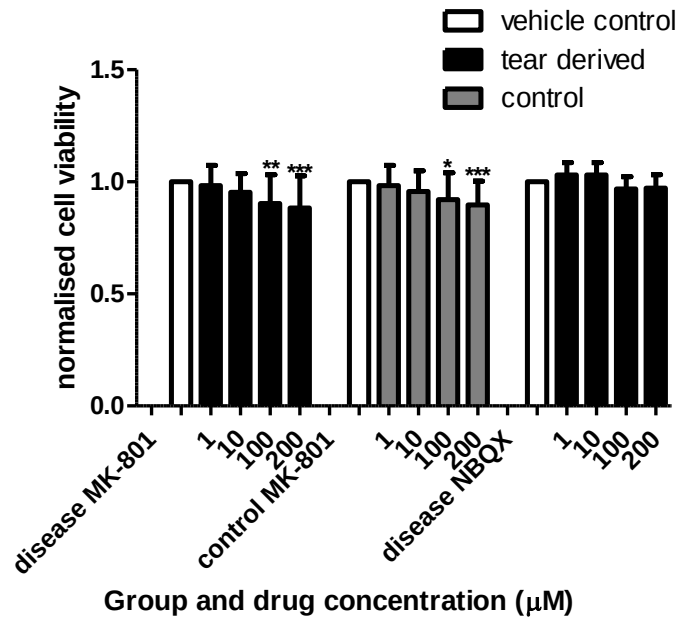


The boxes represent median +/- interquartile range, while the whiskers represent range. Numbers in outlined boxes represent fold change in gene expression. Statistical significance denoted by *p<0.05, **p<0.01, ***p<0.001 versus glutamate free control. n=4 disease and n=4 control

6.5.7 Glutamate receptor modulation

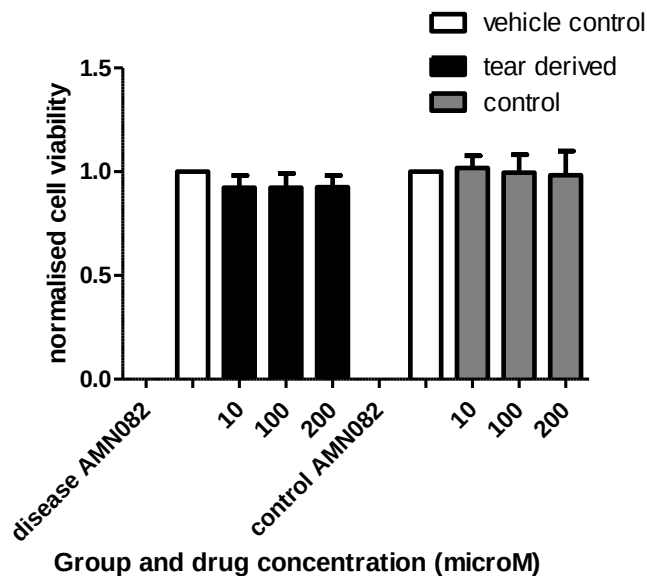
The effects of NMDAR antagonism (MK-801), KA/AMPA antagonism (NBQX) and mGluR7 agonism (AMN082) on cell viability are shown in Figure 6-13 and Figure 6-14. MK-801 only reduced cell viability at the higher concentrations of 100μM and 200μM, while NBQX and AMN082 had no significant effect on cell viability.

Figure 6-13 – Effect of MK-801 and NBQX on tendon derived cell viability at 24 hours



The boxes represent mean $\pm$ standard deviation. All results were normalised with respect to the control. Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle control. $n = 5$ disease and $n = 4$ control

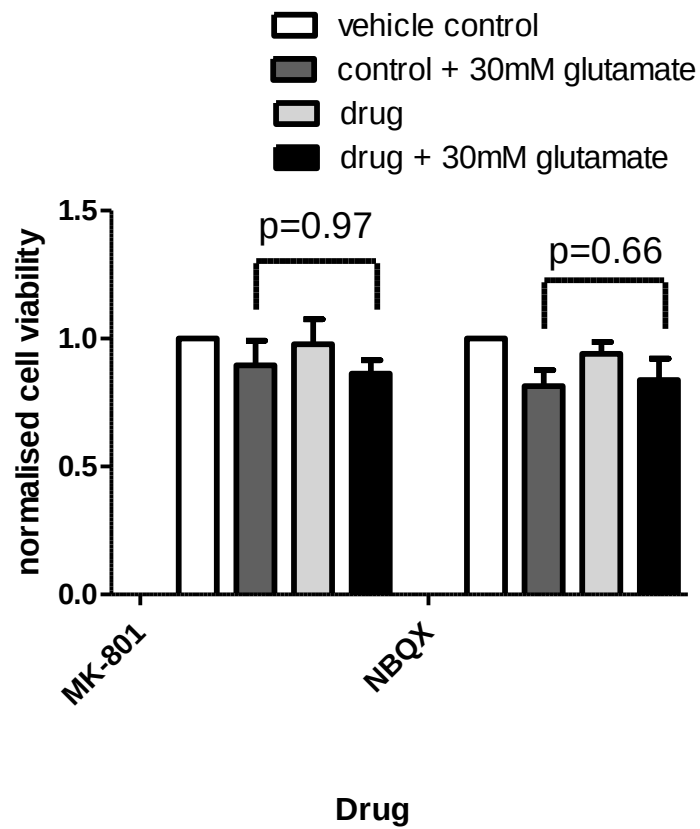
Figure 6-14- Effect of AMN082 on tendon derived cell viability at 24 hours



The boxes represent mean $\pm$ standard deviation. All results were normalised with respect to the control. Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle control. $n = 4$ disease and $n = 4$ control

The deleterious effect of 30mM glutamate was unaffected by the administration of MK-801 (10 μ M) or NBQX (100 μ M) demonstrating that this effect is likely non-specific and unrelated to the ionotropic glutamate receptors (Figure 6-15).

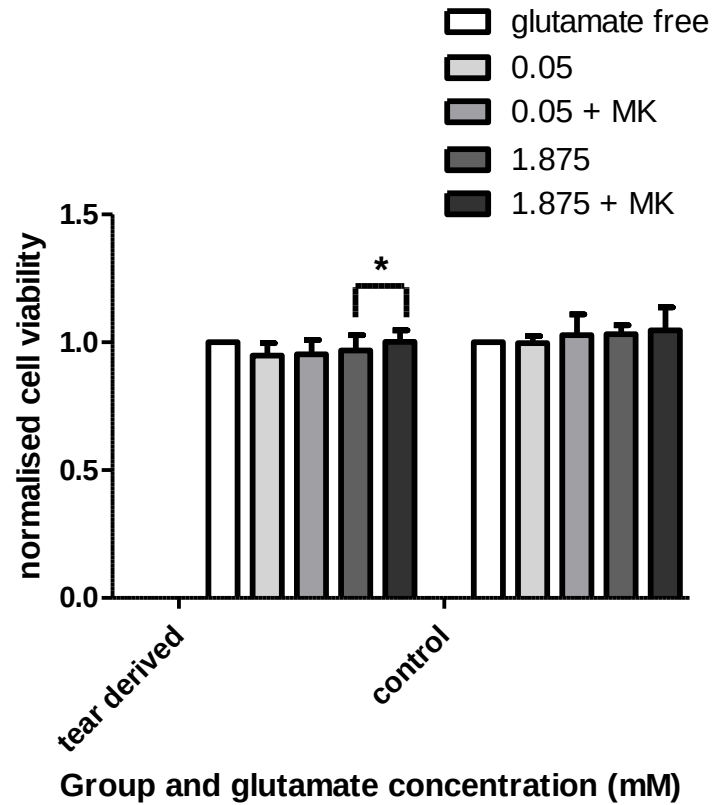
Figure 6-15 – Bar graph depicting the effect of drug treatment on the deleterious effect of 30mM glutamate on tendon tear derived cell viability at 24 hours



The boxes represent mean $\pm$ standard deviation. All results were normalised with respect to the control. Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n=4$

Figure 6-16 demonstrates that tendon tear derived cell viability was slightly reduced after 24 hours of 1.875mM glutamate exposure and this was attenuated with 10 μ M MK-801 administration.

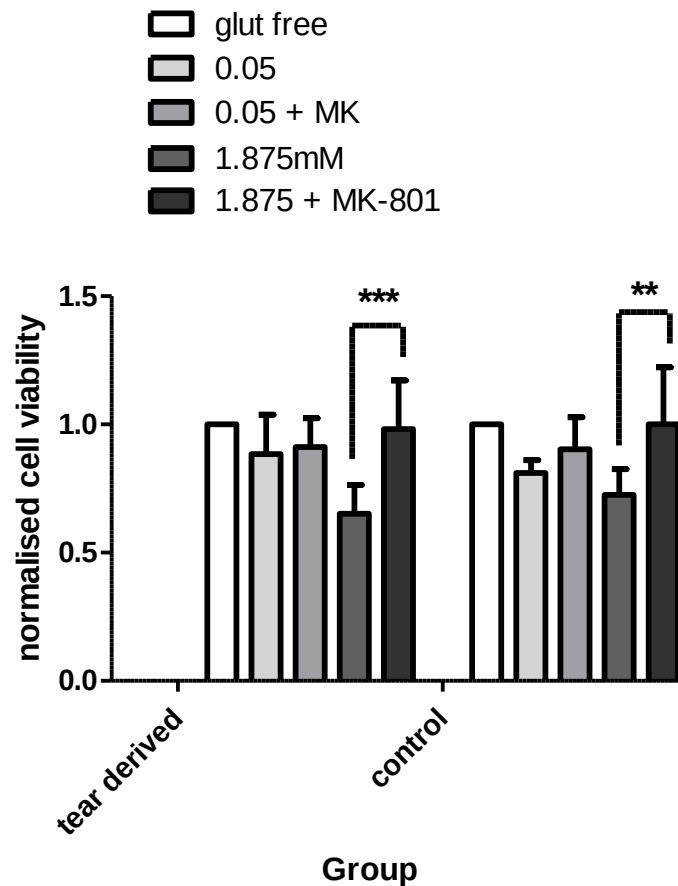
Figure 6-16 - Bar graph depicting the effect of NMDAR antagonism and glutamate exposure at 24 hours in tendon tear derived and control cells



The boxes represent mean $\pm$ standard deviation. All results were normalised with respect to the control. Statistical significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 4$ disease and $n = 4$ control

Figure 6-17 demonstrates that 1.875mM had a significantly deleterious effect on cell viability at 72 hours in both cell groups which was attenuated by 10 μ M MK-801. Cell viability was reduced with 0.05mM and 1.875mM glutamate exposure in the control cells (p=0.008). In tear derived cells viability was only significantly reduced with 1.875mM glutamate exposure (p=0.008) and not with 0.05mM exposure (p=0.1). There was no significant difference between 0.05mM glutamate versus 0.05mM glutamate with 10 μ M MK-801 in both tear derived and control cells (p=0.64 and 0.11 respectively).

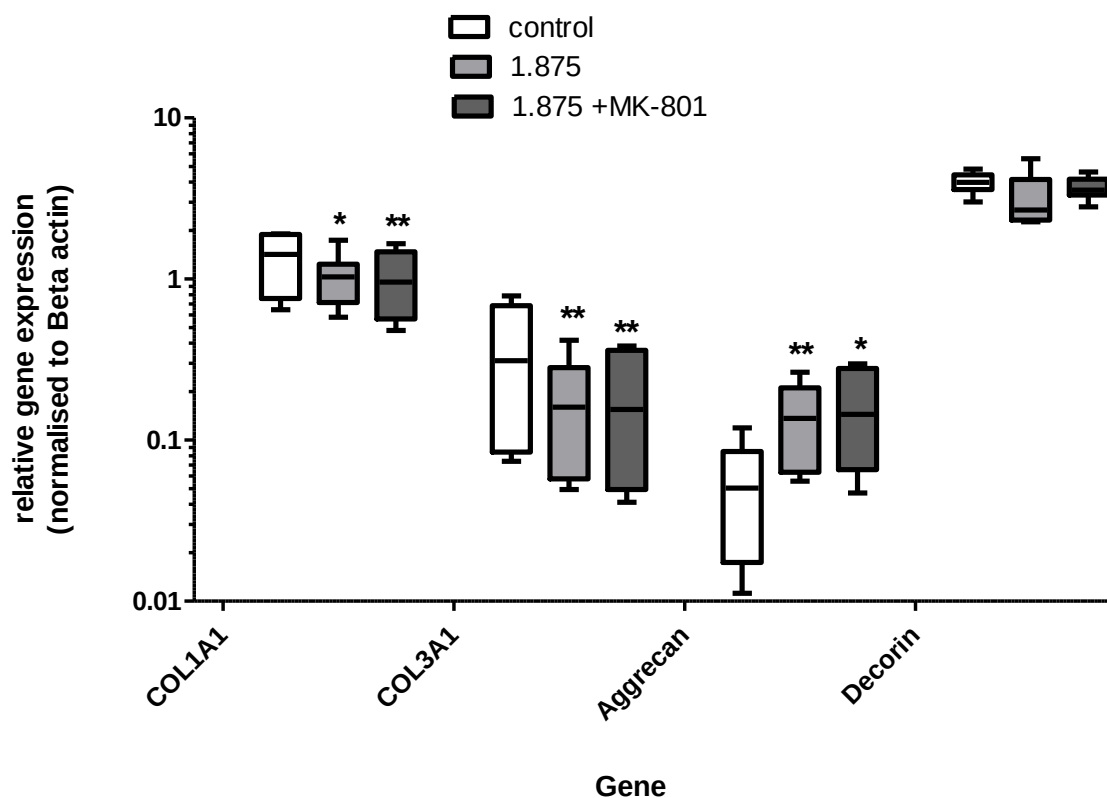
Figure 6-17 - Bar graph depicting the effect of NMDAR antagonism and glutamate exposure at 72 hours in tendon tear derived and control cells



The boxes represent mean $\pm$ standard deviation. All results were normalised with respect to the control. Statistical significance denoted by *p<0.05, **p<0.01, ***p<0.001. n=4 disease and n=4 control

Figure 6-18 shows that the alterations in COL1A1, COL3A1 and Aggrecan gene expression as a result of 1.875mM glutamate were not attenuated by 10 μ M MK-801 administration.

Figure 6-18 Box and whisker plot demonstrating the changes in matrix gene expression after 72 hours of glutamate exposure in tendon tear derived cells



The boxes represent median +/- interquartile range, while the whiskers represent range. Statistical significance denoted by *p<0.05, **p<0.01, ***p<0.001 versus glutamate free control. n=4

6.6 Discussion

Our study provides strong evidence that glutamate has significant effects on tendon derived cells *in vitro* and therefore that high levels of glutamate may contribute to the pathogenesis of tendinopathy.

The effect of 1.875mM glutamate on cell viability is small at 24 hours but has significantly increased after 72 hours. The 1.875mM glutamate exposure for 24 hours resulted in a significant down regulation of collagen gene expression (COL1A1 and COL3A1) and an upregulation of aggrecan expression; of note type I collagen expression is typically reduced in RCT, while aggrecan is increased^[38]. This finding also provides an explanation of how chronically elevated glutamate concentrations may lead to catabolic change in the tendon matrix and hence contribute to the pathogenesis of tendinopathy. The attenuation of the deleterious effect of 1.875mM glutamate on cell viability by 10µM MK-801 implies that this effect may be NMDAR dependent. However the fact that changes to COL1A1, COL3A1 and Aggrecan gene expression as a result of 1.875mM glutamate were unaffected by MK-801 suggests that these effects are independent of NMDAR. These changes in matrix gene expression may be mediated by other glutamate receptors such as AMPAR/KAR or they may be non-specific.

The role of the NMDA receptor is complex and highly variable^[254]. It can be protective against both apoptosis and excitotoxicity^[147] with the activation of the PI3K (phosphoinositide-3-kinase)-Akt kinase pathway and the suppression of pro-death genes appearing implicated in these effects. The pro-survival role of NMDAR is demonstrated by the fact that its blockade *in vivo* can promote oxidative damage and neuronal cell death. However at the other end of the toxicity curve excessive NMDAR activity promotes free radical production and oxidative stress *in vitro* and *in vivo*^[141 147]. NMDA

receptor antagonism in human synoviocytes and chondrocytes has been shown to be significantly anti-proliferative^[274 275]. The effect of MK-801 on cell viability may be related to non-specific mechanisms which become more likely as the drug concentration is increased into hundreds of μM .

Several inflammatory genes were up regulated in tendon tear derived cells versus controls; this supports the results of Chapter 5 and the concept that inflammation contributes to the pathogenesis of tendinopathy. The exact phenotype of the tendon-derived cells is unclear, but the pattern of gene expression (CD45/CD206/TNF α /PGP9.5) demonstrated perhaps points to more of a 'synoviocyte' category of cells than resident 'tenocytes'. The correlations between the glutamate receptor expression (KA1, NMDAR1, mGluR1, mGluR2 and mGluR5) and CD206 expression, alongside the lack of correlation between PGP9.5 expression and glutamate receptor expression, supports the findings from the dual labelling work in Chapter 5. It seems likely that the glutaminergic signaling machinery is predominantly expressed by the macrophage like type A synoviocytes *in vitro* and not the type B cells. The strong correlation between TNF α gene expression and CD206 infers that the TNF α secreted in disease is largely produced by the type A synoviocytes although further work is required to support this observation. It may therefore be postulated that the attenuation of the deleterious effect of 1.875mM glutamate by MK-801 may be related to its action on this more 'inflammatory' cell group, which may then release mediators which indirectly affect the other tendon derived cells.

It is not unsurprising that cell viability at 24 hours was not significantly reduced until hugely supra-physiological concentrations of glutamate were applied. The *in vitro* environment is oxygen rich, unlike the hypoxic environment present in tendinopathy^[35], which means that cells are likely to be far more resistant to glutamate exposure *in vitro*.

It may also be postulated that the majority of tendon derived cells do not express the ionotropic glutamate receptors, hence only the non-specific effects of glutamate are seen at 24 hours in this cell population. The contrasting effect on cell viability in disease and control likely represents the many cellular changes which occur in tendinopathy and combine to make cells more susceptible to extreme stress, as represented by glutamate exposure in this *in vitro* model. Levels of activated caspase-3 were increased by glutamate in both cell groups, demonstrating that the control cells were more resistant to apoptosis than disease cells. The fact that MK-801 and NBQX did not prevent this reduction in cell viability points to an as yet undetermined mechanism independent of the NMDA/AMPA/KA receptors. It could be speculated that increased levels of extracellular glutamate lead to a net efflux of potassium ions, and a net influx of sodium and hydrogen ions as glutamate is transported into cells. Certainly apoptotic cell death is strongly linked with hydrogen and potassium ion levels^[280 281]; these may be key factors by which glutamate increases caspase-3 activation and consequently apoptotic cell death. It has been previously shown that cysteine protects against glutamate induced cell death in human fibroblasts^[136]; this suggests that the cysteine/glutamate antiporter may be involved in the deleterious effects of glutamate seen in these experiments.

The main limitation to this work is related to the fact that the findings of *in vitro* experiments may not translate into real human *in vivo* disease; this is a limiting factor in interpreting the findings of any *in vitro* study. It is known that the *in vitro* environment is oxygen and metabolite rich unlike the *in vivo* disease environment. The absence of normal *in vivo* mechanical forces *in vitro* is another key difference that represents another limitation. The concentrations of glutamate used in the 'low concentration' experiments are at an appropriate level given previous work in this area which has shown that

physiological glutamate concentration is approximately 0.05mM and tendinopathic concentrations are into the hundreds of μM ^[239 276]. The IC50 of MK-801 and NBQX in human derived tendon cells are unknown. The concentrations used were based on previous work on chondrocytes and synoviocytes which are likely to be similar to the cell populations used in this study^[274 275 282]. Hamstring-derived cells are not the ideal control, cells that were matched in terms of patient age and the specific diseased tendon would have been ideal. However age is the strongest risk factor for RCT and it is likely that cellular ageing does play a key role in disease development.

6.7 Conclusions

In conclusion we propose that the effects of glutamate on tendon derived cells, which we have demonstrated in this *in vitro* study, contribute to the pathogenesis of tendinopathy. Novel therapeutics targeting glutaminergic signalling in synoviocytes may provide effective future treatment options for patients with painful tendinopathy.

Chapter 7 Discussion

7.1 Summary of Findings

This thesis has shown for the first time that glutamate and NMDAR1 are increased in rotator cuff tendinopathy. These results add to the work relating to the Achilles and patellar tendons by other groups^[239] ^[172] which have demonstrated that the glutaminergic system is altered in tendinopathy.

The effects of glucocorticoid injection and rotator cuff repair on supraspinatus tendon histology were different and contrasting. NMDAR1 was significantly increased in the glucocorticoid injection group and not in the rotator cuff repair group. Increases in cell proliferation, vascularity and HIF1 α were seen after surgical rotator cuff repair and these features are not seen after glucocorticoid injection.

This work has shown that specific glutaminergic changes (KA1, mGluR7 and mGluR2) are associated with inflammatory cell changes (CD45 and CD206) and the resolution of pain after surgery in rotator cuff tendinopathy. Similar changes in glutaminergic and inflammatory gene expression were seen in tendon tear extracted tendon cells versus healthy controls.

This thesis has demonstrated that glutamate has significant effects on tendon derived cells *in vitro* in terms of cell viability, as well as protein and gene expression. Exposure to 1.875mM glutamate for 72 hours results in reduced cell viability, decreased collagen (COL1A1 and COL3A1) and increased aggrecan gene expression; NMDAR antagonism with MK-801 attenuated this deleterious effect on cell viability but had no effect on the changes to matrix gene expression. These results imply that the deleterious effects of

glutamate on cell viability may be mediated by the NMDAR but that changes in matrix gene expression are independent of the NMDAR.

7.2 Clinical Relevance

My work demonstrates an association between pro-inflammatory cellular changes in tendon cells and pain. The correlations between inflammatory genes and glutamate receptor genes, combined with the co-localisation of NMDAR1 and CD206, suggest that the glutamate receptors are predominantly expressed on 'inflammatory' type cells within tendon. This raises the potential of targeting inflammatory cells as a disease-modifying therapy in rotator cuff tendinopathy. The finding that ionotropic glutamate receptor agonism increases inflammatory cytokine production^[138 274 278] in human synoviocytes and chondrocytes suggests that glutamate receptor modulation may be a viable means of therapeutically targeting these cells in painful tendinopathy, as has been successfully done in an animal model of osteoarthritis^[157]. The association between pain resolution and inflammatory cell numbers also suggests that targeting the TNF α pathway may also be effective in managing painful tendinopathy, as it has been in rheumatoid arthritis^[283]. Recent evidence has demonstrated the efficacy of methotrexate as a disease modifying therapy in knee osteoarthritis^[284], a disease that has historically been seen as a strictly degenerative condition. Disease-modifying anti-inflammatory agents such as methotrexate have also been used successfully in the treatment of rheumatoid arthritis for many years^[284]. Notably the sensitivity of a patient's synoviocytes to *in vitro* apoptosis in response to methotrexate is a predictor of the patient's clinical response to methotrexate therapy^[285]. It may be that specific sub-groups of patients with painful tendinopathy could be treated with disease modifying agents such as methotrexate. However it is worth considering that this type of drug has significant toxicity including liver and bone marrow effects. It would be important to

carefully weigh up the balance between their potential risks and benefits to patients in any future trial.

The *in vitro* effects of glutamate in terms of reducing cell viability, decreasing collagen gene expression and increasing aggrecan gene expression suggest that the raised levels of glutamate may contribute to the pathogenesis of tendinopathy. These results are consistent with several studies have shown that aggrecan synthesis is increased in rotator cuff tendinopathy^[38].

7.3 Future Avenues

There is a relative paucity of work relating to the characterisation of human tendon derived cells. Pauly et al^[286] characterised human supraspinatus and long head of biceps derived cells, comparing them with chondrocytes and osteoblasts largely in terms of matrix gene expression. They found that tendon derived cells expressed significantly less type II collagen and decorin compared to chondrocytes, while type I collagen expression was increased in the tendon derived cells. Taylor et al analysed the gene expression of equine tendon derived cells^[287], finding that COL1A2 was highly expressed *in vitro* and that scleraxis expression is reduced in culture. Collagen and decorin gene expression has been shown to alter as passage number increases^[288 289]. There is a complete lack of work relating to the characterisation cell phenotype in tendon, i.e. which cell surface markers are expressed in tendon derived cells; the characteristics of the 'tenocyte' are relatively unknown. Future work in this area in order to better characterise which cell sub-populations are present in culture would be valuable. It would also be of great value to investigate the protein and gene expression within these different subpopulations of tendon derived cells.

In the context of the increasing evidence supporting the role of inflammation in tendinopathy^[267], specific questions to be answered include whether significant proportions of cultured tendon derived cells are myeloid derived (CD45 positive) and have inflammatory characteristics (CD 206 positive for example). These questions could be answered using techniques such as flow cytometry and cell sorting, which would then enable the development of an understanding as to which cell subpopulations are expressing which particular proteins and genes. It may be hypothesised that a specific subpopulation of tendon derived cells are more inflammatory in terms of cell marker expression and that this group of cells is pivotal in propagating the inflammatory response in tendinopathy by secreting cytokines such as TNF α . Macrophage depletion has been shown to increase the ultimate tensile strength of healing mice Achilles tendons^[290]. Changes in macrophage phenotype and epithelial-to-mesenchymal transition genes have been noted following Achilles tenotomy and during repair^[291]. The development of a better understanding of the role of specific cell subpopulations in the epithelial-to-mesenchymal transition which occurs during tendon healing offers great potential in terms of developing novel therapeutics to augment tendon repair or to treat painful tendinopathy. The role of endothelial cells has been relatively underexplored *in vitro*. In the context of the significant glutaminergic endothelial changes in tendon that this work has shown to be associated with pain symptoms, further work to characterise the role of these cells in terms of their interactions with tendon derived cells using co-culture may be something that could be pursued.

There is also the potential for investigating the role of specific cytokines and their receptors *in vitro*. Gaida et al have demonstrated that TNF α signaling is present in the human Achilles tendon^[292], and TNF α has been shown to reduce cell viability and induce apoptosis in human tendon derived cells *in vitro*^[293]. Campbell et al investigated the role

of the IL-21 receptor (IL-21R) and found that TNF α resulted in an increase in IL-21R expression^[294] in human tendon derived cells. The pro-inflammatory effects of mast cells on human tendon derived cells have been demonstrated by Behzad et al^[295]. While complement split fragment C3a has been shown to increase inflammatory cytokine (TNF α /IL-1 β) production by human tendon derived cells *in vitro*^[296].

The *in vitro* work in this thesis reveals that glutamate has significant effects on tendon derived cells both in terms of viability and gene expression. The determination of changes in protein expression, particularly relating to the extracellular matrix, is an area that could be pursued *in vitro*. It would also be interesting to explore the mechanisms involved in these changes by attempting to delineate the specific glutamate receptors and cellular pathways involved. The deleterious effect of 1.875mM glutamate on cell viability was attenuated by MK-801, implying that this effect may be NMDAR dependent. However the mechanisms underlying the changes in matrix gene expression as a result of 1.875mM glutamate exposure remain undetermined. It would be interesting to determine whether these changes were dependent on AMPAR/KAR by observing the effect of NBQX on this response.

Given the limitations relating to *in vitro* work a logical future step may be to investigate the effect of glutamate receptor modulation *in vivo*, perhaps in an animal model of tendon healing or overuse tendinopathy. A great limitation of the animal models of rotator cuff tendinopathy is that they invariably use tendon detachment, chemical injury or overuse, rather than a chronic age-related degenerative process^[297]. The antagonism of inotropic glutamate receptors has shown promise in an animal model of knee osteoarthritis^[157], it would be fascinating if similar effects were to be observed in an animal model of tendinopathy.

Fundamentally a better understanding of the pathogenesis of tendinopathy and the underlying mechanisms is essential if we are to develop more effective future treatment strategies for the management of painful tendinopathy.

Chapter 8 Appendices

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8.1 Appendix 1: Publications associated with this thesis

A systematic review of the histological and molecular changes in rotator cuff disease. B. J. F. Dean, S. L. Franklin, and A. J. Carr. *Bone Joint Res* July 2012 1:158-166.

Why does my shoulder hurt? A review of the neuroanatomical and biochemical basis for shoulder pain. Dean BJF, Giwilyn SE, Carr AJ. *Br J Sports Med. British Journal of Sports Medicine*. 2013 Feb 21.

The Peripheral Neuronal Phenotype is Important in the Pathogenesis of Painful Human Tendinopathy: A Systematic Review. Floyd Dean BJ, Franklin SL, Carr AJ. *Clin Orthop Relat Res*. 2013 Apr 23.

A Novel Minimally Invasive Ultrasound-Guided Technique to Biopsy Supraspinatus Tendon. RJ Murphy, BJ Floyd Dean, K Wheway, B Watkins, ME Morrey, AJ Carr. *Operative Techniques in Orthopaedics* 23 (2), 56-62.

Tendinopathy: Same Disease Different Results – Why? ME Morrey, BJF Dean, AJ Carr, BF Morrey. *Operative Techniques in Orthopaedics* 23 (2), 39-49.

The risks and benefits of glucocorticoid treatment for tendinopathy: A systematic review of the effects of local glucocorticoid on tendon. Dean BJ, Lostis E, Oakley T, Rombach I, Morrey ME, Carr AJ. *Semin Arthritis Rheum*. 2013 Sep 26. pii: S0049-0172(13)00173-X. doi:10.1016/j.semarthrit.2013.08.006.

Glucocorticoids induce specific ion-channel-mediated toxicity in human rotator cuff tendon: a mechanism underpinning the ultimately deleterious effect of steroid injection in tendinopathy? BJF Dean, SL Franklin, RJ Murphy, MK Javaid, AJ Carr. *Br J Sports Med* 2014. doi:10.1136/bjsports-2013-093178 .

Franklin SL, Dean BJ, Wheway K, Watkins B, Javaid MK, Carr AJ. Up-regulation of Glutamate in Painful Human Supraspinatus Tendon Tears. *Am J Sports Med*. 2014 May 28. pii: 0363546514532754.

8.2 Appendix 2: Laboratory protocols

Details of Staining Protocols

8.2.1 Deparaffinisation and target retrieval

1. Working solution prepared according to manufacturer's instructions: 30mls of EnVision™ Flex high pH target retrieval solution mixed with 1470mls of deionised water to make up 1.5l of solution.
2. PT Link Tank filled with 1.5l of working solution in order to cover tissue sections.
3. PT Link set to preheat solution to 65°C.
4. Formalin fixed paraffin-embedded tissue sections immersed into pre-heated target retrieval solution and incubated at 97°C for 20 minutes.
5. Sections left to cool to 65°C in PT Link.
6. Slide racks removed from PT Link and immersed in PT Link Rinse Station containing diluted, room temperature Dako Wash Buffer (20X).
7. Slides left in the Wash Buffer for 5-10 minutes.

8.2.2 Haemotoxylin and Eosin staining protocol

The programming steps and Incubation times are pre-programmed on the DAKO® Autostainer as follows:

1. Modified Mayer's Hematoxylin – 10 minutes
2. Double Rinse
3. Bluing Reagent – 2 minutes
4. Rinse + Blow
5. Eosin Y – 1 minute
6. Double Rinse
7. 100% ethanol – 1 minute
8. Blow

Use a minimum of 400 µL of reagent per slide. Prior to starting a run, slides are deparaffinised and rinsed in water. The slides are pre-soaked in a working solution of DAKO® Special Stains Wash Buffer for 5 minutes. The slides are then placed on the DAKO® Autostainer and the run is begun. After the run is complete, the slides are dehydrated through 2 changes of 100% alcohol and cleared in 2 changes of xylene. Finally the slides are coverslipped with permanent mounting media.

8.2.3 Immunostaining protocol without linker for mouse/rabbit raised primary antibodies (IS-1)

1. Rinse	1 minute	Buffer
2. Peroxidase Block	5 minutes	200µl EnVision FLEX Peroxidase Blocking Solution
3. Rinse	1 minute	Buffer
4. Primary antibody	Variable time	200µl primary antibody raised in mouse or rabbit
5. Rinse	1 minute	Buffer
6. Detection	20 minutes	200µl EnVision FLEX/HRP
7. Rinse	1 minute	Buffer
8. Rinse	1 minute	Buffer
9. Stain	5 minutes	200µl DAB substrate working solution
10. Stain	5 minutes	200µl DAB substrate working solution
11. Rinse	1 minute	Buffer
12. Counterstain	5 minutes	200µl EnVision FLEX Haemotoxylin
13. Rinse	1 minute	Deionised water
14. Rinse	5 minutes	Buffer
15. Rinse	1 minute	Deionised water

8.2.4 Immunostaining protocol without linker for goat/sheep raised primary antibodies (IS-2)

1. Rinse	1 minute	Buffer
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2. Peroxidase Block	5 minutes	200µl EnVision FLEX Peroxidase Blocking Solution
3. Rinse	1 minute	Buffer
4. Primary antibody	Variable time	200µl primary antibody raised in sheep or goat
5. Rinse	1 minute	Buffer
6. Secondary antibody	20 minutes	200µl Anti Goat or Sheep Ig HRP
7. Rinse	1 minute	Buffer
8. Detection	20 minutes	200µl EnVision FLEX/HRP
9. Rinse	1 minute	Buffer
10. Rinse	1 minute	Buffer
11. Stain	5 minutes	200µl DAB substrate working solution
12. Stain	5 minutes	200µl DAB substrate working solution
13. Rinse	1 minute	Buffer
14. Counterstain	5 minutes	200µl EnVision FLEX Haemotoxylin
15. Rinse	1 minute	Deionised water
16. Rinse	5 minutes	Buffer
17. Rinse	1 minute	Deionised water

8.2.5 Immunostaining protocol with linker for mouse/rabbit raised primary antibodies (IS-3)

1. Rinse	1 minute	Buffer
2. Peroxidase Block	5 minutes	200µl EnVision FLEX Peroxidase Blocking Solution
3. Rinse	1 minute	Buffer
4. Primary antibody	Variable time	200µl primary antibody raised in mouse or rabbit
5. Rinse	1 minute	Buffer
6. Linker	15 minutes	200µl EnVision FLEX+ Rabbit or Mouse Linker
7. Rinse	1 minute	Buffer
8. Detection	20 minutes	200µl EnVision FLEX/HRP
9. Rinse	1 minute	Buffer

10. Rinse	1 minute	Buffer
11. Stain	5 minutes	200µl DAB substrate working solution
12. Stain	5 minutes	200µl DAB substrate working solution
13. Rinse	1 minute	Buffer
14. Counterstain	5 minutes	200µl EnVision FLEX Haemotoxylin
15. Rinse	1 minute	Deionised water
16. Rinse	5 minutes	Buffer
17. Rinse	1 minute	Deionised water

8.2.6 Dehydration, clearing and permanent mounting

1. Slides removed from Autostainer slide racks and placed into Leica slide racks.
2. Dehydrate

1 minute	70% alcohol
1 minute	90% alcohol
1 minute	100% alcohol
3. Clear

5 minutes	Xylene
5 minutes	Xylene
4. Mount Pertex mountant and application of cover slips.

8.2.7 Immunocytochemistry protocol

1. Rinse cells in PBS
2. Fix in 100% ice cold methanol for 5 minutes
3. Block for non-specific binding using 10% normal Donkey serum (or whichever animal your secondary is raised in) in PBS for 1 hour
4. Remove blocking solution without washing
5. Apply primary antibody diluted in 1% normal Donkey serum in PBS
6. Leave overnight at 4°C
7. Wash 2 x 5 minutes in PBS
8. Apply donkey anti-rabbit Texas red and donkey anti-mouse Alexaflour 488 (1:200) for 60 minutes
9. Wash 2 x 5 minutes in PBS
10. Optional: apply 1:1000 DAPI for 3-5 minutes then wash 2 x 5 minutes in PBS
11. Coverslip with Vectashield Mounting Medium with/out DAPI.

8.3 Appendix 3 - Additional Figures

Figure 8-1 Immunohistochemical staining of (A-D) mGluR1, (E-H) mGluR2 and (I-L) mGluR3 in the human rotator cuff tendon

Photomicrographs showing the immunohistochemical staining of different mediators in the rotator cuff tendon in control and tear groups. Scale bar: 20 μ m.

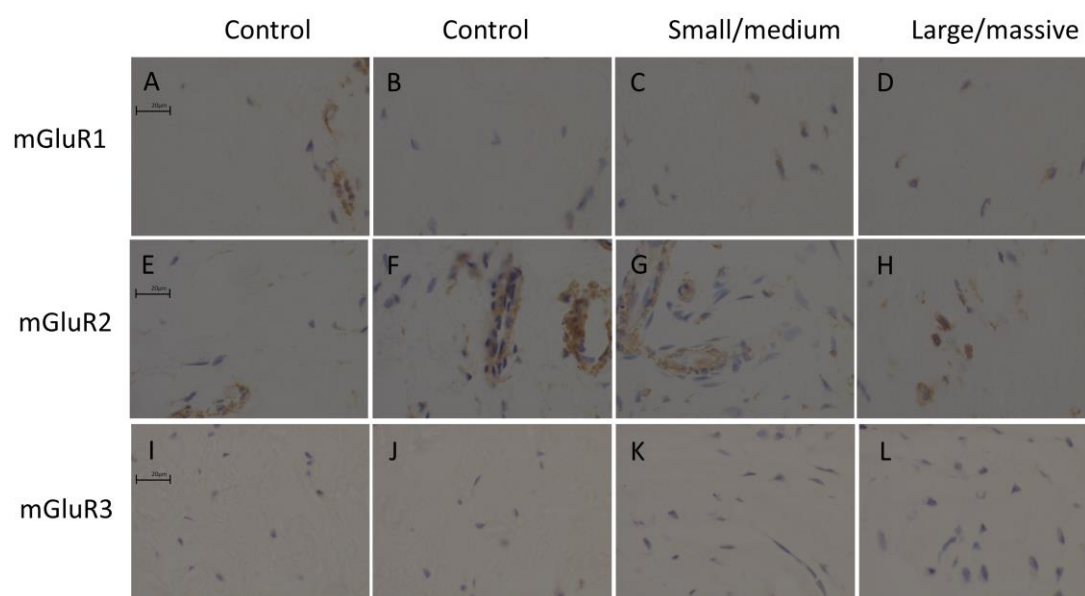


Figure 8-2 Immunohistochemical staining of (A-D) mGluR4, (E-H) mGluR5 and (I-L) mGluR8 in the human rotator cuff tendon

Photomicrographs showing the immunohistochemical staining of different mediators in the rotator cuff tendon in control and tear groups. Scale bar: 20 μ m.

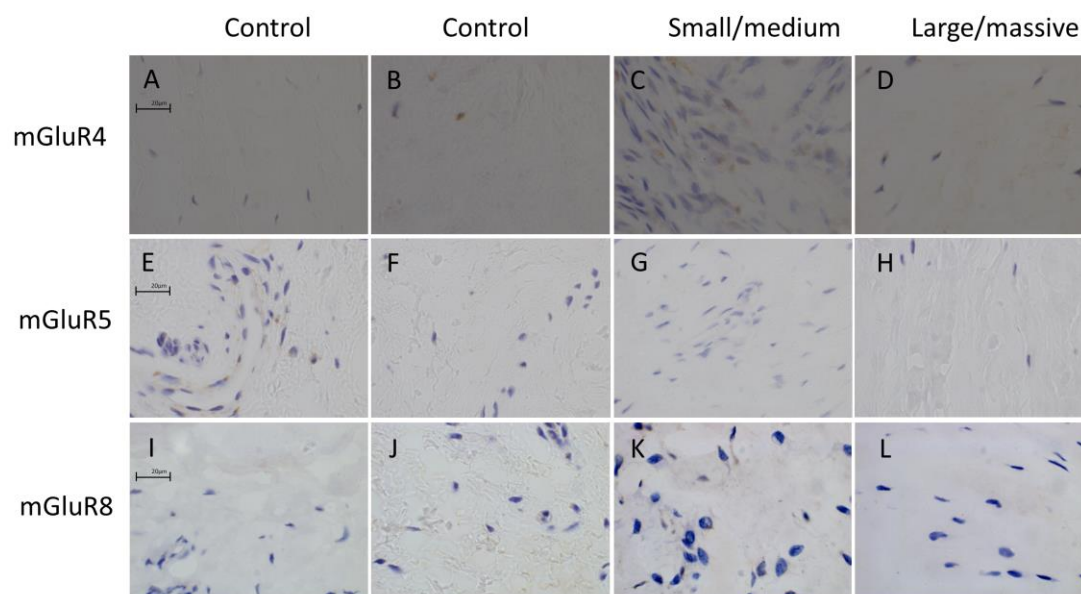


Figure 8-3 Immunohistochemical staining of (A-D) NGF, (E-H) NK-1 and (I-L) NPY in the human rotator cuff tendon

Photomicrographs showing the immunohistochemical staining of different mediators in the rotator cuff tendon in control and tear groups. Scale bar: 20 μ m.

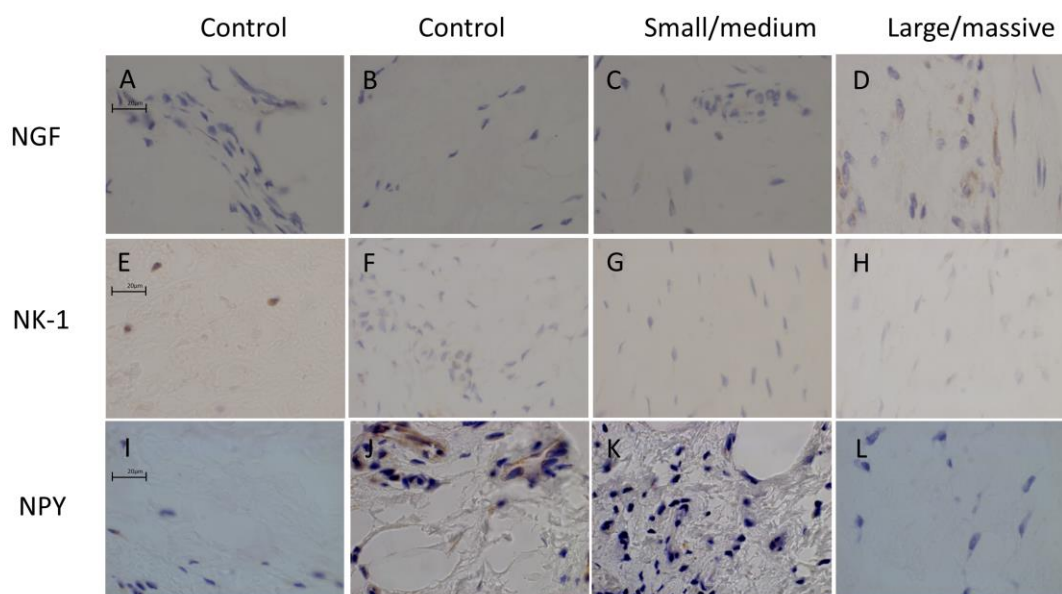
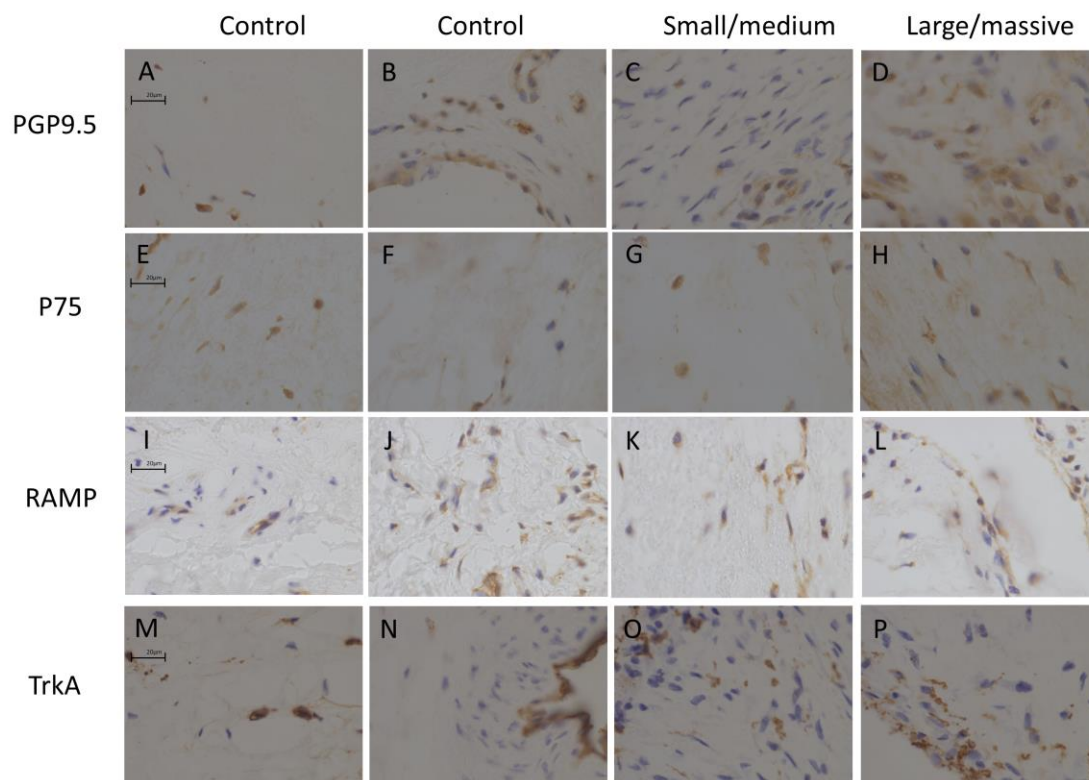


Figure 8-4 Immunohistochemical staining of (A-D) PGP9.5, (E-H) P75, (I-L) RAMP and (M-P) TrkA in the human rotator cuff tendon

Photomicrographs showing the immunohistochemical staining of different mediators in the rotator cuff tendon in control and tear groups. Scale bar: 20 μ m.



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