THE ROLE OF GLUTAMATE IN THE INFLAMMATORY RESPONSE OF THE KNEE

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Dedicated to my parents:

Barbara and Alan Flood

Thank you for all your love and support.

And a special thank you to Dr. Edmund Wadge.

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Abstract

Rheumatoid arthritis (RA) is an autoimmune disease characterised by joint inflammation. The resulting joint destruction is mediated by enhanced secretion of degradative enzymes and pro-inflammatory cytokines. Joint inflammation is accompanied by elevated levels of glutamate in the synovial fluid. Work in this thesis investigates the hypothesis that the increased glutamate concentration in RA synovial fluid can induce pathological changes associated with synovial joint destruction.

To determine whether cells of the synovial joint can respond to glutamate RNA was purified from various cells and tissues of the rat knee. RT-PCR revealed that metabotropic (mGluR4) and ionotropic glutamate receptors (NMDA NR1, KA1, AMPAGluR2, AMPAGluR3) and glutamate transporters (EAATs1 to 3) are expressed in the synovial joint. Differences in expression between RA and normal fibroblast-like synoviocytes (FLS) were observed.

The effect of modulating these receptors and transporters in normal and RA FLS was investigated. RA and normal FLS were treated with a range of glutamate concentrations and inhibitors of glutamate transporters and receptors. Markers of inflammation and matrix degradation were measured. Pro-MMP2, TIMP1, TIMP2 and IL-6 levels were modulated with changes in extracellular glutamate. Elevated production of IL-6, pro-MMP2, TIMP1 and TIMP2 by RA FLS was observed in the presence of glutamate transporter inhibitors. Inhibition of kainate receptors decreased IL-6 production by RA FLS and inhibition of NMDA receptors increased pro-MMP2 in these cells. Glutamate receptor and transporter inhibition caused different responses in normal FLS. The effect of TNFα and IL-6 on expression of the glutamate transporter EAAT1 was also determined. RT-PCR, immunohistochemistry (IHC) and Western blotting revealed that expression of EAAT1 mRNA and protein was increased in RA FLS in response to IL-6. This was not observed in normal FLS. IHC also demonstrated that TNFα increased EAAT1 protein expression in RA FLS but not in normal FLS.

To investigate the function of glutamate receptors, RA FLS were pre-loaded with the calcium indicator fluo-4 and stimulated with glutamate, NMDA or kainate. Upon stimulation, scanning confocal microscopy revealed increases in fluorescence generated by intracellular free calcium indicating functional NMDA and AMPA/kainate receptors in human RA FLS. Using a ^{14}C-glutamate uptake assay it has also been demonstrated that glutamate transporters are functional in RA FLS.

This data demonstrates that glutamate receptors and transporters are expressed in the synovial joint *in vivo* and are functional in human FLS. Furthermore, modulation of glutamate receptors and transporters influences release of IL-6, TIMPs and pro-MMP2 by FLS. Expression and responses differ between RA and normal FLS but the high levels of glutamate found in RA synovial fluid may activate glutamate receptors to induce a pro-inflammatory and degradative phenotype in FLS. This may be influenced through IL-6 and TNFα induced increases in EAAT1 expression, indicating a possible feedback mechanism. It is proposed that the kainate receptor pathway that increases IL-6 release in response to glutamate warrants further investigation as a therapeutic target for RA.

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Abbreviations

AC	adenylate cyclase
Acc No	accession number
ADAMs	A disintegrin and metalloproteinase
ADAMTS	A disintegrin and metalloproteinase with thrombospondin repeats
DAP5	D-(-)-2-Amino-5-phosphonopentanoic acid
AP1	activator protein 1
APS	ammonium persulphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CFM2	1-(4¢-Aminophenyl)-3,5-dihydro-7,8-dimethoxy-4*H*-2,3-benzodiazepin-4-one
DAG	diacylglycerol
DMEM	Dulbecco's modified eagles's medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
EAAC1	excitatory amino acid carrier 1
EAAT	excitatory amino acid transporter
ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
ERK	extracellular signal-regulated protein kinase
FBS	foetal bovine serum
FITC	fluoroscein isothiocyanate
FLS	fibroblast like synoviocyte
g	gravitational force
GLAST	glutamate and aspartate transporter
GLT1	glutamate transporter 1
HRP	Horseradish peroxidase
IL	interleukin
IL-6R	interleukin 6 receptor
IP_3	inositol triphosphate
JAK	janus kinase
LDH	lactose dehydrogenase
LPS	Lipopolysaccharide
Mab227	monoclonal antibody 227, inhibitor of IL-6 signalling
MAPK	mitogen-activated protein kinase
MEK	MAP Kinase Kinase Kinase
MMPs	matrix metalloproteinases
mRNA	messenger ribonucleic acid
NADH	Nicotinamide Adenine Dinucleotide
NBQX	2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f] quinoxaline-7-sulfonamide

NFκB	nuclear factor kappa B
NIH	National institute of health
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
OSM	Oncostatin M
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDZ	PSD-95/Discs-Large/ZO-1
PI3K	phosphoinositide 3 kinase
PK	protein kinase
PLC	phospholipase C
*t*PDC	*trans*-Pyrrolidine-2,4-dicarboxylic acid
RA	rheumatoid Arthritis
RNA	ribonucleic acid
RT	reverse transcription
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
sIL-6r	soluble interleukin 6 receptor
STAT	signal transducer and activator of transcription
TBOA	DL-*threo*-*E*-Benzyloxyaspartic acid
TBS	tris-buffered saline
TEMED	tetramethylethylenediamine
TGF	transforming growth factor
TIMPs	tissue inhibitor of metalloproteinases
TNF	tumor necrosis factor
TRAD	TNF-R1-associated death domain
TRAF	TNF receptor-associated factor
w/v	weight per volume
VCAM1	vascular cell adhesion molecule 1
v	volts
v/v	volume per volume

Chapter 1 Introduction and background

1.1 Introduction

Rheumatoid arthritis (RA) is a painful, debilitating, autoimmune disease resulting in reduced life expectancy due to susceptibility to infection (Baum, J. 1971). The disease was first recorded several thousand years ago in native American populations, but was not reported in Europe until the 17th century (Firestein 2003). It affects more than 350,000 people in the UK and women are more affected than men at a ratio of approximately 3:1 (www.arc.org.uk). RA is a systemic disease because not only does it affect the joints it also causes loss of muscle strength, vasculitis, depression, pulmonary nodules and osteoporosis.

This study focuses on RA disease progression in the peripheral synovial joints and aims to determine whether the elevated glutamate levels observed in the synovial joints of rheumatoid arthritis (RA) patients contributes to disease pathology. Prior to a review of the literature linking glutamate signalling to inflammatory and degradative pathways, the anatomy of synovial joints, the role of inflammatory cytokines and degradative enzymes and an overview of RA treatments will be given.

1.2 The Synovial Joint

A synovial joint is a specialized type of joint whereby juxtaposed bone surfaces are separated by a joint cavity lined by a synovial membrane. The bones' articular surfaces are covered in hyaline cartilage allowing low-frictional movement. The peripheral joints are most affected by RA, the knee joint being one of these (www.arc.org.uk).

The human knee joint, like other synovial joints, is encapsulated in a fibrous capsule which is lined with a synovial membrane or synovium. The joint space contains within this capsule synovial fluid which nourishes the cartilage and lubricates the joint. Surrounding collateral ligaments stabilise the knee by attaching the femur to the fibula and tibia. Within the knee joint the internal anterior and posterior cruciate ligaments also provide support by attaching the femur to the tibia. Within the knee, two half moon

pieces of fibrocartilage, called the menisci, also provide support and help to spread load and generally stabilise the knee joint. The knee joint is protected by the patella, underneath this is the vascularised fat pad which acts as a cushion to absorb load across the joint (Dudhia *et al.* 2004). Figure 1.1 shows the major components of the human knee joint.

1.2.1 Synovium

The lubricating synovial fluid is produced by the synovium which covers the non-cartilaginous surfaces of diarthrodial joints. The synovial membrane is composed of a synovial cell layer and an adipose layer. The adipose layer is extensively vascularised and consists of adipocytes, nerve endings, loose connective tissue and immune cells. Movement of fluid and inflammatory cells into the joint is facilitated by the blood vessels present in the adipose layer. The innermost synovial cell layer of synovium contains mainly macrophages and fibroblasts (Tak *et al.* 2001). The macrophages are called macrophage-like synoviocytes (type A synoviocytes) and the fibroblasts are termed fibroblast-like synoviocytes (type B synoviocytes or FLS). 70% of synoviocytes are FLS. FLS produce many of the proteins found in synovial fluid.

1.2.2. Synovial fluid

Synovial fluid is in direct contact with the synovium and articular cartilage. Normally 1-4ml of synovial fluid is present in the human knee (Dudhia *et al.* 2004). The pH of synovial fluid is rarely less than 7 (Lloyd *et al.* 1990, Brooks *et al.* 2000). Synovial fluid is in dynamic exchange with blood serum and contains electrolytes, hyaluronate, urate, urea, albumin and small globulins. The synovial fluid has many functions. It provides nutrients to joint components and provides lubricating factors for the joint cartilage. Inflammatory cells move into the synovial fluid of the joint after trauma or infection.

1.2.3 Cartilage

A hallmark of RA is destruction of the articular cartilage that lines the articulating bone surfaces within synovial joints, this is accompanied by inflammation. Articular cartilage is a connective tissue consisting of an extracellular matrix (ECM) (80% water,

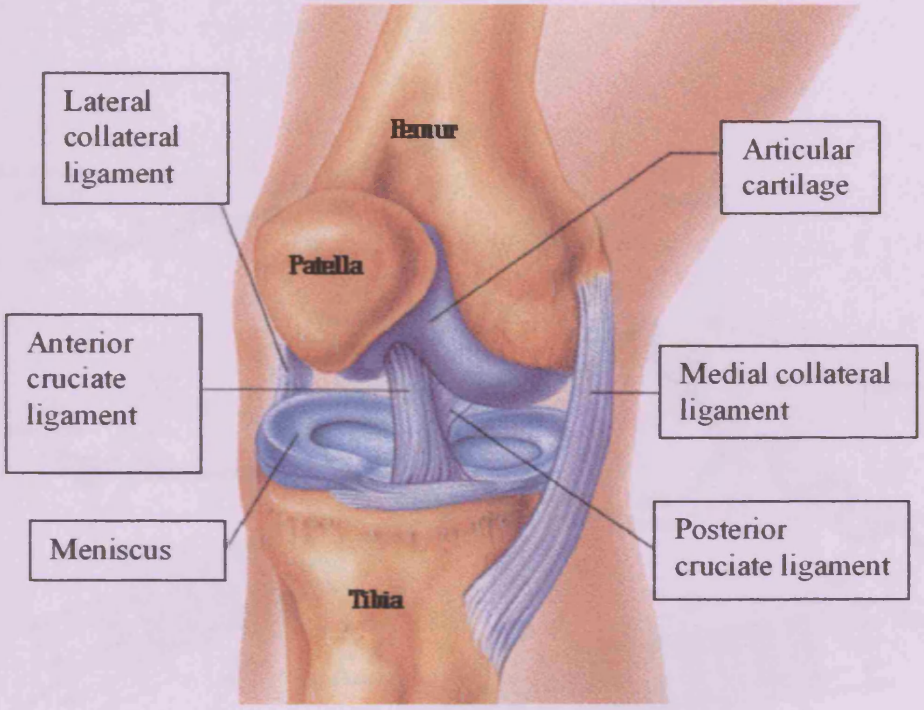
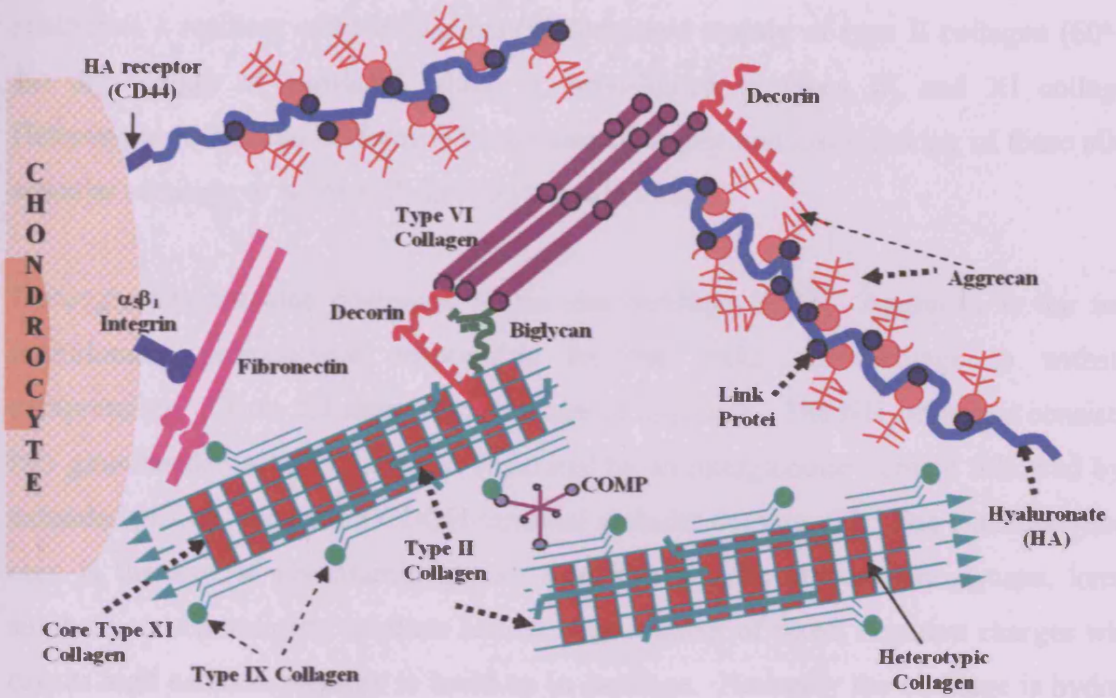


Figure 1.1: Diagram of a healthy knee joint (adapted from www.sky-injury.com and used with permission of Dr. Mike Langran).

Figure 1.2 A schematic diagram showing interactions of proteins in the extracellular matrix of articular cartilage (adapted from a picture kindly donated by Prof. V. Duance, Cardiff University).



proteoglycans and collagens) that is synthesised and maintained by chondrocytes (Lane *et al.* 1975). Figure 1.2 shows a schematic diagram of the protein interactions in the ECM of articular cartilage. There are four zones that articular cartilage is divided into depending on the arrangement of the chondrocytes and their matrix components: superficial, intermediate, deep and calcified (See figure 1.3). Articular cartilage functions to provide smooth surfaces of low resistance that can withstand compressive forces (Morris *et al.* 2000). It can carry out this function because the chondrocytes synthesise a resilient extracellular matrix composed mainly of type II collagen (60% of the dry weight of cartilage) which is cross-linked to types IX and XI collagens. Heterotypic fibrils can be formed from these collagens and cross-linking of these allows articular cartilage to withstand shear and tensile forces.

Proteoglycans are also contained within the cartilage ECM. Aggrecan is the major multidomain proteoglycan responsible for the ability of cartilage to withstand compression. Figure 1.4 shows the structure of aggrecan. The NH_2 terminus consists of two globular domains, G1 and G2, separated by an interglobular domain followed by an extended protein core and a COOH-terminal globular domain, G3. The extended protein core is the site of glycosaminoglycan attachment. The glycosaminoglycans, keratan sulphate and chondroitin sulphate have a large number of fixed, negative charges which causes high osmotic pressure to build up in cartilage. Normally the cartilage is hydrated but under compression the water is squeezed out whilst the cartilage framework remains unaltered thus absorbing shock (reviewed in Heinegard *et al.* 2000). Aggrecan is attached via its N-terminal domain to hyaluronan, this interaction is stabilized by a link protein.

In normal adult cartilage the chondrocytes maintain the rate of synthesis of ECM components at a rate equal to that of degradation by proteases (see section 1.5.1). In RA this process is disrupted and destruction of the cartilage in RA may be followed by destruction of bone.

Figure 1.3 The cellular architecture of articular cartilage.

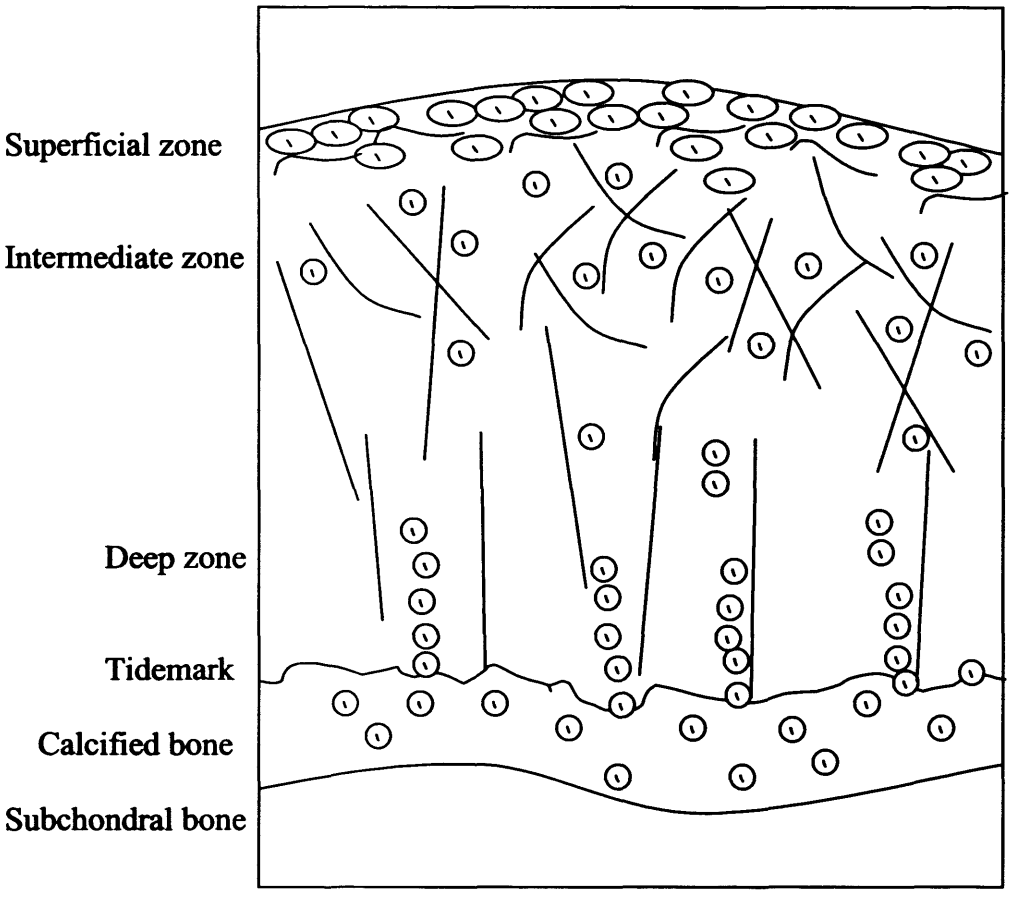
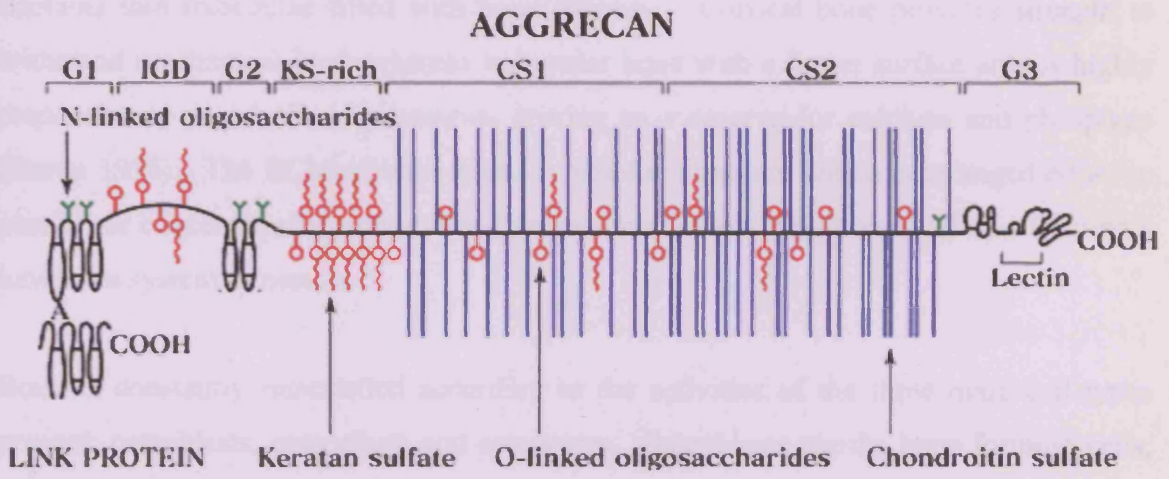


Figure 1.4 The structure of aggrecan
The N-terminus consists of two globular domains, G1 and G2, separated by an interglobular domain (IGD). An extended protein core and a COOH-terminal globular domain, G3, follow the G2 domain. The extended protein core is the site of glycosaminoglycan attachment (chondroitin sulphate:CS, keratan sulphate:KS). In aggregates the G1 domain of aggrecan binds to hyaluronan and this binding is stabilized by link protein, also shown in this figure. (www.glycoforum.gr.jp/).



1.2.4 Bone

There are generally two types of bone in the skeleton: long bones and flat bones. It is the long bones that articulate through synovial joints. At each end of a long bone are the articular cartilage-covered epiphyses separated by the diaphysis. Two bone surfaces are in contact with soft tissue: the external periosteum and the internal endosteum surface. Osteogenic cells organised in layers line these surfaces. The external part of the bone diaphysis is made of cortical bone. Cortical bone is dense and has increased calcification. Between the epiphysis and diaphysis is spongy bone or trabecular bone. Trabecular bone contains thin trabeculae filled with bone marrow. Cortical bone provides strength to withstand mechanical load, whereas trabecular bone with a larger surface area is highly responsive to metabolic requirements serving as a reserve for calcium and phosphate (Baron 1996). The ECM of bone forms a lamellar structure which is arranged either in parallel or concentrically around blood vessels, nerves and lymph vessels, thus forming a haversian system or osteon.

Bone is constantly remodelled according to the activities of the three main cell types present; osteoblasts, osteoclasts and osteocytes. Osteoblasts are the bone forming cells, they deposit osteoid, which is the organic matrix of bone and control its subsequent mineralisation (Schenk *et al.* 2002). The mineral in fully calcified bone is composed mainly of hydroxyapatite crystals (Baron 1996). Upon completion of bone formation, osteoblasts differentiate into bone lining cells to line the periosteum and endosteum, become osteocytes embedded within the mineralized bone matrix, or die (reviewed in Baron 1996). Osteocytes form a network of communicative cells throughout bone matrix and are the most common cell in bone and are currently thought to regulate bone remodelling in response to mechanical loading signals (reviewed in Noble and Reeve 2000).

Osteoclasts are the bone resorbing cells and adhere to the bone surface and seal off the area to be resorbed, in a 'sealing zone'. The bone mineral is dissolved by various enzymes within the acidic environment within the resorption lacunae. This then allows

the bone matrix to be broken down by lysosomal enzymes and metalloproteinases (see section 1.5.1.3) (Schenk *et al.* 2002).

The remodelling or turnover of bone occurs on the surfaces of bone and is regulated by many factors including cytokines (see section 1.4.1.1), hormones and mechanical load.

1.3 Rheumatoid arthritis

1.3.1 The etiology of rheumatoid arthritis

The initial trigger or cause of rheumatoid arthritis is unknown however, RA is considered to be an autoimmune disease. It is clear that both genetic and environmental factors play a role in the onset of RA. The human leukocyte antigen (HLA) allele is the locus at which genetic links have been shown to RA; HLA genes of the major histocompatibility complex (MHC) are on chromosome 6. Mutations in the DR4 gene (also termed HLA DRB1) found in the HLA region appear to confer a predisposition to RA (Stastny, P. 1978). The only known function of the HLA DR4 gene is to present peptides to CD4+ T cells. However HLA-DR4 susceptibility mutations occur in about 20 - 30% of the population and yet only 2% develop RA. Other genes of HLA region have been connected to a susceptibility to RA however only 40% of the genetic contribution to RA is accounted for by genes in the HLA region (Fife *et al.* 2000). This together with the fact that monozygotic twin studies have shown concordance to be approximately 15% (if one twin has RA, there is a 15% chance that the other twin will also suffer from RA), demonstrates that non-genetic factors play a significant role in RA (Silman *et al.* 1993).

A role for bacterial or viral infection as the onset of RA has been the topic of much research, however no individual infectious agent has yet been identified. Bacterial or viral infection might initially trigger autoimmunity in RA via molecular mimicry or bystander activation of IgG antibodies thus accounting for the presence of rheumatoid factor in 80% of RA patients (Kingsley 2000). Rheumatoid factor (RF) is the term for IgM autoantibodies specific for antigenic epitopes on the Fc portion of IgG antibodies. RF is useful in the diagnosis of RA but it is not exclusive to patients with rheumatoid arthritis and not all patients with RA express RF. Approximately 20% of RA patients

are sero-negative for rheumatoid factor. Indeed, RF can be detected in other autoimmune disorders and even in healthy individuals; however RF from RA patients has a higher affinity and is specific for human IgG (Kyburz and Carson 2000). RFs promote inflammatory processes and accelerate tissue destruction (Kyburz and Carson 2000) because they are autoantibodies and therefore recruit macrophages and neutrophils to the synovial joint.

1.3.2 Pathology of rheumatoid arthritis

RA is an inflammatory disease, primarily causing inflammation of the synovial joints, spine and can also affect the internal organs. Early in the disease, following the unknown initiating event for RA, oedema occurs and hypertrophy of the synovium. White blood cells, mainly CD4+ T cells, infiltrate the joint and cause an inflammatory response by releasing cytokines, prostaglandins and other inflammatory mediators. The infiltrated T-cells release cytokines causing activation of macrophage-like synoviocytes and FLS. This in turn causes proliferation of the synoviocytes, which form an invasive tissue called the pannus. The pannus is defined as "a granulomatous mass of proliferating synovial tissue, derived from the synovium" (Sweeney and Firestein 2004). The point where the invading pannus meets the cartilage is called the cartilage-pannus junction (CPJ). The CPJ consists mainly of synoviocytes and macrophages, which are the major source of inflammatory cytokines that perpetuate the joint destruction (see section 1.4.1.1). Adhesion molecules facilitate the migration of T cells to the joint and the adherence of the pannus to cartilage and bone where the process of cartilage and bone degradation begins.

The activated macrophages release the pro-inflammatory cytokines IL-1 and TNFα (see section 1.4.1.1) which allow the FLS to be permanently activated to release cytokines, prostaglandins and proteases (the actions of these proteins in RA are discussed further in sections 1.4 and 1.5). The degradation of aggrecan and collagen (see section 1.2.3) by these proteases leads to loss of cartilage function and hence arthritic joint disease. The FLS produce the pro-inflammatory cytokine IL-6 (see section 1.4.1.1.3) which may travel via the circulation to the liver where, in conjunction with IL-1 and TNFα, it can activate

the acute phase response (see section 1.4.1) (McNiff 1995; Okamoto *et al.* 1997). It is activation of the acute phase response that contributes to the systemic effects of RA.

1.3.2.1 Destruction of cartilage and bone in rheumatoid arthritis

It is the destruction of cartilage and bone that causes the disabling conditions observed in RA patients. In the rheumatoid synovium cathepsins (section 1.5.1.1) and MMPs (section 1.5.1.3.1) are abundant (Firestein 1996: Keyszer *et al.* 1995). The release of proteases by FLS causes cartilage matrix turnover to become dysregulated, which causes the characteristic lesions of RA (Reviewed in Pap *et al.* 2000). Chondrocytes (see section 1.2.3) also contribute to synovial joint destruction because *in vitro*, chondrocytes behave similarly to FLS; they both have IL-1 and TNFα receptors and respond to these cytokines with an increased production of proteases (Cunnane *et al.* 1998).

1.3.3 Animal models of rheumatoid arthritis

There are a number of animal models used to investigate rheumatoid arthritis where local or systemic injection of antigens induces an RA-like immune response.

1.3.3.1 Bacteria-induced arthritis

The injection of live-bacteria can induce RA-like symptoms in experimental animals. *Staphylococcus aureus* (Abdelnour *et al.* 1994) and *Borrelia burgdorferei* (Schaible *et al.* 1991), mycobacterium (Pearson 1956), or purified bacterial products (muramyl dipeptide or lipopolysaccharide) injection (Terato *et al.* 1996; Chang *et al.* 1981) can be used to induce arthritis in various animal models, predominantly rats and mice. In addition, arthritis can be induced in rats by the intraperitoneal injection of streptococcal cell-wall fragments, which causes acute inflammatory arthritis (Cromartie *et al.* 1977).

1.3.3.2 Antigen-induced arthritis

Antigen-induced arthritis (AIA) has been induced in rats, mice, rabbits and primates by injection of an antigen into the knee joint of a pre-sensitised animal (Courtenay *et al.* 1980, Trentham *et al.* 1977, Yoo *et al.* 1988). This results in an immune-complex-mediated arthritis similar to human RA (Linton and Morgan 1999). Various antigens

have been used. For example methylated bovine serum albumin (mBSA) is frequently used. The animals are pre-immunised with the mBSA (injected systemically), then a few weeks later, the same antigen is injected locally into a joint and a T-cell dependent immune complex-mediated arthritis ensues (Holmdahl 2000).

1.3.3.3 Collagen-induced arthritis

Another common model is the type II collagen induced arthritis where the collagen is injected into the knee along with an adjuvant usually 21 days after sensitisation with type II collagen. This type of RA causes a systemic response and can be induced in rats, mice and primates. It is characterised by erosive joint inflammation, which is mediated by T and B cells, pannus formation and synovial hyperplasia (Lindqvist *et al.* 2002). Anti type II collagen antibodies are found in the synovial fluid of this model (Linton and Morgan 1999) showing an autoimmune response has occurred. Other cartilage proteins such as cartilage oligomeric matrix protein (COMP) (Carlsen *et al.* 1998), aggrecan (Glant *et al.* 1987), type XI collagen (Morgan *et al.* 1983) or aggrecan link protein (Zhang *et al.* 1998) have also been used in a similar manner to induce arthritis in animal models.

1.3.3.4 Adjuvant-induced arthritis

The adjuvant-induced arthritis is a destructive, relapsing polyarthropathy causing joint-specific inflammation and is commonly carried out in rats. Non-immunogenic compounds are used; mineral oil (Holmdahl *et al.* 1992), pristane (Vingsbo *et al.* 1996) and avridine (Chang *et al.* 1980) have all been used as adjuvants to induce arthritis. The adjuvant spreads throughout the body with (usually symmetrical) arthritic symptoms occurring in the peripheral joints after 1-2 weeks. The arthritides each adjuvant induces are very similar but differ mainly in the severity and chronicity of the disease (Holmdahl *et al.* 2000).

1.3.3.5 Genetically susceptible animal models of arthritis

Other models of RA include those mouse strains that are inbred (Corthay *et al.* 2000) or genetically manipulated so that they spontaneously develop arthritis. Mice that over-express the human TNFα transgene spontaneously develop acute arthritis; treatment with

TNFα antibodies can prevent the onset of the disease (Keffer *et al.* 1991) and if administered after arthritis has set in, cartilage degradation can be reversed in young mice (Shealy *et al.* 2002). Mice that have the IL-1 receptor antagonist knocked out also spontaneously develop arthritis (Horai *et al.* 2000) and indeed mice that are manipulated to over-express IL-1 develop a severe polyarthritic phenotype (Niki *et al.* 2001). All of these models can provide a useful insight to the disease mechanism and treatment of RA.

1.3.3.6 Knock-out mice

Knock-out mice are also used as a study tool for RA. Cytokines deemed important in RA such as interleukin 6 (IL-6), tumour necrosis factor α (TNFα) and IL-1 have been knocked-out and arthritis induced to see the effects of specific inflammatory mediators in the disease (see sections 1.4.1.1.3, 1.4.1.1.1 and 1.4.1.1.2).

1.3.4 Therapy and treatments for rheumatoid arthritis

There is currently no cure for RA, therapies thus far developed merely attempt to limit destruction of the joint, reduce pain and hence prevent loss of function. Many classes of drugs are used to treat RA. Because of its ability to kill mycobacteria *in vitro,* gold was once used as a treatment for RA. In fact gold does reduce the production of IL-6 *in vitro* by an unknown mechansim (Cunnane *et al.* 1998) and also reduces serum levels of IL-6 (Madhok *et al.* 1993).

1.3.4.1 Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are a common course of treatment for RA sufferers. They affect the inflammatory response by acting upon chemical mediators of inflammation. They inhibit prostaglandin (see section 1.4.1.2) production by blocking COX function. COX-1 is involved in synthesis of prostaglandins involved in normal physiological functions whereas the COX isoform, COX-2, is an enzyme that produces prostaglandins that mediate inflammation. The potency of different NSAIDs to inhibit the two isoforms varies (Luong *et al.* 1996). Despite their popularity, the beneficial effects of NSAIDS on the pain and swelling are however short-lived. Other drugs used with anti-inflammatory action are corticosteroids; they are capable of significantly

alleviating the symptoms of inflammation. They reduce the synthesis of IL-1, IL-2, IL-6 and TNFα (Grabstein *et al.* 1986, Buttgereit *et al.* 1995) and inhibit the expression of MMPs in the synovial joint (Werb 1978). However, the long-term effects of glucocorticoids such as risk of osteoporosis, infection and diabetes have made them less popular.

1.3.4.2 Immunosuppressive drugs

Immunosuppressive drugs such as cyclophosphamide, target cells involved in regulation of the immune response. Cyclophosphamide reduces the proliferation of lymphocytes, plasma cells and mononuclear cells at bone lesion sites (Williams 1974) reducing effects of the inflammatory response. However, the use of cyclophosphamide is restricted due to severe side-effects such as hemorrhagic cystitis, nausea, vomiting, thrombocytopenia, alopecia and herpes zoster infections (Suarez-Almazor *et al.* 2000).

1.3.4.3 Disease-modifying anti-rheumatic drugs

Methotrexate is a folate antagonist, it belongs to the class of disease-modifying anti-rheumatic drugs (DMARDs) and was approved for treating RA in 1988. It is taken orally or injected and its mechanism of action is to inhibit DNA synthesis and in RA has been shown to inhibit proliferation of activated blood lymphocytes (Genestier *et al.* 1998). Patient treatment is optimized by using methotrexate in combination with other anti-rheumatic drugs/therapies.

1.3.4.4 Treatment with biological agents

Anti-TNF therapy is one of the more recent and successful approaches to treating RA. This was chosen as a therapeutic target for three main reasons: 1. TNFα is required for expression of pro-inflammatory cytokines in rheumatoid synovial cultures, 2. in murine collagen induced arthritis, anti-TNFα reduces inflammation and joint destruction and 3. TNFα and TNF-receptor are upregulated in the synovium and cartilage/pannus junction of inflamed joints (Feldmann and Maini 2001). The first anti-TNFα treatment used and therefore most studied is Infliximab. This is a chimeric antibody comprised of a mouse Fv and human IgG1κ antibody and is administered systemically. It binds to TNFα with

high affinity and hence neutralises its action by preventing it from binding to TNFRs. Clinical trials have shown strong evidence that Infliximab is effective in reducing symptoms of rheumatoid arthritis however further research is required into the long-term effects of Infliximab treatment (Maini *et al.* 1999, Feldmann and Maini 2001, Shergy *et al.* 2002). Longterm treatment with anti-TNFα drugs would not be ideal due to the key role of TNFα in the immune system and therefore the possibility of it leading to an increase in infection.

Another anti-TNFα drug is Etanercept. This is a soluble TNF receptor Fc fusion protein which binds to TNFα and blocks its interaction with TNF-R. It is effective in both animal models of RA and in clinical trials in RA patients (Breedveld 1999). Having shown good efficacy in clinical trials Etanercept is also now used as a treatment for RA (Klareskog *et al.* 2001, Klareskog *et al.* 2004).

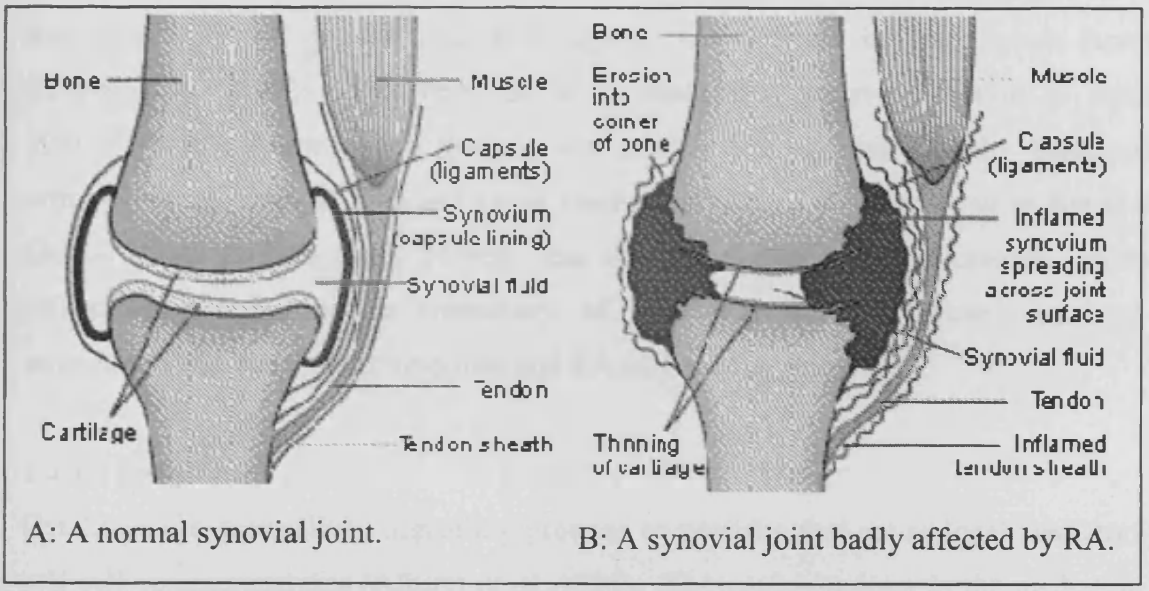
Only two-thirds of patients with methotrexate-resistant RA respond to anti-TNF therapy (Mariette 2004) therefore other anti-cytokine therapies have been investigated. Placebo controlled human trials using a recombinant IL-1 receptor antagonist (Anakinra) have been carried out with a successful outcome due to the observed slowing of cartilage and bone destruction compared to placebo controls (Bresnihan *et al.* 1998; Jiang *et al.* 2000). Anti-IL-6 and anti-IL-6 receptor antibodies have been used successfully as a therapeutic strategy in RA animal models (Mihara *et al.* 1991). More recently a humanized anti-IL-6 receptor (IL-6r) antibody called MRA (monoclonal receptor antibody) has been developed. MRA consists of the complementarity-determining regions from mouse anti-IL-6R antibody grafted to human IgG1, it inhibits the binding of IL-6 to its receptor (Sato *et al.* 1993). In placebo-controlled studies it has shown overall promising results (Nishimoto *et al.* 2003, Choy *et al.* 2002, Nishimoto *et al.* 2004). After 4 weeks of treatment at least a 20% improvement was observed according to the American college of rheumatology criteria, this included a 20% improvement in tender and swollen joints (Nishimoto *et al.* 2004).

1.4 Inflammation

Inflammation of the synovium and degradation of the articular cartilage and bone result in synovial joint destruction in RA. In a healthy state the synovial membrane is composed of an intimal layer, which is one or two cells thick. Underneath this is a sub-lining layer consisting of adipocytes, blood vessels and immune cells (Sibbitt 1999). In RA this synovial membrane turns into a proliferating tissue (becoming up to 20 cells thick) that has many layers of intimal macrophages and FLS (Berg and Bresnihan 1999). The pannus invades the underlying cartilage and bone. In RA, FLS show characteristics only seen in the RA synovium; a prominent nucleus and dispersed chromatin and the cells become surrounded by dense collagen fibres (Cunnane *et al.* 1998). The inflamed synovium becomes infiltrated with lymphocytes and plasma cells that synthesise immunoglobulins which activate the complement system. This in turn causes the release of cytokines (see section 1.4.1.1), prostaglandins (see section 1.4.1.2), proteolytic enzymes (see section 1.5.1) and superoxides including nitric oxide (see section 1.4.1.3) (Tak 2001). It is these agents that mediate the inflammatory response. Osteoclasts, especially where the synovium is attached to bone, become stimulated by inflammatory cytokines causing bone resorption and the characteristic erosions of rheumatoid arthritis (See figure 1.5). If the inflammatory process persists the synovium is gradually replaced by pannus. Pannus can spread to all parts of the joint and can cause total destruction of the joint.

1.4.1 The Inflammatory Response

The inflammatory response functions to restore and maintain homeostasis after injury, whereby cells and other factors are recruited from the bloodstream to the injured site to remove antigenic factors and dead cells. Infiltrated macrophages release cytokines and prostaglandins and other inflammatory mediators, which cause the three major events of the inflammatory response: vasodilation, an increase in capillary permeability, which causes oedema and, an influx of phagocytes (reviewed in Kinne *et al.* 2000). Macrophages, monocytes and neutrophils carry out phagocytosis. During phagocytosis these cells release lytic enzymes which damage nearby healthy cells. If the inflammatory response is severe these infiltrated cells release the pro-inflammatory cytokines IL-1,



A: A normal synovial joint.

B: A synovial joint badly affected by RA.

Figure 1.5: Schematic diagram of a healthy synovial joint (A) and an arthritic synovial joint (B). (www.arc.org.uk, obtained by permission of the Arthritis Research Campaign.)

TNFα and IL-6 (see section 1.4.1.1) which travel via the blood stream to the liver to cause the release of acute phase proteins and thus induce the acute phase response (reviewed in Arend and Gabay 2000).

The acute phase response is when protein levels in the plasma change reflecting an alteration in hepatocyte biosynthesis; albumin levels decrease and amongst others, complement system proteins, IL-1R antagonist and protease inhibitor levels increase (Kinne *et al.* 2000). This response is an attempt to survive infection or chronic inflammation. Acute phase proteins are synthesized and released by hepatocytes primarily in response to IL-6 and cause systemic effects of RA (reviewed in Arend and Gabay 2000). Acute phase proteins can activate the complement cascade, promote inflammation and stimulate chemotaxis of phagocytes. The cytokines involved in induction of the acute phase response and RA will now be discussed.

1.4.1.1 Cytokines

Cytokines are extracellular signalling proteins or peptides that act as local mediators in cell-cell communications (Alberts *et al.* 1994). They are important in the mediation of inflammation in RA. Cytokines include interferons, interleukins, growth factors, colony-stimulating factors, and chemotactic factors and often work synergistically. They are released from various cells in response to a variety of signals and are usually secreted and bind to cell surface receptors on target cells. TNFα and IL-1 are the main mediators of inflammation in the RA process (Feldmann *et al.* 1996; Arend and Dayer 1995). However, IL-1, IL-1 receptor antagonist (IL-1RA), TNFα, IL-6, transforming growth factor β (TGFβ), IL-8, IL-10, IL-11, IL-13, IL-15, interferon β (IFNβ), IL-17 and IL-18 are all produced in RA synovial tissue (Reviewed in Vervoordeldonk and Tak 2002). Evidence of cytokines acting in RA pathogenesis comes from the efficacy of therapies aimed at blocking or modulating cytokine activity (see section 1.3.4). These cytokines have either pro- or anti-inflammatory properties or sometimes both. The proposed actions of these cytokines in RA are summarised in table 1.1.

Table 1.1 The actions of cytokines important in RA

Cytokine	Main cells producing cytokine in synovial joint	Major action of cytokine in synovial joint
IL-1	Macrophages, FLS (see section 1.4.1.1.2).	Induces proteoglycan degradation, production of MMPs and enhances bone resorption (see section 1.4.1.1.2).
IL-1RA	Macrophages, FLS and lymphocytes.	Anti-inflammatory. Inhibits IL-1. (Horai *et al.* 2004)
IL-4	T-cells.	Anti-inflammatory. Inhibits the production of pro-inflammatory cytokines and proteases (Miossec *et al.* 1992).
IL-6	FLS and macrophages (see section 1.4.1.1.3).	Stimulates degradation of cartilage and bone, induces MMP and pro-inflammatory cytokine production (see section 1.4.1.1.3).
IL-8	Macrophages, osteoclasts, chondrocytes, fibroblasts and neutrophils.	Pro-inflammatory. Potent chemoattractant and activator of neutrophils (Taha *et al.* 2003).
IL-10	Macrophages, FLS, B cells.	Anti-inflammatory. Suppresses macrophage production of pro-inflammatory cytokines (Driessler *et al.* 2004).
IL-11	FLS, chondrocytes and bone marrow stromal cells.	Anti-inflammatory. Induces osteoclastogenesis (Girasole *et al.* 1994), induces TIMP production by chondrocytes (Trontzas *et al.* 1998).
IL-12	Antigen-presenting cells.	Pro-inflammatory, is a major inducer of T helper 1 (Th1) responses by stimulating Th1 lymphocyte proliferation and differentiation and by inducing interferon (IFN)-γ production from natural killer and T cells (Elenkov *et al.* 2001).
IL-13	Activated T cells.	Anti-inflammatory, modulates monocyte and B-cell functions (Zurawski and de Vries 1994).
IL-15	Monocytes, osteoclasts, FLS.	Pro-inflammatory. Promotes chemokine release and activation of T-cells, fibroblasts, neutrophils, and macrophages (McInnes and Gracie 2004).
IL-17	T cells.	Pro-inflammatory. Induces pro-inflammatory cytokine release by FLS, chondrocytes, neutrophils, macrophages, osteoblasts and osteoclasts (Miossec 2004). Also induces MMP1 production (Bessis and Boissier 2001).
IL-18	Macrophages, monocytes, FLS, chondrocytes.	Pro-inflammatory. Activates T cells, induces cytokine production by macrophages (Liew and McInnes 2002).

TNFα	Neutrophils, macrophages, FLS, osteoblasts, fibroblasts, T lymphocytes and B lymphocytes (see section 1.4.1.1.1).	Induces macrophages and FLS to release pro-inflammatory cytokines (see section 1.4.1.1.1).
TGFβ	Macrophages and T cells.	Anti-inflammatory, but high concentrations can be pro-inflammatory. Inhibits proliferation of lymphocytes and cartilage and bone destruction (Vervoordeldonk and Tak 2002).

1.4.1.1.1 TNFα

TNFα is an important mediator of changes associated with RA. It is elevated in RA synovial fluids at concentrations of 94pg/ml (compared to OA synovial fluid levels of 39pg/ml) (McNearney et al. 2004). OA is not an inflammatory arthritis compared to RA. TNFα is released by neutrophils, macrophages, FLS, osteoblasts, fibroblasts, T lymphocytes and B lymphocytes in response to a wide variety of stimuli (reviewed in Maini 2004).

TNFα can exist as a biologically active membrane-bound molecule or as a soluble molecule. A membrane metalloproteinase (TNFα converting enzyme [ADAM17], see section 1.5.1.3.2) enzymatically cleaves the membrane-bound TNF to produce the soluble form. TNFα functions as a trimer, and can activate two TNFα receptors: TNFα receptor-1 (TNFR-1 also known as p55) and TNFR-2 (p75). Both TNFα receptors are expressed by most types of nucleated cells (Ledgerwood *et al.* 1999, Vandenabeele *et al.* 1995). The TNF receptors can also be shed in a soluble form and therefore neutralise the activity of TNFα upon binding, thus acting as a negative feedback mechanism (reviewed in Piecyk and Anderson 2001).

TNFα activates its receptors which signal via 2 major transcription factors, AP1 and NFκB (Baud and Karin 2001). TNFα can also signal via protein kinase R and ceramide to increase MMP expression by chondrocytes (Gilbert *et al.* 2004). Following binding of TNFα to its receptor, cytoplasmic signalling proteins called TNF receptor-associated factors (TRAFs) are recruited which then activate protein kinases and transcription

factors. These upregulate genes involved in inflammation (Piecyk and Anderson 2001). The TNFα signalling pathways important in RA are shown in figure 1.6. TNFR-2 also acts via this pathway but can interact with TRAF2 without interacting with TRADD first (Ichijo 1999). Furthermore, TNFα can induce ERK, p38 and MAP kinases in RA FLS (Schett *et al.* 2000).

Expression of TNFα can be regulated at the transcriptional, post-transcriptional and translational levels. Proteins that bind to AU-rich elements in the 3' untranslated region increase the stability of TNFα mRNA. Conversely, other studies have shown that deletion of these AU-rich elements in mice causes the development of polyarthritis (Kontoyiannis *et al.* 1999). The TNFα promoter also contains binding sites for AP1 and NFκB and can therefore regulate its own expression because TNFα can activate both of these transcription factors (Baud and Karin 2001).

TNFα is thought to play a very central role in the pathophysiology of RA. Macrophages and endothelial cells can be induced by TNFα to produce IL-1, IL-6, IL-8, IL-10 and prostaglandin E_2 (PGE_2) (Piecyk and Anderson 2001). NFκB and AP1 can also upregulate MMPs (reviewed in Overall and Lopez-Otin 2002). FLS are also induced by TNFα to produce an array of cytokines; IL-1 (Brennan *et al.* 1989), IL-6, and IL-8 (Butler *et al.* 1995). Furthermore, TNFα and its receptors are highly expressed in areas of inflammation and tissue destruction in the synovium and pannus (Chu *et al.* 1991).

TNFα transgenic mice also demonstrate the involvement of this cytokine in an arthritic phenotype. Spontaneous arthritis develops in mice that over-express TNFα (Keffer *et al.* 1991). Furthermore, TNF-α is crucial for the development of autoimmune arthritis in IL-1 receptor antagonist–deficient mice (Horai *et al.* 2004). In addition, TNFα-knock-out mice have reduced joint swelling and cartilage loss upon induction of streptococcal-cell wall induced arthritis (reviewed in van den Berg and Bresnihan 1999).

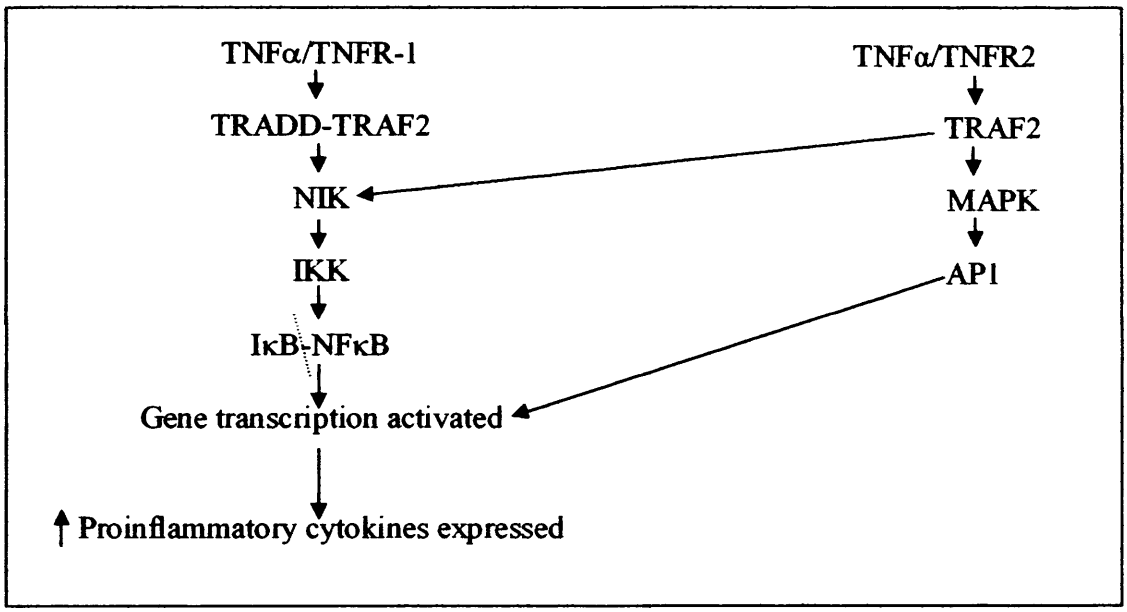


Figure 1.6: The TNFα/TNFR-1 complex interacts with TRAF2 via TNFR-1 associated death domain protein (TRADD). Nuclear factor kappa B (NF-κB)- inducing kinase (NIK) associates with TRAF2 and activates inhibitor of inhibitor kappa B (IκB) kinase (IKK). IKK phosphorylates IκB causing it to dissociate from NF-κB. NF-κB can then translocate to the nucleus and activate gene transcription of proinflammatory cytokines involved in RA. TNFR2 can interact with TRAF2 directly which in turn can activate AP1 via MAPK or NIK (Reviewed in Piecyk and Anderson 2001).

TNFα clearly plays a pro-inflammatory role in RA and the demonstrated efficacy of anti-TNFα and anti-TNF receptor therapies in treatment of RA enforces this (see section 1.3.4.4). Most importantly, TNFα is not the only cytokine involved in RA.

1.4.1.1.2 Interleukin 1

Humans express two isoforms of IL-1, IL-1α and IL-1β encoded by 2 genes found in the IL-1 cluster on chromosome 2q (March *et al.* 1985). Less than 30% of the amino acid sequence is identical between cytokines IL-1α and IL-1β however both work via the same receptors (March 1985). IL-1α is a major cytokine in the early stages of inflammation whereas IL-1β is the more dominant cytokine in advanced inflammation (Berg and Bresnihan 1999). The major type of IL-1 that is found in RA synovium is IL-1β; generally IL-1β is secreted whereas IL-1α is either retained within the cell or expressed on the cell surface (Kay and Calabrese 2004). Therefore, IL-1α is active as an intracellular precursor, as a membrane-associated cytokine and only to a lesser extent as a secreted molecule (Apte and Voronov 2002). *In vivo* studies have shown that IL-1α and IL-1β have equal potency in causing chondrocyte proteoglycan synthesis inhibition (Saklatvala 1986). IL-1β levels are significantly elevated in RA. It is found at concentrations of 130pg/ml in RA synovial fluid compared to osteoarthritis levels of 28pg/ml and has a well established pro-inflammatory role in RA (Westacott *et al.* 1990). IL-1α is also elevated in RA synovial fluid (Hopkins *et al.* 1988).

IL-1α and IL-1β both signal via three types of receptors, type I, type II and IL-1R accessory protein. Activation of these receptors by IL-1 can activate NFκB, c-Jun, AP-1, p38 MAPK and ERK1/ERK2 transduction pathways (reviewed in Gabay 2004). However, IL-1 signal transduction is dependent on the presence of IL-1R accessory protein (Huang *et al.* 1997).

In addition to TNFα and IL-6, IL-1α and IL-1β play a significant role in the pathogenesis of RA. Among the many effects that IL-1 has on cells of the synovial joint, IL-1 stimulates FLS and chondrocytes to produce MMPs, aggrecanases, nitric oxide and prostaglandin E_2 (see section 1.5.1.3, 1.4.1.3 and 1.4.1.2) leading to cartilage degradation

(Abramson and Amin 2002). In addition, IL-1 can work synergistically with oncostatin M (OSM, see section 1.4.1.1.4) to cause destruction of the synovial joint. Treatment of human chondrocytes *in vitro* with IL-1α in combination with OSM caused induced expression of the degradative enzymes ADAMTS4, MMPs 1, 8 and 13 (Koshy *et al.* 2002).

IL-1 receptor antagonist (IL-1ra) is the natural soluble inhibitor of IL-1 signalling and is structurally similar to IL-1α and IL-1β. By binding to the IL-1 receptor it prevents IL-1 from binding. It is present in the RA synovial membrane but is deficient compared to the amount of IL-1 present (Firestein *et al.* 1994). Therefore an imbalance occurs, whereby the greater presence of IL-1 compared to IL-1ra results in the inflammatory effects of RA (Arend *et al.* 2001). The recombinant IL-1 receptor antagonist, Anakinra, is used in RA therapy (see section 1.3.4.4).

In vivo studies have also demonstrated the importance of IL-1 in arthritis. Collagen-induced arthritis in mice can be suppressed by a combination of anti-IL-1α and anti-IL-1β antibodies (Joosten *et al.* 1996). Furthermore, cartilage damage is reduced in streptococcal cell wall induced arthritis in IL-1β knock-out mice and addition of anti-IL-1α antibodies ameliorates all symptoms (reviewed in van den Berg and Bresnihan 1999).

1.4.1.1.3 Interleukin 6

IL-6 is a 212 amino acid protein that is cleaved close to the N-terminus by signal peptidases to produce a 184 amino acid active protein with a molecular weight of approximately 23kD (Wong *et al.* 2003). Within the synovial joint it is mainly produced by FLS and to a lesser extent by macrophages (Vervoordeldonk and Tak 2002) but it is also expressed by chondrocytes, fibroblasts, monocytes, T cells and B cells (Shinmei *et al.* 1989, al-Janadi *et al.* 1993). TNFα, IL-1 and IL-17 all increase IL-6 production by FLS *in vitro* (Guerne *et al.* 1989, Chabaud *et al.* 1998). IL-6 levels are elevated in the synovial tissue and fluid (24.9ng/ml in RA compared to 0.914ng/ml in OA) during active rheumatoid arthritis (Desgeorges *et al.* 1997). Nowell *et al.* further confirmed these

results demonstrating levels of 51.7ng/ml in RA synovial fluid compared to 6.39ng/ml in OA synovial fluid (Nowell *et al.* 2003).

1.4.1.1.3.1 Interleukin 6 signalling pathways

IL-6 interacts with two receptors, one being membrane bound (IL-6R) and the other soluble (sIL-6r), but both acting via the gp130 membrane receptor (Jones *et al.* 2001). The sIL-6r is a 55kD protein and is not expressed by FLS (Nishimoto *et al.* 2000) but IL-6 can still act on these cells because sIL-6r is found in the RA synovial fluid at high levels: 24.7ng/ml (Desgeorges *et al.* 1997). The sIL-6r allows cells that express gp130 but not the IL-6R to respond to IL-6 (Jones *et al.* 2001). The soluble form of the IL-6 receptor is generated by differential splicing of IL-6R mRNA or by proteolytic shedding of the IL-6R from the cell membrane (Horiuchi *et al.* 1994, Mullberg *et al.* 1997).

The IL-6 and IL-6 receptor complex, acts via the gp130 receptor unit. It has been proposed that the receptor/IL-6 complex causes dimerisation of the gp130 receptor so that a tetramer is formed (IL-6, IL-6R/sIL-6r and 2x gp130) (Pflanz *et al.* 2000). However others have proposed that a hexamer is formed (2xIL-6, 2xIL-6 receptor and 2x gp130) (Ward *et al.* 1994, Boulanger *et al.* 2003), and this proposal has recently been confirmed (Skiniotis *et al.* 2005). This multimerisation results in phosphorylation of gp-130-associated janus kinases (JAKs) that attracts signal transducer and activator of transcription -1 (STAT-1)/STAT-3 factors to gp-130, which then become phosphorylated (Reviewed in Jones *et al.* 2001) (Figure 1.7). The phosphorylated STAT proteins then form homomers and heteromers and translocate into the nucleus to induce gene expression (Reviewed in Jones *et al.* 2001). Another signalling pathway activated by IL-6 is the Src homology-2 domain-containing protein tyrosine phosphatase (SHP2)-mediated extracellular signal-regulated kinase (ERK)/ mitogen activator protein kinase (MAPK) pathway. SHP2 is tyrosine phosphorylated by the IL-6/IL-6 receptor/gp130 multimer which activates the ERK/MAPK pathway via the Ras-Raf signalling cascade (Hirano *et al.*1997). This can then activate the transcription factors activator protein 1 (AP-1) and nuclear factor IL-6 (NF-IL-6) (Nakajima *et al.* 1993, Solis-Herruzo *et al.* 1999).

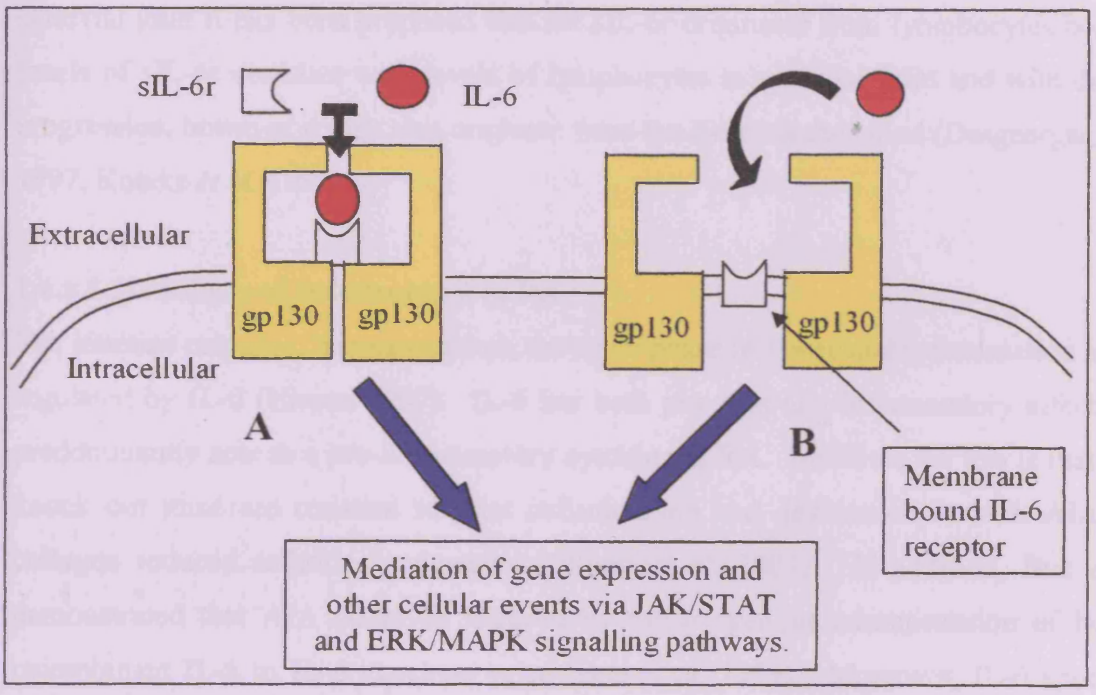


Figure 1.7: Schematic diagram of how IL-6 interacts with the soluble IL-6 receptor (A) and the membrane bound receptor (B) to activate gp130 and hence activate an intracellular cascade of events that mediate inflammation and joint destruction in RA.

ADAM17 (see section 1.5.1.3.2) may be responsible for IL-6R shedding, however other metalloproteinases (see section 1.5.1.3) may be involved (Althoff *et al.* 2000, Franchimont *et al.* 2005). For example cathepsin G, a serine protease (see section 1.5.1.2) has been identified as a possible protease involved in shedding of the sIL-6r from neutrophils (Bank *et al.* 1999). Furthermore, the acute phase response protein, C-reactive protein, can increase shedding of sIL-6r from neutrophils (Jones *et al.* 1999). In the RA synovial joint it has been proposed that the sIL-6r originates from lymphocytes because levels of sIL-6r correlate with levels of lymphocytes in synovial fluid and with disease progression, however it may also originate from the liver via the blood (Desgeorges *et al.* 1997, Kotake *et al.* 1996).

1.4.1.1.3.2 Effects of Interleukin 6 in RA

The immune response, haematopoiesis, the acute phase response and inflammation are all regulated by IL-6 (Hirano 1997). IL-6 has both pro- and anti-inflammatory effects but predominantly acts as a pro-inflammatory cytokine in RA. Evidence for this is that IL-6 knock out mice are resistant to joint inflammation and destruction in both AIA and collagen induced arthritis (reviewed in Wong *et al.* 2003). In addition, Boe *et al.* demonstrated that AIA could be induced by the exogenous administration of human recombinant IL-6 to IL-6 knockout mice (Boe *et al.* 1999). Moreover, IL-6 knockout mice display less cartilage destruction during zymosan-induced arthritis when compared to wild-type mice (van de Loo *et al.* 1997). The fact that clinical trials using antibodies targeted to inhibit the IL-6 signalling system and decrease RA symptoms strengthens this argument (see section 1.3.4.4).

IL-6 influences the activities of cytokines and enzymes important in RA. The intracellular adhesion molecule 1 is an adhesion molecule involved in leukocyte recruitment from blood vessels; its expression is increased when endothelial cells are stimulated with IL-6 *in vitro* (Romano *et al.* 1997). IL-6 may play a direct role in joint destruction by stimulating osteoclasts to increase bone resorption (reviewed in Heymann and Rousselle 2000). In addition, IL-6 enhanced IL-1 induced production of degradatory MMPs (pro-MMP1 and pro-MMP3, see section 1.5.1.3.1) by RA FLS (Ito *et al.* 1992).

Since IL-6 and sIL-6r synovial fluid levels correlate with the extent of joint destruction in RA patients (Kotake *et al.* 1996) it is likely that these local effects of IL-6 are important. In addition, IL-6 in conjunction with sIL-6r has been demonstrated to potentiate aggrecanase-associated catabolism of bovine cartilage explants (Flannery *et al.* 2000).

IL-6 also possesses anti-inflammatory properties. IL-6 increases release of IL-1ra and the soluble TNFα receptor (p55) by macrophages to inhibit these pro-inflammatory pathways (Tilg *et al.* 1994). In addition, IL-6 decreases LPS-induced release of IL-1 and TNFα by monocytes *in vitro* and in serum levels in mice (Schindler *et al.* 1990, Aderka *et al.* 1989). Finally, IL-6 inhibits the proliferation of fibroblastic synovial cells from rheumatoid arthritis patients in the presence of soluble IL-6 receptor (Nishimoto *et al.* 2000). Taken together, this evidence clearly demonstrates that IL-6 plays a pivotal role in both local and systemic effects important in the pathogenesis of rheumatoid arthritis.

1.4.1.1.4 Other members of the IL-6 family

Oncostatin M (OSM), IL-11, leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and cardiotropin are all members of the IL-6 family and all act via the gp130 receptor unit. Although all molecules possess a similar helical structure, their association is due to their functional redundancy and receptor interactions (Hibi *et al.* 1996). Functional redundancy is brought about because they all act via the gp130 receptor. An example of their redundancy of activity is that IL-6, IL-11, LIF and OSM all induce acute phase protein synthesis in hepatocytes (reviewed in Hirano 1997). However, even though gp130 is ubiquitously expressed, signalling via this receptor is controlled by the regulation of expression of the associating α-receptors eg. IL-6R, IL-6sr and leukaemia inhibitory factor receptor (LIFR) (Heinrich *et al.* 2003). Of this family, OSM, IL-11, IL-6 and LIF have all been associated with RA pathology.

Oncostatin M is produced by T cells and monocytic cells and can regulate proliferation of many different cells. Levels of OSM are elevated in the synovial fluid of RA patients compared with fluid from healthy and OA patients indicating a role for this cytokine in inflammatory arthritis (Cawston *et al.* 1998). Recombinant human OSM can inhibit

proteoglycan synthesis in porcine articular cartilage explants (Hui *et al.* 1996) and OSM, in combination with TNFα, can increase collagen breakdown of bovine cartilage in explant culture (Hui *et al.* 2003). In addition OSM can work synergistically with IL-1α to increase release of collagen from cartilage *in vitro* possibly by increasing MMP production and decreasing tissue inhibitor of the matrix metalloproteinases (TIMP) (see section 1.5.1.3.1 and 1.5.2 respectively) production (Cawston *et al.* 1998). Furthermore OSM increases production of IL-6 and TIMP1 by mouse FLS *in vitro* (Langdon *et al.* 2000). Therefore OSM is considered to act as a pro-inflammatory cytokine in RA.

The role of IL-11 in RA is under debate. Within the synovial joint, IL-11 is produced by lymphocytes, FLS, monocytes, osteoblasts and chondrocytes (reviewed by Wong *et al.* 2003). IL-11 may cause bone destruction because it induces osteoclastogenesis (Girasole *et al.* 1994). However, IL-11 may also have a protective role in RA by decreasing MMP1 and MMP3 synthesis (see section 1.5.1.3.1) and increasing TIMP1 synthesis (Taki *et al.* 2000).

Levels of LIF are also elevated in RA synovial fluid compared to OA synovial fluid (Lotz *et al.* 1992). It can stimulate MMP production by osteoblasts (Varghese *et al.* 1999) and can increase IL-1 and IL-6 mRNA production by chondrocytes and FLS *in vitro* (Villiger *et al.* 1993). Furthermore murine LIF binding protein (mLBP) inhibited proteoglycan release from porcine articular cartilage explants incubated with RA patient synovial fluid (Bell and Carroll 2000) thus demonstrating that LIF plays a destructive and inflammatory role in RA.

1.4.1.2 Prostaglandins

Prostaglandins are unsaturated carboxylic acids attached to a five-membered carbon ring. They are produced by most tissues and are involved in many processes throughout the body including reproduction, smooth muscle contraction and the inflammatory response. They are similar to hormones in that they act as messengers to stimulate cells but differ from hormones because they are produced and act locally and are metabolised very quickly.

The two enzymes involved in prostaglandin synthesis are cyclooxygenase-1 (COX-1) and COX-2. Cyclooxygenases convert the substrate, arachidonic acid, to the intermediates prostaglandin G_2 (PGG_2) and prostaglandin H_2, which are then converted by specific tissue prostaglandin synthases to PGE_2 and PGF_2. COX-1 is constitutively expressed and is stimulated by hormones or growth factors to regulate normal cellular processes (Simon and Strand 2004). COX-2 is expressed by most tissues and is induced during inflammation by pro-inflammatory cytokines (although it is constitutively expressed in bone) (Simon and Strand 2001), e.g. IL-1 and TNFα (Anderson 2001). COX-2 synthesises the production of prostaglandins that mediate inflammation (Vane 1994) hence, COX-2 inhibitors, also known as NSAIDS, are prescribed as a treatment for RA (see section 1.3.4.1). Prostaglandins produced by COX-2 mediate inflammation by increasing vasodilation and increase pain by inducing the release of bradykinin (Tannenbaum *et al.* 1996). Bradykinin acts on nociceptors to amplify pain impulses. In addition, prostaglandins lower the threshold of the nerve endings, making them more sensitive to bradykinin, thus enhancing the painful stimulus (Smith *et al.* 2000). Prostaglandins can also regulate bone formation and resorption (Yoshida *et al.* 2002).

1.4.1.3 Nitric oxide

Nitric oxide is a free radical. Levels of nitric oxide (NO) are increased in the synovial joint in RA due to increased activation of the inducible nitric oxide synthase (iNOS) pathway (Grabowski *et al.* 1996). Within the synovial joint, FLS, macrophages, osteocytes, osteoblasts, chondrocytes and endothelial cells can be induced to produce NO (Grabowski *et al.* 1996; Ralston *et al.* 1994): TNFα, IFNβ and IL-1 have all been shown to induce iNOS expression (Stadler *et al.* 1991; Palmer *et al.* 1993). NO reacts with the enzyme guanylyl cyclase to produce the second messenger molecule cyclic guanosine monophosphate (cGMP) and thus, mainly through protein kinase G, it effects phenotypic changes (Matsunobu *et al.* 2000). Nitric oxide has been shown to be involved in both degradative and pro-inflammatory events in RA and this role is demonstrated by the fact that inhibition of iNOS suppresses the development of AIA in rats (Stefanovic-Racic *et al.* 1994). The degradative role of nitric oxide has been demonstrated by experiments showing collagenolytic and caseinolytic activity are both activated by NO in articular

cartilage (Murrell *et al.* 1995). In addition, NO can inhibit osteoblast proliferation (Ralston *et al.* 1994). Furthermore, NO can potentiate TNFα and IL-1 release by leukocytes (Lander *et al.* 1993) thus contributing to inflammatory pathways.

1.5 Degradation

Degradation of the synovial joint causes loss of joint function in RA patients. MMPs and their inhibitors, the TIMPS, have been investigated as markers of degradation in this study therefore this literature review focuses mainly on these. It should be noted however that other proteases are involved in the pathogenesis of RA.

1.5.1 Proteases

Proteases are divided into 4 groups depending on their active site residues: metallo, serine, cysteine and aspartate proteases (Murphy and Reynolds 2002). The primary cause of cartilage and bone destruction in arthritis is by enhanced protease activity degrading collagens and proteoglycans. In RA, FLS, osteoclasts, macrophages, chondrocytes and neutrophils all produce proteases and therefore contribute to joint destruction. The proteases most prominently involved in RA are the cathepsins, serine proteases and MMPs (Kyburz and Carson *et al.* 2000).

1.5.1.1 Cathepsins

Cathepsins are lysosomal, and the cysteine cathepsins generally work intracellularly at acidic pH. The pH of synovial fluid ranges from 7.2-7.4 (Trzenschik and Marx 1987). However, the local environment at the pannus cartilage/bone junction at sites of macrophage and osteoclast attachment is acidic which is ideal for the working conditions of cathepsins rather than MMPs, which work at neutral pH.

Cathepsins B, K and L are all cysteine proteases and are involved in the pathophysiology of RA joint destruction. Cathepsin K is produced by FLS in areas of high proliferation and vascularisation within the RA synovium where it can degrade phagocytosed intra-lysosomal collagen fibrils (Hou *et al.* 2001) however it is predominantly associated with a bone degrading role. The fact that cathepsin K antisense oligodeoxynucleotides inhibit

bone resorption of rabbit osteoclasts cultured on dentine slices (Inui *et al.* 1997) demonstrates that it is essential for osteoclast bone resorption and is involved in bone degradation. Cathepsin K expression is upregulated in RA synovium compared to normal and it is mainly expressed where the synovium attaches and degrades the underlying bone (Hummel *et al.* 1998).

RA synovial fluid contains significantly higher levels of active Cathepsin B compared with osteoarthritic synovial fluid (Hashimoto *et al.* 2001). Cathepsin B can hydrolyse proteoglycans and collagen (Hashimoto *et al.* 2001). Chondrocytes express cathepsins B and L, which can degrade collagen types II, IX and XI (Maciewicz *et al.* 1990), the major cartilage collagens. Cathepsin B is also produced by bone cells and FLS (Aisa *et al.* 2003, Lemaire *et al.* 1997).

This evidence suggests that up-regulation of cathepsin function and cathepsin expression regulation may contribute to tissue destruction in RA.

1.5.1.2 Serine proteases

Serine proteases function at neutral pH. The plasminogen activators and plasmin are believed to be important regulators of connective tissue turnover (Andreason *et al.* 1997). The important role of serine proteases relevant to this study is the initiation of MMP activation (Nagase *et al.* 1991). Serine proteases involved in MMP activation are plasmin, chymase, tryptase, tissue kallikrein, plasma kallikrein, cathepsin G and neutrophil elastase (reviewed in Murphy and Reynolds 2002).

1.5.1.3 Metalloproteinases

Metalloproteinases are part of a superfamily and contain a metal ion in their active site. They can activate pro-enzymes, activate cytokines, degrade many components of cartilage and bone and are involved in the destruction of RA synovial joints (reviewed in Pap *et al.* 2000). The metalloproteinases attack the ECM components such as aggrecan and collagen. Aggrecan is the main proteoglycan of the cartilage ECM and therefore its degradation leads to the destruction of articular cartilage. Hence, the cartilage structural

integrity and its ability to withstand mechanical loading is lost. There are many types of metalloproteinases but the main three involved in RA are the zinc metalloproteinases: the MMPs, the 'a disintegrin and metalloproteinases' (ADAMs) and the ADAM with thrombospondin repeats (ADAMTSs). In this section the TIMPs, the specific inhibitors of the MMPs, will also be discussed.

1.5.1.3.1 MMPs

MMPs are zinc endopeptidases, functional at neutral pH, most of which can be grouped as the stromelysins (MMPs 3, 7, 10 and 11), the collagenases (MMPs 1, 8 and 13), the gelatinases (MMPs 2 and 9) and the membrane-type metalloproteinases (MMPs 14, 15, 16, 17, 24 and 25) (Shaw, T. 2000). MMPs can degrade most components of the ECM; table 1.2 shows all MMPs known to date and their substrates. MMPs require calcium and zinc for activity.

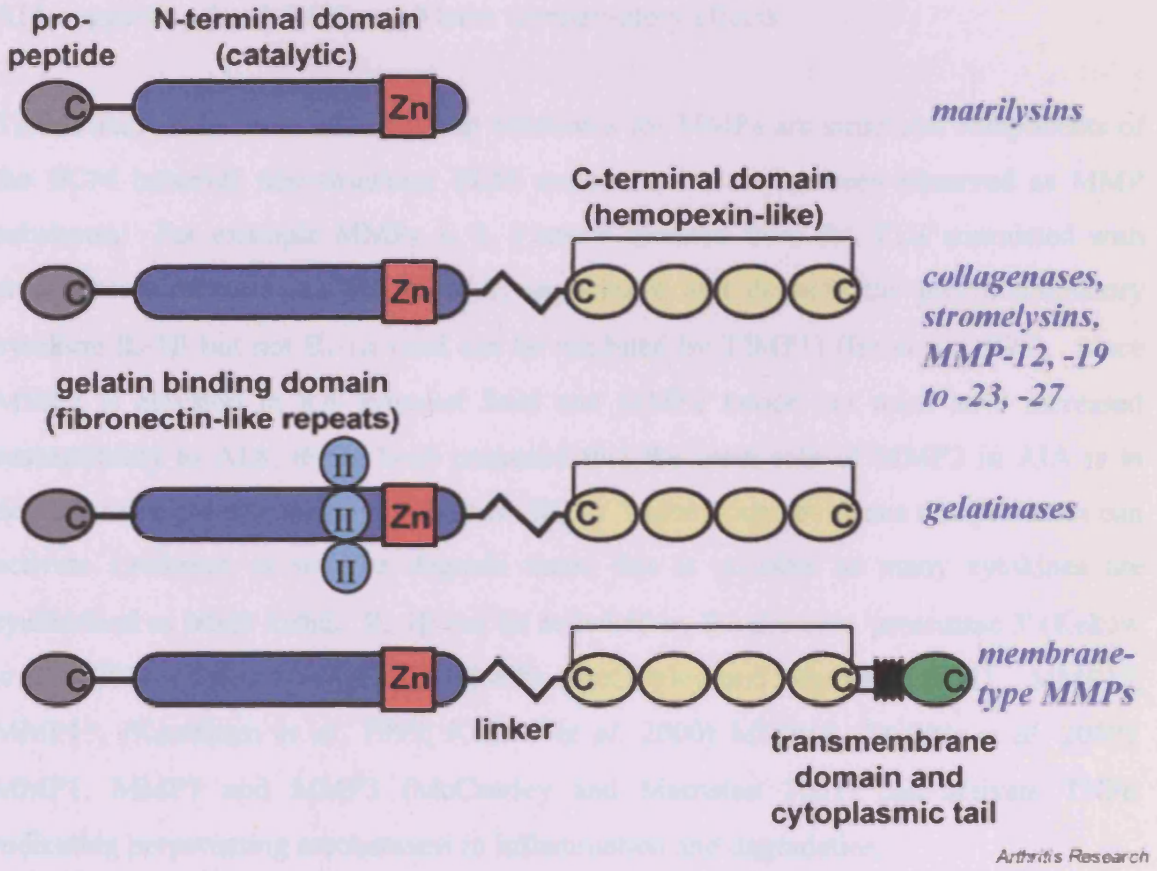
The domain structure of the MMPs is visualized in figure 1.8. All MMPs contain a pro-peptide sequence and an N-terminal catalytic domain. MMPs are secreted as inactive pro-enzymes resulting from the formation of a complex between a single cysteine residue in the pro-peptide domain and the zinc atom in the catalytic domain. The zinc atom in the catalytic domain forms a catalytic complex with three histidine residues also in the catalytic domain. The pro-peptide domain blocks the active site. Activation is achieved by proteolysis or by other means of activating the cysteine switch mechanism by dissociation of the cysteine residue from the complex such as conformational perturbants (eg SDS) or heavy metals (Van Wart and Birkedal-Hansen 1990). This reveals the catalytic complex. The control of activation of MMPs by proteolysis is complex; MMPs can activate other MMPs, for example MMP 3 can activate pro-MMP 1, 8, 9 and 13 (Sternlicht and Werb 1999).

Table 1.2 The substrates of all MMPs known to date (adapted from Cawston 1998; Pelletier *et al.* 2001 and Sommerville 2003).

MMP	MMP Type	Size latent/ active (kDa)	Substrates
1	Collagenase	52/43	Collagens I, II, III, VII, X, Gelatins, aggrecan, tenascin and link protein
8	Collagenase	75/55	Collagens I, II, III, VII, VIII, X, link protein and aggrecan
13	Collagenase	52/42	Collagens I, II, III, VII, X, gelatins and aggrecan
2	Gelatinase	72/62	Gelatin, Collagens I, II, III, IV, V, VII, X, XI, fibronectin, elastin, tenascin, laminin, aggrecan and vitronectin
9	Gelatinase	92/82	Gelatin, Collagens I, III, IV, V, VII, X, XI, XIV, vitronectin, elastin and aggrecan
3	Stromelysin	52/43	Aggrecan, link protein, decorin, elastin, transin, gelatins, fibronectin, laminin, proteoglycanase, collagens I, III, IV, V, VIII, IX, XI, activates MMP-1, vitronectin and tenascin
10	Stromelysin	52/44	Gelatins, Collagens, I, III, IV, V, VIII, IX, activates procollagenase, fibronectin, laminin, elastin and aggrecan
11	Stromelysin	51/46	Cleaves casein, fibronectin, laminin, aggrecan, gelatin, α-1-antitrypsin and serpin
7	Stromelysin	28/19	Gelatins, elastin, aggrecan, entacin, laminin, fibronectin, link protein, activates procollagenase, vitronectin, tenascin,
12	Elastase	52/20	α1-proteinase inhibitor, elastin
14	MT-MMP	64/54	Activates MMP-2, collagens I, II, III, dermatan sulphate, fibronectin, gelatin, vitronectin and laminin
15	MT-MMP	71/61	Fibronectin, vitronectin, fibrillar collagens, aggrecan
16	MT-MMP	66/56	Gelatin, Casein and activates MMP-2
17	MT-MMP	62/51	Fibronectin, α2-macroglobulin, gelatin, fibrinogen, fibrin, activation of pro-TNFα (English *et al.* 2000)
19	RASI-1	56/48	Collagens I and IV, gelatin, aggrecan, casein, fibronectin, laminin, tenascin
20	Enamelysin	54/22	Enamel matrix
23	CA-MMP	58/45	Gelatin
24	MT-MMP	63/28	Gelatin, chondroitin sulphate, fibronectin, activation of pro-MMP2 and pro-MMP13
25	MT-MMP	34/28	Gelatin, collagen IV, fibronectin, activation of pro-MMP2
26	Matrilysin	30/18	Collagen IV, gelatin, casein, fibrinogen, fibronectin
28	Epilysin	62/58	Casein

Figure 1.8 The domain structure of MMPs.

MMPs consist of: a propeptide (grey), a catalytic domain (blue) with the active site and the catalytic zinc (Zn) (red); and a COOH-terminal domain (C) (yellow) with homology to the serum protein hemopexin (not present in the matrilysins). The catalytic domain and the C-terminal domain are connected by a linker peptide. Gelatinases have an insert of three fibronectin type II repeats (turquoise) in the catalytic domain. A transmembrane domain (black) and a cytoplasmic tail (green) at the COOH terminus, which anchors these enzymes in the cell membrane, is only present in the Membrane-type MMPs (obtained by permission of the Arthritis Research Campaign).



Cytokines and growth factors regulate the expression of MMPs. MMP 9 is induced by cytokines such as IL-1 and TNFα; however MMP 2 another gelatinase is constitutively expressed in cartilage and relatively unresponsive to cytokines and growth factors (Konttinen *et al.* 1999). The other constitutively expressed MMPs in the synovial membrane are 3, 11 and 19 (Konttinen *et al.* 1999). The levels of pro and active MMPs 2 and 9 are elevated in the synovial fluid of RA patients (Yoshihara *et al.* 2000) and their roles in RA have been investigated by knock out studies in mice (Itoh *et al.* 2002). The symptoms of antibody-induced arthritis (AIA) are elevated in MMP2 knock out mice compared to wild type and decreased in MMP9 knock out mice compared to wild type mice. The double knock out mouse has a similar phenotype to the wild type mice after AIA suggesting that MMP2 and 9 have compensatory effects.

Traditionally it has been thought that substrates for MMPs are structural components of the ECM however non-structural ECM molecules have now been observed as MMP substrates. For example MMPs 1, 2, 3 and 9 secreted from RA FLS stimulated with aminophenylmercuric acetate (APMA) can cleave and degrade the pro-inflammatory cytokine IL-1β but not IL-1α (and can be inhibited by TIMP1) (Ito *et al.* 1996). Since MMP2 is elevated in RA synovial fluid and MMP2 knock out mice have increased susceptibility to AIA, it has been proposed that the main role of MMP2 in AIA is to degrade inflammatory factors (Itoh *et al.* 2002). There is also evidence that proteases can activate cytokines as well as degrade them, this is possible as many cytokines are synthesised as latent forms. IL-1β can be activated by the protease 'proteinase 3' (Kekow *et al.* 2000), MMP2, MMP3 and MMP9 (McCawley and Matrisian 2001). MMP13, MMP15, (Konttinen *et al.* 1999; Kekow *et al.* 2000) MMP19 (English *et al.* 2000), MMP1, MMP7 and MMP3 (McCawley and Matrisian 2001) can activate TNFα indicating perpetuating mechanisms in inflammation and degradation.

1.5.1.3.2 ADAMs

A disintegrin and metalloproteinases (ADAMs) are transmembrane proteinases consisting of a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain, a transmembrane domain, and a cytoplasmic tail. The ADAMs,

like the MMPs are also zinc metalloproteases. In humans there are 19 ADAMs genes but they are not as well characterized as the MMPs (Seals and Courtneidge 2003). Some ADAMs are commonly referred to as sheddases and function to proteolytically cleave cell-surface proteins. ADAM17 also known as TNFα converting enzyme (TACE) converts pro-TNFα to its active form and inhibitors of TACE have shown efficacy, equivalent to treatments that neutralise TNFα, in a mouse model of collagen-induced arthritis (Newton *et al.* 2001).

1.5.1.3.3 ADAMTSs

The ADAMTSs are a family of secreted proteases (Kuno *et al.* 1997) of which there are at least 19 members (Hashimoto *et al.* 2004). The ADAMTS proteins are characterised by the presence of an N-terminal pro-sequence, a metzincin domain, a disintegrin-like domain and a variable number of thrombospondin repeats (Sandy *et al.* 2000). The ADAMTSs have many functions including degradation of versican and brevican, roles in fertilization and angioinhibitory properties (reviewed in Llamazares *et al.* 2003). However, the functions of many recently discovered ADAMTSs have not yet been characterised.

Importantly for RA pathology, a subset of the ADAMTS proteases degrade aggrecan and these include ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS8, ADAMTS9 and ADAMTS15 (Little *et al.* 2005). ADAMTS4 and ADAMTS5 (aggrecanase 1 and aggrecanase 2 respectively) are both elevated in RA (Murphy and Reynolds *et al.* 2002). Furthermore, IL-1 and TNFα can stimulate ADAMTS4 production in chondrocytes (Tortorella *et al.* 2001). Aggrecan is the main proteoglycan in cartilage (see section 1.2.3) and is cleaved at two main sites. MMPs are responsible for cleavage at one site and aggrecanase is responsible for the other. Aggrecanase cleaves aggrecan within the interglobular domain (IGD) at the Glu^{373} – Ala^{374} site (Abbaszade *et al.* 1999). Recent studies show that there are four additional cleavage sites within the C-terminal region, which are cut by ADAMTS4 (Tortorella *et al.* 2000). Cleavage at the NITEGE – ARGSVIL (Glu^{373}-Ala^{374} site) sequence renders the bulk of aggrecan to be free from the cartilage matrix and enabling it to diffuse into the synovial joint fluid. In fact, elevated

levels of aggrecan fragments, which have the ARGSVIL N-terminus, have been identified with monospecific antipeptide antibodies in the synovial joint fluid of patients with rheumatoid arthritis (Lark *et al.* 1997). In addition, deletion of ADAMTS5 can prevent cartilage degradation in a mouse model of arthritis (Glasson *et al.* 2005) thus indicating that aggrecanases play an important role in arthritis. Furthermore, it may be necessary for aggrecanases to deplete cartilage of aggrecan before the MMPs can degrade the collagen network (Pratta *et al.* 2003), therefore aggrecanases are considered important in the early stages of cartilage destruction in rheumatoid arthritis (Nagasi and Kashiwagi 2003).

1.5.2 TIMPs

The natural inhibitors of MMPs are the tissue inhibitors of metalloproteinases (TIMPs) (See table 1.6) however they can also inhibit ADAMs and ADAMTSs. They form a 1:1 complex with MMPs, binding to the active site, to inhibit them. TIMPs are not just involved with MMP inhibition; TIMP2 is needed for activation of pro-MMP2 by MMP14 (a MT-MMP) (Sommerville 2003). TIMP3 is involved in the regulation of the ADAM 17 (ADAM17, see section 1.5.1.3.2) (Murphy *et al.* 2003). Despite this, it is generally recognised that if an imbalance of MMPs and TIMPs occurs (i.e. more MMPs) the cartilage and bone become degraded as in rheumatoid arthritis. TIMP1 but not TIMP2 has been demonstrated to be elevated in RA synovial fluid (Yoshihara *et al.* 2000).

Table 1.6 The TIMPs known to date (adapted from Cawston 1998, Kashiwagi 2001).

TIMP	MMP-inhibition	Protein size (Da)	Localisation	Expression	Major tissue sites	Pro-MMP binding
1	All	20243	Diffusible	Inducible	Bone, ovary	MMP-9
2	All	21729	Diffusible	Constitutive	Lung, placenta, ovary, testis, heart	MMP-2
3	All, ADAMTS4, ADAMTS5, ADAM 17	21676	ECM bound	Inducible	Kidney, brain, lung, heart, ovary	unknown
4	All	22609	Unknown	Unknown	Kidney, placenta, colon, brain, skeletal muscle, ovary, testis, heart	unknown

1.6 Glutamate

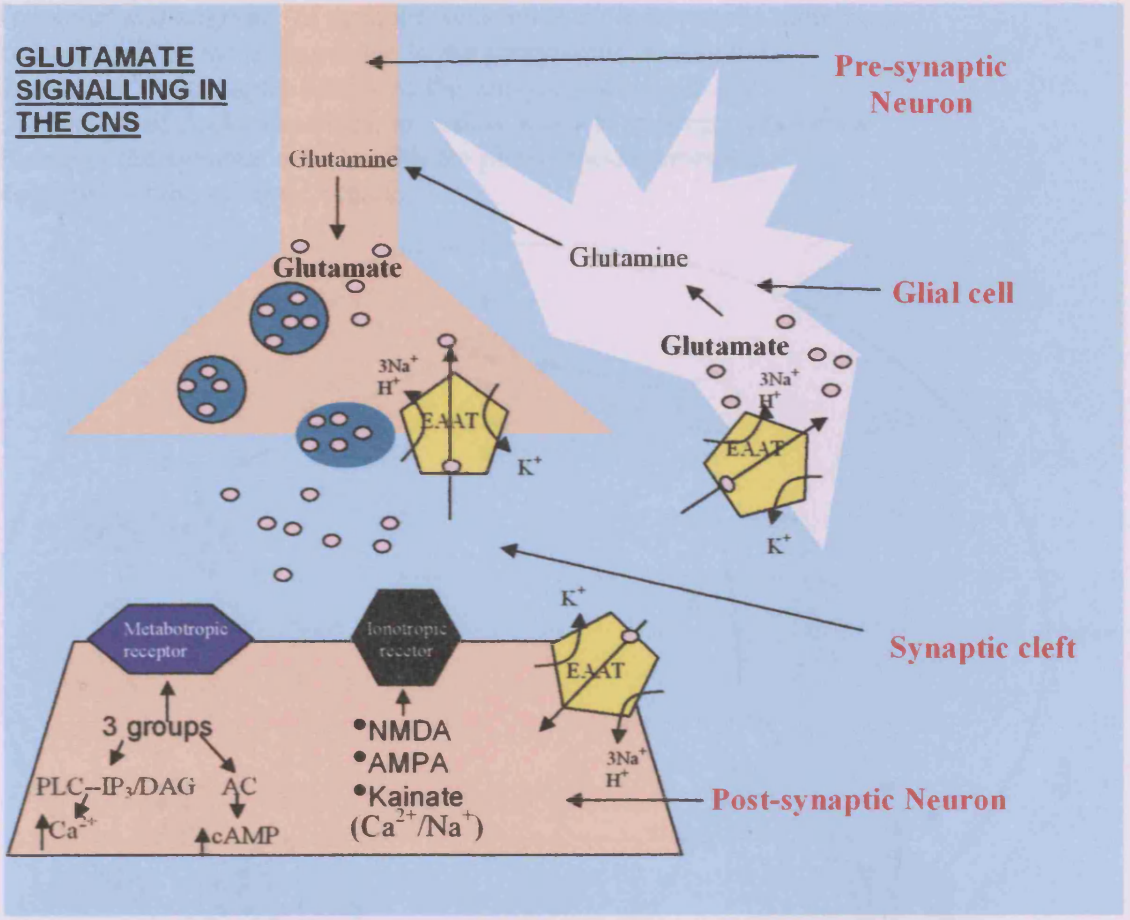
1.6.1 Introduction to glutamate and RA

The concentration of glutamate is elevated in the synovial fluid of human joints with synovitis compared to normal (McNearney *et al.* 2000). Furthermore, RA synovial fluid levels of glutamate have been shown to correlate with pro-inflammatory markers (McNearney *et al.* 2004). Glutamate levels also become elevated following induced inflammation. Studies in rats have shown that knee joint inflammation doubles the amount of glutamate in the synovial fluid (Lawand *et al.* 2000). This study explores whether this elevated glutamate modifies the phenotype of cells within the synovial joint. To date, most research involving glutamate signalling has focused on its role as a neurotransmitter; therefore the mechanism of glutamate signalling within the central nervous system will be summarised prior to discussing its potential role in RA.

1.6.2 Glutamate signalling within the central nervous system

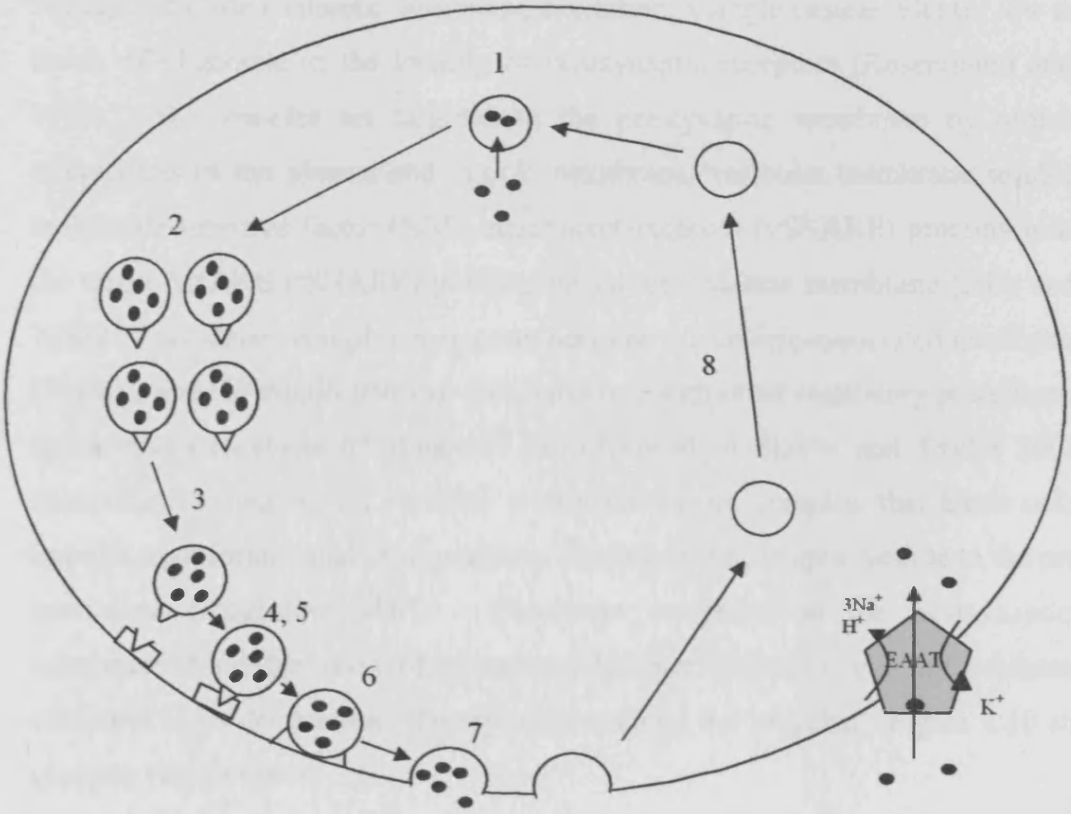
Glutamate signalling has been well studied in the central nervous system (CNS) where L-glutamate is the major neurotransmitter at excitatory synapses. To introduce the glutamate signalling mechanism, the functioning of a glutamatergic synapse (see figure 1.9) will be briefly described.

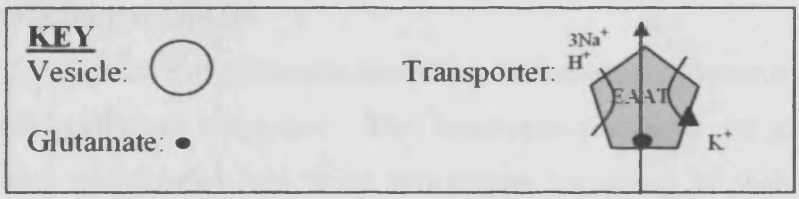
Figure 1.9 A glutamatergic synapse in the CNS



Upon excitation of the presynaptic neuron, glutamate is released into the synaptic cleft. This activates glutamate receptors on the postsynaptic neuron to continue the excitatory signal. Glutamate transporters predominantly via the associated glial cells rapidly take up the glutamate and recycle it via glutamine (by the enzymes glutamine synthetase, glutaminase and glutamate dehydrogenase) back to the presynaptic neuron (refer to abbreviations page for definition of acronyms).

Figure 1.10 The synaptic vesicle cycle (Adapted from Bhangu 2003).
1) Synaptic vesicle loading glutamate
2) Generation of synaptic vesicle pools
3) Transport and targeting of synaptic vesicles to the presynaptic membrane
4) Tethering of the synaptic vesicle to the presynaptic membrane
5) Docking of the synaptic vesicle to the presynaptic membrane
6) Conversion of docked vesicles, to vesicle ready to undergo exocytosis
7) Fusion of the synaptic vesicle with the presynaptic membrane
8) Recycling of the synaptic vesicle





Glutamate is stored in the presynaptic neuron in vesicles. The vesicles in the presynaptic neuron take up glutamate via vesicular glutamate transporters (VGLUT) 1 and 2. As a driving force for glutamate uptake, these transporters use the electrochemical proton gradient generated by H^+-ATPase (reviewed in Danbolt 2001).

Upon excitation of the presynaptic neuron, glutamate is released from intracellular vesicles into the synaptic cleft. Excitation occurs when the presynaptic neuron depolarizes in response to an action potential. This leads to an influx of Ca^{2+} through voltage gated ion channels. Following excitation, a single vesicle releases low millimolar levels of glutamate in the locality of postsynaptic receptors (Rosenmund and Stevens 1996). The vesicles are targeted to the pre-synaptic membrane by protein-protein interactions of the plasma and vesicle membrane; vesicular membrane soluble N-ethyl maleimide-sensitive factor (NSF) attachment receptor (vSNARE) proteins interact with the target SNARE (tSNARE) proteins on the cell plasma membrane (Jahn and Hanson 1998). This allows complex formation between the vesicle-associated membrane protein (VAMP) and tSNARES that can then associate with other regulatory proteins to regulate fusion and exocytosis of glutamate (as reveiwed in Skerry and Taylor 2001). It is synaptotagmin that is the receptor within the fusion complex that binds calcium and through a conformational change allows binding of the synaptic vesicle to the presynaptic membrane (Augustine 2001). Glutamate receptors on the postsynaptic neuron membrane, bind the released glutamate, become activated and the excitatory signal continues to its destination. The synaptic vesicles are recycled. Figure 1.10 shows this synaptic vesicle cycle.

1.6.2.1 Glutamate receptors

There are 2 sub-groups of glutamate receptors, the ionotropic glutamate receptors and the metabotropic glutamate receptors. The ionotropic receptors are glutamate-gated ion channels and are divided into three sub-groups according to their responses to the pharmacological ligands: *N*-Methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate. The binding of glutamate to the ionotropic receptors allows the entry of cations into the postsynaptic cell. The

metabotropic receptors are G-protein coupled receptors. Activation of both types of receptor allow perpetuation of the exctitatory signal.

1.6.2.1.1 NMDA receptors

Three families of NMDA receptor subunits exist. The NMDA receptor subunits are NR1 (1 gene), NR2A to D (4 genes) and NR3A and B (2 genes). NR1 has a much shorter C-terminus compared to the NR2 and NR3 subunits (the length depends on splice variation) and is an essential subunit for NMDA receptor formation (Ishii *et al.* 1993). Figure 1.11a highlights the topology of the NMDA receptor subunit NR1.

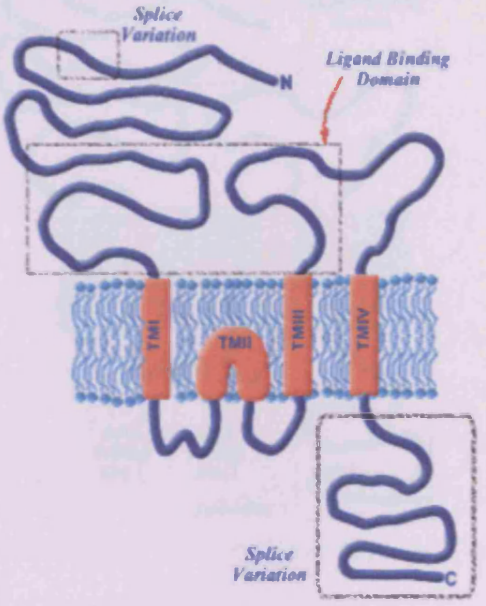
Eight functional isoforms of the NR1 subunit exist, all from differential mRNA splicing (Moriyoshi *et al.* 1991, Sugihara *et al.* 1992). Two sites undergo splice variation, the C-terminal and the N-terminal. The C-terminal region is involved in multiple protein-protein interactions, such as PSD-95/Discs-Large/ZO-1 (PDZ)-domain containing proteins, which allows clustering of the NMDA receptor subunits. The NR2 and NR3 subunits are modulatory, however only when NR1 is expressed with an NR2 subunit can active channels be formed (Ishii *et al.* 1993). This is because the binding site for glycine is on the NR1 subunit and glycine is an essential co-agonist for NMDA receptor function (Kleckner and Dingledine 1988). In addition, NR1 and NR2 subunits are essential for formation of the ion channel pore. Furthermore the NR2 subunits contain the glutamate binding sites (Anson *et al.* 1998). The role of the NR3 subunits is not fully understood. NMDA receptors form tetrameric complexes, and it is likely that they form tetrameric heterooligomers (Schorge and Colquhoun 2003).

Due to the multiple combinations of individual subunits (there are also splice variants of NR2B, NR2C and NR2D, NR3A [Sun *et al.* 1998]), many different NMDA receptor subtypes can exist with different functional and pharmacological properties (Dingledine *et al.* 1999). For example, splicing at the N-terminal region of NR1 can result in variation in potentiation by polyamines, and variation in the C-terminal region of NR1 can affect binding to PDZ-binding proteins (Putzke *et al.* 2000).

Figure 1.14 Representative topology of glutamate receptor or receptor subunits
Pictures obtained by permission of Dr. Andrew Doherty, Bristol University.

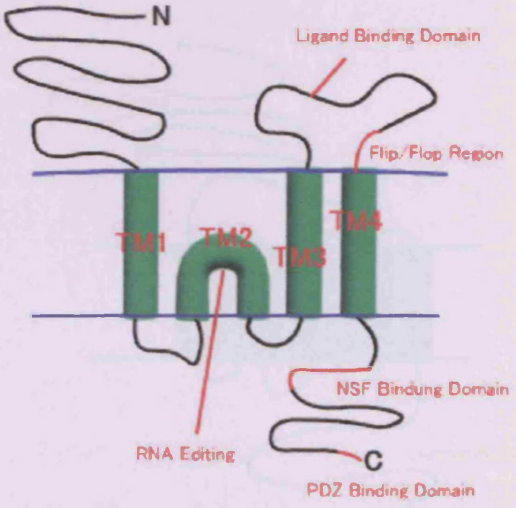
A: NMDA

Topology of an NMDA receptor subunit NR1 (of which there are 8 splice variants). NMDA receptor subunits have an extracellular N-terminus and an intracellular C-terminus. Both undergo extensive splice variation. The ligand binding domain is also highlighted.



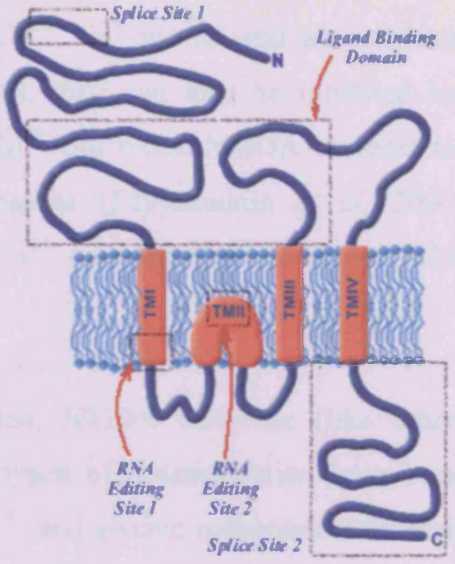
B: AMPA

Topology of an AMPA receptor subunit (AMPA GluR2). AMPA receptor subunits have an extracellular N-terminus and an intracellular C-terminus that undergoes splice variation. The NSF and PDZ binding domains are shown. The 'flip-flop' region and RNA editing site are also highlighted.



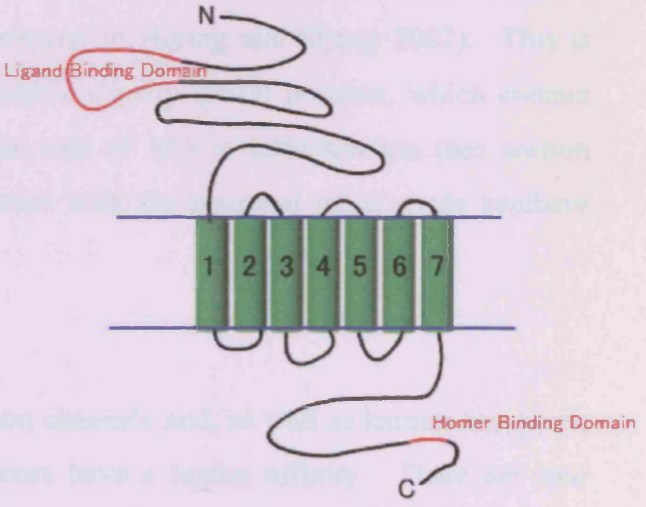
C: Kainate

Topology of a kainate receptor subunit (GluR5 subunit). Kainate receptor subunits have an extracellular N-terminus and an intracellular C-terminus that both undergo splice variation. The two RNA editing sites are also highlighted.



D: Metabotropic

Topology of a representative metabotropic receptor. Metabotropic receptors are G-coupled proteins with 7 transmembrane domains. Generally the C-terminus undergoes extensive splice variation.



In the CNS the NMDA receptors are expressed differentially across the brain, except the NR1 subunit which is ubiquitously expressed (Liu and Zhang 2000). The NMDA receptors play a key role in neurotransmission, synaptic plasticity and synaptogenesis. The NMDA receptors are permeable to Ca^{2+}, Na^{+} and K^{+} and are sensitive to voltage-gated Mg^{2+} block (Liu and Zhang 2000), they can also be inhibited by protons at physiological pH (Cull-Candy 2002). Zn^{2+} can block NMDA currents too in a non-competitive and voltage-independent manner (Fayyazuddin *et al.* 2000). NMDA receptors have higher permeability to Ca^{2+} compared to the non-NMDA ionotropic glutamate receptors.

In the prolonged presence of an agonist, NMDA receptors (like other membrane receptors) become desensitised. Three types of desensitisation have been identified: Ca^{2+}-dependent, glycine-sensitive and Ca^{2+}- and glycine-independent desensitisation (Liu and Zhang 2000). The role of NMDA desensitisation is not fully understood.

In the CNS, stimulation of NMDA receptors causes signal transduction events involving protein kinases, phosphatases and small GTPases which lead to adaptive responses in the postsynaptic cell such as changes in the cytoskeleton, membrane excitability, cell adhesion, transcription and translation (reviewed in Hering and Sheng 2002). This is often through interaction with the post-synaptic density (PSD) proteins, which contain PDZ domains. Of interest, because of the role of NO in inflammation (see section 1.4.1.3), the PSD protein PSD95 can interact with the neuronal nitric oxide synthase (Brenman *et al.* 1996).

1.6.2.1.2 AMPA receptors

The AMPA receptors are non-selective cation channels and, as well as kainate receptors, can bind to kainate, but the kainate receptors have a higher affinity. There are four different subunits, GluR1 to 4. The AMPA subunits form homomeric or heteromeric receptors and are encoded by 4 separate genes (Cull-Candy 2002). At present the stoichiometry of these receptor complexes is unknown. *In vivo* AMPA receptors are thought to be heteromeric (Stricker and Huganir 2002).

The variation in glutamate receptor potencies varies widely (Hollmann and Heninemann 1994). Alternative-splicing renders different AMPA subunits with different properties. Figure 1.11b shows the topology of a representative AMPA subunit. The AMPA receptor subunits all exist in at least two different alternatively spliced forms: flip and flop. The difference between the flip and flop variants being 9-11 amino acids (Stricker and Huganir 2002). Flip variants, which desensitise less and slower, are the most abundant form before birth, whereas flop variants tend to be more abundant after birth (Swanson *et al.* 1997, Dingledine *et al.* 1999). The C-terminal tails of AMPA receptor subunits also undergo alternative splicing, which may affect the role of the C-terminal tail in signal transduction and membrane trafficking (reviewed in Palmer *et al.* 2005). RNA editing also provides subunits with different properties. For example, it is certain RNA edited variants of the AMPAGluR2 subunit make some AMPA receptors impermeable to Ca^{2+} (Washburn *et al.* 1997). AMPA subunits are expressed differentially throughout the brain. GluR2 and GluR3 are ubiquitously expressed but GluR1 is only found in approximately 50% of synapses. Only basal dendrites express GluR4 subunits (reviewed in Stricker and Huganir 2002).

Like NMDA receptors, AMPA receptors can also activate non-ionotropic signalling pathways which are independent from ion-flux through the channel. NMDA and AMPA receptors can inhibit each other via these non-ionotropic mechanisms. In rat hippocampal neurons activation of AMPA receptors decreases NMDA-induced current, moreover glutamate-induced current via NMDA receptors increased when AMPA receptors were blocked (Bai *et al.* 2002). AMPA receptors, via the C-terminal, can interact with proteins that contain a PDZ-binding domain, these include PSD95, AMPA receptor binding protein (ABP), glutamate receptor interacting protein (GRIP) and Protein interacting with C-kinase (PICK1). In addition AMPA receptors can interact with non-PDZ domain proteins such as N-ethylmaleimide sensitive fusion protein (NSF), Lyn (a tyrosine phosphatase) and soluble NSF attachment proteins (SNAP) (reviewed in Braithwaite *et al.* 2000). These interacting proteins provide potential mechanisms for regulation of AMPA receptor activity and activation of intracellular signalling pathways.

AMPA receptors can also activate G-protein signalling pathways as demonstrated in rat cortical neurons in the absence of intracellular Ca^{2+} and Na^{+} (Wang *et al.* 1997). AMPA receptors have been shown to activate the MAP kinase signalling in neurons, a pathway which could be blocked by the lack of extracellular calcium, thus indicating that calcium entry through AMPA receptors activates MAP kinase (Wang and Durkin 1995).

1.6.2.1.3 Kainate receptors

The kainate receptors are also non-selective cation channels, however research has focused less on these receptors compared to the other ionotropic receptors. It should be noted that in addition to kainate, AMPA can also activate some kainate receptors (Herb *et al.* 1992). The kainate receptor subunits are GluR5 to 7, KA1 and KA2. KA1 and KA2 however, can only form functional receptors when co-expressed with the GluR5 to 7 subunits (Egebjerg *et al.* 1991). Kainate receptors are composed of four subunits (Bleakman *et al.* 2002). The exact stoichiometry of kainate receptors is not fully understood.

Unlike the AMPA receptors, kainate receptor subunits do not undergo flip-flop splicing but instead are alternatively spliced at the N and C-terminal domains. Figure 1.11c shows the topology of a representative kainate receptor subunit, the kainate subunits have similar topology to the AMPA receptor subunits. The GluR5 and GluR6 subunits also undergo RNA editing (like the GluR2 subunit does, section 1.6.2.1.2), this editing occurs in the channel pore ('RNA editing site 2' see figure 1.11c) and regulate ion flow properties (Sommer *et al.* 1991) and calcium permeability (Burnashev *et al.* 1995). GluR6 undergoes further RNA editing at an additional site in the first transmembrane domain ('RNA editing site 1' see figure 1.11c), this is also involved in Ca^{2+} permeability (Kohler *et al.* 1993). Furthermore, the GluR5 subunit undergoes alternative splicing at the N-terminus which produces two GluR5 variants.

Like the AMPA receptors, receptors of different subunit composition have different pharmacological properties. For example, homomeric GluR5 and heteromeric

GluR6/KA2 receptors are AMPA-sensitive whereas GluR5 heteromers, GluR6 homomers and GluR7 homomers are not (Howe *et al.* 1996, Schiffer *et al.* 1997).

Kainate receptors are expressed on presynaptic neurons and may modulate neurotransmitter release (reviewed in Huettner 2003), whereas postsynaptically they can mediate excitatory transmission. Research into the function of kainate receptors is limited although involvement of kainate receptors in nociception (Ruscheweyh and Sankuhler 2002) and seizures (Smolders *et al.* 2002) has been described. In addition, interaction of kainate receptors with PDZ binding domain proteins has been described, with the associated activation of JNK (Savinainen 2001).

1.6.2.1.4 Metabotropic receptors

Metabotropic glutamate receptors (mGluR) are monomeric receptors coupled to G-proteins and are expressed in both pre-and postsynaptic membranes. They have 7 transmembrane domains connected by three intracellular and three extracellular loops (see figure 1.11d) with their glutamate-binding domain at the N-terminal (Egebjerg, J. *et al.* 2002). There are 8 known metabotropic glutamate receptors divided into 3 groups according to the intracellular signalling pathways that they activate. Group 1 consists of mGluR1 and 5, Group 2 consists of mGluR 2 and 3 and Groups 3 consists of mGluR3, 4, 6, 7 and 8. Variation is increased due to splicing.

The group one receptors are positively coupled to phospholipase C and increase the intracellular concentration of calcium via the IP_3/DAG (inositol 1, 4, 5 trisphosphate/ diacylglycerol) pathway (Aramori and Nakanishi 1992). The Ca^{2+} is released from intracellular stores and can also potentiate voltage-dependent calcium channels and inhibit K^+ conductances (Gubellini *et al.* 2004). Phospholipase C can also activate protein kinase C, which is responsible for a wide variety of cellular events through the phosphorylation of proteins (Egebjerg *et al.* 2002). In addition group 1 metabotropic receptors have also been shown to activate the MAPK pathway (Ferraguti *et al.* 1999).

The group 2 and 3 metabotropic receptors are negatively coupled to the cAMP cascade (Tanabe *et al.* 1992) and can also modulate ion channel activity (Conn and Pin 1997). For example group 2 and 3 metabotropic receptors can inhibit voltage-gated calcium channels in neurons (Anwyl 1999). It should be noted that Ca^{2+} has been shown to activate the metabotropic glutamate receptors mGluR1, mGluR3 and mGluR5 (Kubo *et al.* 1998) however other studies have shown this to enhance only the stimulatory effect of glutamate on these receptors (Saunders *et al.* 1998).

To date, roles of metabotropic receptors in the CNS include direct modulation of neurotransmitter release in all brain structures and modulation of postsynaptic ionotropic receptor activity (reviewed in Gubellini *et al.* 2004). The metabotropic glutamate receptors can also interact with NMDA receptors; activation of mGluR5 can enhance NMDA receptor-mediated responses (Domenici *et al.* 2003).

1.6.3 Glutamate transport

Glutamate release can occur by vesicular release (section 1.6.2), exocytosis, cystine-glutamate antiport and by volume-regulated anion channels (reviewed in Shigeri *et al.* 2004). Glutamate transport can be Na^+-dependent, Na^+-independent and of low or high affinity. Low-affinity uptake is usually Na^+-independent and may supply brain cells with amino acids for metabolic purposes (Erecinska and Silver 1990). When compared to high-affinity transport in the CNS this mechanism of transport appears redundant (Danbolt 2001).

Cystine (the oxidised form of cysteine)-glutamate exchangers are Na^+-independent transporters. This transporter has been investigated in human fibroblasts (cell line derived from foetal lung fibroblasts) and been shown to act as a cystine transporter that is driven by the transmembrane gradient of glutamate (Bannai 1986). In fact TNFα has been shown to enhance this glutamate-mediated transport of cystine in mouse peritoneal macrophages (Sato *et al.* 1995).

In the CNS there are also the vesicular glutamate transporters (see 1.6.2) which take up and store glutamate ready for release from the presynaptic neuron upon excitatory stimulation.

Finally there are the high-affinity Na^+-dependent transporters. These are very important in the CNS as they allow rapid uptake of glutamate and subsequent termination of the excitatory signal (see section 1.6.2). Due to their ability to rapidly take up glutamate and regulate extracellular glutamate concentrations, this thesis has focused more on these transporters. They will now be discussed in greater detail.

1.6.3.1 EAATs

The high-affinity Na^+-dependent transporters are known as the excitatory amino acid transporters (EAATs). Located predominantly in synapse associated glial cells, they maintain low extracellular levels of glutamate in the CNS. The EAATs regulate extracellular glutamate thus preventing toxic levels from being reached and also terminating excitatory signals. Inhibition of EAATs *in vitro* and *in vivo* increases extracellular glutamate and hence neuronal death via excitotoxicity (reviewed in O'Shea 2002).

Five genes encoding EAATs are known to exist: GLAST-1 (EAAT1), GLT-1 (EAAT2), (Storck *et al.* 1992) EAAC1 (EAAT3), EAAT4 and EAAT5 all of which carry out Na^+ and K^+ coupled transport of glutamate and aspartate. Table 1.7 shows the nomenclature for these transporters. EAATs contain eight potential transmembrane domains, one or two re-entrant loops and an N-terminal and C-terminal, both of which are cytoplasmic. However, the exact topology at the C-terminal end is in dispute between Amara's group (Seal *et al.* 1999) and Kanner's group (Kanner and Borre 2002).

There are splice variants of EAAT1 and EAAT2. Alternative splicing of the EAAT2 gene yields at least three different splice variants (Rauen *et al.* 2004). However, the functional significance of these variants remains to be decided. A splice variant of EAAT1 (termed GLASTla) is expressed in bone and brain *in vivo* (Huggett *et al.* 2000)

and also in retina (Huggett and Mason, personal communication). This splice site occurs in three of the other EAATs therefore potential variants of these transporters may also exist (Huggett, personal communication).

Table 1.7 Nomenclature of EAATs

Transporter name Rodent	**Transporter name Human**	**Other names**
GLAST	EAAT1	Slc1a3, GluT-1
GLT1	EAAT2	Slc1a2
EAAC1	EAAT3	Slc1a1
EAAT4	EAAT4	Slc1a6
EAAT5	EAAT5	Slc1a7

The stoichiometry of glutamate transport by EAATs is complex. Sodium is required for glutamate binding and potassium is required for the electrogenic transport process (reviewed by Danbolt 2001). The process is driven against the glutamate concentration gradient by ionic gradients of Na^+, K^+ and H^+ which are maintained by the sodium pump. Two or three sodium ions and one proton are co-transported with the glutamate and one potassium ion is counter-transported therefore making this process electrogenic (reviewed in Amara and Fontana 2002). The transport of substrates by EAATs can occur bi-directionally, into or out of the cell (Kanner and Marva 1982). However under normal conditions in the CNS glutamate is taken up into the cell due to ion gradients.

Glutamate transporters (most apparent in EAAT4 and EAAT5) also have a constitutive ion channel function (Billups *et al.* 1996). In addition, glutamate can bind to the transporters and can trigger anion conductance independently of the glutamate transport process (reviewed in Danbolt 2001). Furthermore, glutamate transporters may also function as receptors. It has been demonstrated previously that glutamate can activate the MEK/ERK pathway in astrocytes via glutamate transporters (Abe and Saito 2001). Furthermore in a myogenic cell line exposed to 2-chloro adenosine, glutamate caused an increase in intracellular Ca^{2+} levels, an effect that was inhibited by specific glutamate

transporter antagonists (Frank *et al.* 2002). It is suggested that this is caused by glutamate transport-associated currents activating voltage-gated calcium channels (Frank *et al.* 2002). A further role for the EAATs is that they provide glial cells in the CNS with glutamate for metabolism in the glutamate-glutamine cycle (Rae *et al.* 2000).

The glutamate transporters are expressed by different cell types and in different regions of the brain (Danbolt *et al.* 2001). Glutamate transporter protein expression is regulated by many factors. In primary astrocytes cAMP increased expression of EAAT1 and EAAT2 (Gegelashvili *et al.* 1996, Swanson *et al.* 1997). Glutamate can up-regulate EAAT1 expression in cultured astrocytes, possibly via kainate receptors (Gegelashvili *et al.* 1996). Neuronal factors can alter expression of glutamate transporters in cultured astroglia (Gegelashvili 1997). In addition glutamate transporters are down-regulated in ischaemia, Huntingdon's disease, epilepsy and amyotrophic lateral sclerosis (reviewed by Su *et al.* 2003).

Glutamate transporter activity can also be regulated directly by phosphorylation, sulphydryl oxidation and indirectly by arachidonic acid. The transit of glutamate transporters can also be regulated between intracellular compartments and the cell plasma membrane. For example, the actin cytoskeleton and protein kinase C phosphorylation have been implicated in control of EAAT trafficking (reviewed in Danbolt, 2001). In addition, the increased expression of cell-surface GLAST is inhibited by cytochalasins, which inhibit actin polymerisation (Duan *et al.* 1999). Regulation of glutamate uptake can also take place by glutamate; activation of group 1 metabotropic receptors in astrocytes leads to the down-regulation of GLAST-1 protein (Gegelashvili *et al.* 2000).

Table 1.8 Summarry of glutamate receptor and transporter expression in cells and tissues outside of the CNS.

Glutamate gene	keratinocytes	cardiomyocytes	intestinal epithelial cells	megakaryocytes	mouse testis	lympho-cytes	pancreatic islet cells	mammary gland
EAAT1								R, P[10]
EAAT2	P[1]	P, R[3]						R, P[10]
EAAT3	P[1]	P, R[3]	P[4]		R[6]			
EAAT4								
NMDA NR1	P[1,2]			P[5]			R[9]	
NMDA NR2A	P[1]						R[9]	
NMDA NR2B	P[1]							
NMDA NR2C							R[9]	
NMDA NR2D							R[9]	
AMPAGluR1							P[8]	
AMPAGluR2	P[1]						P[8], R[9]	
AMPAGluR3	P[1]						P[8], R[9]	
GluR6							P[8], R[9]	
GluR7							P[8], R[9]	
KA2							P[8], R[9]	
mGluR1	P[1]					P, R[7]		
mGluR2	P[1]							
mGluR3	P[1]							
mGluR4								
mGluR5						P, R[7]		

KEY: R= mRNA, P= protein

(1) Genever *et al.* 1999a, (2) Morhenn *et al.* 1994, (3) Kugler 2004, (4) Fan *et al.* 2004, (5) Genever *et al.* 1999b, (6) Wagenfeld *et al.* 2002, (7) Pacheco *et al.* 2004), (8) Weaver *et al.* 1996), (9) Inagaki *et al.* 1995), (10) Marinez-Lopez *et al.* 1998).

1.6.4 Expression of glutamate signalling outside of the central nervous system

Glutamate signalling is not limited to the CNS as previously thought. All components of glutamate signalling (vesicular transporters, vesicle docking proteins, transporters and receptors) are expressed in bone and cartilage cells and some transporters and receptors have been shown to be functional in these cells. The glutamate signalling components expressed outside of the CNS are shown in table 1.8 (bone, cartilage and fibroblasts are described in a separate section). Thus it is clear that many cell types have the potential to regulate extracellular glutamate concentrations and respond to glutamate via both ionotropic and metabotropic receptors.

1.6.4.1 Glutamate signalling in bone

Recent studies have suggested that glutamate plays a role in intercellular communication within bone (Mason *et al.* 1997, Chenu 2002, Hinoi *et al.* 2004, Kalariti and Koutsilieris 2004, Mason 2004) and in fact, evidence of glutamate signalling in bone has increased greatly over the last eight years.

Expression of glutamate receptors and transporters has been observed in osteoclasts, osteoblasts, osteocytes (see sections 1.6.4.1.1, 1.6.4.1.2 and 1.6.4.1.3), cartilage (section 1.6.4.2), fibroblasts and macrophages (section 1.6.4.3). Functional studies have revealed that activation of glutamate receptors can influence both bone formation and bone resorption. A GLAST-1 knock out mouse has been used to observe changes in bone at different stages of development but no differences in bone length, trabeculation or resorption lacunae of bone were detected compared with wild type siblings (Gray *et al.* 2001). However this work has been criticised due to the fact that compensatory mechanisms from other glutamate transporters were not taken into account (Skerry *et al.* 2001). Neither was the fact that the role of the gene may only be observed if functional studies are carried out (Chenu *et al.* 2001), for initially GLAST-1 was discovered in rat bone by its regulation under mechanical loading (Mason *et al.* 1997). GLAST-1 also has a splice variant called GLAST-1a and this was not taken into account either in the studies by Gray *et al.* (Skerry *et al.* 2001). GLAST1a does not contain exon 3 and is expressed in bone, brain (Huggett *et al.* 2000) and retina (Dr. D. J. Mason, Cardiff university,

personal communication). In addition, other neurotransmitters are present in bone: bradykinin, calcitonin gene related peptide, VIP and NO (reviewed in Skerry 2002).

Because levels of glutamate are elevated in RA and bone is degraded in RA the role of glutamate signalling in these cell types will be discussed. Table 1.9 summarises the data discussed in the following sections.

1.6.4.1.1 Glutamate signalling in osteocytes

GLAST-1 mRNA and protein is expressed in osteocytes *in vivo* and its expression is regulated by osteogenic mechanical load (Mason *et al.* 1997). GLAST-1 mRNA is expressed in bone *in vivo* and has the same open reading frame as that expressed in rat brain and it encodes a 60kDa protein in both tissues (Huggett *et al.* 2000). Expression of GFP tagged GLAST-1 in transfected MLOY4 cells (an osteocyte-like cell line) showed that GLAST-1 was expressed at the cell surface, a requirement for glutamate transport into and out of cells (Mason and Huggett 2002). In addition, the cell surface expression of GLAST is influenced by extracellular glutamate concentration in these cells (Mason and Huggett 2002).

Glutamate receptors are also expressed in osteocytes. NMDA NR1, AMPAGluR1 and AMPA GluR2 protein are expressed in rat osteocytes *in vivo* (Chenu *et al.* 1998).

1.6.4.1.2 Glutamate signalling in osteoblasts

Primary cultured osteoblasts release glutamate at concentrations sufficient to activate receptors expressed on bone cell surfaces (2-7 nmoles/mg protein) (Genever and Skerry 2001) and many studies have demonstrated the expression of receptors and transporters that could interact with this glutamate (see table 1.9). Glutamate release is Ca^{2+}-dependent and induced by K^+ (Genever and Skerry 2001). The machinery for targeted vesicular glutamate exocytosis is also expressed in osteoblasts (Bhangu *et al.* 2001). Namely tSNAREs and vSNAREs necessary for initial interaction of the vesicular and cellular plasma membranes as well as the complex forming VAMP synaptobrevin. The regulatory proteins SNARE protein-25 (SNAP-25) and syntaxin 4 which regulate the

fusion and exocytosis of glutamate containing vesicles and the plasma membrane are also expressed. Osteoblasts also express the mRNA for proteins involved in vesicular glutamate transport; BNPI (brain-specific sodium-dependent inorganic phosphate transporter) and DNPI (differentiation-associated sodium-dependent inorganic phosphate cotransporter) (Hinoi *et al.* 2002).

Functional studies have been undertaken on these glutamate signalling components. Patch-clamping methods have shown that the NMDA, AMPA and metabotropic types are functional in osteoblastic cell lines (Ljubojevic *et al.* 1999; Gu and Publicover 2000; Hinoi *et al.* 2002). In addition, limiting the release of glutamate with riluzole (a member of the benzothiazole class of drugs) by osteoblast-like cell lines induced apoptosis (Genever and Skerry 2001). Hence it was concluded that for *in vitro* survival of osteoblasts glutamate signalling appears essential (Genever and Skerry 2001). Conversely, Gray *et al.* have shown that activating or blocking NMDA receptors has no effect on bone formation by rat primary osteoblasts (Gray *et al.* 2001) however, only the non-competitive NMDA antagonist AP-5 was used, and at a concentration that did not take into account the fact that osteoblasts constitutively release glutamate (Skerry *et al.* 2001, Chenu *et al.* 2001).

1.6.4.1.3 Glutamate signalling in osteoclasts

The bone resorbing cells, osteoclasts also express glutamate receptors (summarised in table 1.9) like those expressed at glutamatergic synapses in the central nervous system (Chenu *et al.* 1998; Patton *et al.* 1998; Hinoi *et al.* 2002). Osteoclasts express ionotropic receptors (Genever *et al.* 1999, Ljubojevic *et al.* 1999), specifically subunits NMDA NR1, NR2B, NR2D, AMPA GluR 1, 2 and 4 (Chenu *et al.* 1998; Itzstein *et al.* 2001). The fact that both osteoblasts and osteoclasts express several regulatory NMDA subunits in addition to NMDA NR1 suggests a molecular diversity of NMDA receptor channels similar to that seen in brain (Itzstein *et al.* 2001).

The NMDA receptors in osteoclasts have been shown to be functional, because blocking these glutamate gated ion channels with pharmacological antagonists (D-AP5 or MK

801) inhibits bone resorption *in vitro* (Espinosa 1999). This leads to the conclusion that glutamate affects receptor-associated functions in osteoclasts such as bone remodelling, differentiation and cell activity (Chenu *et al.* 1998). MK801 has also been shown to inhibit osteoclastogenesis *in vitro* suggesting that osteoclastogenesis is dependent on constitutive glutamate signalling of osteoclasts (Peet *et al.* 1999). Furthermore MK801 inhibits actin ring formation in osteoclasts which is a prerequisite for the adhesion of osteoclasts to bone (Itzstein *et al.* 2000).

Other neuropeptides such as substance P and vasoactive-intestinal peptide have been implicated in osteoclast formation and bone formation (reviewed in Lerner 2002). This all provides evidence that neurosignals and in particular glutamate, can influence bone resorption.

Table 1.9 Summary of glutamate signalling apparatus expressed in bone cells (adapted from Mason 2004).

Glutamate release by exocytosis		Glutamate receptors		Glutamate transporters	
Vesicle fusion		**Ionotropic**		GLAST-1 (EAAT1)	Osteocytes and bone forming osteoblasts *in vivo* (12). Primary osteoblasts and MLO-Y4 and SaOS-2 cells (13,14). MG63 (16).
VAMP-1	MG63, SaOS-2, (1)	NMDAR1	Osteoclasts, osteoblasts,osteocytes *in vivo* (5). MG63 (5,6) SaOS-2 (6). Cultured osteoclasts (5). Bone marrow (6,9). Primary osteoblasts (7,10)		
SNAP-25	MG63,SaOS-2(1),osteoblasts *in vivo* (2)				
Syntaxin 4	MG63,SaOS-2(1)		Bone marrow (9)		
Syntaxin 6	SaOS-2,MG63 (1)	NMDAR2A & B	Bone marrow (6,9) osteoclasts (11) Primary osteoblasts (7,10)	GLAST-1a	Osteocytes *in vivo* (13), MLO-Y4 cells (15)
Vesicle docking		NMDAR2D	Osteoclasts (11)		
Munc-18	MG63,SaOS-2 & primary osteoblasts(1)	NMDAR3A	MG63 (16).		
DOC2	MG63,SaOS-2(1)	AMPAGluR1	Osteocytes,osteoblasts *in vivo* (5)	GLT-1 (EAAT-2)	Mononuclear cells of bone marrow *in vivo* (12).
RSec8	MG63,SaOS-2(1)	AMPAGluR2	Osteocytes,osteoblasts *in vivo* (5)		
Synaptophysin	MG63(3),SaOS-2(1)	AMPAGluR3	Primary calvarial osteoblasts (10)		
Regulatory proteins		KA1 and KA2	Primary calvarial osteoblasts (10)		
Rab3A,B,D	Primary osteoblasts(1) rat calvarial osteoblasts (2)	**Metabotropic** mGluR1b	Primary femoral osteoblasts (8) MG63 (16)		
Rab3A,C	Osteoclasts (4) SaOS-2 (2)	mGluR2	MG63 (16).		
Synapsin 1	MG63,SaOS-2(1), rat calvarial oblasts	mGluR3	MG63 (16).		
		mGluR4	Primary osteoblasts (7). MG63 (16).		
Synaptogamin I	Osteoblasts (2)	mGluR8	Primary osteoblasts (7). MG63 (16).		

Key (1) Bhangu *et al.* 2001, (2) Bhangu 2003, (3) Barry 2000, (4) Abu-Amer *et al.* 1999, (5) Chenu *et al.* 1998, (6) Patton *et al.* 1998, (7) Hinoi *et al.* 2001, (8) Gu and Publicover 2000, (9) Merle *et al.* 2003, (10) Hinoi *et al.* 2002,(11) Izstein *et al.* 2001, (12) Mason *et al.* 1997, (13) Mason and Huggett 2002, (14) Huggett *et al.* 2002, (15) Huggett *et al.* 2000, (16) Kalariti *et al.* 2004.
NB. SaOs-2 and MG63 cells are an osteosarcoma cell line (osteoblast-like). MLOY-4 cells are an osteocyte-like cell line.

1.6.4.2 Glutamate signalling in cartilage

EAAT1 mRNA is expressed in human chondrocytes (Mason and Huggett, unpublished data). mRNA for EAAT1, EAAT2 and EAAT3 has also been observed in rat primary chondrocytes (Hinoi *et al.* 2005). Protein expression of EAAT1 and EAAT2 was also observed in this study (Hinoi *et al.* 2005). Furthermore, Na^+-dependent uptake was also observed in these cells indicating that the glutamate transporters are functional (Hinoi *et al.* 2005).

mRNA and protein of NR1 and NR2A NMDA receptor subunits are expressed in chondrocytes (Salter *et al.* 2004). A difference in receptor responses was observed between normal and OA chondrocytes; NMDA receptor antagonists inhibited the hyperpolarisation response of normal FLS to mechanical stimulation but no effect was observed in OA chondrocytes (Salter *et al.* 2004).

1.6.4.3 Glutamate signalling in fibroblasts and macrophages

The invasive pannus in RA is made up of macrophage-like and fibroblast-like synoviocytes. Evidence of glutamate signalling has been observed in both of these cell types. Cultured monocyte-derived macrophages express EAAT1 and 2 (Rimaniol *et al.* 2000). In addition, in the alveolar macrophage cell line, NR8383, NMDA receptor subunit (NR1, NR2C and NR2D) mRNA is expressed (Dickman *et al.* 2004).

Na^+-dependent glutamate uptake has been demonstrated in macrophages and fibroblasts. TNFα-stimulated monocyte derived macrophages (from spleen and lung) show Na^+-dependent and Na^+-independent glutamate transport but only Na^+-independent glutamate transport in unstimulated cells (Rimaniol *et al.* 2000). Na^+-dependent glutamate transport was also observed upon differentiation of the macrophages into fibroblast-like macrophages (Rimaniol *et al.* 2000).

EAAT 1, 2, 3 and 4 proteins are expressed in cultured human skin fibroblasts (Cooper *et al.* 1998). EAAT 1, 2 and 3 proteins are also expressed by skin fibroblasts from alzheimer's disease patients (Zoia *et al.* 2005). The ability of fibroblasts to take up

glutamate has been recognised since 1983 when skin-derived human fibroblasts were shown to transport glutamate (Dall'Asta et al 1983). Cultured human fibroblasts from embryonic muscle, skin and peripheral nerve tissues and a fibroblast cell line (3T3) were shown to demonstrate a Na^+-dependent glutamate uptake process with K_m valued between 5-20μM (Balcar 1992, Balcar *et al.* 1994). Cooper *et al.* have also demonstrated glutamate uptake by skin fibroblasts via EAATs (Cooper *et al.* 1998).

1.6.5 Glutamate and Rheumatoid arthritis

Glutamate levels become elevated following induced inflammation. Studies in rats have shown that using kaolin and carrageenan injected into the knee joint to induce inflammation, doubles the glutamate concentration in the synovial fluid from 3μM to 6μM within ten minutes (Lawand *et al.* 2000). Glutamate is also found elevated in human joints with synovitis; compared to the average concentration from normal autopsy cases (range = 0.82-22μM) glutamate in synovial fluid from patients with synovitis were 54 times higher at an average of 326μM (range = 4-608μM) (McNearney *et al.* 2000, McNearney *et al.* 2004). If cells within the synovial joint are responsive to glutamate, these studies suggest that glutamate may be involved in mediating or prolonging the inflammatory response. In addition, recently it has been reported that glutamate levels in synovial fluid correlate with Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES), IL-8 and macrophage inhibitory protein alpha (MIPα) in RA synovial fluid (McNearney *et al.* 2004).

The blood plasma levels of glutamate are also elevated in human RA patients at an average of 50μM compared to 41μM in the normal controls (Partsch *et al.* 1978). This suggests that glutamate is actively removed from the synovial joint in normal conditions because the normal level of glutamate in synovial fluid is 6μM, hence some mechanism must be present in cells of the synovial joint to remove glutamate. The levels of glutamate in the blood of rats are between 100-200μM (De Cristobal *et al.* 2001), which is far higher than the K_D values of the glutamate receptors expressed in the rat brain. This suggests that peripheral glutamate receptors outside of the CNS may have protection mechanisms against exposure to such high glutamate concentrations (Hinoi *et al.* 2002).

In the central nervous system it is the high affinity glutamate transporters that take on the protective role by quickly removing extracellular glutamate released by synapses.

In addition, ionotropic and group I metabotropic receptors play a role in inflammation in the brain; activation of these receptors causes an increase in intracellular calcium. This calcium can then activate, amongst others, the generation of free radicals, stimulation of inflammatory cascades and eventually neuronal death (O'Shea *et al.* 2002). The calcium also causes activation of NO synthase, which in turn produces nitric oxide (Riedel and Neeck 2001). Even though this has been observed in neuronal cells, a potential link can still be seen between glutamate and inflammatory processes of the synovial joint because NO is a mediator of inflammation (section 1.4.1.3). NO can also damage DNA, which causes the p53 tumour supressor gene to be activated. Mutations in p53 have been observed in longstanding RA patients that cause impaired apoptosis which contributes to pannus formation (Tak *et al.* 2001). It should be noted that NO, like glutamate, also acts as a neurotransmitter within the central nervous system however CNS NO is produced by a constitutively produced neuronal NO synthase.

1.6.5.1 Source of glutamate in the synovial joint

The source of glutamate in the RA synovial joint is not yet determined. In bone, it may come from nerve endings that are able to secrete glutamate. Glutamate can excite peripheral neurons and glutamate signalling components are expressed in the peripheral nervous system (Jackson *et al.* 1995). mGluR1 and mGluR5 proteins have been shown to be expressed in mouse peripheral unmyelinated sensory afferents (Bhave *et al.* 2001). In addition, in the rat dorsal root ganglion AMPAGluR1-4, the kainate receptor GluR5 and NMDA NR1 protein is expressed (Sato *et al.* 1993). The nerve endings in bone may therefore be able to secrete glutamate. In fact the presence of a dense network of nerve processes in bone has been observed, some of which contain glutamate (Serre *et al.* 1999). Alternatively it may come from the constitutive release of glutamate by osteoblasts. White blood cell release of glutamate has been investigated but levels of white blood cells in the synovial joint do not correlate with glutamate levels suggesting that they are not the source (McNearney *et al.* 2000). McNearney *et al.* therefore suggest

that the source of glutamate in synovial fluid during inflammation is from blood, mast cells, FLS or nerve endings or from degradatory products of inflammatory events.

1.6.5.2 Links between the CNS and RA

Interestingly, a possible link between the neural system and arthritis can be seen *in vivo* in patients who develop a stroke or paralysis. In some patients inflammatory or degenerative arthritis only develops on the non-paralysed side of the body suggesting that these processes may be affected by neuronal activity (Thomason and Bywater 1962). To further support this, in some patients that develop a stroke after the onset of arthritis incomplete reversal of the arthritis was observed on the affected side of the body (Thomason and Bywater 1962).

Delgado *et al* have also shown a link between the neural system and inflammation. Treatment of arthritis experimentally induced in rats, with the neuropeptide vasoactive intestinal peptide (VIP) show decreased joint swelling and inhibition of cartilage and bone destruction (Delgado *et al.* 2001).

1.6.5.3 Glutamate and pain in RA

Studies investigating nociception in animal models of inflammation have shown increases in the release of excitatory amino acids (including glutamate) and concluded that glutamate in peripheral nociceptive signalling is involved in models of arthritis (Lawand *et al.* 2000, Westlund *et al.* 1992, Sluka and Westlund 1992, Sorkin *et al.* 1992). Furthermore, glutamate receptors have been associated with the pain experienced in RA. Both NMDA and non-NMDA receptors are involved in inflammatory pain in the CNS (Carlton and Coggeshall 1999, Li and Neugebauer *et al.* 2004). Zhang *et al.* also demonstrated that NMDA and non-NMDA contribute to the induction of adjuvant-induced arthritic pain in rats, furthermore pain-related behaviour could be prevented by injection of MK801 (a non-competitive NMDA receptor inhibitor) and 2, 3-dihydroxy-6-nitro-7-sulfamoylbenzoquinoxaline (NBQX, a competitive antagonist of AMPA/kainate receptors) (Zhang *et al.* 2003).

1.6.5.4 Glutamate signalling and pro-inflammatory cytokines in the CNS

Pro-inflammatory cytokines have been investigated within the CNS; where there is some evidence linking glutamatergic signalling to inflammatory responses. For example there is evidence that the pro-inflammatory cytokine IL-6 is linked to CNS diseases associated with glutamate signalling irregularities. Various neuropathologies such as ischaemia (Szczudlik *et al.* 2004, Chen *et al.* 2003), amyotrophic lateral sclerosis (Hensley *et al.* 2003, Sekizawa *et al.* 1998), HIV infection (Laurenzi *et al.* 1990, Perrella *et al.* 1992 and Tyor *et al.* 1992), meningitis (Waage *et al.* 1989) Parkinsons disease (Ciesielska *et al.* 2003, Blum-Degen *et al.* 1995), systemic lupus erythematosus with CNS involvement (Hirohata and Miyamoto 1990), Alzheimer's disease (Blum-Degen *et al.* 1995), and stroke (Dziedzic *et al.* 2004) are associated with elevated IL-6 levels. These diseases are also associated with excessive glutamate receptor activation; ischaemia (Simon *et al.* 1984, Meldrum *et al.* 1994), amyotrophic lateral sclerosis (Niebroj-Dobosz *et al.* 1999), HIV (Lipton *et al.* 1991), meningitis (Spranger *et al.* 1996), Parkinson's disease (Meyerson *et al.* 1990), systemic lupus erythematosus (McNearney *et al.* 2000), Alzheimer's disease (Mattson *et al.* 1991) and stroke (Choi and Rothman 1990). This therefore indicates a potential link between the synchronous elevated levels of IL-6 and glutamate in these diseases.

Pro-inflammatory cytokines are known to influence glutamate signalling in the CNS. IL-6 (5ng/ml) enhances NMDA receptor activation in rat cortical neuron cultures (Qiu *et al.* 2003). However, IL-6 also appears to prevent over-stimulation of NMDA receptors because pre-treatment with IL-6 (50ng/ml) for 15 hours inhibits glutamate-induced death in primary cultures of rat hippocampal neurons by an unknown mechanism (Yamada 1994). In addition, IL-6 (5 to 50ng/ml) with sIL-6r (2.5 to 25ng/ml) prevents NMDA receptor-induced apoptosis in retinal ganglion cells *in vivo* possibly by activation of the STAT3, MEK/MAPK or PI3K/Akt signalling pathways and the same study also demonstrated that NMDA injection (20nmol) in rats caused up-regulation of gp130 in retinal tissue (Inomata *et al.* 2003). Furthermore, the increased susceptibility to seizures upon injecton of kainate (a specific activator of the ionotropic kainate receptors) of IL-6 knock out mice indicates that IL-6 also inhibits over-stimulation of these receptors

(Penkowa *et al.* 2001). IL-6 has also been shown to have a neurotoxic role; Qui *et al.* demonstrate that IL-6 (5ng/ml) enhances NMDA neurotoxicity in developing CNS neurons by enhancing the calcium signal to NMDA (Qui *et al.* 1998 and 1995).

TNFα and IL-1 and their interaction with glutamate signalling has also been investigated in the CNS. After 24 hours TNFα (20ng/ml) and IL-1β (20ng/ml) inhibit transport-mediated glutamate uptake (1mM) in rat glial cultures probably via the EAATs because the glutamate transporter inhibitor, *t*PDC (2.5mM), prevented these inhibitory effects (Liao and Chen 2001). This is of relevance as these are seen as the two major cytokines involved in RA. It was also observed that free radicals and oxygen species which are also present in the RA joint, can impair glutamate transport into astrocytes (Liao and Chen 2001). The suggested mechanism by which these pro-inflammatory cytokines regulate glutamate transport was that they were causing an increased production in free radicals and active oxygen species. These were then altering the redox potential of the astrocytes which in turn was altering the sodium pump that generates electrochemical gradients used by the transporter to drive glutamate uptake (Liao and Chen 2001). In addition, glutamate transporters in the CNS have been shown to be oxidant vulnerable and their activity is directly affected by oxidation (Trotti 1998).

Treatment for more than 2 hours with TNFα (2ng/ml) or IL-1β (1ng/ml) can attenuate glutamate uptake in primary rat astrocytes *in vitro* through a pathway that involves the liberation of nitric oxide by the stimulation of nitric oxide synthase (Ye and Sontheimer 1996). In addition, TNFα (60-1000U/ml [3ng-50ng/ml] for 60 minutes) reduces ionotropic glutamate receptor activation in astrocytes *in vitro* (Koller *et al.* 2001).

1.6.5.5 Glutamate signalling and pro-inflammatory cytokines outside the CNS

Limited research has revealed links between glutamate signalling and pro-inflammatory cytokines outside of the CNS. For example, NMDA receptors interacting with IL-6 can be linked to osteoclastogenesis. The fact that MK801 has also been shown to inhibit osteoclastogenesis *in vitro* (Peet *et al.* 1999) and inhibit actin ring formation in osteoclasts which is a prerequisite for the adhesion of osteoclasts to bone (Itzstein *et al.*

2000) and that IL-6 and sIL-6r can trigger osteoclast formation in vitro (Tamura *et al.* 1993) indicates that signalling via this cytokine and glutamate may converge to regulate osteoclastogenesis. Finally, outside of the CNS IL-6 has been shown to induce NF-κB activation in cultured intestinal epithelial cells within 2 hours (Wang *et al.* 2003) and PDTC, an inhibitor of NFκB activation causes a decrease in glutamate uptake (Liao and Chen 2001). With regards to the effects of TNFα on glutamate outside of the CNS, treatment of osteoblasts with TNFα inhibits glutamate release from osteoblasts (Genever and Skerry 2001).

These studies therefore demonstrate that cytokines may directly influence glutamate signalling pathways outside of the CNS.

1.6.5.6 Glutamate and degradative enzymes

There is some evidence linking glutamatergic signalling to degradative responses in cells of the CNS. Yong *et al.* indicates a role for MMPs in several CNS diseases such as multiple sclerosis and Alzheimer's disease (Yong *et al.* 2001), where glutamate transporter expression is dysregulated. Kainate receptor activation increases MMP9 mRNA and protein expression within hours in the rat hippocampus *in vivo* (Szklarczyk *et al.* 2002). Rats systemically injected with kainate also demonstrate increases in MMP2 protein expression in the brain (Zhang *et al.* 2000). Furthermore, kainic acid –induced excitotoxic seizures increases expression levels in the rat brain of MMP 9 (Jourquin *et al.* 2003). The kainate and AMPA ionotropic glutamate receptors are directly implicated in these responses since kainate has been shown to mediate up-regulation of MMP9 in mouse retinal astrocyte cells *in vivo* (Zhang *et al.* 2004). In addition, inhibition of MMPs in the rat prevents kainic acid-induced cell death in the brain (Campbell *et al.* 2004) demonstrating that MMPs may have an affect on glutamate receptors. Moreover, TIMP1 protects against excitotoxic death in neurons (Tan *et al.* 2003) thus demonstrating a link between TIMPs and glutamate signalling.

1.6.5.7 Glutamate and RA summary

Taken together these findings clearly demonstrate a role for glutamate signalling in non-neuronal tissues and in bone. Furthermore, glutamate signalling pathways have been shown to interact with cytokines and degradative enzymes in the CNS. Therefore, the elevated glutamate in synovial fluid that accompanies RA may induce phenotypic changes associated with synovial joint inflammation and destruction. The overall purpose of this present study is to investigate the role of glutamate signalling in the disease mechanisms in inflammatory arthritis. As yet there is no cure for rheumatoid arthritis. Identification of novel signalling pathways involved in RA pathogenesis will enable new and maybe more effective therapeutic targets to be identified.

1.7 Hypothesis and aims of project

Hypothesis:

The increased levels of glutamate in RA synovial fluid mediate the inflammatory and catabolic responses associated with joint destruction.

To investigate this hypothesis there were four specific aims:

1. To determine which cells of the synovial joint express glutamate receptors and transporters.

2. To determine which cells of the synovial joint are responsive to glutamate and to characterize those responses with respect to joint destruction.

3. To determine whether pro-inflammatory cytokines influence glutamate receptor and transporter activity in FLS.

4. To determine whether glutamate receptors and transporters are functional in FLS.

CHAPTER 2: Materials and Methods

All reagents were obtained from Sigma Aldrich, unless otherwise stated.

2.1 Dissection and homogenisation of tissues

Rats were dissected immediately after death and the parts of the knee joint required (meniscus, patella and fat pad) were dissected, snap frozen in liquid nitrogen and stored at –80°C until required. Tissues were powdered in 1ml Trizol® reagent (Invitrogen) using a B. Braun Biotech Dismembrator at 2000rpm for 3 minutes, cooled with liquid nitrogen. Both the medial and lateral menisci from the same joint were homogenised together and two or three fat pads were combined in order to obtain enough RNA for analysis.

2.2 RNA extraction

Total RNA was extracted in Trizol® (Invitrogen) following the manufacturer's instructions. Briefly 0.2 volumes chloroform was added to the Trizol® extract containing either $1x10^6$ cells or homogenised tissue, mixed and separated by centrifugation (12000rpm) for 15 minutes at 4°C (Universal 16R centrifuge, Hettich Zentrifugen). The aqueous, upper phase was transferred to a clean tube and an equal volume of 0.5% isopropanol was added and incubated overnight at –20°C to precipitate the RNA. The RNA was collected by centrifugation, washed twice in 1ml ethanol (100% followed by 75% ethanol), air-dried and resuspended in 86µl dH_2O. (Fat pad samples, prior to addition of chloroform were subjected to an additional isolation step in Trizol®; these samples were centrifuged at 12000g for 10 minutes at 4°C and the cleared homogenate saved for addition of 0.2 volumes chloroform as before).

2.2.1 DNase treatment of RNA

Contaminating genomic DNA was removed by digestion with 2 units of RQ1 DNase (Promega) in the presence of 40U RNasin® ribonuclease inhibitor (Promega) at 37°C for 15 minutes. RNA was purified by re-extraction in 300µl Trizol®, resuspended in 60µl dH_2O and stored at –80°C.

2.3 Generation of cDNA by reverse transcription

Approximately 1μg RNA dissolved in 11μl dH_2O was mixed with 1μl (0.5μg) oligo$(dT)_{15}$ (Promega) and denatured for 10 minutes at 65°C. The reaction was briefly chilled on ice and 4μl 5x first strand buffer (Invitrogen), 2μl 0.1M DTT (Invitrogen) and 1μl 10mM dNTPs (Promega) added. The reaction was mixed and incubated at 42°C for 2 minutes and 1μl Superscript II (Invitrogen) added. The reaction was incubated for 50 minutes at 42°C and terminated by heating to 70°C for 15 minutes. All incubations were carried out in a thermal cycler (PCR Express, Hybaid). cDNA was stored at –20°C until use.

2.4 PCR

All primers were designed using Primer express™ 1.0 (Applied Biosystems, Perkin Elmer) to sequences homologous between rat, human and mouse unless otherwise stated. To prevent amplification of genomic DNA that had not been degraded by DNase treatment, all primers were designed to amplify products spanning intron/exon boundaries. β-actin was used as a housekeeping gene to confirm successful cDNA preparation. Table 2.1 shows all primer sequences used in this study.

PCR reagents were made up as master mixes and positive (rat brain cDNA, GLAST plasmid or β-actin plasmid) and negative controls (no template) were used for each group of PCR reactions. The PCR conditions consisted of one cycle of 95°C for 5 minutes, 40 cycles of 95°C for 30 seconds (strand separation), specific primer pair annealing temperature (see table 2.1) for 30 seconds, 72°C for 1 minute (amplicon extension) and one cycle at 72°C for 10 minutes (final extension) followed by incubation at 4°C in a thermal cycler (PCR Express, Hybaid). The appropriate $MgCl_2$ concentration was optimised for each primer pair (see table 2.2).

Table 2.1 List of primers used

Primer name and sequence it aligns to.	Sequence
β-actin [Ac.No.NM_031144] (Araki *et al.* 1993)	Forward:5'-TGTATGCCTCTGGTCGTACCAC (506bp-527bp) Reverse:5'-ACAGAGTACTTGCGCTCAGGAG (1076bp-1097bp)
GLASTcc (EAAT1) [Ac.No.NM_019225] (Huggett *et al.* 2000) {AF265360}	Forward:5'-TGAAGAGCTACCTGTTTCGGAA (325-346bp) {243-264bp of GLAST1a} Reverse:5'-TACATGTTTTCTTTCGTGCCC (650bp-670bp) {430-450bp of GLAST1a}
GLAST-1a specific [Ac.No.AF265360] (Hugget *et al.* 2000)	Forward:5'-CAGCGCTGTCATTGTGGGAATGGC (286-309bp) Reverse:5'-AGGAAGGCATCTGCGGCAGTCACC (472-495bp)
Human EAAT2 [Ac,No.NM_004171] (Zoia *et al.* 2004)	Forward:5'-ACGAGGAGGCCAACGCAACAAG (888-906bp) Reverse:5'-GATGCCCCCGTGGATGATGAGG (1252-1273bp)
Rat EAAT2 [Ac.No.NM_017215] (GLT1) (gift from Dr. J. Huggett, Cardiff University)	Forward:5'-GAGCCAAAGCACCGAAAC (177-194bp) Reverse:5'-GGAAATGATGAGAGGGAGGAT (375-395bp)
Human EAAT3 [Ac.No.U03506] (Zoia *et al.* 2004)	Forward:5'-CGTCCTGGGCTTGATTGTCTTT (651-672bp) Reverse:5'-TGCACCAACGGGTAACACGA (1088-1107bp)

Rat EAAT3 [Ac.No.NM_013032] (EAAC1) (gift from Dr. J. Huggett, Cardiff University)	Forward:5'-CCCCGATTCCTCACAAAC (90-107bp) Reverse:5'-TGCTGACTTCAGGGGTTTTGC (512-532bp)
Rat EAAT4 [Ac.no.RNU89608] (gift from Dr. J. Huggett, Cardiff University)	Forward:5'-CGCGGGTTCTGGCTTTTG (69-84bp) Reverse:5'-CATCCTCCCTGTTGCCTTGTT (505-525bp)
NMDA NR1 [Ac.No.NM_017010]	Forward:5'-CAGGAGCGGGTAAACAACAG (1724-1743bp) Reverse:5'-CCTGGTACTTGAAGGGCTTG (1855-1874bp)
KA1 [Ac.No.NM_014619) (Sokolv *et al.* 1998)	Forward5'-GACTGCAGAAACCATGTGTCAGATCC (234-259bp) Reverse:5'-GGTGCAGTTGAAGAAGTTCAGGATCC (457-480bp)
AMPA GluR2 [Ac.No.M85035]	Forward:5'-GGTTGTCACCCTAACTGAGCTC (1500-1521bp) Reverse:5'-AGTAGCCCTCGTAACGCTCATT (1630-1651bp)
AMPA GluR3 [Ac.No.NM_032990]	Forward:5'-AATTCCCTGAAGCCAAGAATG (919-939bp) Reverse:5'-GGATTTGCTAAGCAGTCTCCA (1049-1069bp)
mGluR4 [Ac.No.NM_000841]	Forward:5'-AGACCTTCAACGAGGCCAAG (2506-2525bp) Reverse:5'-CGCTCAGACTCACGGAGACC (2639-2658bp)

Table 2.2 PCR conditions and product sizes for each primer pair

Primers	Annealing Temp. (°C)	$MgCl_2$ concentration (mM)	Primer concentration (μM) forward and reverse	Product size (bp)
β-actin	58	1.5	0.016	592
EAAT1(GLAST)	58	1.5	0.016	348
GLAST-1a	58	1.5	0.016	210
EAAT2 human	59	3	0.016	385
EAAT2 rat	55	2	0.016	219
EAAT3 human	58	2	0.016	457
EAAT3 rat	56	2	0.016	443
EAAT4	55	1.5	0.016	436
NMDA NR1	58	2	0.016	151
KA1	56	2	0.016	247
AMPA GluR2	58	1.5	0.016	152
AMPA GluR3	58	2	0.016	151
mGluR4	58	2	0.016	153

2.4.1 Agarose gel electrophoresis

The DNA amplicons generated by the PCR reactions were combined with loading dye (Promega) at a 10:1 ratio and resolved on an appropriate percentage agarose (Promega) gel (see table 2.3). Gels containing 10μg/ml ethidium bromide in 1x Tris Borate EDTA (TBE) buffer (89mM Tris-borate, 2mM EDTA, pH8.3) were run at 80v for 1 hour. DNA standards (100bp ladder, Promega) were used to identify the size of products.

Table 2.3: Percentage of agarose used in gels for running different sized PCR products

% w/v agarose in gel	Size of PCR amplicon
2	100-200bp
1.5	200-350bp
1	>350bp

2.4.2 Gel electrophoresis image generation

Images of DNA within agarose gels were visualised using a GelDoc system (BioRad). The results section of chapters 3 and 5 contain images of gels that have had irrelevant lanes cropped out using Adobe photoshop to enable lanes containing amplicons to be adjacent to DNA standards. The full pictures of these gels can be found in the appendix.

2.4.3 Cloning and sequencing of PCR amplicons

Amplicons from newly designed primers were sequenced to confirm that the primers were functioning correctly. Under *uv* light, amplicons were cut out of agarose gels and purified using a PCR purification kit (Qiagen). Manufacturer's instructions were followed to remove dNTPs, salts, agarose, proteins, ethidium bromide and polymerases.

The PCR products were TA cloned into the pGEM-T vector (50ng) by incubating in 2X Rapid ligation buffer (5µl), 3 Weiss units of T4 DNA ligase (1µl) and the reaction volume made up to 10µl with distilled water. Ligation reactions were incubated for 1 hour at room temperature prior to transformation.

The ligated plasmid was transformed into *Escherichia coli* (JM109 competent cells), by heatshocking. Transformed cultures were grown up overnight on LB, ampicillin (100µg/ml), IPTG (0.5mM), X-GAL (50µg/ml) plates overnight at 37°C. Successfully ligated plasmids resulted in white colonies due to interruption of the β-galactosidase gene. White colonies were picked and placed in 10ml of LB broth (1.1g LB tablet in 50ml dH_2O supplemented with 100µg/ml ampicillin) and incubated overnight at 37°C whilst shaking (150rpm).

In order to obtain the ligated plasmids for sequencing, cultures were prepared using a Wizard plus SV midiprep kit (Promega). Briefly, 200µl of the overnight culture was amplified further by inoculation of 250ml LB broth and incubated overnight at 37°C with shaking (230rpm). Cells were harvested by centrifugation at 3300rpm, resuspended and lysed. Neutralisation buffer was added to the lysed cells and incubated for 10minutes followed by centrifugation at 3300rpm for 15 minutes. An equal volume of isopropanol was added to the retained supernatant and incubated at -20°C overnight to precipitate the DNA. Samples were centrifuged (1500g, 1 hour) and the supernatant discarded. The precipitated plasmid was washed twice with 75% ethanol and repelleted (centrifugation at 1500g, 1 hour) to remove isopropanol. The plasmid pellet was air dried prior to resuspension in distilled water. 5µl was restricted with 5 units of *Eco*RI (37°C, 1 hour, 1 times buffer containing 10µg bovine serum albumin [Promega]), to confirm the presence of the insert. Clones were then sequenced using M13 vector primers.

2.5 Tissue culture

2.5.1 Bovine chondrocytes

Primary bovine chondrocytes were isolated from articular cartilage of the metacarpalphalangeal joint of 7 day old calves using a sterile scalpel first to remove the cartilage. Following dissection, the articular cartilage samples were placed in 25ml DMEM (Gibco) supplemented with 2mM glutamine, 1200units/ml penicillin, 1200µg/ml streptomycin and 20µg/ml fungizone and left for 10 minutes. The tissue was washed twice for 10 minutes in 25ml DMEM supplemented with 2mM glutamine, 300U/ml penicillin, 300µg/ml streptomycin and 5µg/ml fungizone.

The articular cartilage slices were cut into small pieces, placed in a T75 flask (Corning Ltd.) and incubated for 30 minutes at 37°C with gentle agitation in 25ml (4mg/ml) pronase (from *Streptomyces griseus*) in DMEM (containing 2mM glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 2.5µg/ml fungizone and 10% dialysed foetal bovine serum [SLI Ltd.]) to provide the cells with nutrients. Dialysed foetal bovine serum was used to minimise the presence of glutamate. The pronase solution was removed and

replaced with 25ml DMEM containing 3mg/ml collagenase (from *Clostridium histolyticum*), 100U/ml penicillin, 100μg/ml streptomycin, 2.5μg/ml fungizone and 10% FBS and agitated at 37°C for 2 hours to release the chondrocytes from the collagen matrix. The medium was taken off every 30 minutes and centrifuged at 1500g (IEC Centra CL2 centrifuge) for 10 minutes to collect the chondrocytes and passed through a 40μm cell strainer to remove cellular debris before being pooled. The chondrocytes were seeded at a density of $1x10^6$ cells per T25 flask (Corning Ltd.) in 7ml DMEM containing 2mM glutamine, 100U/ml penicillin, 100μg/ml streptomycin, 2.5μg/ml fungizone and 10% FBS and incubated at 37°C in a 5% CO_2 atmosphere.

Cells were removed from T25 (Corning Ltd.) flasks by adding 1ml Trizol and the extract used for glutamate signalling apparatus mRNA expression studies.

2.5.2 Fibroblast-like synoviocytes

Primary human FLS kindly provided by Dr. A. S. Williams in collaboration with Prof. B. Williams (Department of Rheumatology, Cardiff University) were established from the synovial fluid of RA or non-arthritic patients taken from a synovectomy. Table 2.4 shows the details of each patient from whom synoviocytes were used.

Table 2.4: Details of patients from whom synoviocytes were used in this study

Patient number	Age	Sex	Condition
1	Unknown	Unknown	RA
2	56	Female	RA
3	62	Female	RA
4	64	Female	Non-arthritic
5	67	Female	RA
6	64	Female	RA
7	43	Female	RA
8	55	Male	RA
9	27	Female	RA

FLS were not used before passage 5 due to the possible presence of macrophages and only cultured up to passage 8 due to the loss of phenotype observed beyond this passage (Dr A. S. Williams, personal communication). FLS seeded into 6-well plates (Corning Ltd.) were grown at 37°C, 5% CO_2 in Dulbecco's modified essential medium (DMEM) Nut mix F-12 supplemented with 10% foetal bovine serum (FBS, Serum Laboratories Incorporated), penicillin (100U/ml), streptomycin (100μg/ml) and 4mM L-glutamine. To minimise the presence of glutamate, dialysed FBS was used. It should be noted that the DMEM used contained 50μM glutamate. Synoviocytes were cultured to confluency in 48-well plates (Corning Ltd.), 6-well plates, 60mm petri dishes (Corning Ltd.) or 8-well chamber slides (Fisher Scientific) seeded at a density of 2.5 X 10^4, 1 X 10^5, 1.5 X 10^5 and 2.5 X 10^4 respectively. Prior to stimulation with glutamate, cytokines or inhibitors the cells were incubated for 24 hours with serum-free medium.

2.5.2.1 Passaging of FLS

FLS were only cultured up to passage 8 due to the loss of phenotype observed beyond this passage (Dr A. S. Williams, personal communication). FLS were cultured in T75 and T225 flasks (Corning Ltd.) until confluent and trypsinised using 1ml 10x trypsin EDTA (Gibco) after the media had been removed. Cells were then added to fresh media and split 1 in 3 into fresh T75 flasks or into an appropriate experimental vessel.

2.6 Immunohistochemistry

2.6.1 Methanol fixation of slides for immunostaining

FLS on 8-well chamber slides, following stimulation with cytokines (details in experimental chapters) were washed three times with 0.5ml phosphate buffered saline (PBS) per well. 0.5ml methanol was aliquoted into each well and incubated for 5 minutes before being removed and the slides allowed to air dry. Chamber slides were stored at –20°C until used. Experimentation with paraformaldehyde fixing of cells revealed that this process did not show any binding of the polyclonal anti-GLAST (EAAT1) antibody therefore all immunohistochemical analysis presented in this study has been methanol fixed.

2.6.2 Immunostaining of FLS

Experimental FLS, stored at –20°C following methanol fixation were defrosted and allowed to reach room temperature before incubating in non-immune goat serum for 1 hour. Slides were incubated for 1 hour in 100µl of rabbit serum containing a rabbit polyclonal GLAST antibody (made by Dr. Eryl Liddell, Cardiff University) used at an optimal dilution of 1/100, diluted with PBS. Slides were washed 3 times in PBS before incubating for 1 hour with 100µl of a goat, anti-rabbit FITC conjugate secondary antibody diluted to 1/200 with PBS. Cells were washed 3 times in PBS to remove unbound secondary antibody. All incubations were carried out at room temperature.

2.6.3 Mounting of immunolabelled FLS

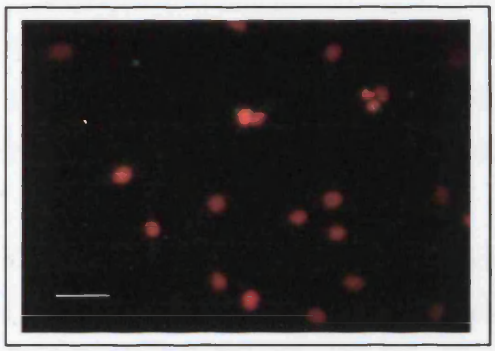
Cells were mounted with Vectashield® mounting medium containing propidium iodide (Vector Laboratories, USA). Slides were sealed with nail varnish and stored at 4°C until viewed with a fluorescence microscope (Leitz Laborlux 12). Propidium iodide was used as a counterstain for cell nuclei. Images were captured using a Coolsnap digital camera (R S Photometrics). Two images of different areas of each well were taken. The optimal exposure time was determined using the automatic function and this value was then set in manual mode so that all images being compared were exposed for the same length of time.

2.6.4 Optimisation of the polyclonal GLAST antibody

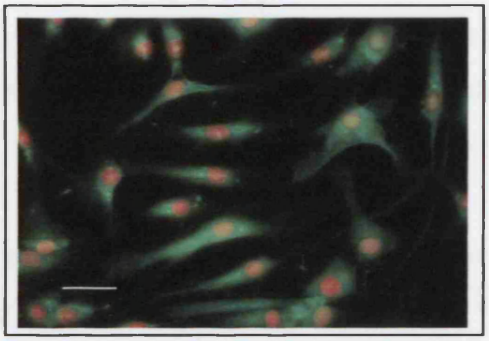
The GLAST antibody had previously been partially characterized for Western blotting by Dr J. Huggett and Sian Kneller, Cardiff University. A range of dilutions for both the primary (polyclonal rabbit anti-human, GLAST) and secondary (goat anti-rabbit, FITC-conjugated) antibodies were tested until the optimal concentration was obtained. Immunostaining with different concentrations of the secondary antibody is shown in figure 2.1. A 1/200 dilution (figure 2.1, panel D) was deemed optimal for the secondary antibody.

Figure 2.1 Optimisation of the secondary antibody (goat, anti-rabbit FITC conjugate), using 1/100 primary anti-GLAST antibody.

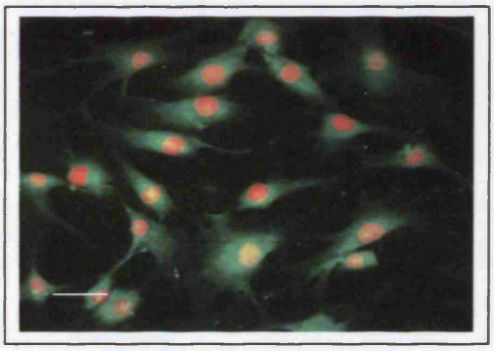
A.Primary negative



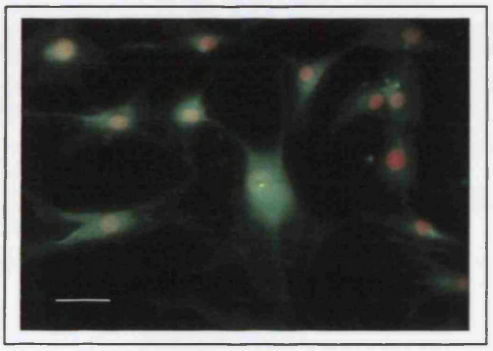
B.1/50 Secondary



C.1/100 secondary



D.1/200 secondary



2.7 Western Blotting

2.7.1 SDS PAGE

Cell extract proteins alongside 5µl of pre-stained molecular weight marker (Biorad) were resolved on 10% SDS-polyacrylamide gels (see table 2.5 for components) at 200v in Laemmli buffer. (Laemmli, 1970) Resolved protein was then transferred to polyvinyldifluoride membrane (Immobilon-PVDF, Millipore) for 1 hour at 100v in transfer buffer (20% v/v methanol in Laemmli buffer). To prevent overheating, ice packs were placed in electrophoresis tanks. Prior to blotting membranes were washed in methanol (15 seconds), water (2 minutes) and transfer buffer (5 minutes).

Table 2.5 Components of 10% SDS PAGE gel

Components of gel	10% resolving gel	4% stacking gel
40% bis-acrylamide	3.83ml	575µl
1M Tris/HCl pH8.8	3.63ml	-
1M Tris/HCl pH6.8	-	1.3ml
10% (w/v) SDS	100µl	50µl
10% (w/v) APS	75µl	37µl
TEMED	15µl	7.5µl
dH_2O	7.05ml	4.075ml

2.7.2 Blotting

The polyclonal GLAST antibody used was raised to amino acids 24-40 (made by Dr. Eryll Liddell, Cardiff University). Non-specific sites on the membrane were blocked by incubating in 5% (w/v) skimmed milk power, 0.05% Tween 20 in TBS (0.05M Tris-HCl, pH8.0, 0.15M NaCl) for 1 hour. The membrane was sequentially incubated in 5ml antiserum to GLAST-1 (1:2000 in TBS containing 1% milk) for one hour and 5ml goat anti-rabbit horse radish peroxidase (HRP) conjugate 1:20000 for 1 hour. Membranes were washed 3 times in 150ml TBS-Tween 20 for 15 minutes after each antibody incubation. Duplicate membranes were prepared in which the primary antibody was omitted to control for non-specific binding of the secondary antibody.

2.7.3 Chemiluminescent detection of bound antibody

Specific binding of the GLAST antibody was detected by enhanced chemiluminescence on Hyperfilm-ECL (Amersham, UK) according to manufacturer's instructions.

2.7.4 Stripping a Western blot for reprobing

Some membrane blots were stripped and reprobed with a different antibody concentration. Blots were incubated at 50°C, for 30 minutes in stripping solution (62.5mM Tris HCl [pH6.7], 2% SDS, 100mM 2-mercaptoethanol). Membranes were washed in dH_2O and incubated in blocking solution as above prior to reprobing.

2.7.5 BCA assay to determine how much protein was loaded onto SDS PAGE gels

To ensure that equal protein was loaded onto each SDS PAGE gel for Western blotting the amount of protein in each sample was determined using a BCA assay (Pierce, Perbio). A microplate assay was carried out according to manufacturer's instructions. Albumin standards were prepared to produce a standard curve ranging from 25µg/ml to 1500µg/ml. 25µl of standard or RA FLS protein extract were aliqouted in triplicate on a 96-well plate and incubated at 37°C for 30 minutes with 200µl of working reagent (50 parts reagent A [containing sodium carbonate, sodium bicarbonate, bicinchoninic acid, sodium tartrate in 0.1M NaOH] with one part reagent B [containing cupric sulfate]). The protein present in the standards and samples reduces Cu^{2+} to Cu^{+} which produces a purple coloured reaction product that can be measured by reading the absorbance at 562nm.

2.8 IL-6 ELISA

Analysis of IL-6 levels in FLS media was quantified using a matched pair IL-6 enzyme linked immunosorbent assay (ELISA) Duo set kit (R&D Systems). Manufacturer's instructions were followed and all incubations were carried out at room temperature. Briefly, the 96 multiwell plate (ICN Biomedicals Inc.) was coated with 100µl (2µg/ml) capture antibody (mouse anti-human IL-6 in PBS) overnight. The plate was washed three times with 300µl wash buffer (0.05% Tween 20 in PBS pH 7.4) and blocked with 300µl blocking solution (1% bovine serum albumin. 5% sucrose and 0.05% Sodium nitrite in PBS) for one hour. The wells were washed three times with 300µl wash buffer and

100µl of FLS media or standards was added and incubated for 2 hours. The wells were washed three times with 300µl wash buffer and 100µl of the biotinylated detection antibody (200ng/ml, goat anti-human IL-6) was added for 2 hours. The wells were washed three times as before and 100µl of streptavidin HRP (R&D Systems) was added to each well for 20 min. The wells were washed three times with 300µl wash buffer and 100µl substrate solution (a 1:1 mixture of hydrogen peroxide and Tetramethylbenzidine) was added to each well for 20 min away from direct light. The reaction was stopped with 50µl 1N sulphuric acid and the absorbance read at 450nm (Multiskan plate reader, Anthos 2001 series). A standard dilution curve of $\log_{10}$ Absorbance v $\log_{10}$ concentration was used to determine the concentration of IL-6 in the sample media. The term 'production of IL-6' used throughout this thesis is defined as the IL-6 released and measured in FLS media.

2.9 Zymography

2.9.1 Analysis of MMP levels using gelatin zymography

The media collected from the RA FLS was mixed with an equal volume of 2x sample buffer (0.06M Tris/HCl pH 6.8, 2% (w/v) SDS, 10% (w/v) glycerol, 2mg/ml Bromophenol blue in distilled H_2O [dH_2O]). 10µl of the samples were run on polyacrylamide gels: 7.5% resolving gel and 4% stacking gel (see table 2.6 for components). The gels were resolved for approximately 90 min at 100v in 1x Laemmli buffer (0.1% w/v SDS, 25mM Tris and 195mM glycine) (Laemmli, 1970) and placed overnight at 37°C in MMP proteolysis buffer (50mM Tris, pH 7.8, 50mM $CaCl_2$ and 0.5M NaCl) to activate MMP proforms. Gels were stained with Coomassie Brilliant Blue (2.5g Coomassie Brilliant Blue, 100ml concentrated acetic acid, 450ml methanol in 450ml water) for 30 minutes and destained (100ml methanol, 75ml concentrated acetic acid in 825ml water) until lysis bands could be seen. Relative quantities of MMPs were quantified by comparison to a standard (conditioned media) and analysed by scanning densitometry (UMAX magic scan) and NIH image software (National Institutes of Health, Bethesda, MD). The term 'production of MMPs' used throughout this thesis is defined as the MMPs released and measured in FLS media.

Table 2.6 Components of gels for gelatin zymography

Components of gel	7.5% resolving gel	4% stacking gel
40% bis-acrylamide	2.72ml	575µl
1M Tris/HCl pH8.8	3.63ml	-
1M Tris/HCl pH6.8	-	1.3ml
10% (w/v) SDS	100µl	50µl
10% (w/v) APS	75µl	37µl
TEMED	15µl	7.5µl
7.5mg/ml Gelatin	1ml	-
dH_2O	6.16ml	4.075ml

2.9.2 Reverse zymography

Media collected from FLS was mixed with an equal volume of 2X sample buffer and resolved on 10% gelatin zymography gels as above (see table 2.7 for components), incorporated with conditioned media (1ml per gel) containing active MMP2/9. Reverse zymograms were visualised in the same way as gelatin zymograms (section 2.9.1). The term 'production of TIMPs' used throughout this thesis is defined as the TIMPs released and measured in FLS media.

Table 2.7 Components of gels for reverse zymography

Components of gel	12.5% resolving gel	4% stacking gel
40% bis-acrylamide	4.53ml	575µl
1M Tris/HCl pH8.8	3.63ml	-
1M Tris/HCl pH6.8	-	1.3ml
10% (w/v) SDS	100µl	50µl
10% (w/v) APS	75µl	37µl
TEMED	15µl	7.5µl
Conditioned media	2ml	-
7.5mg/ml Gelatin	1ml	-
dH_2O	3.35ml	4.075ml

2.9.3 Conditioned media used as standards and as source of gelatinase activity in reverse zymography.

This media was prepared from a bovine skin fibroblast cell line (BOVS-1) and was tested for gelatinase activity prior to incorporation into reverse zymograms. Conditioned media was also used as a standard on gelatin and reverse zymograms.

2.10 Fluorescent measurement of intracellular calcium

Confluent RA FLS (approximately 1.5 x 10^5 cells) in a 60mm petri dish (Corning ltd.) were incubated for 60-90 minutes at 37°C with 5μM fluo-4 AM (Molecular Probes, Eugene, OR, USA). All experiments were performed in 2ml of buffer (120mM NaCl, 16mM $NaHCO_3$, 2mM KCl, 1.25mM KH_2PO_4, 1mM or 0 $MgSO_4$ and 2mM $CaCl_2$) perfused with 95% O_2 and 5% CO_2, at room temperature. Buffer containing equimolar amounts of $CaCl_2$ instead of $MgSO_4$ was used for experiments carried out in the absence of Mg^{2+}. Following a recording period, to measure background activity 1mM glutamate, 400μM TBOA, 1mM NMDA, 100μM Thimerosal or 1mM kainate was pipetted onto the cells in a 2ml volume, therefore the final concentration of these treatments being 500μM, 200μM, 500μM, 50μM and 500μM respectively. Background activity was measured by observing RA FLS prior to addition of stimulant to see if $[Ca^{2+}]_i$ fluxes occurred randomly. No random $[Ca^{2+}]_i$ fluxes occurred during these experimental periods therefore all $[Ca^{2+}]_i$ changes subsequent to stimulation are considered to be effected by stimulation.

2.10.1 Confocal fluorescence imaging

The recording chamber and manipulators were mounted on a moveable top plate platform (MP MTP-01; Scientifica, Harpenden, UK). Fluorescence was measured using a Noran Odyssey confocal unit (Thermo Noran, USA) fitted to a Nikon E600FN (Nikon, Kingston, UK) upright microscope. Wholefield images were acquired every 3 or 5 seconds with a x40 objective lens. Acquisition and image analysis were performed using Noran Intervision software and fluorescence intensity data values were imported into Excel for change in % fluorescence plot production.

2.11 Glutamate uptake assay

Confluent RA FLS (patient 7, passage 7) in 8-well chamber slides were cultured as described in section 2.5.2. RA FLS were serum starved for 24 hours prior to incubation with 250µl ^{14}C-labelled glutamate (the specific activities for each glutamate concentration are shown in table 2.8) for approximately 30 minutes at room temperature. Glutamate uptake was terminated by washing cultures 3 times in ice-cold PBS. Cells were dissolved in 500µl 2% SDS and half were used for scintillation counting (section 2.11.1).

In some experiments (as stated in experimental chapter) net glutamate uptake was determined by subtracting the amount of glutamate bound to RA FLS after 0 time from uptake after 30 minutes. It was assumed that binding of glutamate to cell surface receptors and transporters would occur immediately and contribute to final measurements of radioactivity. Therefore duplicate experiments were set up whereby the ^{14}C-labelled glutamate was pipetted onto the cell layer and immediately aspirated. Not all experiments took bound glutamate into account, therefore in this case only total uptake after 30 minutes was measured.

Table 2.8: Specific activity of each labelled glutamate concentration used in the radioactive glutamate uptake assay

Glutamate concentration (µM)	Specific activity
0 (only ^{14}C-labelled glutamate added)	45mCi/mmol
5	45mCi/mmol
150	22.5mCi/mmol
300	22.5mCi/mmol
500	9mCi/mmol

2.11.1 Scintillation counting

250µl of FLS extract in 2% SDS were aliquoted into scintillation vials and 10ml scintillant (Emulsifier Safe ™, Perkin Elmer) added. Each sample was counted for 15 minutes on an LS6500 Multi-purpose scintillation counter (Beckman Coulter).

2.12 Cytotoxicity Assay

To confirm that responses to treatments in FLS were real and not due to cell death a cytotoxicity assay was carried out following manufacturers' instructions (Cytotox 96, non-radioactive cytotoxicity assay, Promega). Lactate dehydrogenase (LDH) activity is quantified because it is a stable cytosolic enzyme, which upon cell lysis is released. Briefly, triplicate wells of 50µl of media on a 96-well plate were measured and the LDH produced by the cells was used to convert 50µl substrate mix (NAD^+ and lactate) to pyruvate and NADH. The NADH produced after incubation in the dark for 30min was used to convert INT (a tetrazolium salt) by the enzyme diaphorase to NAD^+ and formazan. The colour change due to formazan production is measured using a spectrophotometer at 492nm. Serum free media was used as a blank control and the absorbance reading of this media subtracted from all other readings. Absorbance readings were compared to the absorbance reading of 100% lysis of duplicate cultures treated the same. 2% SDS was added to cells to initiate 100% cell lysis. The level of cell death was considered insignificant if death caused by treatments compared to control (no inhibitor) was lower than 5% of total cell death caused by 2% SDS.

2.13 Statistics

Statistical analysis was carried out using Minitab 13.32 (Minitab Inc., RA, USA) statistics package. Data was tested for normality and equal variance, before significant differences between data points were determined using a student t test. Data that was not normally distributed and/or demonstrated equal variance were logarithmically transformed. If the number of control samples were equal to the number of experimental samples a paired t-test was used, otherwise an unpaired 2-sample t-test was used.

Chapter 3 Expression of glutamate receptors and transporters in cells and tissues of the synovial joint

3.1 Introduction

Glutamate receptors and transporters were initially studied as entities solely found within the central nervous system (CNS) however it is now well accepted that glutamate plays a role in signalling outside of the CNS. The initial discovery of the upregulation of GLAST-1 in bone (Mason *et al.* 1997) was the first indication of glutamate functioning in cells of the synovial joint.

Expression of four excitatory amino acid glutamate transporters and the three types of glutamate receptor was determined in cells of the synovial joint. Reports of glutamate receptor expression in tissues outside of the nervous system were used to decide which receptors and receptor subunits to explore (see section 1.6.4). Expression of the metabotropic glutamate receptor mGluR4 mRNA has previously been shown to be expressed in osteoblasts *in vitro* (Hinoi *et al.* 2001) so this was chosen to illustrate expression of metabotropic receptors within the synovial joint. Likewise, expression of the AMPA glutamate receptor subunits AMPAGluR2 [protein in cultured pancreatic islet cells (Weaver *et al.* 1996)], AMPAGluR3 [mRNA in osteoblasts (Hinoi *et al.* 2002) and protein in cultured pancreatic islet cells (Weaver *et al.* 1996)] and the kainate receptor KA1 [mRNA in osteoblasts (Hinoi *et al* 2002) and protein in spermatids (Hayashi *et al.* 2003)] were used as an example of expression of these receptor types within the synovial joint. Only expression of the NR1 subunit of the NMDA receptor subunits was determined because it is required for all functional forms of NMDA receptors and all other NMDA receptor subunits are regulatory. NMDA NR1 has also been shown to be expressed in non-CNS tissues including bone. Section 1.6.4 provides a comprehensive overview of all glutamate transporter and receptor expression outside of the CNS. Expression of all of the EAATs was explored except EAAT5 because to date, expression of this transporter has only been shown in the retina.

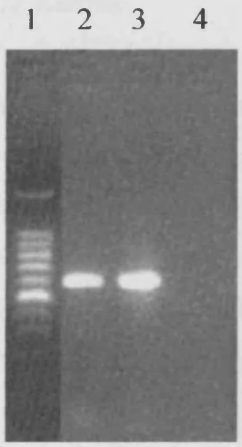
The elevation of glutamate levels in the synovial fluid of RA patients (326μM) compared to that from normal patients (6μM), and the fact that glutamate levels

become elevated in rat synovial joints upon injection of an adjuvant, suggest that it may play a role in inflammation, degradation or even protection of the synovial joint. This study therefore determines which cells of the synovial joint have the ability to respond to glutamate by using RT-PCR, immunohistochemistry and Western blotting to look for expression of glutamate receptors and transporters.

3.2 Methods

Various tissues of the rat knee joint (see figure 1.1, chapter 1, for diagram of the human knee joint), human RA and normal FLS, and bovine chondrocytes (cultured as in section 2.5) were subjected to RNA extraction and RT-PCR (sections 2.2, 2.3 and 2.4). The rat tissues analysed were patella, fat pad, menisci, collateral ligaments and cruciate ligaments. However expression of glutamate signalling components could not be determined in the collateral and cruciate ligaments due to insufficient amounts of RNA being obtained for RT-PCR analysis. Rat tissues were dissected from 3 to 6 month old rats, snap frozen in liquid nitrogen and RNA extracted after homogenisation in 1ml Trizol® reagent (see section 2.1). Bovine chondrocyte and FLS RNA (RA: patient 2, passage5; Normal: patient 4, passage 7) were also obtained through the Trizol® method of extraction (see section 2.2). Expression of glutamate receptors and transporters was also determined in human OA cartilage cDNA (kindly donated by Samantha Hurst, Cardiff University) and in a human chondrocyte cDNA library (Clontech). The library had been generated from pooled mRNAs extracted from unstimulated, IL-1β stimulated and TGFβ stimulated human adult, knee primary chondrocytes.

RT-PCR was carried out on all tissue and cell samples collected to determine mRNA expression of glutamate receptors/receptor subunits (mGluR4, AMPAGluR2 and 3, NMDA NR1 and KA1) and transporters (EAATs1-4). All cDNA samples used tested positive for β-actin PCR, thus confirming cDNA integrity (Figure 3.1 shows representative β-actin amplicons from rat menisci cDNA separated on an agarose gel). Rat brain cDNA was used as a positive control for all primers able to amplify rat sequences. For the PCR amplification of EAAT 4, rat retina cDNA (a kind gift from Dr. David Carter, Cardiff University) was used as a positive control. It should be noted that RT-PCR products less than 100bp are caused by primer dimers.



Key:
1- 100bp DNA ladder
2- Rat menisci cDNA
3- β-actin plasmid (positive)
4- water (negative)

Figure 3.1 Image of agarose gel showing RT-PCR product demonstrating mRNA expression of β-actin in rat menisci. This confirmed cDNA integrity and is representative of all cDNA samples. See appendix 3 for full gel.

Products from primers that had been newly designed were sequenced to confirm that the correct sequence was being amplified (see section 2.4.3).

Immunohistochemistry (section 2.6) and Western blotting (section 2.7) were also utilised to determine protein expression of EAAT1 in FLS.

3.3 Results

Products of primers that had been designed specifically for this project and had therefore not been used before were sequenced. Sequence data was entered into a blast search (www.ncbi.nlm.nih.gov/BLAST) and homology to published sequence determined. NMDA NR1 (95%/98% homology, appendix 28), mGluR4 (100% homology, appendix 29), AMPAGluR2 (100% homology, appendix 30) and AMPAGluR3 (partial sequence showed 100% homology, appendix 31) primers were thus confirmed to amplify the correct sequence.

3.3.1 Expression of glutamate receptors/transporters in the rat patella

RT-PCR confirmed that the rat patella expressed GLAST-1, GLAST-1a, GLT1 and EAAC1 (EAATs 1, 1a, 2 and 3) (Figure 3.2 panels A, B, C and D respectively). EAAT 4 was not expressed in the rat patella however EAAT 4 has never been shown to be expressed outside of the CNS. All receptors/receptor subunits determined (NMDA NR1, mGluR4, KA1, AMPAGluR2 and AMPAGluR3) were expressed in the rat patella (figures 3.2 panels E to I). All patella cDNA used tested positive for β-actin mRNA expression.

3.3.2 Expression of glutamate signalling receptors and transporters in the rat menisci

GLAST-1 (EAAT1) and its splice variant GLAST-1a are the only glutamate transporters expressed in the meniscus (figures 3.3 panels A and B). GLT1, EAAC1 (EAATs 2 and 3) and EAAT4 were not expressed in the rat menisci. The NMDA NR1 subunit was not expressed in the rat menisci therefore no functional NMDA NR1 receptors are present in this tissue. The KA1 receptor subunit was not expressed in the menisci. AMPAGluR2 and AMPAGluR3 were both expressed (figures 3.3 panels D and E respectively) in the rat menisci as well as the metabotropic receptor

mGluR4 (figure 3.3 panel C). β-actin mRNA expression confirmed cDNA integrity (see figure 3.1).

3.3.3 Expression of glutamate signalling apparatus in the rat fat pad

EAAT1, 1a, 2 and 3 (GLAST-1, GLAST-1a, GLT-1 and EAAC1 respectively) glutamate transporter mRNAs were all expressed in the rat fat pad (figures 3.4 panels A to D). The CNS specific transporter EAAT4 was not expressed in the fat pad. No expression of the NMDA (NR1 subunit) could be detected; therefore no functional NMDA receptors are present in the rat fat pad because NR1 is required for a functioning NMDA receptor. mGluR4 mRNA could not be detected either in the rat fat pad. Kainate (KA1) and AMPA (GluR2 and GluR3) receptor subunit mRNAs were expressed in the rat fat pad (figures 3.4 panels E to G). All fat pad cDNA samples tested positive for β-actin mRNA expression.

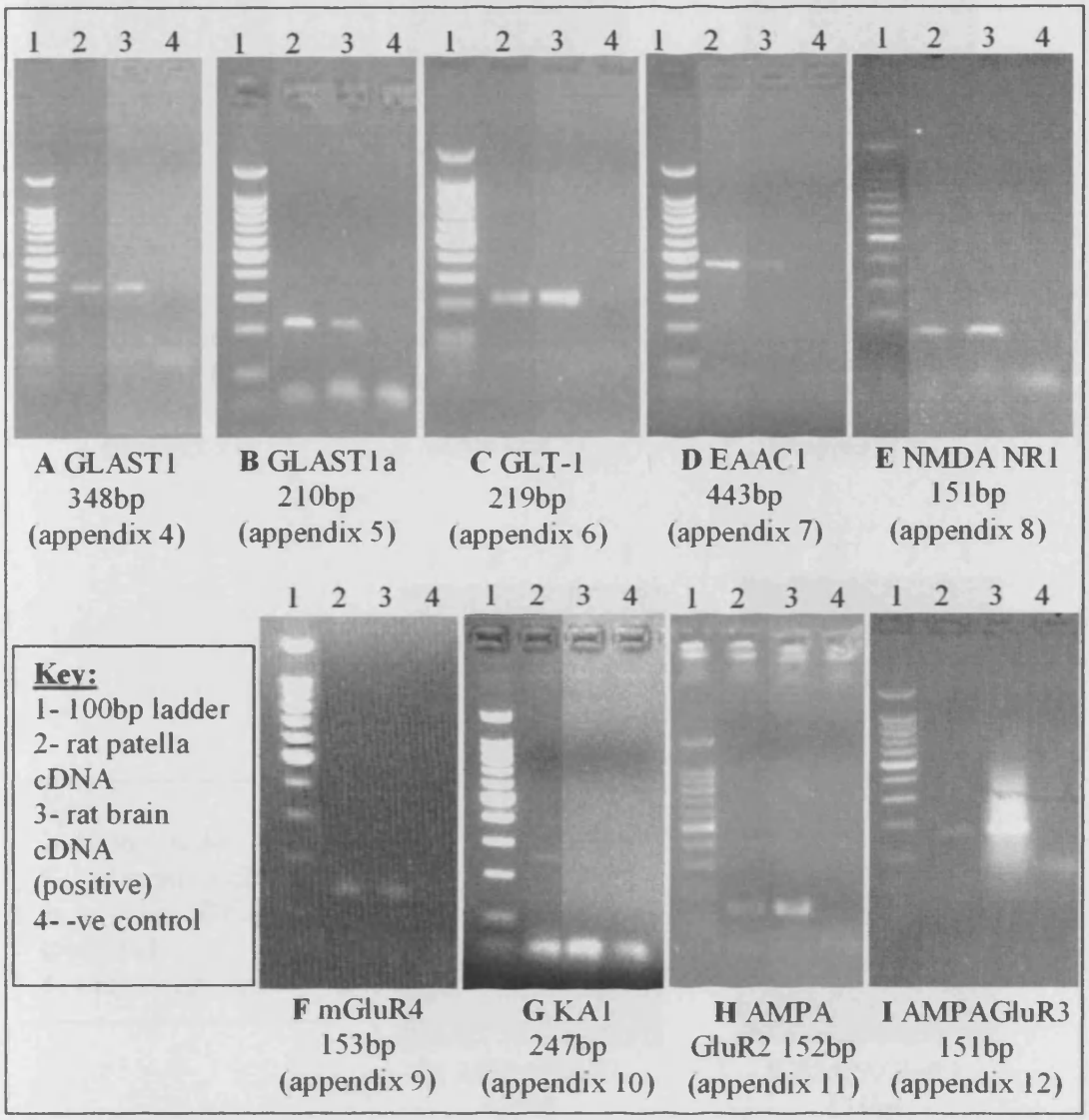


Figure 3.2 Images of agarose gels showing RT-PCR products demonstrating mRNA expression of glutamate signalling components in the rat patella. Products less than 100bp are caused by primer dimers. The full picture of each gel can be seen in the appendix indicated.

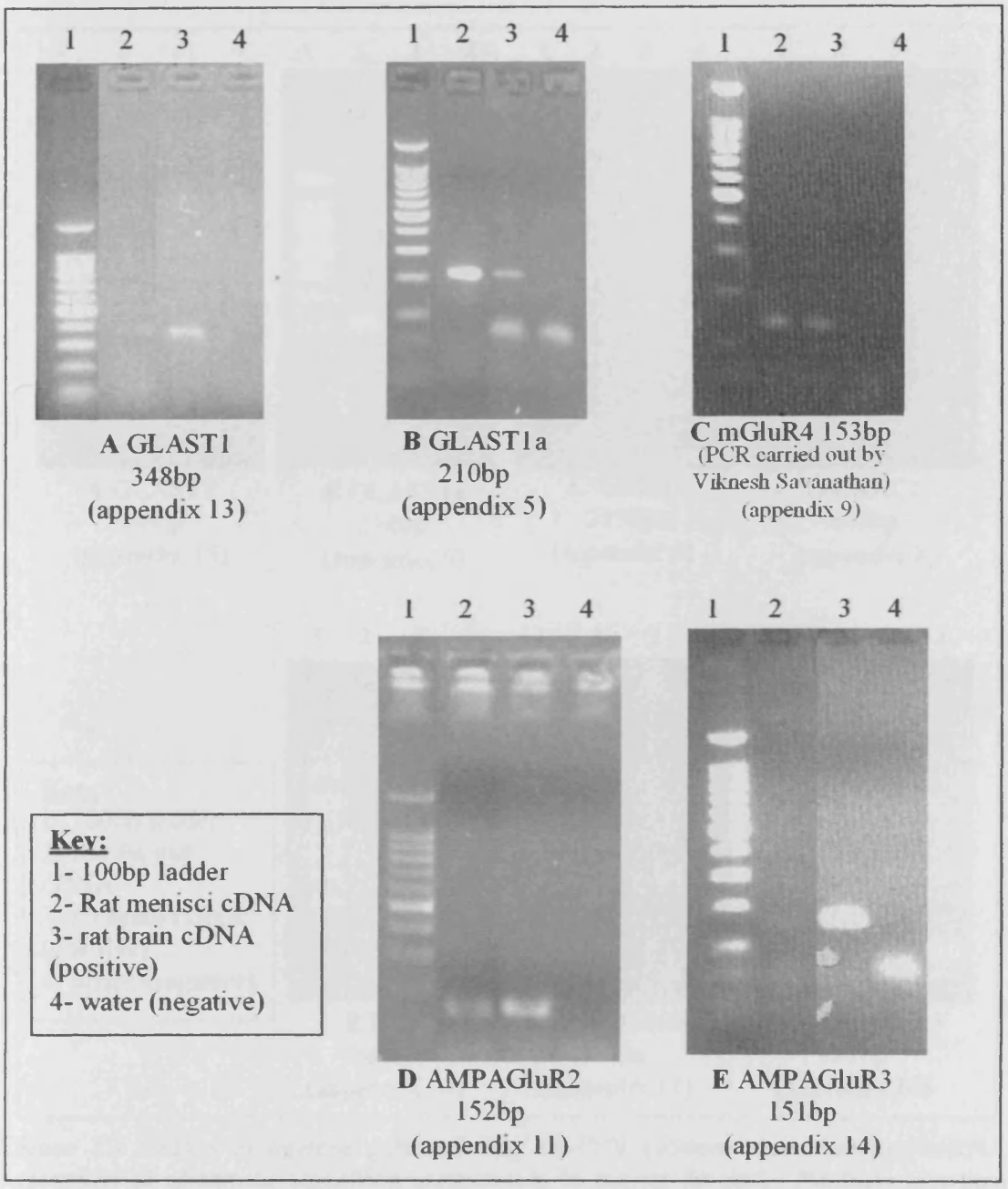


Figure 3.3 Images of agarose gels showing RT-PCR products demonstrating mRNA expression of glutamate signalling components in rat menisci. Products less than 100bp are caused by primer dimers. The full gel picture can be seen in the appendix indicated.

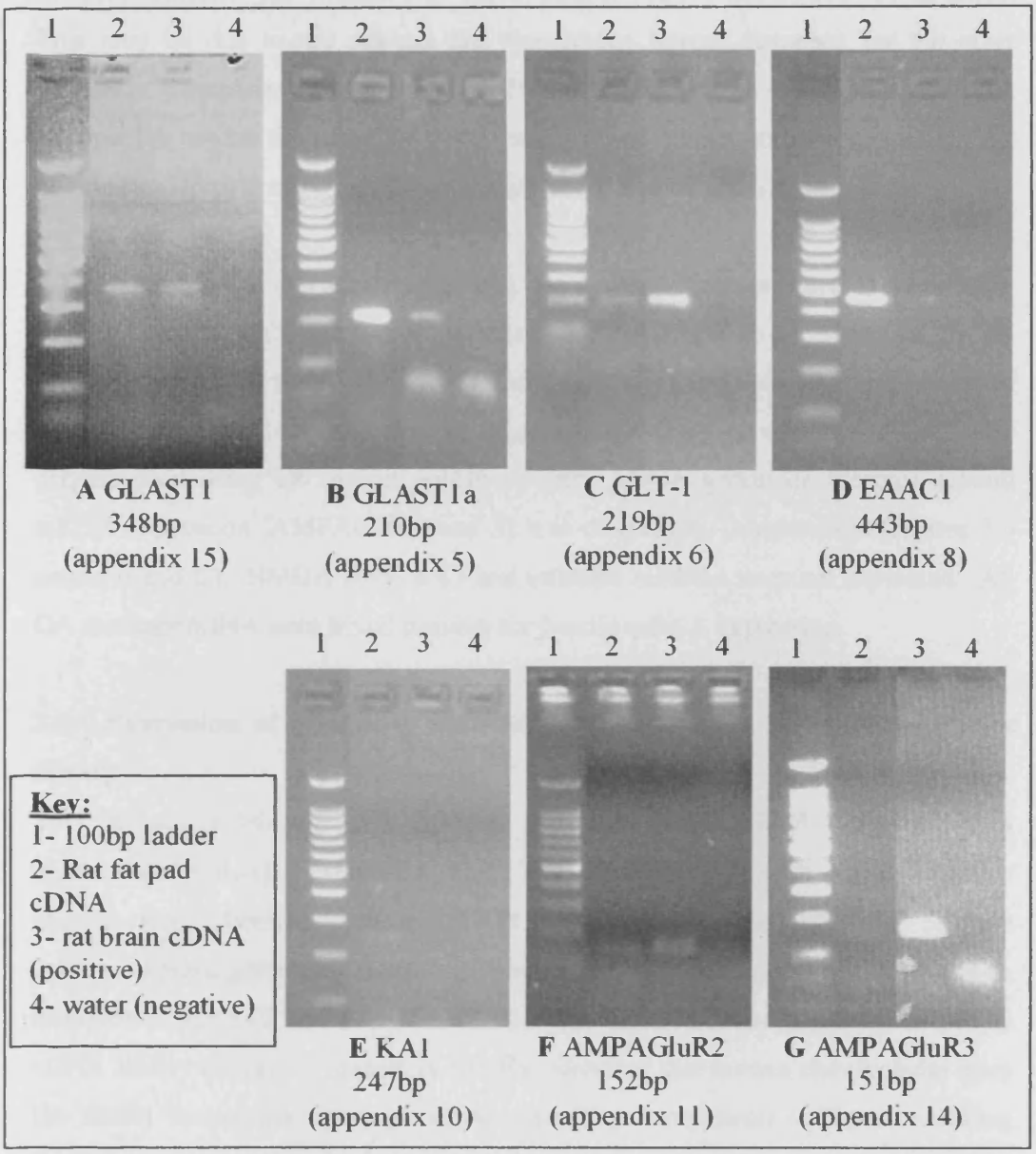


Figure 3.4 Images of agarose gels showing RT-PCR products demonstrating mRNA expression of glutamate signalling components in the rat fat pad. Products less than 100bp are caused by primer dimers. The full gel picture can be seen in the appendix indicated.

3.3.4 Expression of glutamate signalling apparatus in bovine chondrocytes

Glutamate transporters (EAAT1, GLAST1a and 2) were the only mRNAs expressed in bovine chondrocytes (figures 3.5 A to C) despite EAAT3 and EAAT4 being tested. This may be due to the primers not recognising bovine sequence for the other glutamate transporters and receptors. Primers could not be designed specifically because the bovine sequence for these receptors and transporters are unknown. All bovine chondrocyte cDNA used tested positive for β-actin mRNA expression.

3.3.5 Expression of glutamate signalling apparatus in human, knee, OA cartilage

EAAT3 was the only glutamate transporter mRNA found to be expressed in OA cartilage (figure 3.6 panel A), EAAT1 and 2 were not expressed. The expression of EAAT4 and the human equivalent of GLAST1a could not be determined due to the primers used being rat species specific. Only AMPA glutamate receptor subunit mRNA expression (AMPAGluR2 and 3) was detected in OA cartilage (figures 3.6 panels B and C). NMDA NR1, KA1 and mGluR4 mRNAs were not expressed. All OA cartilage cDNA used tested positive for β-actin mRNA expression.

3.3.6 Expression of glutamate signalling apparatus in a human chondrocyte library

The human chondrocyte library was generated from mRNA extracted from unstimulated, IL-1β stimulated and TGFβ stimulated adult, knee primary chondrocytes. Therefore a positive RT-PCR result only indicates the ability of these cells to express glutamate signalling components. Clones encoding the glutamate transporters, EAAT2 and EAAT3 mRNA were detected in the human chondrocyte cDNA library (figure 3.7 panels A and B) indicating that human chondrocytes have the ability to express these glutamate signalling components. Clones encoding EAAT1 were not detected. Expression of EAAT4 and EAAT1a was not determined due to the primers being rat species specific. The ability of chondrocytes to express mRNAs for the metabotropic glutamate receptor mGluR4 and the AMPA glutamate receptor subunit AMPAGluR3 has been shown in the human chondrocyte library (figure 3.7 panels C and D). Clones encoding NMDA NR1, KA1 and AMPAGluR2 were not detected in the human chondrocyte library.

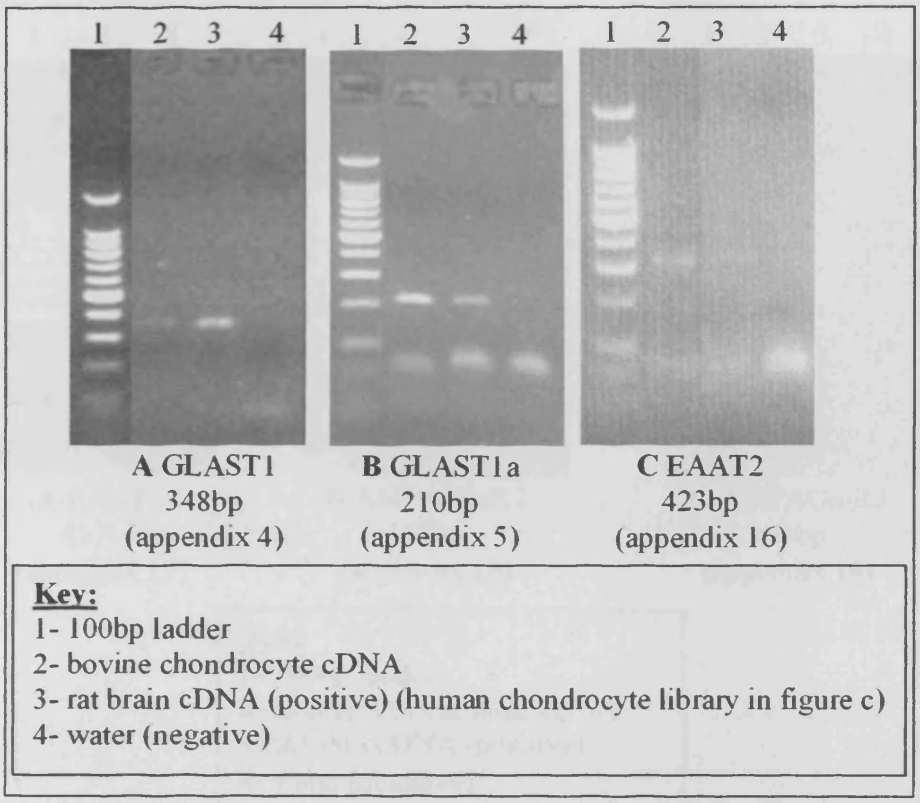


Figure 3.5 Images of agarose gels showing RT-PCR products demonstrating mRNA expression of glutamate signalling components in bovine chondrocytes. Products less than 100bp are caused by primer dimers. The full picture of these gels can be seen in the appendix indicated.

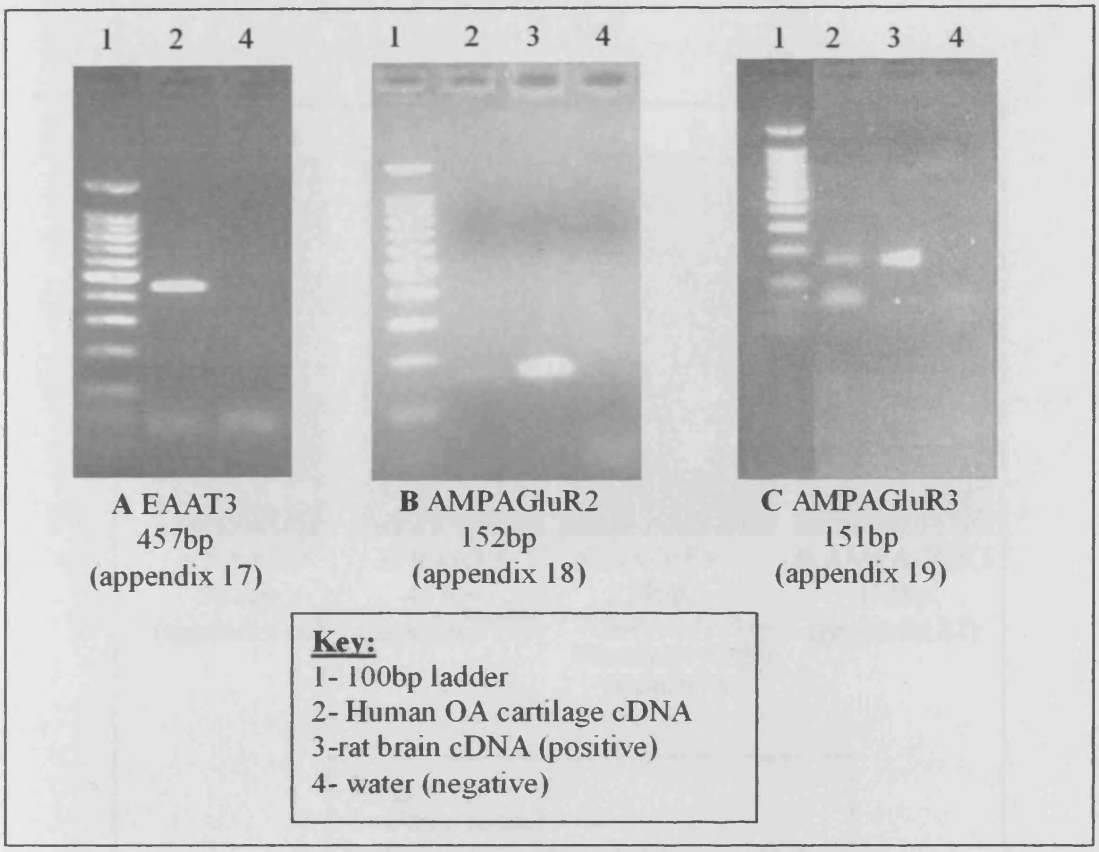


Figure 3.6 Images of agarose gels showing RT-PCR products demonstrating mRNA expression of glutamate signalling components in human OA cartilage. Products less than 100bp are caused by primer dimers. The full picture of these gels can be seen in the indicated appendix.

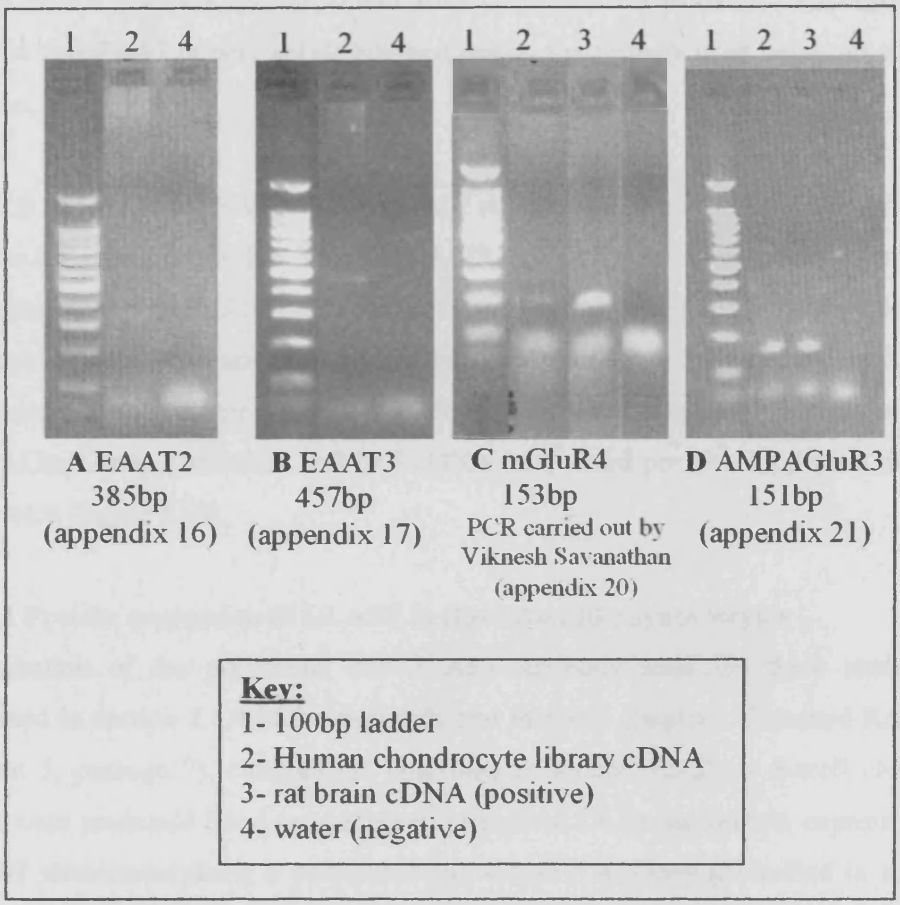


Figure 3.7 Images of agarose gels showing RT-PCR products demonstrating mRNA expression of glutamate signalling components by a human chondrocyte library. Products less than 100bp are caused by primer dimers. The full picture of each gel can be seen in the indicated appendix.

3.3.7 Expression of glutamate signalling apparatus in human FLS

RT-PCR confirmed that human RA FLS expressed the glutamate transporters EAAT1 and EAAT3 (figures 3.8 panels A and B) but not EAAT 2. Normal, human FLS also expressed the glutamate transporters: EAAT1 and EAAT 3 (figures 3.9 panel A and C) but unlike RA FLS also expressed EAAT2 (figure 3.9 panel B). Expression of EAAT4 and EAAT1a was not determined due to the primers used being rat species specific.

RA FLS expressed mRNA for the glutamate receptor subunits NMDA NR1 and KA1 (figures 3.8 panels C and D). The NMDA NR1 subunit mRNA was also shown to be expressed in normal FLS (figure 3.9 panel D). In addition normal FLS also expressed the metabotropic glutamate receptor, mGluR4 mRNA (figure 3.9 panel E) but did not express the KA1 receptor subunit. Neither RA FLS nor normal FLS expressed the AMPAGluR2 and 3 subunits. All FLS cDNA used tested positive for β-actin mRNA expression (figure 3.10).

3.3.7.1 Protein expression of GLAST in fibroblast-like synoviocytes

Optimisation of the polyclonal anti-GLAST antibody used for these studies is discussed in section 2.6.4 in the materials and methods chapter. Untreated RA FLS (patient 5, passage 7), cultured (as described in section 2.5.2) on 8-well chamber slides were methanol fixed (as described in section 2.6.1) and protein expression of GLAST determined using a polyclonal anti-GLAST antibody (described in section 2.6.2).

3.3.7.1.1 Characterisation of the polyclonal antibody to GLAST for immunohistochemistry

Control tests were performed with the polyclonal GLAST antibody to confirm that interactions were specific. The controls were:

1. Primary negative. To confirm that interactions were not due to non-specific binding of the secondary antibody incubations were performed in the absence of the anti-GLAST primary antibody. This control was carried out with every immunostaining experiment and revealed no non-specific secondary binding (figure 3.11, panel A).

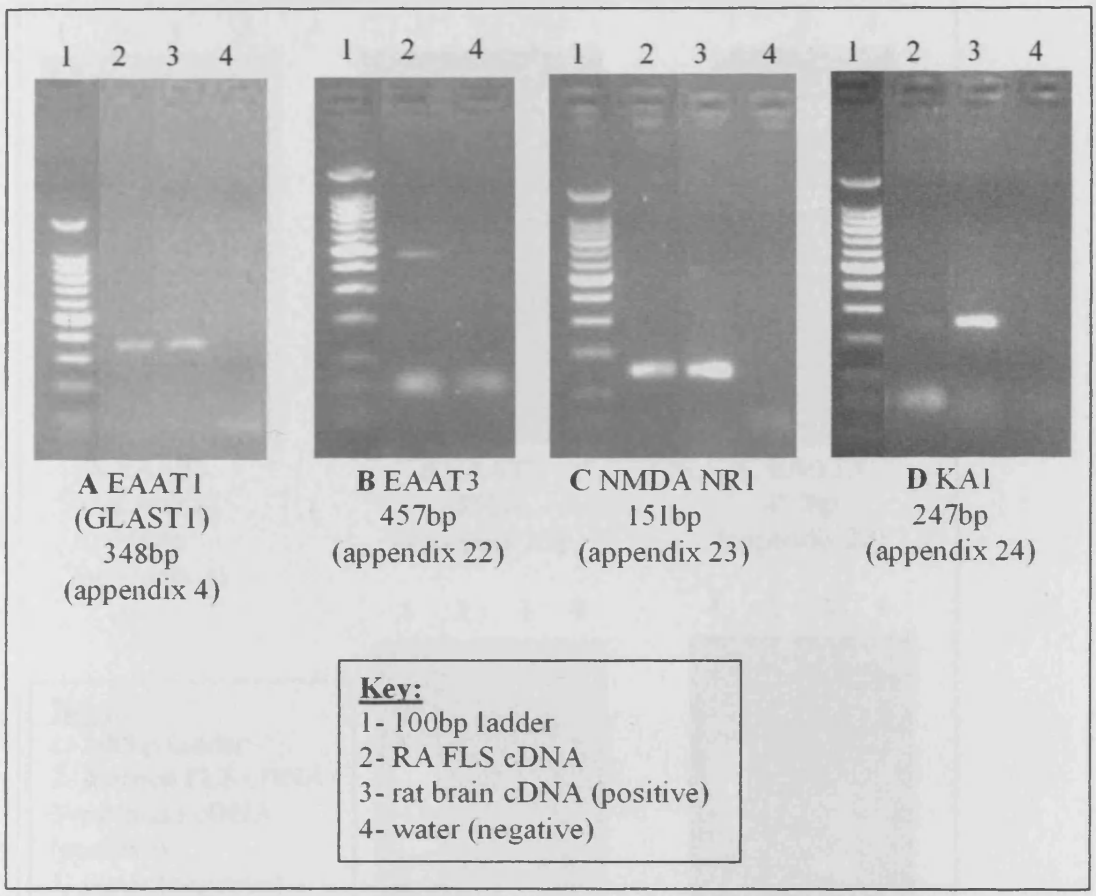


Figure 3.8 Images of agarose gels showing RT-PCR products demonstrating mRNA expression of glutamate signalling components in RA FLS. Products less than 100bp are caused by primer dimers. The full gel pictures can be seen in the appendix indicated.

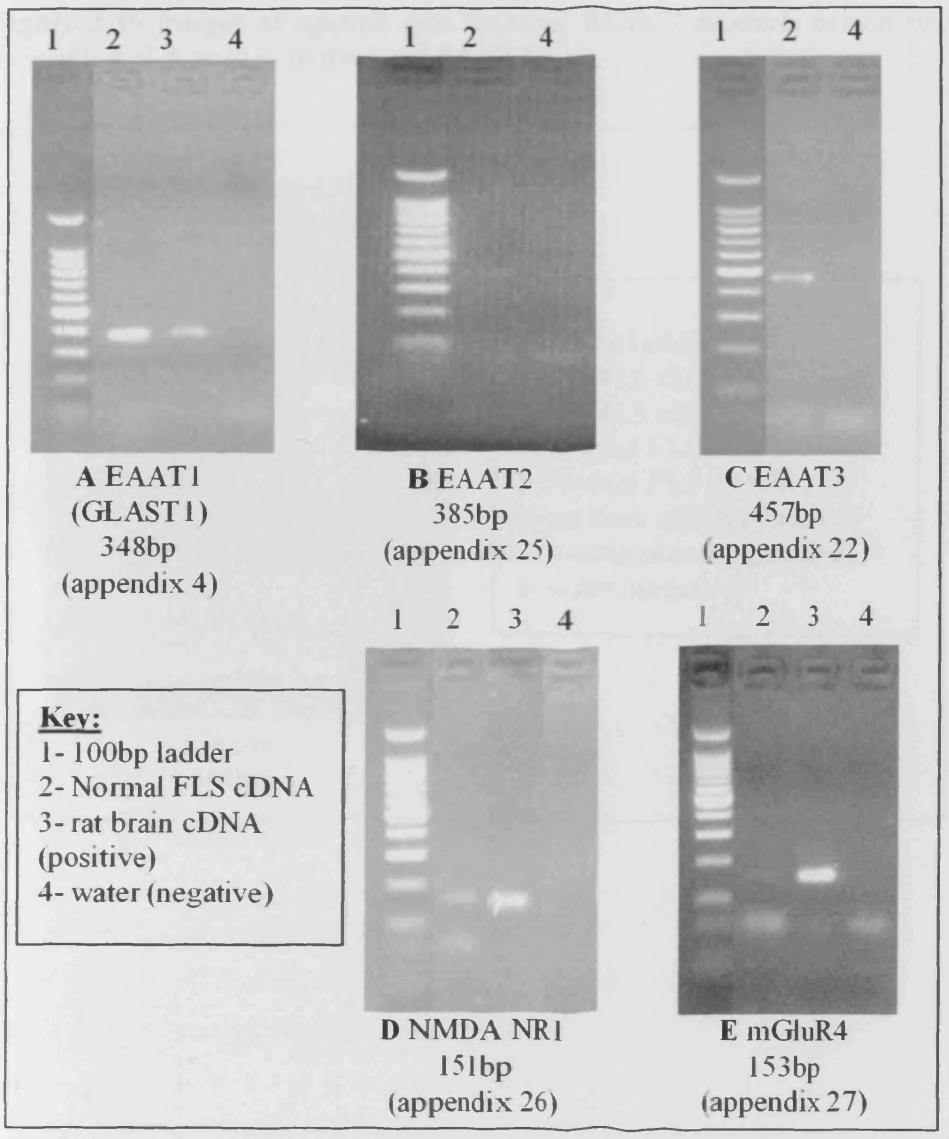
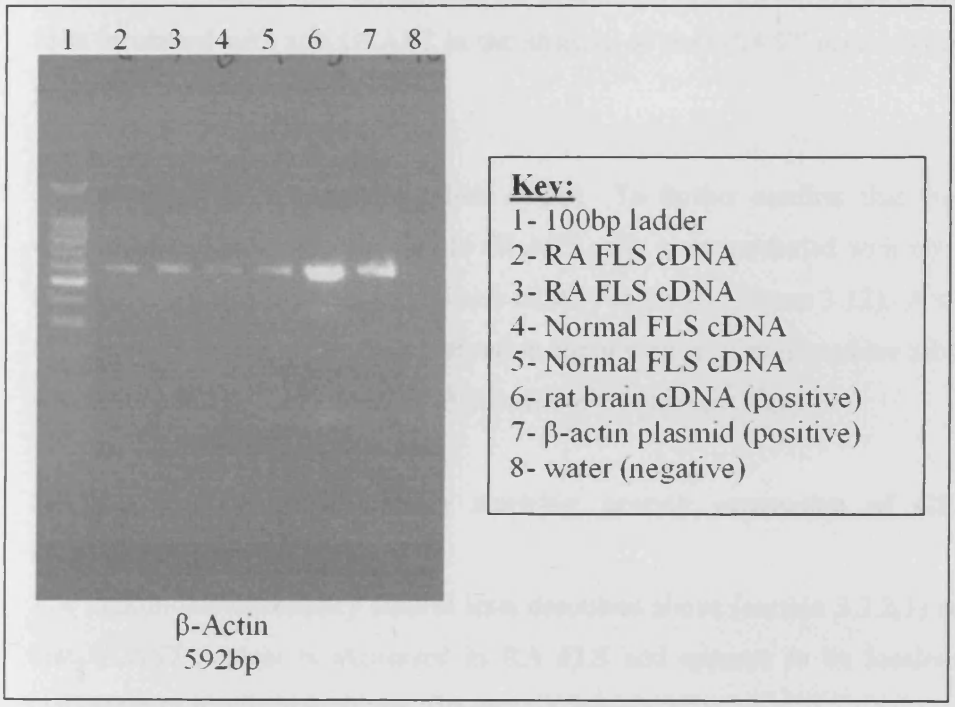


Figure 3.9 Images of agarose gels showing RT-PCR products demonstrating mRNA expression of glutamate signalling components in normal FLS. Products less than 100bp are caused by primer dimers. The full gel picture can be seen in the appendix indicated.

Figure 3.10 Images of agarose gels showing RT-PCR products demonstrating mRNA expression of β-actin in normal and RA FLS.



2. Blocking anti-GLAST binding with the peptide that the antibody was raised against. Different concentrations of the N-term peptide of GLAST-1 were used in incubations with the primary antibody to confirm that the GLAST antibody was specifically binding to GLAST protein (Figure 3.11 panels B to D). A significant reduction in staining was observed in the presence of 100μg/ml peptide compared to cells incubated with anti GLAST in the absence of the GLAST peptide (figure 3.11, panel E).

3. Blocking with non-immune rabbit serum. To further confirm that the primary antibody was specifically binding to GLAST cells were incubated with non-immune rabbit serum rather than polyclonal anti GLAST antibody (Figure 3.12). A significant reduction in fluorescence was observed in the presence of non-immune rabbit serum confirming that the polyclonal GLAST antibody binds specifically.

3.3.7.1.2 Immunohistochemistry showing protein expression of GLAST in synoviocytes

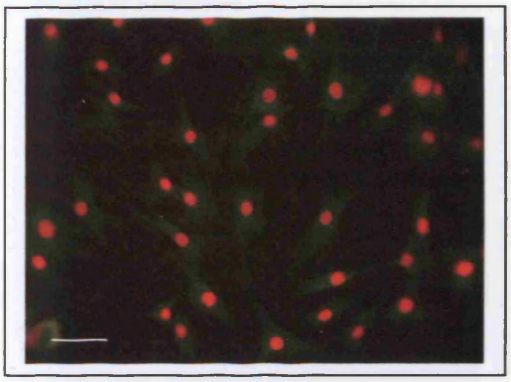
The immunohistochemistry control tests described above (section 3.2.2.1) confirmed that GLAST protein is expressed in RA FLS and appears to be localised to the cytoplasm or plasma membrane (figure 3.12, panel C).

3.3.7.2 Western blotting showing protein expression of GLAST in FLS

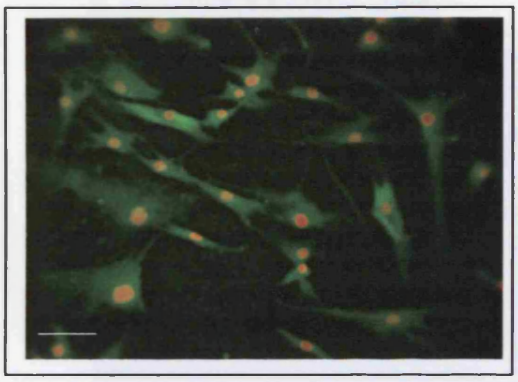
Western blotting (according to section 2.7) confirmed the presence of GLAST protein in protein extracts from human RA FLS (patient 7, passage 7). Protein extracted from rat brain was used as a positive control. The 69kDa monomer of GLAST-1 was the only form present in RA FLS (figure 3.13); higher molecular weight primary specific bands in the rat brain extract identified represent multimeric forms of GLAST protein described previously (Haugeto *et al.* 1996, Huggett *et al.* 2000). The 54.4kDa GLAST-1a splice variant was not detected in RA FLS. This was not tested in NFLS.

Figure 3.11 Blocking interaction of the GLAST antibody and GLAST in RA FLS with GLAST peptide to confirm specificity of polyclonal anti-GLAST primary antibody (measure bar corresponds to 100μm). The red is propidium iodide counter-staining of the nuclei. The green is FITC-labelled EAAT1.

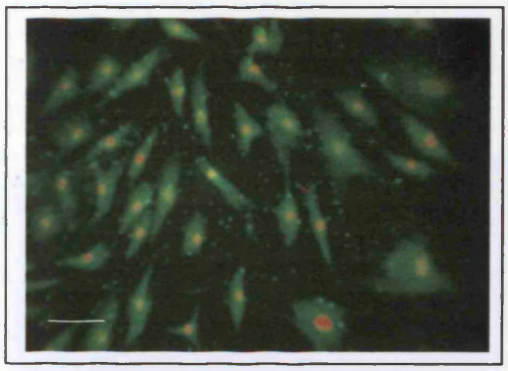
A. Primary negative



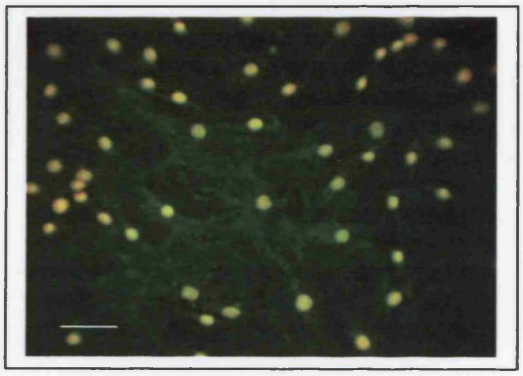
B. 1/100 Primary and 1μg/ml peptide



C. 1/100 Primary and 10μg/ml peptide



D. 1/100 Primary and 100μg/ml peptide



E. 1/100 Primary (GLAST peptide not present)

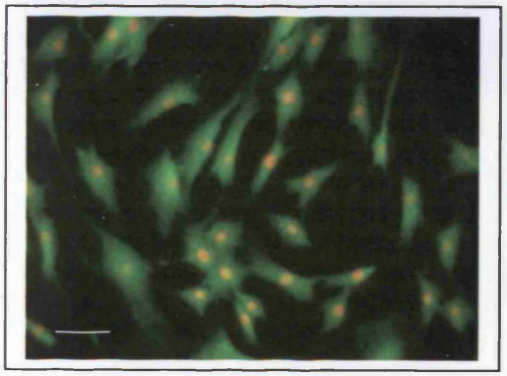
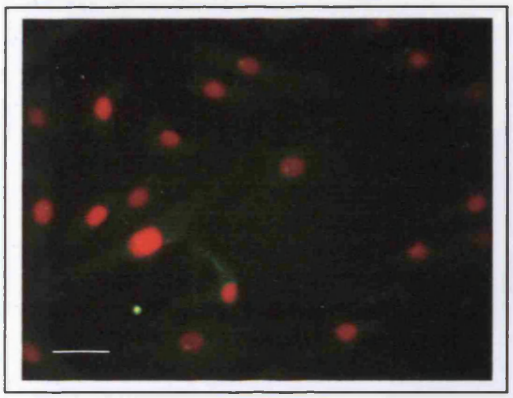
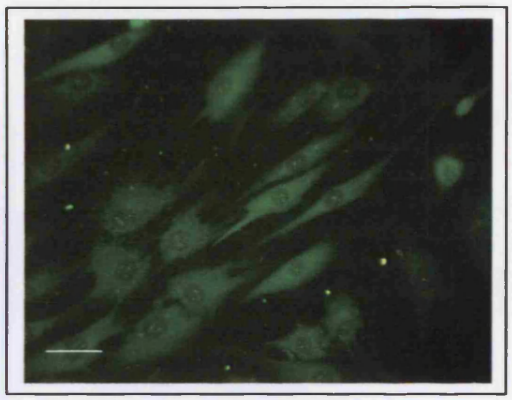


Figure 3.12 To confirm specific interaction of the primary polyclonal anti-GLAST antibody, non-immune rabbit serum was used instead of the anti-GLAST antibody in RA FLS (measure bar corresponds to 100μm). The red is propidium iodide counter-staining of the nucleii. The green is FITC-labelled EAAT1.

A. Primary negative



B. non-immune rabbit serum



C. 1/100 primary antibody

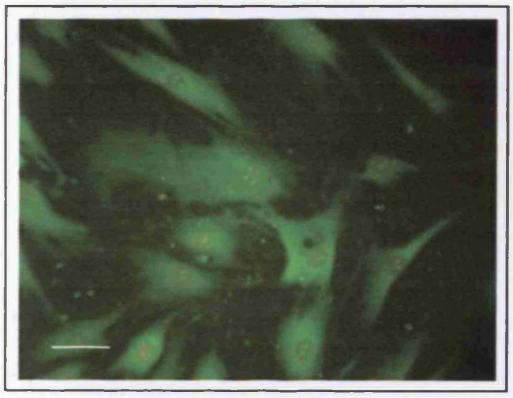
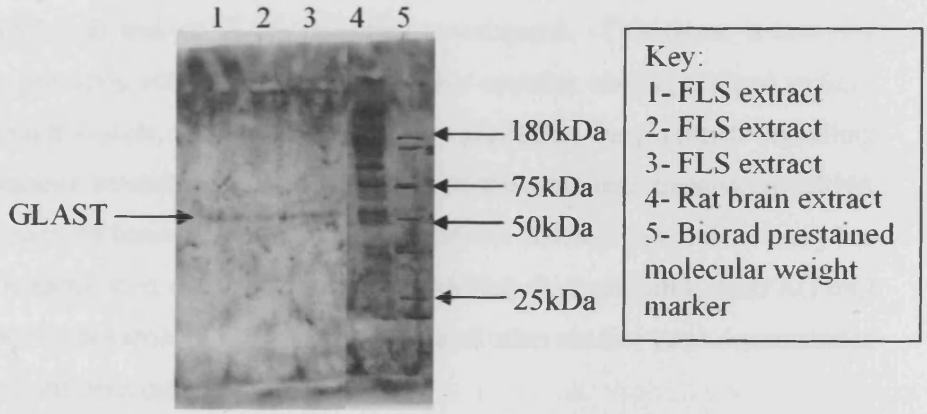


Figure 3.13 Expression of EAAT1 protein in RA FLS as shown by Western blotting

Western blot of protein extracts from human RA FLS (patient 7, passage 7). The 69kDa monomer of EAAT1 expressed in RA FLS is indicated by an arrow, higher molecular weight primary specific bands in the rat brain extract represent multimeric forms of GLAST proteins. The 54.4kDa GLAST-1a variant was not detected in these cells. (For full picture of this blot see figure 5.5).



3.4 Discussion

Table 3.1 shows a summary of glutamate signalling apparatus expression in the cells and tissues of the synovial joint. Glutamate transporter and receptor mRNA has been shown to be expressed in tissues and specific cells of the synovial joint thus indicating that the necessary machinery for glutamate signalling is present and hence that these cells have the ability to respond to extracellular glutamate within the synovial joint.

RT-PCR revealed that the rat patella expressed mRNA for GLAST-1, 1a, GLT1, EAAC1 (EAATs1-3) and all of the receptors investigated. This tissue is however multicellular, primarily consisting of bone but also contains cartilage, blood vessels, nerves and lymph vessels, thus the exact cell type producing the glutamate signalling components cannot be ascertained. It is likely that the glutamate transporter mRNA is being expressed by bone cells or by the chondrocytes because this present study has shown mRNA expression of EAATs 1, 1a (GLAST1a), 2, 3 mGluR4, AMPAGluR2 and AMPAGluR3 in cartilage and chondrocytes and other studies have demonstrated expression in bone cells (see section 1.6.4.1).

The glutamate transporters GLAST-1, 1a, GLT1 and EAAC1 (EAATs1-3) were all expressed in the rat fat pad. AMPA and KA receptor subunit types were also expressed in the rat fat pad. This tissue consists of vascularised adipocyte tissue so does not consist of one cell type. Sensory nerves are also found within adipose tissue, therefore the glutamate receptors shown to be expressed in this tissue may originate from the peripheral nervous system.

Only GLAST1 and 1a (EAAT1) were expressed in the rat menisci. The mRNA from this tissue is likely to be from a pure population of cells due to the menisci not being an innervated tissue and having only 10-30% peripheral vacularisation. The cells of the menisci are fibrochondrocytes because they produce a fibrocartilage matrix and yet resemble chondrocytes (Dudhia, J *et al.* 2004). Thus, it is likely to be these cells that are producing the glutamate transporter and receptor mRNA. Both AMPAGluR2 and AMPAGluR3 mRNA were expressed by the rat menisci; in addition, expression of the metabotropic glutamate receptor, mGluR4, was also demonstrated. This is the

first observation of expression of these glutamate receptors and transporters in fibrochondrocytes.

Bovine chondrocytes, a pure population of cells, demonstrated expression of EAAT1, 1a and 2 mRNA which is also a novel observation. Thus, these cells have the ability to regulate extracellular and intracellular glutamate levels. Expression of the other glutamate transporters and receptors could not be determined due to the bovine sequences being unknown. Despite testing primers designed to both human and rat sequences, a negative result was obtained. However, this does not prove that these glutamate signalling components are not present in bovine chondrocytes due to the possibility that the primers are species specific.

The human knee OA cartilage is likely to be without nerves and therefore an almost pure population of chondrocyte cells, however because it is from an OA patient it may have vascularisation. The only glutamate transporter expressed in human OA cartilage was EAAT3, however EAAT4 was not determined due to the primers not cross-reacting with human sequence. However it is unlikely that EAAT4 would be expressed in this tissue because to date expression of this transporter has been restricted to localised areas of the CNS. OA cartilage has the ability to respond to extracellular glutamate because mRNA expression of both AMPAGluR2 and 3 subunits were detected.

Expression of EAAT2 and EAAT3 clones were detected in the human chondrocyte library. In addition expression of clones for the mGluR4 metabotropic receptor and the AMPAGluR3 subunit were detected. The human chondrocyte library consisted of approximately 3-6 x 10^5 clones which corresponds to the number of genes expressed by a chondrocyte at any one time so it is likely that all genes were represented within the library. However because the library was generated from mRNA extracted from pooled (unstimulated, IL-1β stimulated and TGF-β1 stimulated) primary chondrocytes the expression of these glutamate signalling components detected in this study does not mean that human chondrocytes express these normally *in vivo*. It merely demonstrates the ability of these cells to express mRNA for these components.

Both normal and RA FLS showed a similar complement of glutamate signalling components. Both expressed EAAT1 and EAAT3, however only normal FLS expressed EAAT2. This demonstrates a difference in the ability of these cells to regulate glutamate levels within the synovial joint because the different EAATs have various uptake properties. EAAT2 has a higher probability of transporting bound glutamate than releasing it compared to EAAT1 in the CNS (Otis and Kavanaugh 2000). This could influence the elevated levels of glutamate in RA synovial fluid if the normal FLS express a transporter more likely to bind and transport glutamate. In addition the stoichiometry of uptake and the affinity for glutamate varies between transporter subtypes (Danbolt 2001). The presence of EAAT1 mRNA in RA FLS was further confirmed by immunohistochemistry and Western blotting (confirmed in one patient) which revealed the presence of EAAT1 protein. The apparent localisation of the transporter to the plasma membrane demonstrates that it is in the necessary location for glutamate transport. Exact localisation of the transporter could not be determined, further investigation using confocal microscopy is required. Both RA and normal FLS expressed the essential NMDA NR1 subunit, indicating the potential for these cells to form functional NMDA receptors. Only the normal FLS expressed mGluR4, whereas only the RA FLS expressed KA1. Again this demonstrates a difference in how RA and normal FLS have the ability to respond to extracellular glutamate and the signalling pathways that extracellular glutamate may activate within the FLS. For example, mGluR4 is a G protein which is negatively coupled to the cAMP pathway, whereas kainate receptors are ion channels. It is not certain that these differences between RA and normal FLS are true for all RA patients, however it is true for the patients in this study because cDNA integrity was confirmed by demonstrating β-actin mRNA expression. More patients need to be tested in order for full conclusions to be drawn.

Expression of the AMPA receptor subunits 2 and 3 was detected in the patella, fat pad, menisci and human OA cartilage. A splice variant of the AMPAGluR2 subunit controls AMPA receptors permeability to calcium (Egebjerg 2002), thus these tissues may therefore have the ability to control calcium permeability via AMPA receptors, however the primers used in this study were unable to detect whether this splice variant was present.

Expression of at least one glutamate transporter, EAATs1-3, was detected in all tissues and cells investigated, thus these cells and tissues all have the potential ability to regulate extracellular glutamate levels. The primers used for EAAT 4 and GLAST1a were rat specific. EAAT4 was not expressed in the rat tissues. This was expected as these transporters have only been reported to be expressed within the CNS.

This is the first study to report glutamate receptor and transporter expression in FLS, previously only glutamate transporter expression has been reported in epidermal fibroblasts (see section 1.6.4.3). Until recently this was also the first observation of mRNA expression of glutamate signalling components in cells and tissues of the synovial joint other than bone although NMDA NR1 has been shown to be expressed by chondrocytes (Salter *et al.* 2004). More recently McNearney *et al.* inferred expression of NMDA and metabtropic glutamate receptors in FLS but did not specify which subunits/types (McNearney *et al.* 2004) and EAAT and NMDA NR2 subunit mRNA expression has also been reported (Hinoi *et al.* 2005, Salter *et al.* 2004) (see section 1.6.4.2).

The most interesting result of this study is the difference in glutamate signalling component expression between RA and normal FLS, highlighting the potential difference in how FLS may respond to and control extracellular levels of glutamate in the normal and diseased state.

Table 3.1 Summary of mRNA expression of glutamate signalling apparatus in cells and tissues of the synovial joint

	Rat patella	Rat fat pad	Rat menisci	Bovine chondrocytes	Human RAFLS	Human OA cartilage	Human normal FLS	Human chondrocyte library
GLAST-1	+	+	+	+	+	-	+	-
GLAST-1a	+	+	+	+	ND	ND	ND	ND
EAAT2	+	+	-	+	-	-	+	+
EAAT3	+	+	-	ND	+	+	+	+
EAAT4	-	-	-	ND	ND	ND	ND	ND
NMDA NR1	+	-	-	ND	+	-	+	-
mGluR4	+	-	+	ND	-	-	+	+
KA1	+	+	-	ND	+	-	-	-
AMPAGluR2	+	+	+	ND	-	+	-	-
AMPAGluR3	+	+	+	ND	-	+	-	+

Key: + = mRNA expressed

\- = mRNA not detected

ND= Not determined

Chapter 4 The effect of glutamate on Interleukin 6 production by fibroblast-like synoviocytes

4.1 Introduction

Glutamate levels are increased 54 fold in human RA synovial fluid compared to normal synovial fluid (McNearney *et al.* 2000) and antigen induced arthritis in rats doubles the amount of glutamate present in synovial fluid (Lawand *et al.* 2000). Whilst the responses of bone cells to glutamate stimulation have been well characterised (recently reviewed in Mason 2004), little is known about how the cartilage and synovium respond. It is unclear what effect this increased glutamate will have on FLS phenotype and whether this is relevant to the pathogenesis of RA. Recently, it has been reported that glutamate levels correlate with levels of specific pro-inflammatory cytokines in RA synovial fluid (McNearney *et al.* 2004). Furthermore, glutamate treatment of human synoviocytes derived from RA patients increased release of TNFα, demonstrating that changes in glutamate may contribute to inflammatory processes in RA.

IL-6 (section 1.4.1.1.3) levels are elevated in the synovial tissue and fluid during active rheumatoid arthritis (Desgeorges *et al.* 1997). Anti-IL-6 therapy has shown good efficacy in treating RA in clinical trials, thus demonstrating the importance of IL-6 in this disease (see section 1.3.4.4). Anti-IL-6 and anti-IL-6 receptor antibodies have been used successfully as a therapeutic strategy in RA animal models (Mihara *et al.* 1991). In addition clinical trials have demonstrated evidence for the efficacy of anti-IL-6 receptor antibodies for RA (Nishimoto *et al.* 2004, Choy *et al.* 2002).

Within the central nervous system (CNS) glutamate has been well studied as a neurotransmitter (section 1.6.2) but the effects of glutamate and expression of components of the glutamate signalling pathways have not been well characterized in other tissues (see chapter 3 for glutamate signalling components expressed in cells and tissues of the synovial joint). The effects of glutamate on pro-inflammatory cytokine production within the CNS are not well documented. However there are some studies into the effects of glutamate on pro-inflammatory cytokines (section 1.6.5.4 and 1.6.5.5).

These previously published studies link glutamate signalling to pro-inflammatory cytokine release and indicate that the increased glutamate in RA patient synovial fluid may mediate or prolong the inflammatory responses associated with RA.

Having demonstrated in chapter 3 that FLS express glutamate receptors and transporters, and therefore have the ability to respond to extracellular glutamate, the purpose of this study was to investigate the role of glutamate signalling on the pro-inflammatory phenotype of FLS. To this end the effects of glutamate and inhibitors of glutamate receptors and transporters on synoviocyte expression of the pro-inflammatory cytokine IL-6 were determined. Because IL-6 has a major role in the pathogenesis of RA, this was chosen as an indicator of induction of an inflammatory response by glutamate.

4.2 Methods

4.2.1 Cell culture treatments

To determine the effect of glutamate on human FLS, cells were cultured in DMEM (50μM glutamate) as in section 2.5 and 1μM, 10μM, 100μM, 500μM, 1000μM or 2000μM glutamate (Sigma Aldrich) added for 15 hours. 15 hours was chosen because it was long enough for changes in protein expression to occur. These concentrations of glutamate were chosen because the levels of glutamate in normal synovial fluid and synovial fluid from RA patients are on average 6μM and 326μM respectively and therefore glutamate concentrations around this range were tested. Due to the 50μM glutamate present in the culture media experiments less than this concentration could not be carried out. Each culture condition was repeated between 3 and 6 times with cells derived from the same patient and the same passage (See table 2.4 for patient details). After 15 hours at 37°C the media was aspirated from the cell layer and stored at -20°C for analysis of IL-6 and LDH (sections 2.8 and 2.12). Statistical analysis was carried out using Minitab (see section 2.13). Measurements outside of two standard deviations from the mean were considered outliers and removed.

4.2.2 Glutamate transporter inhibitors

To demonstrate the effects of extracellular glutamate on FLS it is necessary to prevent clearance of glutamate via high-affinity transport. Therefore to block glutamate uptake by RA FLS cells were cultured as above (section 4.2.1) in the presence of either 100μM DL-*threo*-*E*-Benzyloxyaspartic acid (TBOA) (TOCRIS), a competitive, non-transportable inhibitor of excitatory amino acid transporters (EAATs) 1-5 or 100μM *trans*-Pyrrolidine-2,4-dicarboxylic acid (*t*PDC) (TOCRIS), a competitive transportable inhibitor of EAATs 1-4 and non-transportable inhibitor or EAAT5. The studies described in chapter 3 demonstrated that normal FLS express EAAT1, 2 and 3 and RA FLS express EAAT1 and EAAT3, therefore these transporters would be inhibited by TBOA and *t*PDC. The inhibitors were added immediately prior to the addition of glutamate. The number of glutamate transporters expressed by FLS and their binding affinity for glutamate in FLS is unknown therefore the concentration of inhibitor used was based on previously published work using these inhibitors (Cooper *et al.* 1998, Balcar 1992, Waagepetersen *et al.* 2001). After 15 hours at 37°C the media was aspirated from the cell layer and stored at -20°C for analysis of IL-6 and LDH (sections 2.8 and 2.12).

4.2.3 Glutamate receptor inhibitors

To explore whether effects of extracellular glutamate are mediated by ionotropic glutamate receptors, FLS were cultured in DMEM with various concentrations of glutamate (as in section 4.2.2) for 15 hours with ionotropic glutamate receptor antagonists. The NMDA receptor inhibitors used were 100μM MK801 (TOCRIS), a non-competitive NMDA receptor antagonist and 10μM D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5) (TOCRIS), a competitive NMDA receptor antagonist. The non-competitive inhibitor 1-(4'-Aminophenyl)-3,5-dihydro-7,8-dimethoxy-4*H*-2,3-benzodiazepin-4-one (CFM-2, 10μM) or the competitive inhibitor 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f] quinoxaline-7-sulfonamide (NBQX, 150μM) were used to inhibit AMPA and kainate receptors. CFM-2 inhibits AMPA receptors and NBQX inhibits both AMPA and KA receptors. Previous studies in osteoblasts and non-CNS tumour cells were considered to determine concentrations of receptor antagonists (Chenu *et al.* 1998, Hinoi *et al.* 2002, Laketic-Ljuobojevic *et al.* 1999, Rzeski *et al.* 2002, Genever and

Skerry 2001). After 15 hours at 37°C the media was aspirated from the cell layer and stored at -20°C for analysis of IL-6 and LDH (sections 2.8 and 2.12).

4.3 Results

4.3.1 Cytotoxicity of the glutamate receptor and transporter antagonist treatments

The amount of LDH released into the media by FLS during each 15 hour treatment was measured using a cytotoxicity assay (section 2.12). Total cell lysis by treatment with 2% SDS of one well of a 6-well plate of FLS seeded at the same density as used in this experiment gave an absorbance reading of between 0.9 and 1.1 (n=6, data not shown). Thus an absorbance reading of approximately 1 reflects 100% cell death. The cytotoxicity results of the controls (50μM glutamate, no antagonist) after 15 hours in this experiment produced low absorbance readings within the range of 0.073 – 0.084 (see figure 4.1); therefore an average of 8% of the cells in total were dying during culture. Treatment with *t*PDC, TBOA, (figure 4.1a) DAP5, MK801 (figure 4.1b) CFM2 and NBQX (figure 4.1c) and up to 2000μM glutamate (over and above the 50μM glutamate present in the media) produced absorbance readings ranging 0.07 to 0.11 (7-11% of cells dying). Treatments therefore changed cell number by a maximum of approximately 4% (n=1 well, triplicate measurements taken) compared to the control (50μM glutamate, no inhibitor added) therefore none of these treatments greatly influence cell number.

4.3.2 Extracellular glutamate increases the release of IL-6 by human RA synoviocytes but not normal synoviocytes

The effect of extracellular glutamate on the levels of the pro-inflammatory cytokine IL-6, produced by human primary RA FLS was explored. An increase in IL-6 production was observed at increased glutamate concentrations in cells cultured from patient 3 (figure 4.2a). IL-6 production was significantly increased in the presence of 550μM (IL-6 103pg/ml, p=0.005) and 2050μM glutamate (IL-6 = 96pg/ml, p=0.011) compared to the RA FLS cultured in 50μM glutamate (IL-6 = 60pg/ml). Increasing extracellular glutamate concentration to 150μM did not affect IL-6 release (figure 4.2a). However in patient 8 extracellular glutamate 150μM (82pg/ml, p=0.024) and 2050μM (70pg/ml, p=0.034) glutamate caused a significant reduction in IL-6 production compared to the

control (115pg/ml) (figure 4.2c). The constitutive levels (in the presence of 50μM glutamate) of IL-6 produced by this cell line were 2 times greater than levels of IL-6 produced by the cell line derived from patient 3. Thus, extracellular glutamate had varying effects on IL-6 production in these 2 patients.

Increasing the passage of the cells also varied IL-6 responses to glutamate. Conversely to results at passage 5, patient 3 cells increased IL-6 production at passage 7 (figure 4.2b) between 50μM glutamate and 60μM glutamate ($p=0.048$) and between 550μM glutamate and 1050μM glutamate ($p=0.014$). However, a decrease in IL-6 production was observed in these cells between treatment with 60μM and 550μM ($p=0.036$) glutamate and between 1050μM and 2050μM glutamate ($p=0.017$).

Extracellular glutamate had no effect on IL-6 production by RA FLS from patient 2 at passage 6 (data not shown). However, constitutive levels of IL-6 produced by cells from patient 2 were too low to be detected (data not shown).

The effect of glutamate on IL-6 production was very different in normal synoviocytes, which were unresponsive to changes in extracellular glutamate concentration (figure 4.3a and b). IL-6 released by normal FLS was very low (4-24pg/ml) compared to that released by RA FLS (60-120pg/ml) except for the RA patient number 2 whose levels were too low to detect using the IL-6 ELISA.

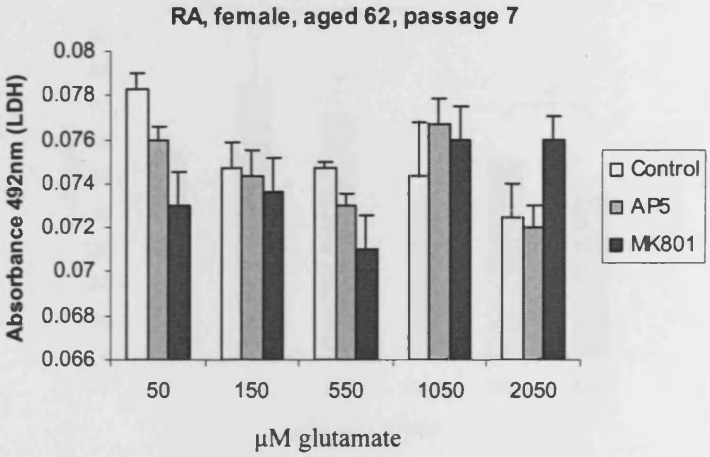
4.3.3 EAAT inhibitors increase IL-6 release by FLS

To determine whether the high affinity glutamate transporters (EAATs1-5) have an effect on IL-6 production by FLS by altering extracellular glutamate concentration, the inhibitors TBOA (100μM) or *t*PDC (100μM) were added to the cultures. Transporter inhibition by TBOA increased IL-6 released by RA FLS from patient 3, passage 5 exposed to 50μM (122pg/ml compared to 61pg/ml without TBOA, $p=0.012$) and 150μM (119pg/ml compared to 63pg/ml without TBOA, $p=0.034$) extracellular glutamate treatments (figure 4.4a). At higher concentrations of glutamate (550μM and 2050μM) no difference could be observed between TBOA treated cultures and the controls.

Figure 4.1 Measurement of LDH production by FLS using a cytotoxicity assay demonstrating the effects of glutamate and the glutamate transporter and receptor antagonists on cell number
(All error bars show SEM and demonstrate error in the triplicate readings of the assay).

a: Treatment of FLS with 100μM *t*PDC or 100μM TBOA and up to 1050μM glutamate changed the total cell population by a maximum of 1%. Therefore these treatments are not considered to have a significant effect on total cell number (n=1 well).

b: Treatment of FLS with 10μM DAP5 or 100μM MK801 and up to 2050μM glutamate changed the total cell population by a maximum of 1%. Therefore these treatments are not considered to have a significant effect on total cell number (n=1 well).



c: Treatment of FLS with 10μM CFM2 or 150μM NBQX with 50μM to 2050μM glutamate changed the total cell population by a maximum of 3%. Therefore these treatments are not considered to have a significant effect on total cell number (n=1 well).

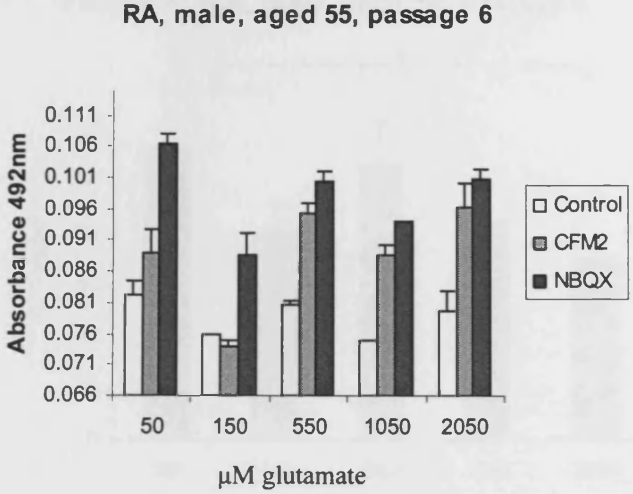
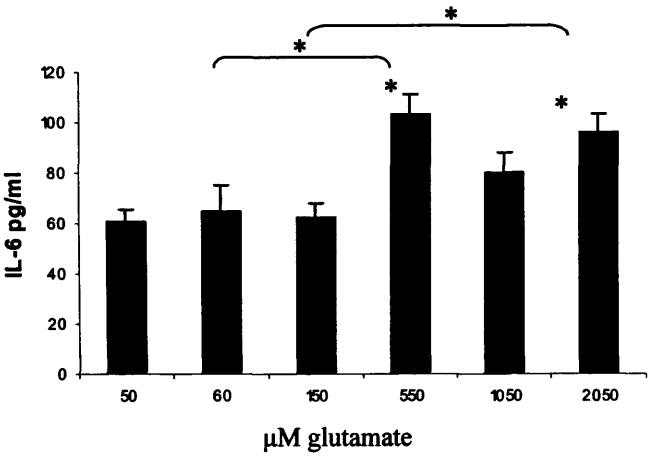


Figure 4.2 The effect of glutamate on the release of IL-6 by RA FLS.

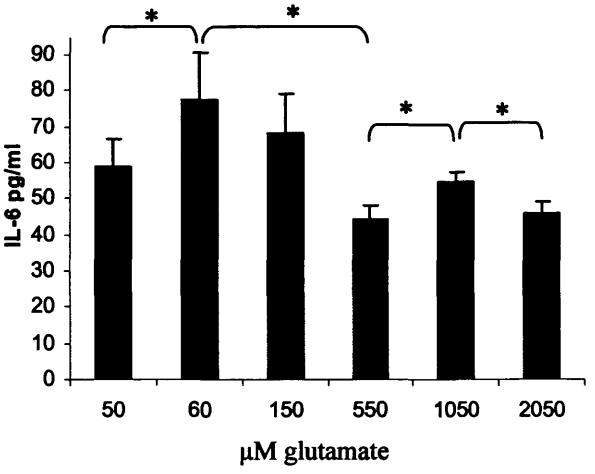
a: Treatment of RA FLS from patient 3, passage 5 with 550μM (p=0.005) and 2050μM (p=0.011) glutamate caused an increase in IL-6 production compared to IL-6 production at 50μM glutamate. Significant differences are compared to the 50μM control unless otherwise indicated by brackets (*:p<0.05, SEM error bars, 2-sample t-test used except comparison of 550μM and 2050μM glutamate to the control where a paired t-test was used, n=6, n=3 for 60μM samples SEM error bars).

Patient 3: RA, female, aged 62, passage 5



b: Treatment of RA FLS from patient 3, passage 7 with 60μM to 2050μM glutamate had varying effects on IL-6 production. An increase in IL-6 production was seen between 50μM and 60μM glutamate (p=0.048) and between 550μM and 1050μM glutamate (p=0.014). A decrease in IL-6 production was observed between treatment with 60μM and 550μM (p=0.036) glutamate and between 1050μM and 2050μM glutamate (p=0.017) (*: p<0.05, data transformed (log_{10}) due to unequal variances prior to analysis with a paired t-test, n=6, SEM error bars).

Patient 3: RA, female, aged 62, passage 7



c: Treatment of RA FLS from patient 8, passage 6 with 150μM (p=0.024) and 2050μM (p=0.034) glutamate caused a decrease in IL-6 production (80pg/ml) compared to the level of IL-6 (115pg/ml) produced with 50μM glutamate. Significant differences are compared to the 50μM control unless otherwise indicated by brackets. (paired t test, *:p<0.05, n=6, SEM error bars).

Patient 8: RA, male, aged 55, passage 6

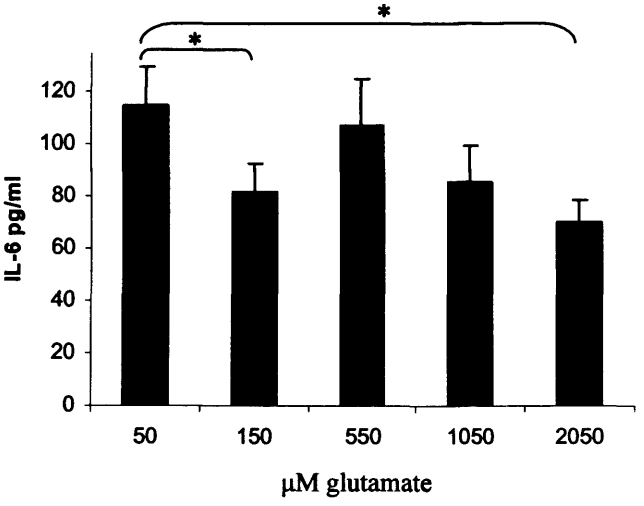
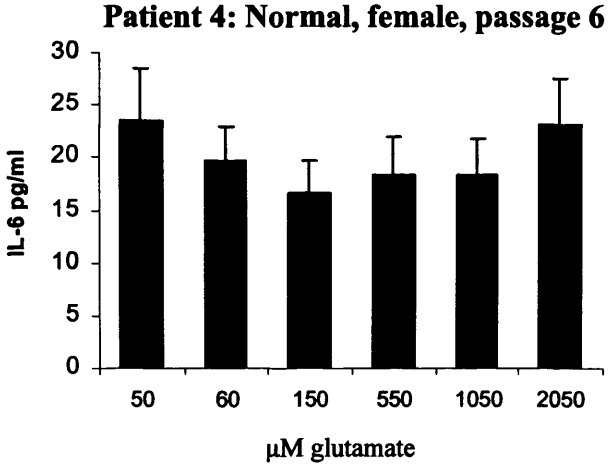
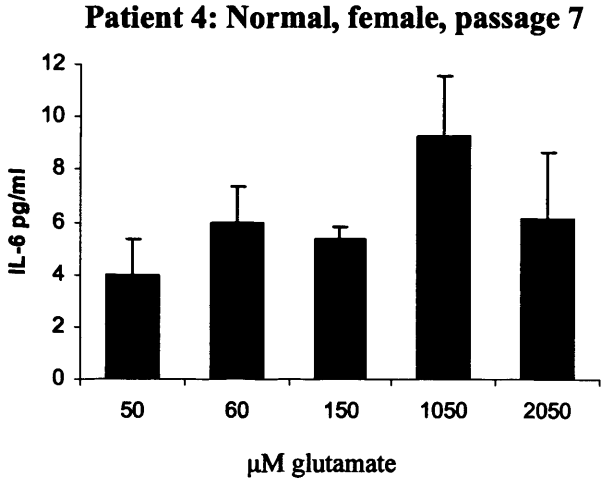


Figure 4.3 The effect of glutamate on the release of IL-6 by normal FLS.

a: Treatment of normal FLS from patient 4, passage 6 with 60μM to 2050μM glutamate caused no significant differences in IL-6 production compared to the control cultures grown in 50μM glutamate (paired t-test, n=6, SEM error bars).



b: Treatment of normal FLS from patient 4, passage 7 with 60μM to 2050μM glutamate caused no significant differences in IL-6 production compared to the control cultures grown in 50μM glutamate (paired t-test, n=6, SEM error bars).



The glutamate transporter inhibitors *t*PDC (competitive, transportable inhibitor) and TBOA (competitive, non-transportable inhibitor) also appeared to induce IL-6 production by RA FLS from patient 2, passage 6 (figure 4.4b). Levels of IL-6 produced by the control RA FLS from patient 2 were too low to be detected (subtraction of the absorbance value of the media blank reduced control sample absorbance readings to zero).

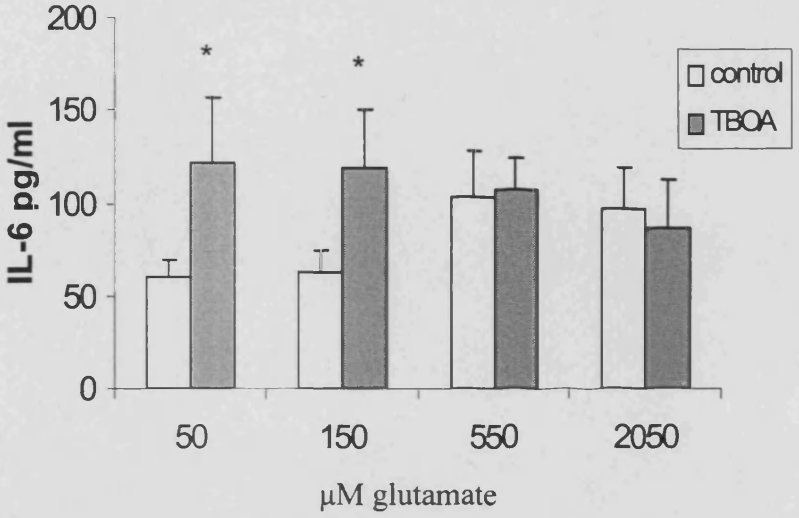
Whereas in RA FLS, glutamate transporter inhibition caused an increase in IL-6 production, TBOA caused a reduction in IL-6 production in normal FLS at 60μM (11pg/ml compared to 20pg/ml without TBOA, p=0.045), 1050μM (6pg/ml compared to 18pg/ml without TBOA, p=0.018) and 2050μM (13pg/ml compared to 23pg/ml without TBOA, p=0.04) glutamate (figure 4.5). The different constitutive levels of IL-6 in normal FLS (20pg/ml) and RA FLS (60pg/ml) and the significant increase in IL-6 release by RA cells in response to EAAT inhibitors compared to the decrease in normal FLS, strongly indicates an increased sensitivity to glutamate and a difference in glutamate signalling in the disease state.

4.3.4 NMDA receptor inhibitors influence IL-6 release by RA and normal synoviocytes

To determine whether the effect of increased IL-6 production in the presence of high extracellular glutamate was being mediated by NMDA receptor activation, FLS were treated with a range of glutamate concentrations in the presence of the NMDA receptor antagonists MK801 or D-AP5. NMDA receptor inhibition with the competitive inhibitor DAP5 had no effect on RA or normal FLS. The non-competitive inhibitor MK801 increased IL-6 production by RA FLS (figure 4.6a) only in the presence of 550μM glutamate (65pg/ml compared to 44pg/ml without MK801, p=0.031). These cells produced constitutively low levels of IL-6. MK801 increased IL-6 production in normal FLS in the presence of 50μM (11pg/ml compared to 4pg/ml without MK801, p=0.016) and 60μM (11pg/ml compared to 6pg/ml without MK801, p=0.016) glutamate (figure 4.6b).

Figure 4.4 The effect of glutamate and inhibition of glutamate transporters on IL-6 levels of RA FLS

a: The effect of extracellular glutamate concentration (white bars) and TBOA treatment (grey bars) on the release of IL-6 by RA FLS from patient 3, passage 5. TBOA treatment significantly increased IL-6 release at 50μM (p=0.012, paired t-test) and 150μM (p=0.034, 2-sample t-test) glutamate. P-values are derived by comparing IL-6 levels in treated and control media at each glutamate concentration (data transformed [log_{10}] prior to analysis, *: p<0.05, n=6, SEM error bars).



b: The effect of extracellular glutamate concentration (white bars, not visible because are at zero), *t*PDC (grey bars) and TBOA treatment (black bars) on the release of IL-6 by RA FLS from patient 2, passage 6. *t*PDC significantly increased IL-6 release between 60μM and 1050μM glutamate. TBOA treatment significantly increased IL-6 release at all concentrations of glutamate. P-values are derived by comparing IL-6 levels in treated and control media at each glutamate concentration (*: p<0.05, n=6, n=3 at 60μM, SEM error bars).

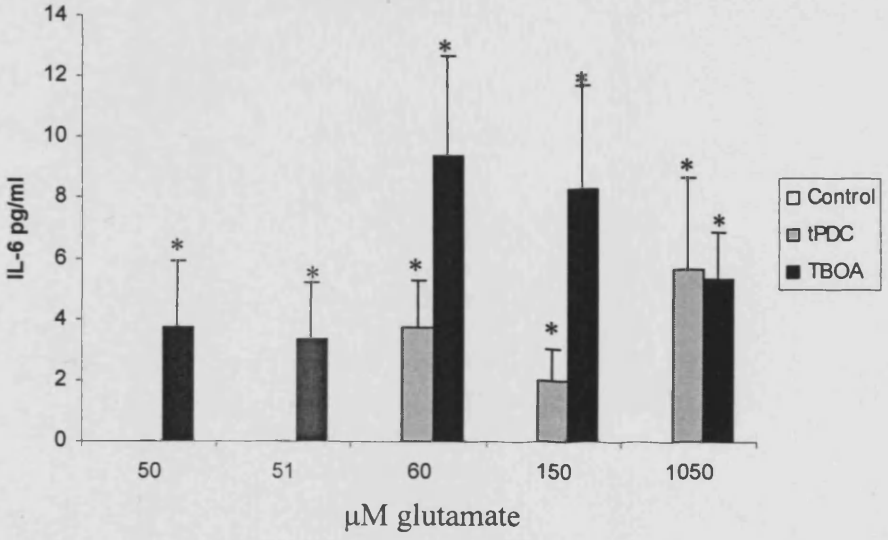


Figure 4.5 The effect of glutamate transporter inhibition on IL-6 production by normal FLS

Normal FLS (patient 4, passage 6) treated with 60μM (p=0.045, 2-sample t-test), 1050μM (p=0.018, paired t-test) and 2050μM (p=0.04, 2-sample t-test) glutamate and TBOA showed a decrease in IL-6 production (data were transformed [log_{10}] prior to t-test analysis, *:p<0.05, n=6, SEM error bars).

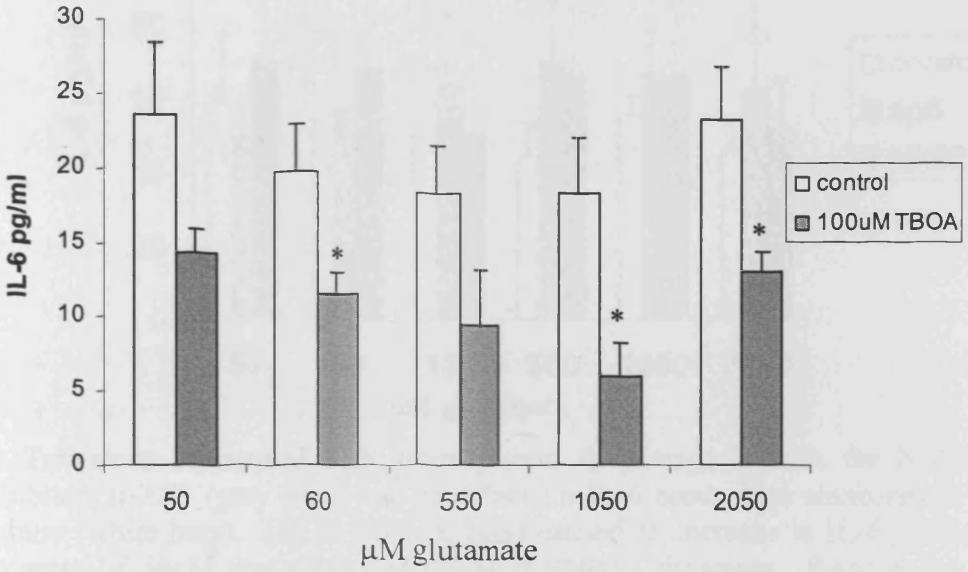
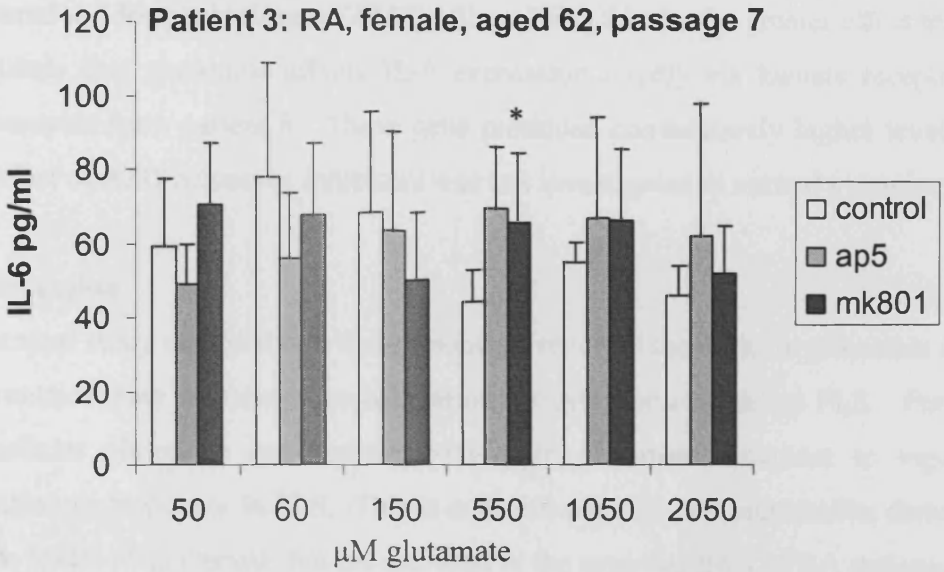
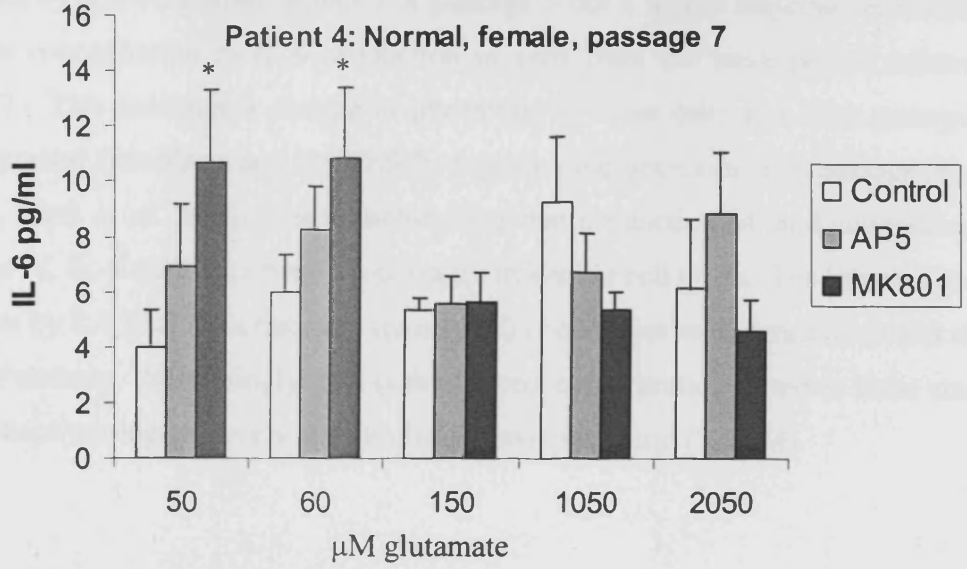


Figure 4.6 The effect of NMDA receptor inhibition on IL-6 production by fibroblast-like synoviocytes

a: Treatment of RA FLS with the NMDA receptor inhibitors D-AP5 (grey bars) and MK801 (black bars) had no effect on IL-6 production when compared to the control (white bars) except at 550μM glutamate, where DAP5 significantly increased IL-6 production ($p=0.031$, data transformed [$\log_{10}$] prior analysis with a paired t-test, n=6, *:$p<0.05$, SEM error bars). P-values are derived by comparing IL-6 levels in treated and control media at each glutamate concentration.



b: Treatment of normal FLS from patient 4, passage 7 with the NMDA receptor inhibitors D-AP5 (grey bars) had no effect on IL-6 production compared to the control culture (white bars). MK801 (black bars) caused an increase in IL-6 production in the presence of 50μM ($p=0.016$) and 60μM ($p=0.016$) glutamate. P-values are derived by comparing IL-6 levels in treated and control media at each glutamate concentration (data transformed [$\log_{10}$] prior to paired t-test analysis, n=6, SEM error bars, *:$p<0.05$).



4.3.5 AMPA/kainate receptor inhibitors decrease IL-6 release by RA synoviocytes

The issue of whether inhibiting AMPA/kainate ionotropic receptors influenced IL-6 release by RA synoviocytes was studied. At all glutamate concentrations except 2050μM the AMPA/kainate receptor inhibitor NBQX significantly reduced IL-6 release by RA FLS from patient 8 (figure 4.7). The competitive, specific AMPA receptor inhibitor CFM2 reduced IL-6 release in RA FLS treated with 550μM glutamate (96pg/ml compared to 136pg/ml without CFM2). Since NBQX had a far greater effect than CFM2, it is likely that glutamate affects IL-6 expression largely via kainate receptors in RA synoviocytes from patient 8. These cells produced constitutively higher levels of IL-6. The effect of AMPA/kainate inhibitors was not investigated in normal synoviocytes.

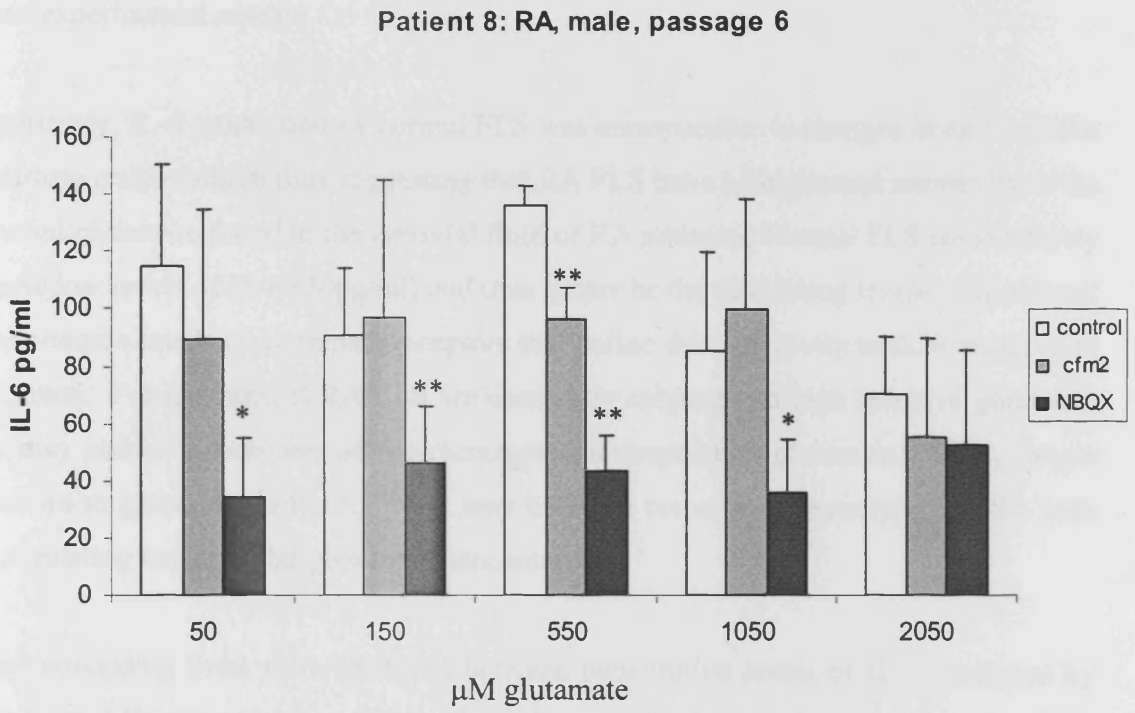
4.4 Discussion

The present study demonstrates that previously reported increases in glutamate in RA and inflammation may influence pro-inflammatory cytokine release by FLS. Furthermore, extracellular glutamate can activate ionotropic glutamate receptors to regulate pro-inflammatory responses in FLS. This is constant with the previous studies demonstrating that the levels of glutamate that are elevated in the synovial fluid of RA patients correlate with the levels of various pro-inflammatory markers (McNearney *et al.* 2004).

The data shows that glutamate influences IL-6 production by FLS although this response is variable between RA patients. High extracellular glutamate caused an increase in IL-6 production by RA FLS from patient 3 at passage 5 but a mixed response depending on glutamate concentration in IL-6 production in cells from the same patient cultured at passage 7. This indicates a change in phenotype in these cells at a later passage. In human gingival fibroblasts and RA FLS IL-6 production decreases with passage (Kent *et al.* 1996, Hirth *et al.* 2002) thus demonstrating that production of, and suggesting that regulation of, IL-6 can differ between passages in similar cell types. The increase in IL-6 production by RA FLS from patient 3 (passage 5) occurred at concentrations at and above 550μM glutamate. Interestingly, this is the nearest concentration tested in these studies, to the pathophysiological levels observed in RA synovial fluid (326μM).

Figure 4.7 The effect of AMPA and KA glutamate receptor inhibition on IL-6 production by RA FLS

IL-6 release by RA FLS is decreased by the AMPA/KA receptor inhibitor NBQX (black bars). The effect of inhibiting AMPA and KA receptors with NBQX significantly decreased IL-6 production by RA FLS at all concentrations of glutamate except 2050μM. The AMPA inhibitor, CFM2 (grey bars), only significantly reduced IL-6 production in the presence of 550μM glutamate. P-values are derived by comparing IL-6 levels in treated and control media at each glutamate concentration. (*:$p<0.05$, **:$p<0.005$, n=6)



In RA FLS from patient 8, high extracellular glutamate caused an overall decrease in IL-6 production. The differences in these responses may be due to variation in constitutive IL-6 production as this was much lower in patient 3 (60pg/ml) than in patient 8 (115pg/ml). The difference in constitutive IL-6 expression between patients has been previously reported (Bucala *et al.* 1991) and is influenced by factors such as the presence of other cytokines, passage and disease state (Hirth *et al.* 2002). It should be noted that patient 8 was male and patient 3 was female, therefore gender may also have an effect on RA FLS phenotype. In addition age and treatments the patients were receiving may affect experimental results.

Importantly, IL-6 production by normal FLS was unresponsive to changes in extracellular glutamate concentration thus suggesting that RA FLS have a heightened sensitivity to the elevated glutamate found in the synovial fluid of RA patients. Normal FLS constitutively release low levels of IL-6 (20pg/ml) and thus it may be the circulating levels of IL-6 itself or expression levels of glutamate receptors that define this sensitivity in IL-6 response to glutamate. For example, if RA FLS are constantly subjected to high levels of glutamate this may induce expression of the receptors that respond to glutamate. If so, longer exposure to glutamate in normal cells may induce a responsive phenotype, i.e. the cells are regulating extracellular glutamate concentration.

Some interesting links were observed between constitutive levels of IL-6 produced by FLS from different patients and the effect glutamate had on IL-6 production. RA FLS from patient 8 had very high constitutive levels of IL-6 which were decreased upon incubation with glutamate. Patient 3 RA FLS produced medium to high levels of IL-6 which were increased further upon incubation with IL-6. Finally the RA FLS from patient 2 and normal FLS from patient 4 had constitutively low levels of IL-6 and glutamate had no effect on IL-6 production by these cells. The presence of glutamate transporter inhibitors increased IL-6 production regardless of constitutive IL-6 levels produced by RA FLS.

Inhibiting glutamate transport had opposing effects in normal and RA FLS. In RA FLS, the non transported competitive inhibitor TBOA caused an increase in IL-6 production in cells from 2 patients; in normal FLS it decreased IL-6 production. TBOA is active against all EAATs but is most potent against EAAT1. Both normal and RA FLS express EAAT1 and EAAT3 but only normal FLS express EAAT2 (see chapter 3). Thus the effects of these inhibitors are likely to influence EAATs 1 and 3 in RA FLS and EAATs 1, 2 and 3 in normal FLS. In addition the number of transporters (and receptors) expressed would determine how RA and normal FLS respond to glutamate transporter inhibitors. The affinity of the inhibitors for the transporters in comparison to glutamate would also affect the effectiveness of inhibition. Previous studies in neurons demonstrate that TBOA (K_i approximately 19μM) has a higher affinity for glutamate transporters than *t*PDC (K_i approximately 38 μM) (Waagepetersen 2001) which could explain why TBOA had a greater effect on IL-6 production in this study (see appendix for calculation of K_i values). It would therefore be of interest to quantify EAAT expression in normal and RA FLS because inhibitors may not have uniform effects if types and levels of expression differed. In addition, the inhibitory potency (IC_{50}) of these inhibitors should be determined in normal and RA FLS prior to future experiments in order to determine exactly the inhibitory effect they have on these cells.

In normal FLS the glutamate transporters may be acting directly as receptors (glutamate-gated ion channel receptors). It has been demonstrated previously that glutamate can activate the MEK/ERK pathway in astrocytes via glutamate transporters (Abe and Saito 2001). Therefore the effects of TBOA and *t*PDC may be preventing transporters acting as ligand-gated ion channel receptors; in normal FLS transporter inhibitors increased IL-6 production. However, the most likely effect of inhibition of glutamate transporters is that sodium dependent uptake of glutamate is prevented causing a build up of extracellular glutamate and enhanced activation of glutamate receptors. To investigate whether this was the possible mechanism of glutamate induction of IL-6 in some RA patients, various groups of ionotropic glutamate receptors were systematically inhibited.

The NMDA receptor antagonist D-AP5 (competitive) had no effect on IL-6 production by RA FLS and normal FLS possibly because the concentration of D-AP5 was too low to compete against glutamate levels. It is difficult to determine the correct concentration of competitive inhibitors to use as the number of receptors expressed by FLS is unknown. In addition, there are further factors that would affect the potency of receptor antagonists. The concentration of glutamate that the cells are exposed to, the Mg^{2+} concentration in the media (Mg^{2+} inhibits NMDA receptor activation), the subunit composition of the receptors and the trafficking of glutamate receptors and transporters in response to changes in extracellular glutamate levels. It has been shown however, that D-AP5 has a rapid rate of dissociation from NMDA receptors (Monaghan *et al.* 1984) which could explain why it had no effect in these relatively long term cultures. DAP5 has a lower binding affinity (K_i approximately 81nM) for NMDA receptors than MK801 (K_i approximately 54nM, calculated in pig cerebral cortex, see appendix for details of calculation) which together with the fact that DAP5 is also a non-competitive inhibitor may explain why greater effects were seen with MK801 (Fritz *et al.* 1996). MK801 (non-competitive) increased IL-6 production by normal and RA FLS. The effect of MK801 on IL-6 production differed with levels of extracellular glutamate. In RA FLS MK801 increased IL-6 production in the presence of 550μM glutamate whereas in normal FLS MK801 increased in IL-6 production in the lower concentrations of 50μM and 60μM glutamate. This demonstrates another difference between RA and normal FLS in the way they respond to glutamate. The observation that normal FLS increase IL-6 production in response to the NMDA receptor inhibitor MK801 may be correlated with the fact that the EAAT inhibitor TBOA caused a decrease in IL-6 production in normal FLS. Therefore in normal FLS, TBOA may decrease IL-6 by preventing uptake of glutamate, thus activating NMDA receptors to down-regulate IL-6 production.

Taken together, these results demonstrate that inhibition of NMDA receptors caused an increase in IL-6 production, thus suggesting that glutamate acts upon NMDA receptors to down-regulate IL-6 production by FLS. Therefore activation of NMDA receptors by glutamate may serve to prevent high IL-6 levels in the synovial fluid. This mechanism

may be less sensitive in RA patients where FLS are conditioned to high extracellular glutamate concentrations.

The AMPA and kainate receptor competitive antagonist, NBQX, significantly reduced IL-6 release in RA FLS at all glutamate concentrations whereas the AMPA receptor-specific non-competitive antagonist CFM-2 was only effective at 550μM glutamate. Thus, it is concluded that control of increases in IL-6 production by glutamate is likely to be via kainate receptors. In fact kainic acid injected into rat brain induces IL-6 mRNA expression after 2 hours (Minami *et al.* 1991, Lehtimaki *et al.* 2003). Furthermore systemic kainic acid administration to rats increases the pro-inflammatory cytokine IL-1β mRNA levels in the brain which may be via kainate or AMPA receptors (Eriksson *et al.* 2000) demonstrating that glutamate receptors have previously been linked to regulation of pro-inflammatory cytokine expression. The effect of metabotropic antagonists on IL-6 production has not been investigated in this study; therefore the control of IL-6 production in RA may also involve this subtype of glutamate receptor.

These studies using human RA synoviocytes have shown that IL-6 is likely to be regulated by changes in extracellular glutamate concentrations. Glutamate-induced changes in IL-6 production are likely to be mediated via NMDA and kainate receptors with kainate receptors increasing IL-6 levels and NMDA receptors decreasing IL-6 levels. The effect of inhibiting kainate receptors on IL-6 production by RA FLS was greater than the effect of inhibiting NMDA receptors. Therefore the high levels of glutamate present in RA synovial fluid may be having a more pro-inflammatory effect on synoviocytes, mediated through the cytokine IL-6. Previously, induction of IL-6 expression in RA synovioyctes has been demonstrated to be via IL-1 (Ogura *et al.* 2002) and TNFα (Piecyk and Anderson 2001). This must be taken into context with the fact that glutamate and TNFα are both elevated in RA synovial fluid compared to OA synovial fluid. Therefore the regulatory effects of glutamate on IL-6 production need to be investigated with respect to these cytokines to determine which is the primary modulator or whether these are distinct pathways that induce expression or production.

The differing responses of RA and normal FLS to glutamate and glutamate receptor and transporter antagonists needs to be investigated further. These differences are important because they highlight a change in phenotype between normal and RA FLS. Repeating the experiments on FLS from more patients would help to characterise the responses observed. It would be interesting also to see if patient synovial fluid glutamate levels affect the phenotype of the FLS derived from them.

The effect of glutamate on IL-6 production by RA FLS presented here has varied between patients. Constitutive glutamate and IL-6 levels, disease stage and treatments received may affect how FLS respond. Therefore these experiments need to be repeated in FLS from more patients in order to conclude fully the effect that glutamate has. This may demonstrate trends in the effect of glutamate on IL-6 production and patient age, sex and treatments received. Repetitions should also be carried out inhibiting more than one signalling component, for example, using MK801 and TBOA. Furthermore, the differences in IL-6 production in response to glutamate with respect to passage number needs to be characterised further. This could be done by repeating experiments at each passage on cells from the same patient and comparing the effects. IL-6 is an important mediator of inflammation in RA, therefore if the increase in IL-6 production caused by glutamate treatment in RA FLS from patient 3 is shown to occur in many patients this could indicate a novel therapeutic approach in which to treat RA. Anti-IL-6 therapy has shown very good efficacy therefore targeting the signalling pathway leading to IL-6 production may improve upon this treatment. Exploring the point of regulation would facilitate this; glutamate may regulate transcription, translation or shedding and secretion of IL-6. Hence, a better understanding of the pathways activated by the elevated glutamate in RA patients may enable new and more effective therapeutic targets to be identified. For example, data presented here demonstrate that kainate receptor inhibitors could have the potential to decrease IL-6 levels in RA patients.

Chapter 5 Regulation of EAAT1 expression by pro-inflammatory cytokines

5.1 Introduction

Important mediators of inflammation in RA include cytokines such as interleukin-6 (IL-6), interleukin 1 (IL-1) and tumor necrosis factor alpha (TNFα). Section 1.4.1.1 provides an overview of these cytokines and their role in RA. IL-6 is elevated in synovial tissue and fluid (from 24.9 to 49.7ng/ml) during active phases of inflammation in RA patients (Desgeorges *et al.* 1997). TNFα is also elevated in RA synovial fluids (94pg/ml compared to OA synovial fluid levels of 39pg/ml) (McNearney *et al.* 2004). Anti IL-6 and IL-6 receptor therapy is now being developed as a treatment for rheumatoid arthritis, and have demonstrated good efficacy in clinical trials (Nishimoto *et al.* 2004 and Choy *et al.* 2002) (see section 1.3.4.4). Furthermore, the effectiveness of anti-TNFα and anti-TNFα receptor therapy which are currently used as treatments for RA (see section 1.3.4.4) demonstrates the important role of pro-inflammatory cytokines such as TNFα in RA. IL-1 levels are also elevated in RA synovial fluid (130pg/ml) compared to OA synovial fluid levels (28pg/ml) and has a well established pro-inflammatory role in RA (Westacott *et al.* 1990). For example, IL-1 induces synovial fibroblasts and chondrocytes to release MMPs, aggrecanases, nitric oxide and prostaglandins (see section 1.4.1.1.2).

The effects of these pro-inflammatory cytokines have been investigated within the CNS where there is some evidence linking glutamatergic signalling to inflammatory and degradative responses (see section 1.6.5.6). This therefore indicates a potential link between the synchronous elevated levels of IL-6 and glutamate in these diseases. Studies have also investigated the direct effects of these cytokines on cells within the CNS (see section 1.6.5.4) and outside the CNS (see section 1.6.5.5), particularly the effect of cytokines on glutamate transport and receptors.

Glutamate levels are elevated in the synovial fluid of RA patients and the synovial fluid of antigen-induced arthritis in rats (McNearney *et al.* 2000, Lawand *et al.* 2000), therefore suggesting a link between inflammation and glutamate regulation (see section 1.6.5.7). These elevated levels of glutamate in RA synovial fluid may be

caused or influenced by direct effects of cytokines on the activity or expression of glutamate transporters and receptors. Therefore the effects of pro-inflammatory cytokines elevated in RA synovial fluid (IL-6, TNFα and IL-1) on EAAT1 (GLAST) mRNA and protein expression was investigated. EAAT1 was chosen because this is more highly expressed in fibroblasts than EAAT 3 and EAAT4 (Cooper *et al.* 1998).

5.2 Methods

5.2.1 Determining the effect of pro-inflammatory cytokines on EAAT1 mRNA expression

To determine whether extracellular pro-inflammatory cytokines can influence the expression of glutamate signalling components, the effect of treating primary human RA FLS with IL-6/sIL-6r on EAAT1 mRNA expression was investigated. Primary human RA FLS (patient 1) were treated with IL-6 (50ng/ml) in conjunction with 20ng/ml sIL-6r or IL-1 (10ng/ml) for 0, 1, 6, 15 or 24 hours and reverse transcribed to cDNA (kindly donated by Dr. S. Jones and Dr M. Nowell, Department of medical biochemistry and immunology, Cardiff University). PCR was carried out to determine β-actin and EAAT1 mRNA expression (see section 2.4).

5.2.2 Determining the effect of pro-inflammatory cytokines on EAAT1 protein expression using immunohistochemistry

To determine whether pro-inflammatory cytokines alter EAAT1 protein expression, primary human RA FLS (patient 5, passage 7) and normal FLS (patient 4, passage 7) cultured in 8-well chamber slides (see section 2.5.2) were treated with 500pg/ml TNFα (PeproTech Inc.) or 50ng/ml IL-6 (PeproTech Inc.) in combination with 20ng/ml sIL-6r (R & D systems). These concentrations of IL-6 and sIL-6r were chosen because an increase in EAAT1 mRNA expression had already been observed with these concentrations (see section 5.3.1) and they are consistent with the level of IL-6 observed in RA synovial fluid (24.9ng/ml and 51.7ng/ml, see section 1.4.1.1.3). RA synovial fluid levels of sIL-6r are 24.7ng/ml (Desgeorges *et al.* 1997). 500pg/ml of TNFα was chosen because this was between RA synovial fluid levels (94pg/ml) and levels used for treatments in other published work on FLS (see section 1.4.1.1.1 and 1.6.5.5). Treatments were carried out for 0, 30 minutes, 1 hour or 3 hours because effects on mRNA had been observed after 1 hour. Duplicate cultures were treated with an inhibitor to the IL-6 receptor, Mab227 (2μg/ml, R&D Systems) which

is a function blocking antibody. EAAT1 protein expression was investigated by immunohistochemistry (see section 2.6) using a polyclonal antibody raised against the N-terminal of GLAST1 (EAAT1) and GLAST1a. Experiments demonstrating specificity of this antibody when used for immunohistochemistry are described in chapter 3 (section 3.3.7.1.1).

5.2.3 Determining the effect of pro-inflammatory cytokines on EAAT1 protein expression using Western blotting

RA FLS (patient 7, passage 7) cultured in 6-well plates (see section 2.5.2) were treated with 50ng/ml IL-6 in combination with 20ng/ml sIL-6r, with or without the IL-6 function blocking antibody, Mab227 (2μg/ml, R& D Systems). Treatments were replicated in three to five wells ($3<n<5$). Cells were treated for 0, 1 and 3 hours. The media was removed and the protein extract subjected to SDS PAGE and Western blotting (section 2.7). The total protein in each extract was measured (section 5.2.3.2 below) to ensure accurate, comparable quantification of EAAT1 levels between samples (section 5.2.3.3 below). Normal FLS were not analysed.

5.2.3.1 Cytotoxicity of the treatments used in the Western blotting experiment (section 5.2.3.3)

The amount of LDH released into the media over the course of treatments was measured using a cytotoxicity assay (section 2.12) and calculated as a percentage of the amount of LDH released after total cell lysis. Results showed that between 0.3 and 2% of cells died during the course of treatments, therefore this would not significantly affect the levels of EAAT1 measured by Western blotting.

5.2.3.2 BCA assay

In order to normalise the amount of protein extract loaded onto each SDS PAGE gel the amount of protein in each RA FLS extract was determined using a BCA assay (section 2.7.5). Protein content was determined prior to concentrating the samples using centricon tubes.

5.2.3.3 Quantifying regulation of EAAT1 protein expression by Western blotting

Western blotting was used to confirm and quantify the immunohistochemical observations. Prior to separation by SDS PAGE, cell protein extracts in 2% SDS

were concentrated in centricon tubes. The concentration factor was taken into account when calculating the relative amounts of EAAT1 protein expressed. The amount of EAAT1 protein detected by Western blotting was normalised to the amount of total protein loaded on the SDS PAGE gel as measured by the BCA assay (see section 5.2.3.2). The relative amounts of EAAT1 per sample per total sample protein were therefore calculated and compared using a student unpaired 2-sample t test (section 2.13).

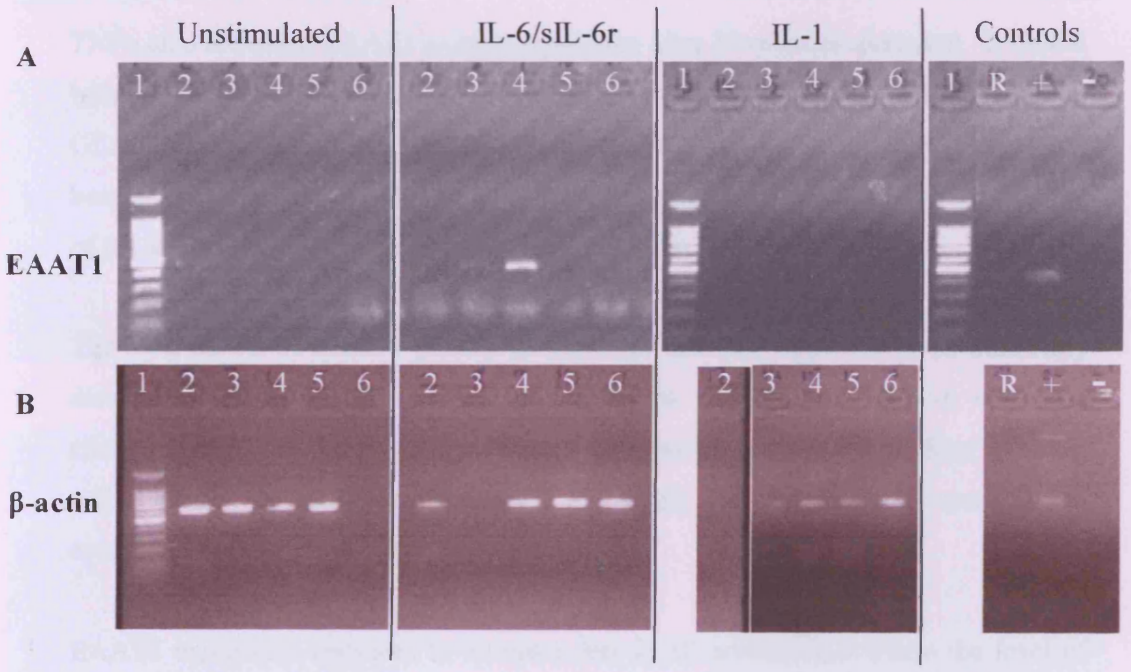
5.3 Results

5.3.1 The effect of IL-6 on EAAT1 mRNA expression in primary human FLS

RT-PCR (section 2.4) revealed the 345bp EAAT1 PCR product after 1 and 6 hours (figure 5.1A) in IL-6/sIL-6r stimulated cells but only in the unstimulated cells after 24 hours of culture. IL-1 failed to induce expression of EAAT1 mRNA and was therefore not investigated further in this study. Successful amplification of β-actin in all but 2 samples (figure 5.1B: unstimulated lane 6, IL-6 lane 3) confirmed cDNA integrity indicating induction of EAAT1 expression by IL-6 (figure 5.1B). Although β-actin was not detected in these two samples cDNA integrity was adequate for amplification of the EAAT1 amplicon in both cases. Positive (plasmid vector containing the full open reading frame of GLAST1 and β-actin) and negative controls (water) were also amplified to confirm specificity and a successful PCR reaction. RNA that had not undergone reverse transcription was also subjected to PCR. The lack of a PCR product confirmed that amplicons originated from mRNA as opposed to any contaminating genomic DNA.

Figure 5.1
A: Agarose gel of EAAT1 RT-PCR products from human RA FLS stimulated with IL-1 (10ng/ml) or IL-6 (50ng/ml)/IL-6sr (20ng/ml) for 0 to 24 hours. EAAT1 expression occurred in the unstimulated controls only after 24 hours but did not occur in the IL-1 treated RA FLS at any time. EAAT1 mRNA expression was induced by IL-6/IL-6sr after 1 and 6 hours, but was not present after 15 and 24 hours treatment.

B: β-actin RT-PCR products in human FLS stimulated with IL-1 or IL-6 and sIL-6r. β-actin expression was detected in all samples except the control after 24 hours and the one hour IL-6 stimulated sample both of which were positive for EAAT1.



Key
1 -100bp ladder
2 - 0 hours
3 - 1 hour
4 - 6 hours
5 - 15 hours
6 - 24 hours
R – RNA control
+ - plasmid, GLAST or β-actin
- - water control

5.3.2 Immunohistochemistry showing the effect of pro-inflammatory cytokines on EAAT1 protein expression in FLS

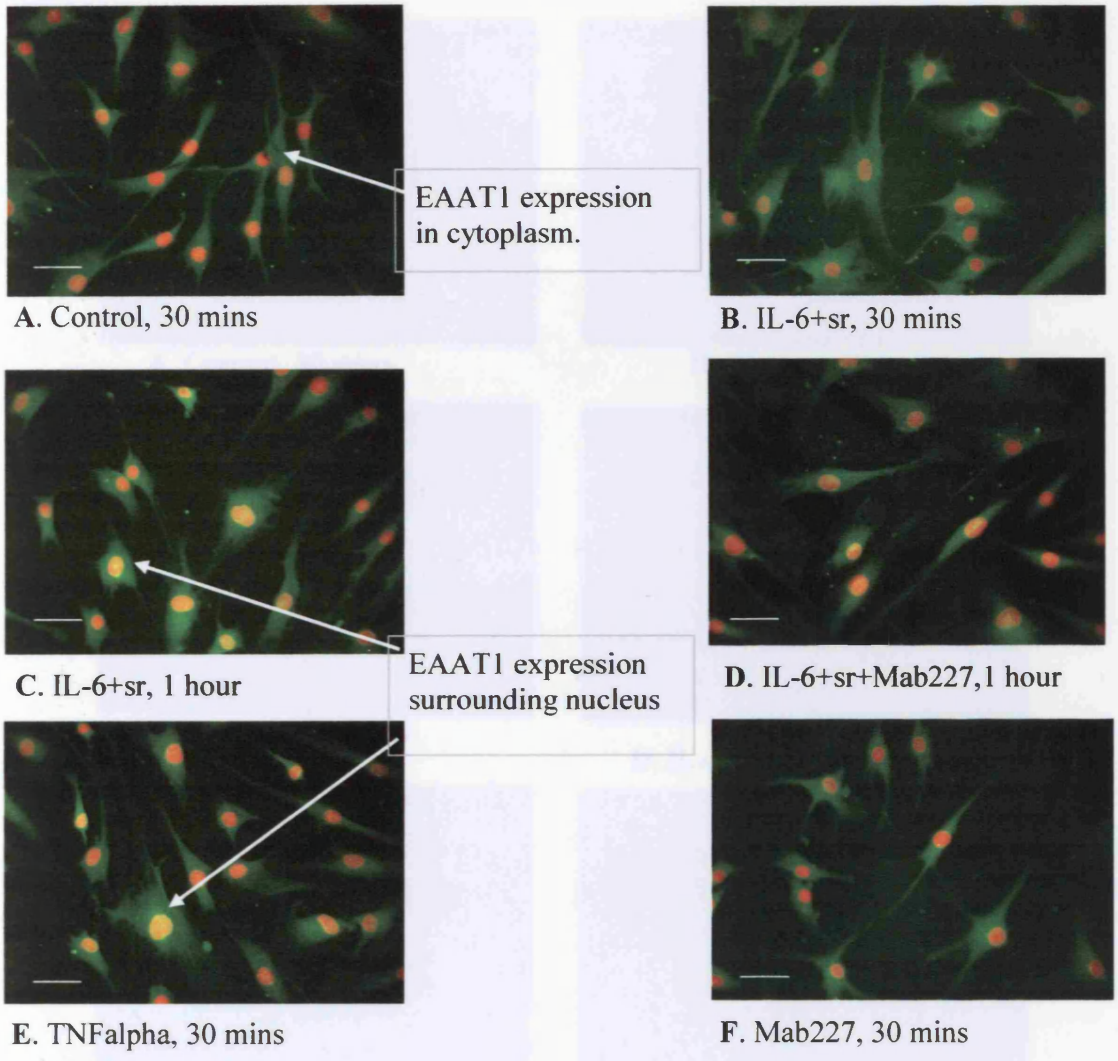
Fluorescence microscopy (figure 5.2) revealed that the low level EAAT1 protein expression in RA FLS appeared to increase after 30 minutes and 1 hour treatment with IL-6/sIL-6r in 100% of the cells (figure 5.2, panels B and C respectively). The IL-6 induced increase in EAAT1 expression was inhibited by Mab227 in 100% of the cells (figure 5.2, panel D), indicating that the effect is likely to be mediated via the sIL-6r since FLS do not normally express an IL-6 receptor. The IL-6 function inhibitor (Mab227) had no affect on RA or normal FLS when administered alone (figure 5.2F and 5.3F respectively).

TNFα also increased EAAT1 protein expression after 30 minutes treatment. It should be noted that the antibody used for immunolocalisation does not discriminate between GLAST (EAAT1) variants, however to date, the splice variant, GLAST1a, has not been shown to be expressed in FLS. Control experiments to confirm specific binding of the antibodies were carried out and are detailed in section 3.2.2.1.

The localisation of EAAT1 protein in untreated RA FLS appeared to be uniformly distributed within the cytoplasm and/or plasma membrane. In IL-6 or TNFα stimulated RA FLS, EAAT1 appeared to concentrate around the nucleus of some cells (highlighted in figure 5.2, panels C and E) and in condensed areas of the cytoplasm or plasma membrane.

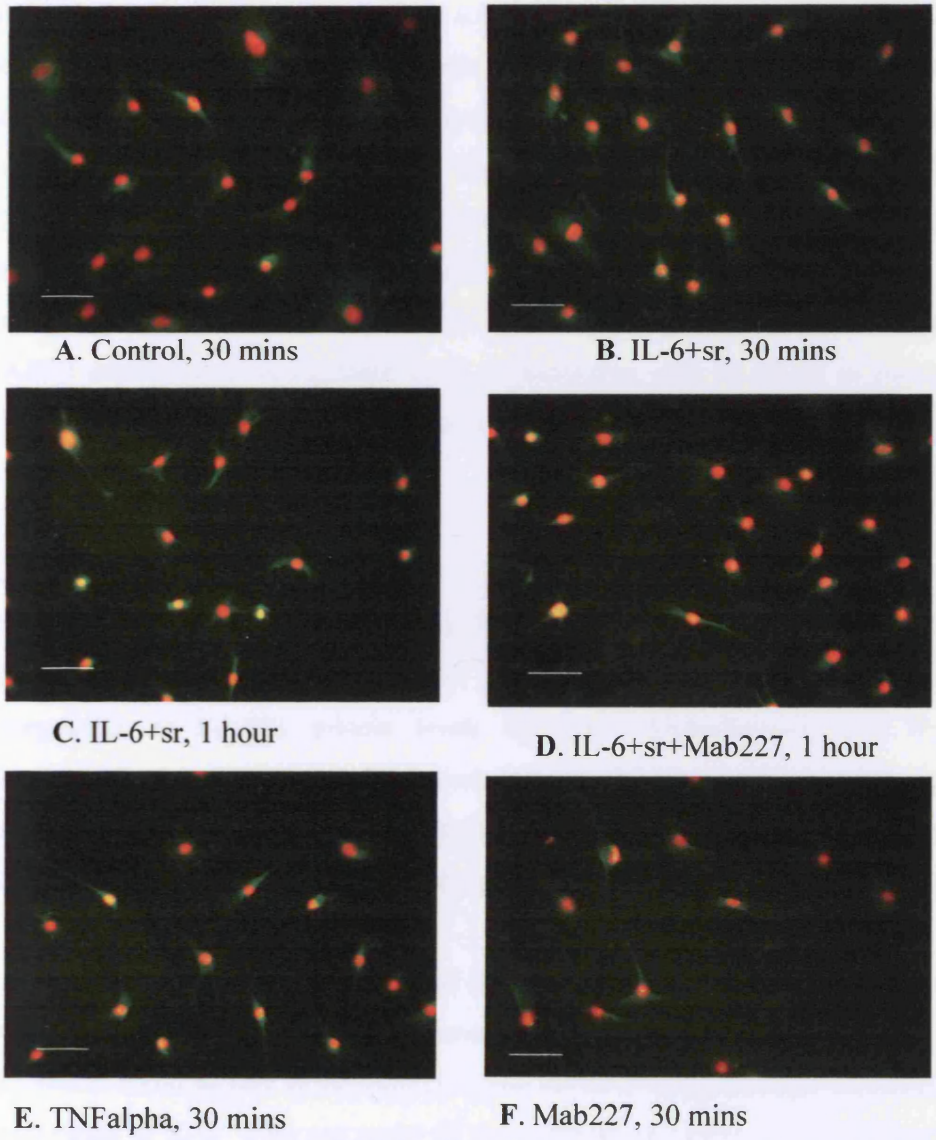
EAAT1 expression appeared to be much less in all normal FLS where the level of expression and localisation of EAAT1 appeared unaffected by treatment with IL-6, TNFα or Mab227 (figure 5.3, compare panels B to D to panel A). Therefore EAAT1 expression in normal FLS is unresponsive to the pro-inflammatory cytokines TNFα and IL-6. These data indicate a decreased level of EAAT1 glutamate transporter expression in normal FLS and a difference in response of normal and RA FLS EAAT1 expression to IL-6 and TNFα treatment.

Figure 5.2 Fluorescence microscopy images reveal that both IL-6 and TNFα increase EAAT1 expression in human RA FLS (patient 5, passage 7) and that the IL-6 effect is prevented by Mab227.



RA FLS were treated for up to 1 hour with TNFα or IL-6 and sIL-6r with or without the inhibitor of IL-6 signalling, Mab227. EAAT1 was detected by a FITC conjugated secondary antibody (green), propidium iodide was used to stain the nuclei (red). EAAT1 expression detected by fluorescence microscopy showed that both proinflammatory cytokines increase EAAT1 expression (panels B, C and E) compared to no treatment (panel A). Mab227 inhibited the induction of EAAT1 expression by IL-6 (panel D) but had no effect when administered alone (panel F). Representative images are shown. (Measure bar = 100μm)

Figure 5.3 Fluorescence microscopy images reveal that expression of EAAT1 in normal FLS (patient 4, passage 7) is not induced subsequent to treatment with IL-6 and TNFα as it is in RA FLS. Neither does Mab227 have an effect on EAAT1 expression by normal FLS.



A. Control, 30 mins

B. IL-6+sr, 30 mins

C. IL-6+sr, 1 hour

D. IL-6+sr+Mab227, 1 hour

E. TNFalpha, 30 mins

F. Mab227, 30 mins

Normal FLS were treated for up to 1 hour with TNFα or IL-6 and sIL-6r with or without the presence of the inhibitor of IL-6 signalling, Mab227. EAAT1 was detected by a FITC conjugated secondary antibody (green), propidium iodide was used to stain the nuclei (red). EAAT1 expression detected by fluorescence microscopy showed that neither pro-inflammatory cytokines alter patterns of EAAT1 expression (panels B, C and E) compared to no treatment (panel A). Mab227 had no effect on EAAT1 expression when administered with IL-6 (panel D) or alone (panel F). Representative images are shown. (Measure bar = 200μm)

5.3.3 Confirmation of regulation of EAAT1 by IL-6 in RA FLS by Western blotting

Western blotting confirmed that EAAT1 expression quantitatively increased upon treatment with IL-6/sIL-6r (a representative gel is shown in figure 5.4a). As expected, no significant differences in EAAT1 protein expression were observed at 0 hours. RA FLS significantly increased EAAT1 expression after 1 hour of IL-6/sIL-6r treatment compared to the untreated cells ($p=0.004$, figure 5.4b). In addition, after 1 hour of IL-6 treatment, RA FLS expressed more EAAT1 compared to the IL-6 treated cells at 0 time ($p=0.003$, figure 5.4b). Treatment with Mab227 significantly reduced EAAT1 expression after 1 and 3 hours compared to treatment with IL-6 alone at each time point ($p=0.000$ and $p=0.021$ respectively) consistent with the immunohistochemistry data (figure 5.2). These data show, for the first time, that EAAT1 expression is upregulated by IL-6 interacting with its sIL-6r in human RA FLS. This indicates a novel control mechanism for the regulation of glutamatergic signalling in synovioyctes.

5.4 Discussion

This study has demonstrated that in RA FLS, the pro-inflammatory cytokine IL-6 can upregulate EAAT1 mRNA expression and has verified this effect by showing upregulation of EAAT1 protein levels by immunohistochemistry and Western blotting. Each experiment was carried out on FLS from different RA patients therefore, the novel effect of IL-6 inducing EAAT1 mRNA or protein expression in RA FLS has been observed in 3 patients (patients 1, 5 and 7).

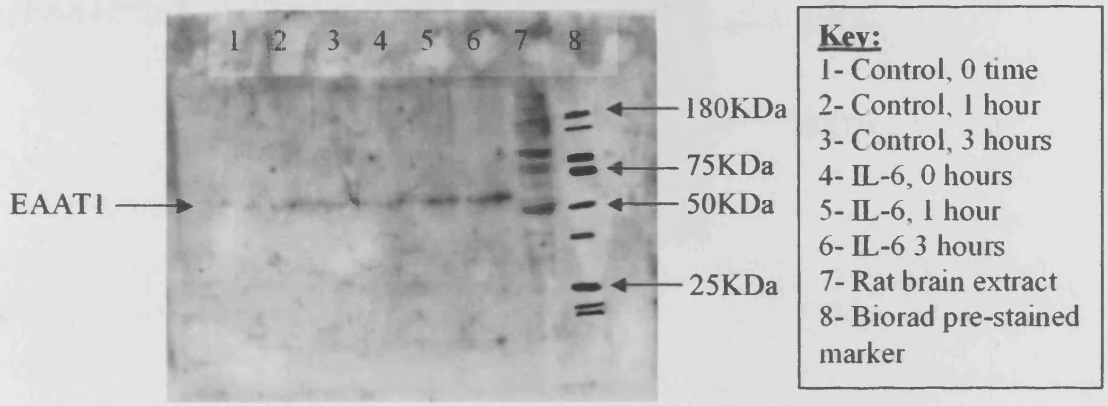
EAAT1 mRNA expression was induced by 1 hour of treatment with IL-6 but after 15 and 24 hours EAAT1 mRNA expression was no longer detected. This transient expression could be due to the FLS lowering the IL-6 levels through degradation or endocytosis of IL-6. I am not aware of any publications where the half life of IL-6, when used in FLS culture, is recorded. Hence, after 15 hours IL-6 may no longer be present in the culture media. In the control cells from this patient (no treatment) EAAT1 mRNA was only detected after 24 hours of culture, which may be due to culture conditions. This indicates that constitutive EAAT1 mRNA expression varies in RA FLS from different patients because results presented in chapter 3 (section 3.2.1.7 and Figure 3.6, panel A) demonstrate constitutive expression of EAAT1 in

Figure 5.4 IL-6 induces EAAT1 protein expression in human RA FLS (patient 7, passage 7).

a. Western blot of protein extracts from human RA FLS. The 69kDa monomer of EAAT1 is indicated by an arrow, higher molecular weight primary specific bands in the rat brain extract represent multimeric forms of GLAST proteins. The 54.4kDa EAAT1a (GLAST-1a equivalent) variant was not detected in these cells.

b. The relative amount of EAAT1 as a fraction of total protein added to each lane shows that EAAT1 protein expression increased significantly after 1hr treatment with IL-6/ IL-6sr and was significantly reduced by Mab 227 compared to treatment with IL-6 at all times. Significant differences are compared to the untreated control at each time point unless otherwise stated by brackets (unpaired 2-sample t-test, *p<0.05, **:p<0.005, SEM error bars, n=3, n=6 for IL-6 only treated cells).

a.



b.

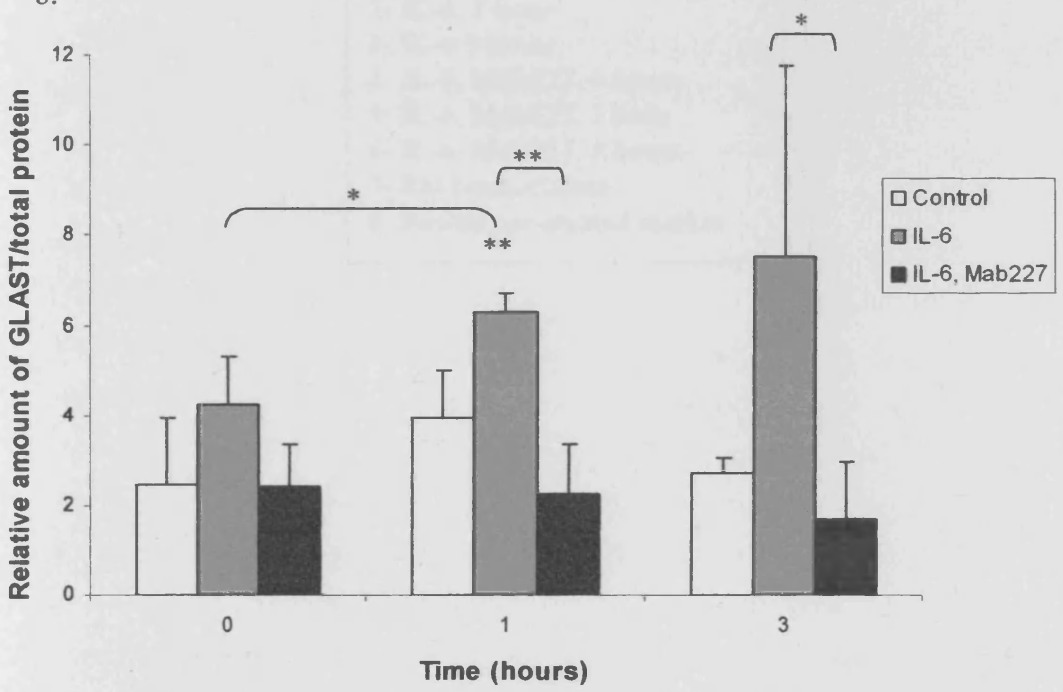
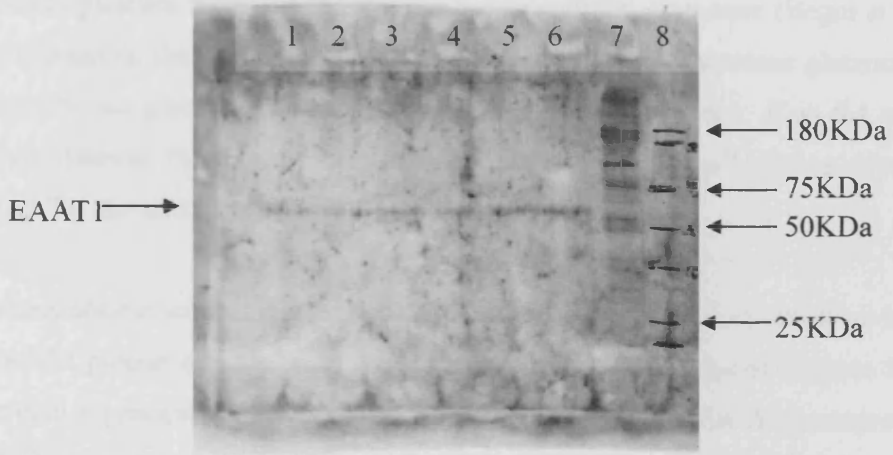


Figure 5.5 Mab227 inhibits IL-6 induced EAAT1 protein expression in human RA FLS (patient 7, passage 7).
Western blot of protein extracts from human RA FLS. The 69kDa monomer of EAAT1 is indicated by an arrow, higher molecular weight primary specific bands in the rat brain extract represent multimeric forms of GLAST proteins. The 54.4kDa EAAT1a (GLAST-1a equivalent) variant was not detected in these cells.



Key:
1- IL-6, 0 hours
2- IL-6, 1 hour
3- IL-6 3 hours
4- IL-6, Mab227, 0 hours
5- IL-6, Mab227, 1 hour
6- IL-6, Mab227, 3 hours
7- Rat brain extract
8- Biorad pre-stained marker

RA FLS from patient 2. Further cases would need to be investigated to confirm this. Constitutive expression of EAAT1 mRNA may depend on medication taken by the patient, disease state or the levels of glutamate in the synovial fluid that the FLS were derived from; however this information for these patients is unknown. Previous studies have shown that the glutamate concentration in RA synovial fluid ranges from 4-608μM (McNearney *et al.* 2000). The effect of IL-6 on EAAT1 mRNA may be mediated via levels of extracellular glutamate since GLAST1 (EAAT1) expression in human platelets increases in response to extracellular glutamate (Begni *et al.* 2005). It is possible that inflammatory signals such as IL-6 may increase glutamate release and activate glutamate uptake in FLS from some RA patients. If an RA patient has high synovial fluid levels of glutamate and hence high cell-surface expression of EAAT1, the induction of EAAT1 by IL-6 may be reduced.

Immunohistochemical studies have shown that after 1 hour IL-6 causes an increase in EAAT1 protein expression as well as mRNA. The IL-6 induced increase in EAAT1 protein expression after 1 hour was inhibited by Mab227. The IL-6 receptor function blocking antibody (Mab227) had no effect on RA or normal FLS EAAT1 expression when administered alone. Thus, abrogation of the increase in EAAT1 expression by IL-6 observed upon treatment with IL-6 and Mab227 is due to Mab227 blocking the interaction of the cytokine with its receptor and preventing interaction with gp130. These data indicate that binding of IL-6 to sIL-6r and the complex to gp130 leads to an increase in transcription of EAAT1 and thus is likely to increase glutamate transport in FLS. The pathway or mechanism through which this regulation occurs needs to be determined (see section 9.3). For example, IL-6 can activate phosphatidylinositol 3 kinase (Chung *et al.* 2000) and this in turn has been shown to regulate EAAT1 expression (Boehmer *et al.* 2003, reviewed in Kim *et al.* 2003). IL-6 can also activate the JAK/STAT pathway (see section 1.4.1.3.1.1) and the EAAT1 promoter contains GC-box motifs that have the potential for binding STAT transcription factors (Kim *et al.* 2003). Alternatively, IL-6 may increase EAAT1 expression indirectly by inducing glutamate release. This may increase EAAT1 expression and also lead to a positive feedback mechanism to up-regulate IL-6 production (data in chapter 4 shows that extracellular glutamate up-regulates FLS production of IL-6 in some RA patients). If IL-6 alters extracellular glutamate levels, this could lead to increased EAAT1 expression via activation of NMDA receptors as

has been demonstrated in primary co-cultures of astrocytes and neurons (Schlag *et al.* 1998). Results of the studies in chapter 3 demonstrate that both RA and normal FLS expressed the NMDA-NR1 subunit of NMDA receptors (section 3.2.1.7).

Immunohistochemistry also demonstrated that TNFα increases EAAT1 protein levels in RA FLS. TNFα is a pro-inflammatory cytokine also elevated in RA synovial fluid. This effect of TNFα conflicts with the study by Liao *et al.* who demonstrated that TNFα causes a decrease in glutamate uptake activity in astrocytes although it is unclear which EAAT subtype the effect is caused by (Liao *et al.*2001). Studies in chapter 3 have shown that RA FLS express EAAT1 and EAAT3 (section 3.3.7) therefore TNFα may also alter EAAT3 expression in RA FLS.

TNFα is a well known inducer of IL-6 in FLS (Piecyk and Anderson 2001, Harigai *et al.* 1991). Therefore, the effect of TNFα on EAAT1 protein expression could also be brought about directly by TNFα increasing IL-6 levels which in turn induce EAAT1 expression. Alternatively TNFα could be acting on EAAT1 expression directly. TNFα increases Na^+-dependent glutamate uptake by monocyte derived macrophages (Rimaniol *et al.* 2000). Therefore several mechanisms of EAAT1 regulation by inflammatory cytokines have been postulated:
i) IL-6 via intracellular signalling pathways directly increases EAAT1 expression,
ii) IL-6 increases extracellular glutamate which in turn increases EAAT1 expression,
iii) TNFα via intracellular signalling pathways directly increases EAAT1 expression,
iv) TNFα increases IL-6 production which (via i or ii above) increases EAAT1 expression.

Further work is necessary to determine the exact mechanism. Treating FLS with TNFα, with or without Mab227 and measuring EAAT1 protein expression would determine whether TNFα is mediating its effects via IL-6. If FLS took longer to respond to TNFα with an increase in EAAT1 expression, this would indicate TNFα was acting via IL-6. Specifically inhibiting and activating the signalling pathways that TNFα and IL-6 activate would determine the exact signalling mechanism that increases EAAT1 expression by these cytokines.

Differences in localisation of EAAT1 protein expression were observed between treatments in RA FLS. In untreated RA FLS EAAT1 appeared to be equally distributed within the cytoplasm; however stimulation with TNFα or IL-6 caused concentrated pools of EAAT1 to be observed in the cytoplasm and around the nucleus. Whilst, the exact location of expression may only be fully determined using confocal microscopy, it is possible that cytoplasmic pools of EAAT1 are present. This is consistent with the storage and trafficking of transporters observed in astrocytes (Duan *et al.* 1999). The method used in this study only observes the cells from above and therefore expression in the cytoplasm and plasma membrane cannot be differentiated.

Immunohistochemistry also indicated that EAAT1 protein expression by normal FLS is unresponsive to both IL-6 and TNFα whereas RA FLS were responsive to IL-6. This indicates a potential difference in the way extracellular glutamate levels are regulated in FLS in the RA diseased state. The IL-6 induced EAAT1 expression may be a response to lower the observed increase in extracellular glutamate in RA synovial fluid. In fact it has previously been reported that RA and normal FLS express different proteins in response to TNFα (Ando *et al.* 2003).

Differences in constitutive expression of EAAT1 protein were noted between RA and normal FLS. RA FLS appeared to express higher levels of EAAT1 protein than normal FLS thus this may in response to the elevated glutamate levels in RA synovial fluid (McNearney *et al.* 2000). However, the method used for comparison of constitutive expression of EAAT1 protein between normal and RA FLS is not quantitative because staining of the cells was carried out at different times. In order to make a conclusive comparison both normal and RA FLS should have been cultured, treated and immunostained concurrently. It should be noted that exposure times and microscope settings were standardised in this study.

Western blotting confirmed the immunohistochemical data in that IL-6 quantitatively increased EAAT1 protein expression by RA FLS after 1 hour by 33%. This increase in EAAT1 protein expression was significantly inhibited by Mab227 thus correlating with the immunohistochemical data and indicating that the response is mediated via the IL-6 receptor and gp130. Whilst immunohistochemistry indicated that this

response apparently did not occur in normal cells, this was not tested by Western blotting.

IL-6 may therefore be decreasing the high levels of extracellular glutamate observed in the synovial fluid of RA patients through increasing expression of GLAST-1. The effect of this reduced glutamate on the phenotype of the cells of the synovial joint however is unknown although data presented in chapters 4 and 6 indicate that pro-MMP2, TIMPs and IL-6 levels are influenced by changes in extracellular glutamate.

If glutamate is acting on RA FLS through glutamate receptors (NMDA-NR1 or KA1) in an anti-inflammatory way then IL-6 would be blocking this interaction by causing an increase in EAAT1 expression and thus decreasing extracellular glutamate levels. This however, is assuming that the increase in EAAT1 expression is causing more glutamate to be taken up into the RA FLS. High levels of extracellular glutamate are excitotoxic to neurons, however data presented in chapter 4 shows that up to 2mM glutamate is not toxic to FLS (see section 4.3.1). GLAST1 does not just transport glutamate into the cell; the direction of transport depends on many factors such as pH and the ion gradients of Na^+, K^+ and H^+ (Danbolt 2001). Therefore the IL-6-induced expression of EAAT1 may elevate extracellular glutamate levels further if glutamate transport were reversed and thus may increase glutamate receptor activation in RA FLS (NMDA and KA receptors, see section 3.2.1.7).

GLAST1 has also been shown to act as a receptor and activate the MEK/ERK pathway (Abe and Saito 2001). This receptor function of EAAT1 may therefore affect gene expression within RA FLS. Use of specific inhibition of the glutamate transporter/receptor or activation of the MEK/ERK pathway are therefore required to predict the effect that increased EAAT1 expression by IL-6 is having on FLS phenotype. Needless to say regulation of EAAT1 expression would be affecting the way glutamate interacts with the receptors that are expressed by RA FLS (chapter 3, NMDA and KA receptors).

Finally it would be interesting to see whether IL-6 affects expression of the other glutamate transporters expressed in normal and RA FLS (EAAT2 and EAAT3)

because each transporter has different properties and up-regulation of one transporter may be compensated by down-regulation of another.

To conclude, data presented in this chapter demonstrate that EAAT1 mRNA and protein expression increases in response to IL-6 and EAAT1 protein increases in response to TNFα. These effects are likely to be of significance because these cytokines play a major role in RA pathogenesis.

Chapter 6 The effect of glutamate on the degradative phenotype of fibroblast-like synoviocytes

6.1 Introduction

Having demonstrated that glutamate alters the pro-inflammatory phenotype of fibroblast-like synovioyctes (FLS) (see chapter 4) the effect of glutamate on the degradative phenotype of FLS was investigated. The primary cause of cartilage and bone destruction in arthritis is the enhanced expression and activation of proteases that degrade the collagenous and proteoglycan component of the ECM. In RA, FLS produce metalloproteinases (see section 1.5.1.3) which degrade the ECM, facilitate invasion of pannus and therefore contribute to joint destruction. MMPs are likely to be the most important matrix degrading enzymes in RA (Vincenti and Brinckerhoff 2002).

The levels of MMPs 2 and 9 are elevated in the synovial fluid of RA patients and contribute to the bone and cartilage destruction that accompanies this disease (Yoshihara *et al.* 2000). In addition to MMPs, TIMP1 and TIMP2 levels are also elevated in RA synovial fluid compared to OA synovial fluid (Klimiuk *et al.* 2002). TIMPs are the natural inhibitors of MMPs (see section 1.5.2) and form a 1:1 complex with MMPs to inhibit them. If an imbalance of MMPs and TIMPs occurs such that MMP levels exceed TIMP levels, the cartilage and bone starts to degrade (Tchetverikov *et al* 2004).

Links between glutamate and MMP and TIMP production and regulation are discussed in section 1.6.5.6. Since MMPs and TIMPs are increased in RA, mediate important processes in the disease pathology (degradation, cytokine activation) and are regulated by glutamate receptor activation in other cell types this study investigated the effect of glutamate on MMPs 2 and 9 and TIMPs and the role of ionotropic glutamate receptors and EAATs in regulation of MMPs 2 and 9 and TIMP production in normal and RA FLS.

6.2 Methods

6.2.1 Cell culture treatments

Media from the treated RA and normal FLS detailed in chapter 4 were used in this MMP and TIMP release study (see 4.2.1, 4.2.2 and 4.2.3). Briefly, FLS were treated with a range of glutamate concentrations (50μM-2000μM) to determine the effect of extracellular glutamate on MMPs 2 and 9 and TIMP production (release into the media). The glutamate transporter inhibitors, *t*PDC (100μM) and TBOA (100μM) were used to investigate the effect of blocking glutamate transport on MMP and TIMP production by FLS. In addition the effects of the glutamate receptor antagonists, MK801 (100μM, non-competitive NMDA receptor antagonist), D-AP5 (10μM competitive NMDA receptor antagonist), CFM2 (10μM, non-competitive AMPA receptor antagonist) and NBQX (150μM, competitive AMPA and KA receptor antagonist) on MMP and TIMP release were investigated. It should be noted that none of these treatments were toxic to the FLS (see section 4.3.1) and concentrations used were based on published data (see section 4.2.2 and 4.2.3). Levels of pro and active MMPs 2 and 9 in the media from treated cells were determined by gelatin zymography (section 2.9.1, see figure 6.4 for representative gel). TIMP levels were measured by reverse zymography (section 2.9.2, see figure 6.5 for representative gel). Quantification of MMP and TIMP levels was achieved by comparison to a standard (conditioned media, section 2.9.3) loaded on each gel. A maximum of 10 samples were loaded on each gel. Treatments were repeated on replicate cultures between 3 and 6 times, statistical comparisons were carried out as described in section 2.13.

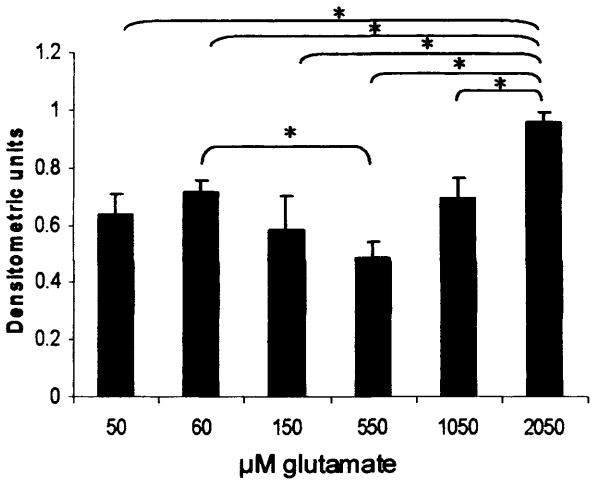
Levels of MMPs could be compared between patients by accounting for the relative amounts of sample media loaded onto each gel.

6.3 Results

It should be noted that in all treatments pro-MMP9, active MMP9 and active MMP2 were expressed at such low levels by FLS that they could not be quantified by gelatin zymography. In addition, only TIMP1 and TIMP2 were observed to be expressed.

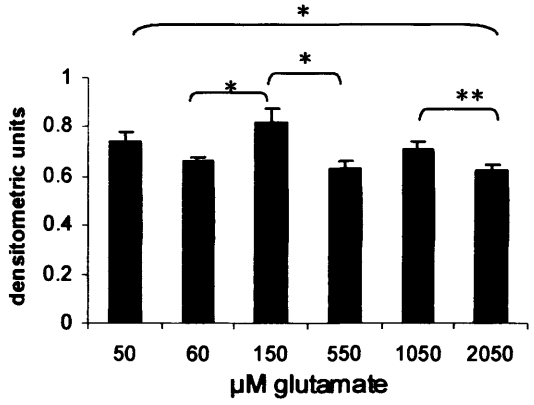
Figure 6.1 The effect of glutamate on pro-MMP2 levels produced by RA FLS
Glutamate had different effects on pro-MMP2 levels produced by RA FLS taken from different patients. (*:$p<0.05$, **:$p<0.005$, n=6, n=3 for 60μM samples, SEM error bars).

Patient 3: RA, Female, aged 62, passage 5



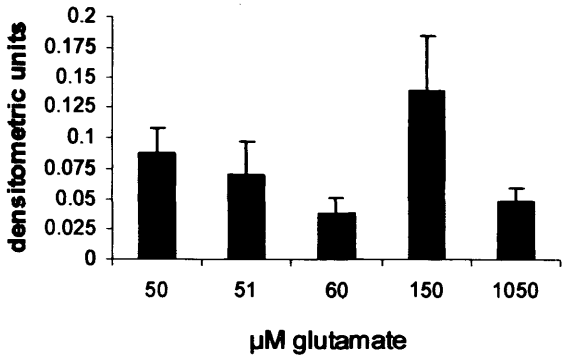
A: Treatment of RA FLS (patient 3, passage 5) with 2050μM glutamate caused an increase in pro-MMP2 production. A significant decrease in pro-MMP2 production was observed between 60μM and 550μM glutamate (2-sample t-test, p=0.016).

Patient 3: RA, Female, aged 62, passage 7

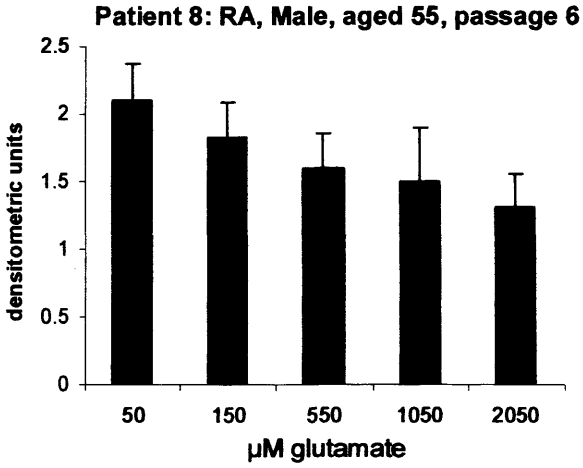


B: Treatment of RA FLS (patient 3, passage 7) with 2050μM glutamate caused a decrease in pro-MMP2 production compared to the control (50μM, paired t-test, p=0.022). However an increase was observed between 60μM and 150μM glutamate (paired t-test, p=0.042). A significant decrease in pro-MMP2 occurred between 150μM and 550μM glutamate (paired t-test, p=0.043).

Patient 2: RA, Female, aged 56, passage 6

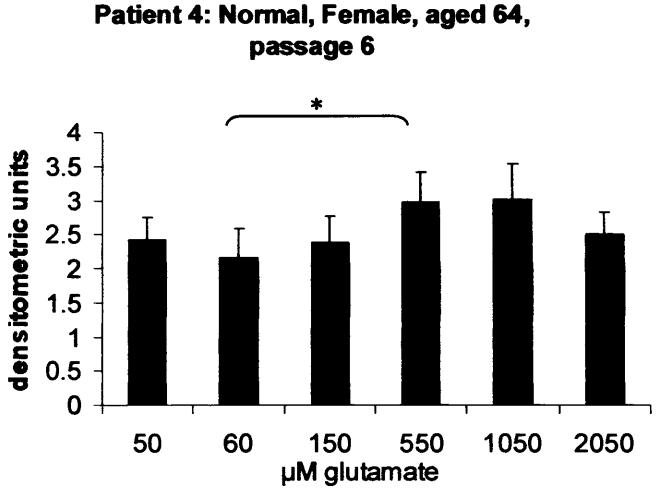


C: Treatment of RA FLS (patient 2, passage 6) with up to 1050μM revealed that glutamate had no significant effects on pro-MMP2 release.

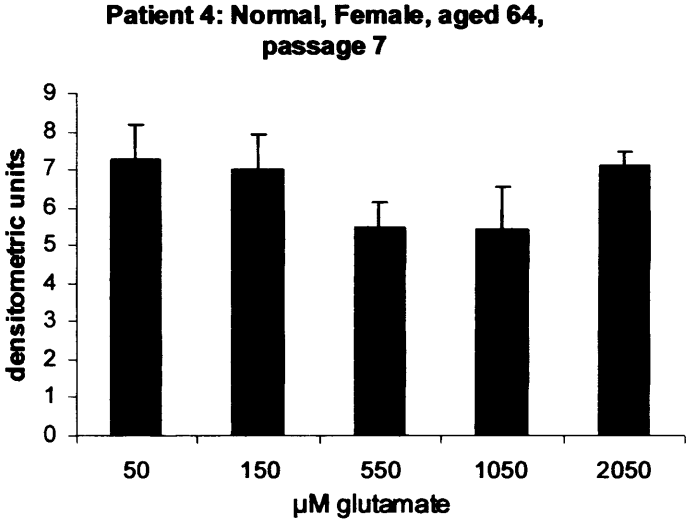


D: Treatment of RA FLS (patient 8, passage 6) with up to 2050μM revealed that glutamate had no significant effects on pro-MMP2 release.

Figure 6.2 The effect of glutamate on pro-MMP2 levels produced by normal FLS
Pro-MMP2 production by normal FLS from patient 4 demonstrated minimal response to extracellular glutamate at passage 6 and 7 (figure A and B respectively). Increasing extracellular glutamate caused an increase in pro-MMP2 production at passage 6 (*:$p<0.05$).



A: Treatment of RA FLS (patient 4, passage 6) with 550µM glutamate caused an increase in pro-MMP2 production compared to treatment with 60µM glutamate (paired t-test, $p=0.018$, $n=6$). No other effects were observed.



B: Treatment of RA FLS (patient 4, passage 7) with up to 2050µM glutamate had no significant effects on pro-MMP2 production.

6.3.1 The effect of glutamate on MMP and TIMP release by fibroblast-like synoviocytes

Glutamate concentration had varying effects on pro-MMP2 release in RA synoviocytes (figure 6.1). On patients 2 and 8 glutamate had no significant effect on pro-MMP2 release (figure 6.1 c and d). However glutamate influenced the release of pro-MMP2 by cells from patient 3, but had different effects on cells from different passages. At passage 5, 2050μM glutamate caused a significant increase in pro-MMP2 production by RA FLS when compared to all other glutamate concentrations (figure 6.1a). However, at passage 7 these RA cells significantly decrease pro-MMP2 production at 2050μM glutamate (figure 6.1b).

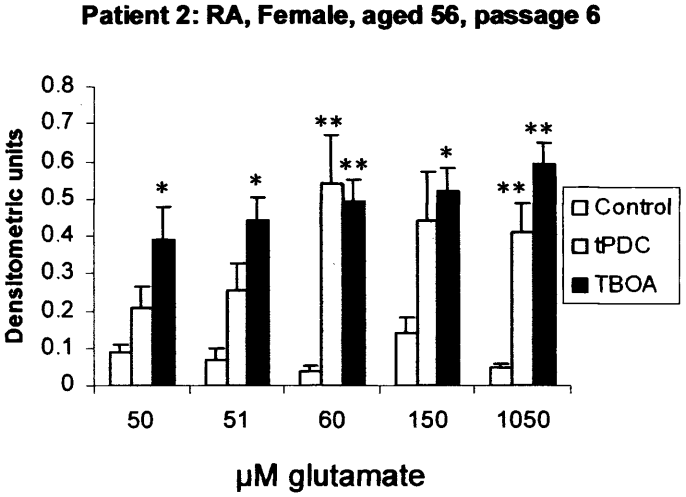
Higher glutamate concentrations (550μM) in normal FLS caused an increase in pro-MMP2 production at passage 6 (figure 6.2a) but had no effect at passage 7 (figure 6.2b).

Since MMP levels were normalised to the same standard on all zymograms, variations in the constitutive levels (at 50μM glutamate) of pro-MMP2 could be compared across patients. The pro-MMP2 produced by RA FLS from patient 3 was more than 20 times lower than the levels produced by RA FLS from patient 8. Furthermore, levels of pro-MMP2 were higher in normal FLS (2.5-7 relative densitometric units) compared to RA FLS (0.08-2 relative densitometric units).

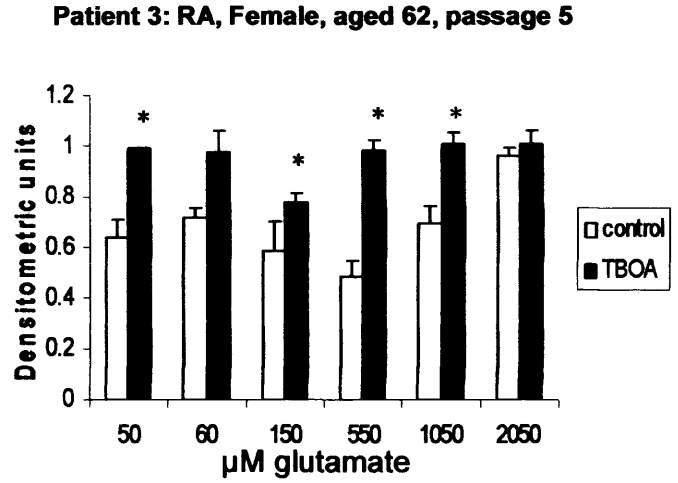
TIMP1 and TIMP2 were detected by reverse zymography in both RA and normal synoviocytes. However, no effect of extracellular glutamate concentration was observed on TIMP levels (data not shown).

Figure 6.3 The effect of glutamate transporter inhibitors on pro-MMP2 production by RA FLS.

15 hours treatment with glutamate transporter inhibitors (*t*PDC or TBOA) significantly increased pro-MMP2 production by RA FLS from patient 2 and 3 (figures A and B respectively) (*:$p<0.05$, **:$p<0.005$, n=6, n=3 for 60μM samples, SEM error bars).

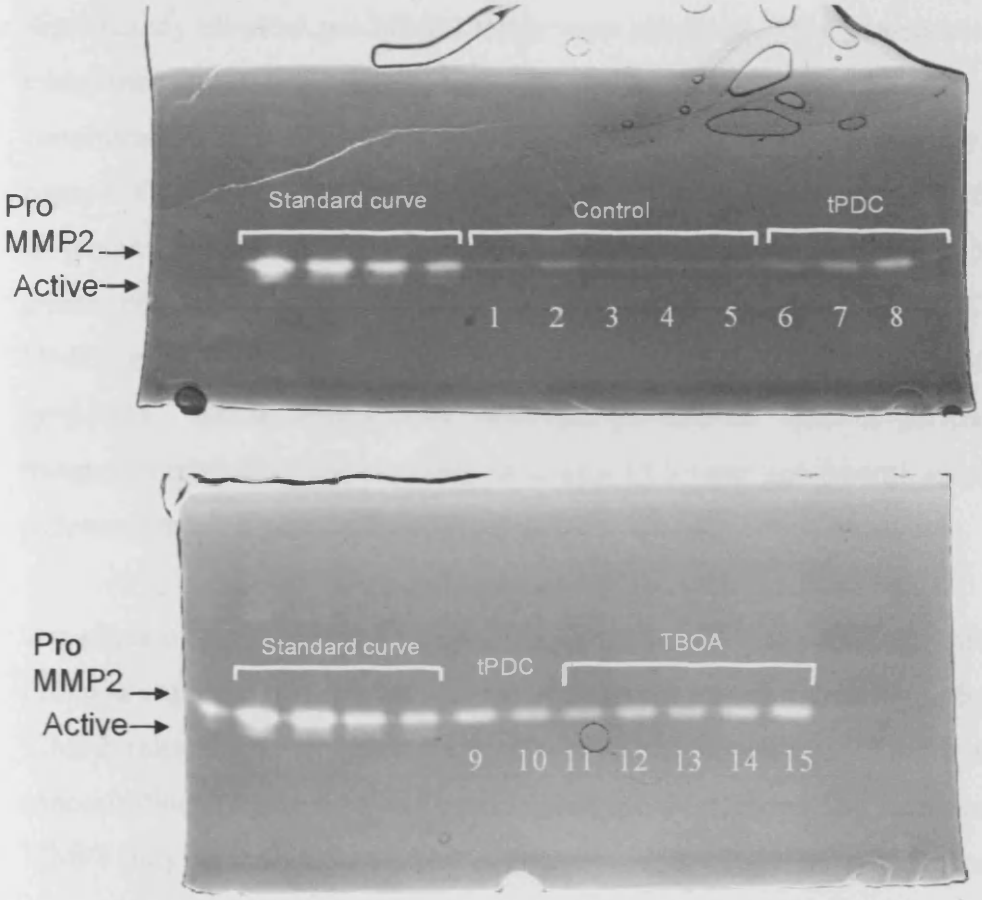


A: Treatment of RA FLS (patient 2, passage 6) with TBOA caused an increase in pro-MMP2 production at all glutamate concentrations compared to the control (no TBOA). *t*PDC increased pro-MMP2 production only in the presence of 60μM ($p=0.002$) and 1050μM glutamate ($p=0.001$). This data set was transformed (log_{10}) prior to statistical analysis with a 2-sample t-test.



B: Treatment of RA FLS (patient 3, passage 5) with TBOA caused an increase in pro-MMP2 production at all glutamate concentrations except 60μM and 2050μM compared to the control (no TBOA). (paired t-test)

Figure 6.4 Representative zymograms showing that glutamate transporter antagonists increase production of pro-MMP2 by RA FLS from patient 2.



Key:

1-50μM glutamate
2-51μM glutamate
3-60μM glutamate
4-150μM glutamate
5-1050μM glutamate
6-50μM glutamate, *t*PDC
7-51μM glutamate, *t*PDC
8-60μM glutamate, *t*PDC
9-150μM glutamate, *t*PDC
10-1050μM glutamate, *t*PDC
11-50μM glutamate, TBOA
12-51μM glutamate, TBOA
13-60μM glutamte, TBOA
14-150μM glutamte, TBOA
15-1050μM glutamate, TBOA

6.3.2 The effect of inhibiting glutamate transporters on the release of pro-MMP2 and TIMPs by FLS

The effect of blocking EAATs 1 to 5 glutamate transporters on MMP release was also determined. EAAT inhibitors did not induce pro-MMP9 nor active MMP2 and MMP9. Significantly elevated pro-MMP2 levels were observed in the presence of the glutamate transporter inhibitors *t*PDC and TBOA in RA synoviocytes at all glutamate concentrations in patient 2 RA FLS (figure 6.3 a, representative zymogram is shown in figure 6.4) and at 50, 150, 550 and 1050μM by RA FLS from patient 3 (figure 6.3b). At all glutamate concentrations the non-transportable inhibitor of EAATs1-5, TBOA, had a greater effect than the transportable inhibitor of EAATs1-4 *t*PDC. *t*PDC increased pro-MMP2 production only in the presence of 60μM ($p=0.002$) and 1050μM glutamate ($p=0.001$). These trends were observed in RAFLS from 2 patients. Glutamate transporter inhibition had no effect on normal FLS (data not shown) which constitutively expressed much higher levels of pro-MMP2.

The effect of EAATs 1 to 5 glutamate transporter inhibitors on TIMP release by human FLS was explored (representative reverse zymogram is shown in figure 6.5). TIMP1 and TIMP2 release was significantly increased in the presence of TBOA at all glutamate concentrations (figure 6.6a and b respectively). *t*PDC caused an increase in TIMP1 and TIMP2 only in RA FLS cultured in 60μM – 1050μM glutamate. Normal synoviocytes released both TIMP1 and 2 but glutamate transporter inhibitors had no effect on levels of expression (data not shown).

6.3.3 The effect of ionotropic glutamate receptor antagonists on the release of pro-MMP2 and TIMPs by FLS

To determine whether the effect of glutamate transporter inhibitors on pro-MMP2 release reflected increased activation of ionotropic NMDA receptors due to a decrease in glutamate uptake, FLS cultures were treated with DAP-5 (competitive NMDA receptor inhibitor) and MK801 (non-competitive NMDA receptor inhibitor) at various glutamate concentrations for 15 hours. At all glutamate concentrations, except 150μM, MK801 significantly increased pro-MMP2 production (figure 6.7a). The competitive inhibitor,

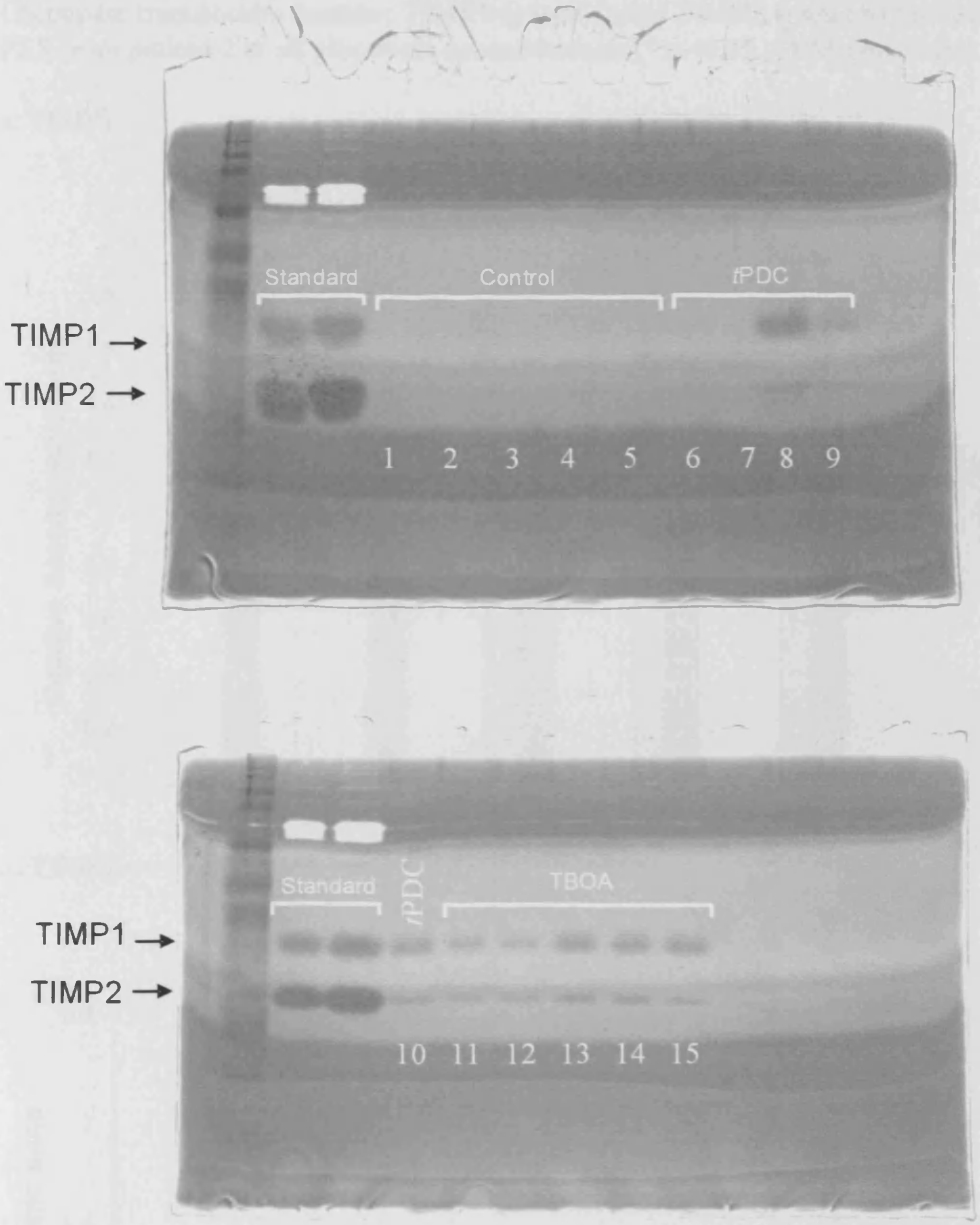
DAP-5 had no significant effect on pro-MMP2 release by RA synoviocytes at any glutamate concentration.

MK801 and DAP-5 had no effect on pro-MMP2 release by normal synoviocytes (figure 6.7b). Furthermore, the AMPA/KA receptor inhibitors, CFM2 and NBQX had no effect on pro-MMP2 release by RA FLS (data not shown, this was not tested in normal FLS). TIMP1 and TIMP2 release by RA synoviocytes did not change at any glutamate concentration when treated with inhibitors of the ionotropic receptors (MK801, D-AP-5, NBQX or CFM2, see figures 6.8 and 6.9 for representative zymograms). This was not tested in normal FLS.

6.4 Discussion

Cytokines and growth factors regulate the expression of MMPs in many cell types and it has previously been shown that cytokine induced increases in expression of MMPs may lead to cartilage degradation (Imai *et al.* 1997, Gilbert *et al.* 2002, 2004). The levels of MMPs 2 and 9 are elevated in the synovial fluid of RA patients (Yoshihara 2000) and contribute to cartilage degradation that accompanies RA. However, the active MMP2 and pro and active MMP9 levels produced by the FLS in this study were too low to quantify. This concurs with previous studies showing that RA FLS secrete more MMP2 than MMP9, and very little active gelatinases (Smolian *et al.* 2001). Furthermore, only pro-MMP2 and not active MMP2 has been detected in RA synovial fluid (Giannelli *et al.* 2004). In order to determine whether the FLS used in this study were expressing these latent and active MMPs and TIMPs, a more sensitive method of detection such as ELISA could be used, or samples should be concentrated.

Figure 6.5 Representative reverse zymograms showing that glutamate transporter antagonists increase production of TIMP1 and TIMP2 by RA FLS from patient 2.

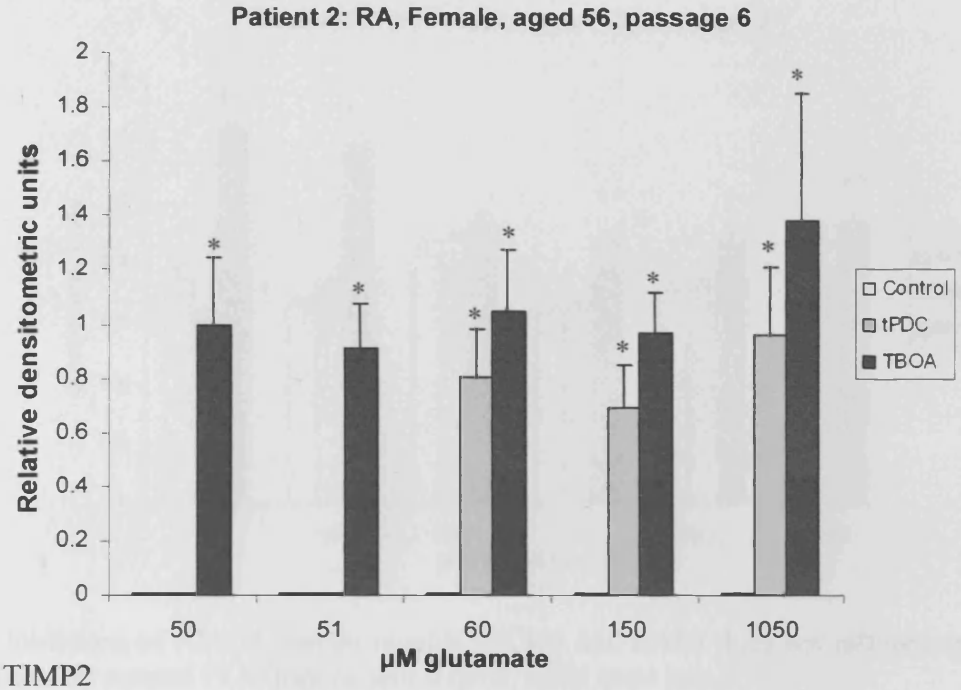


Key:

1-50μM glutamate
2-51μM glutamate
3-60μM glutamate
4-150μM glutamate
5-1050μM glutamate
6-50μM glutamate, *t*PDC
7-51μM glutamate, *t*PDC
8-60μM glutamate, *t*PDC
9-150μM glutamate, *t*PDC
10-1050μM glutamate, *t*PDC
11-50μM glutamate, TBOA
12-51μM glutamate, TBOA
13-60μM glutamte, TBOA
14-150μM glutamte, TBOA
15-1050μM glutamate, TBOA

Figure 6.6: The effect of glutamate transporter inhibitors on TIMP 1 and 2 production by RA FLS
Glutamate transporters increase TIMP1 (panel a) and TIMP2 (panel b) production by RA FLS from patient 2 at all glutamate concentrations (*:$p<0.05$, SEM error bars).

a. TIMP1



b. TIMP2

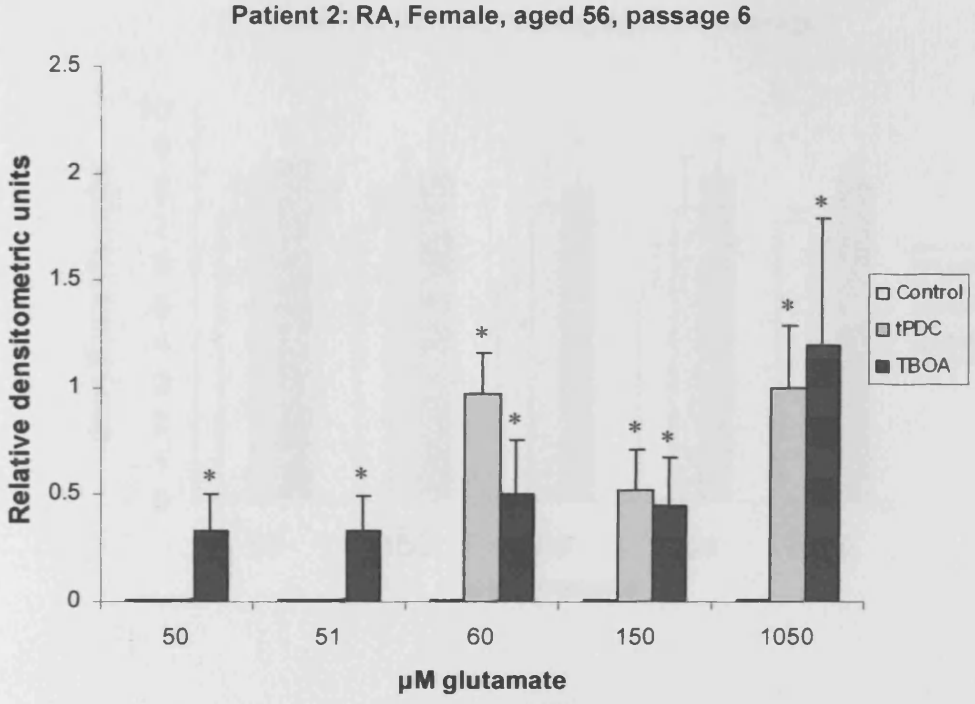
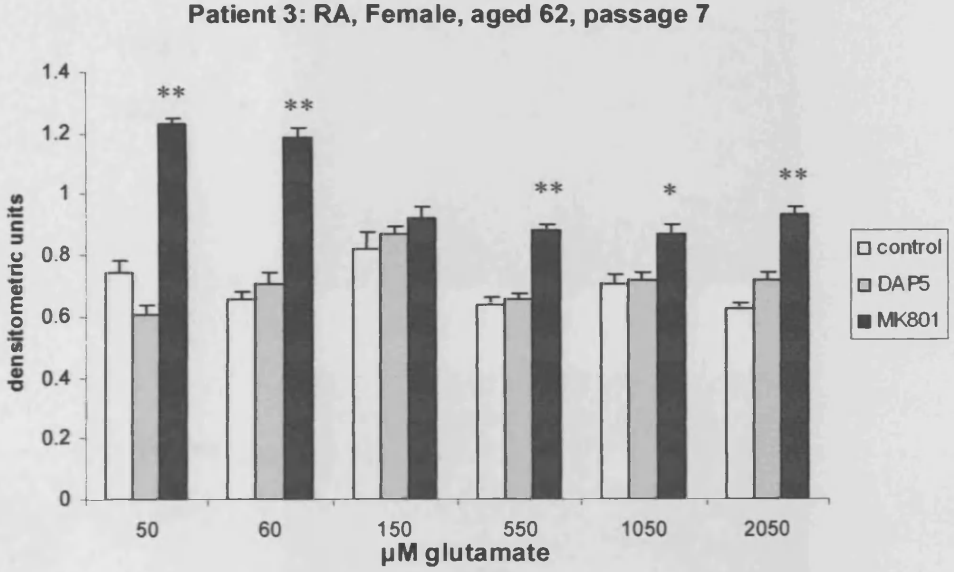


Figure 6.7 The effect of NMDA glutamate receptor inhibitors on pro-MMP2 production by RA and normal FLS

a. The non-competitive NMDA receptor antagonist MK801 (black bars) caused a significant increase in proMMP2 production at all glutamate concentrations except 150μM. D-AP5 (grey bars) had no effect on pro-MMP2 release by RA FLS from patient 3 (*:$p<0.05$, **$p<0.005$ n=6, SEM error bars).



b. Inhibition of NMDA receptors with MK801 and DAP5 does not influence pro MMP2 release by normal FLS from patient 4 (n=6, SEM error bars).

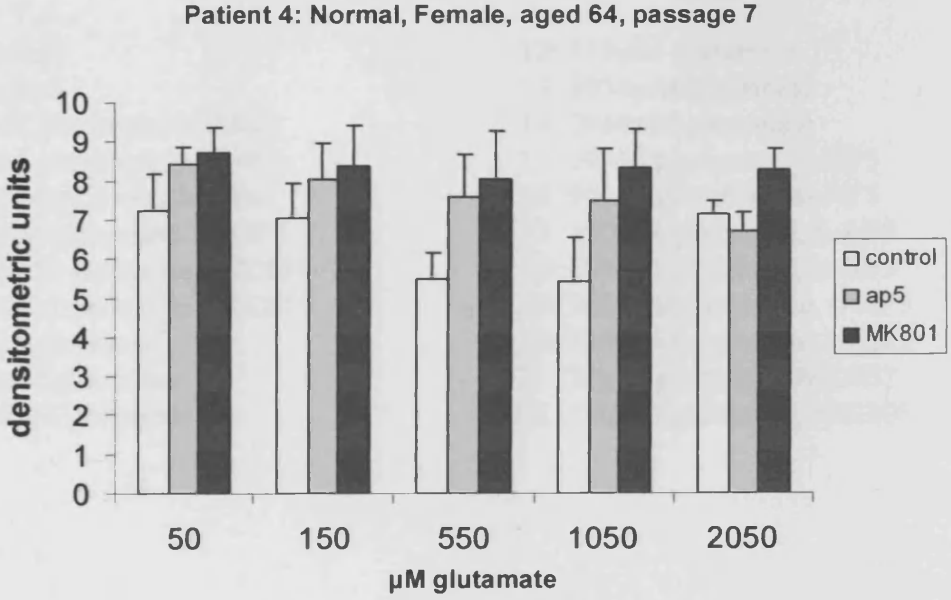
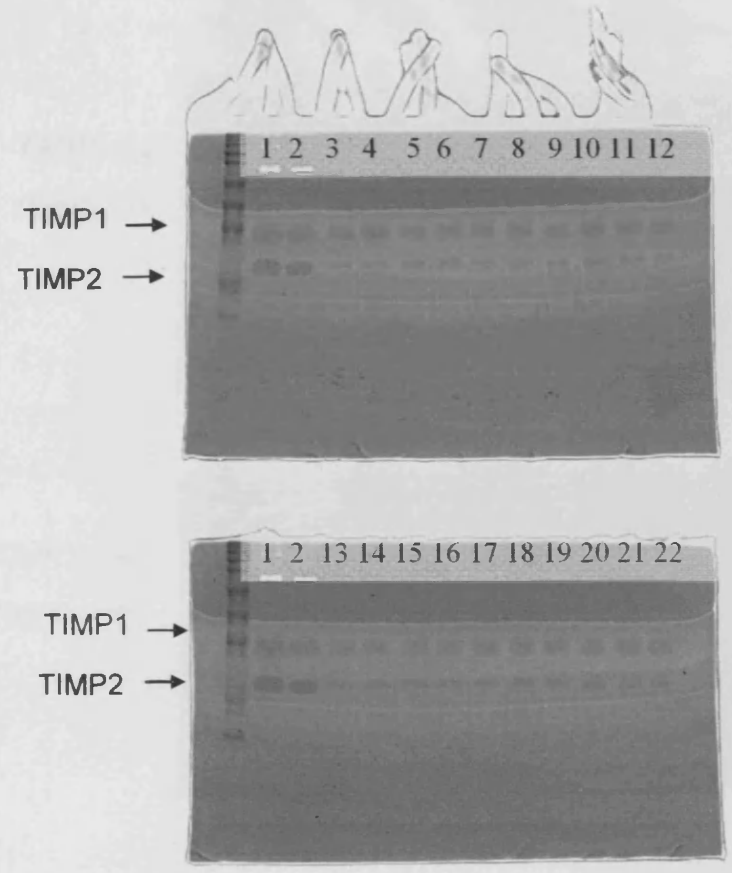


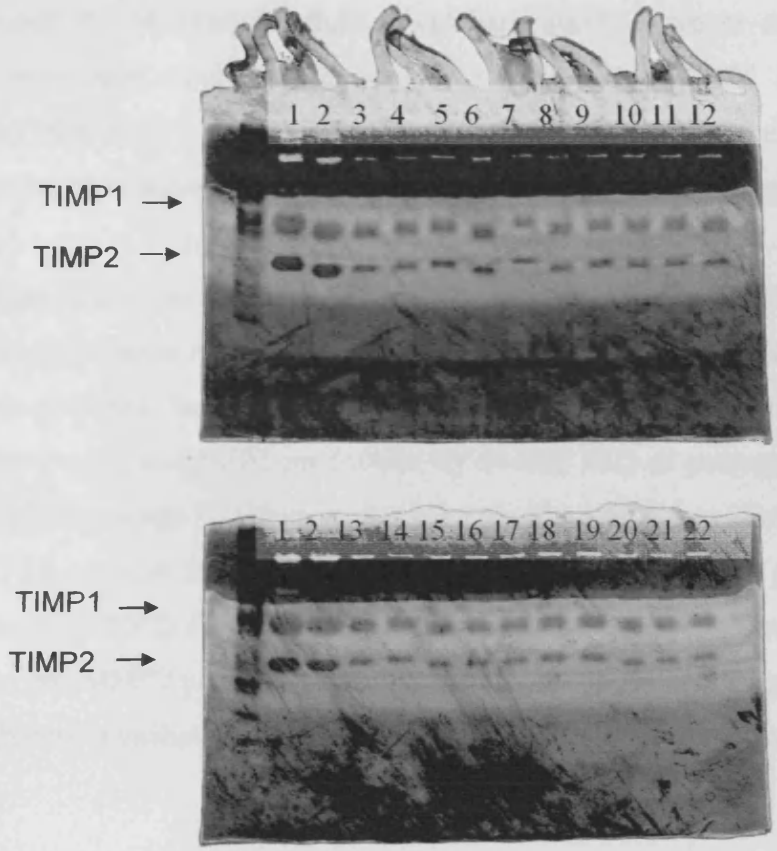
Figure 6.8 Representative reverse zymograms showing that glutamate receptor antagonists (D-AP5 and MK801) have no effect on the production of TIMP1 and TIMP2 by RA FLS from patient 3.



Key

1: Standard
2: Standard
3: 50μM glutamate, MK801
4: 60μM glutamate, MK801
5: 150μM glutmate, MK801
6: 550μM glutamate, MK801
7: 1050μM glutamate, MK801
8: 2050μM glutamate, MK801
9: 50μM glutamate
10: 60μM glutamate
11: 150μM glutamate
12: 550μM glutamate
13: 1050μM glutamate
14: 2050μM glutamate
15: 50μM glutamate, D-AP5
16: 60μM glutamate, D-AP5
17: 150μM glutamate, D-AP5
18: 550μM glutamate, D-AP5
19: 1050μM glutamate, D-AP5
20: 2050μM glutamate, D-AP5
21: 50μM glutamate, MK801
22: 150μM glutamate, MK801

Figure 6.9 Representative reverse zymograms showing that glutamate receptor antagonists (CFM2 and NBQX) have no effect on the production of TIMP1 and TIMP2 by RA FLS from patient 8.



Key:

1: Standard
2: Standard
3: 50μM glutamate, CFM2
4: 150μM glutamate, CFM2
5: 550μM glutamate, CFM2
6: 1050μM glutamate, CFM2
7: 2050μM glutamate, CFM2
8: 50μM glutamate, NBQX
9: 150μM glutamate, NBQX
10: 550μM glutamate, NBQX
11: 1050μM glutamate, NBQX
12: 2050μM glutamate, NBQX
13: 50μM glutamate
14: 150μM glutamate
15: 550μM glutamate
16: 1050μM glutamate
17: 2050μM glutamate
18: 50μM glutamate, CFM2
19: 150μM glutamate, CFM2
20: 550 μM glutamate, CFM2
21: 1050μM glutamate, CRM2
22: 2050μM glutamate, CFM2

Constitutive levels of pro-MMP2 were higher in normal FLS (2.5-7 relative densitometric units) compared to RA FLS levels (0.075-2 relative densitometric units). This was surprising because levels of pro-MMP2 have been shown to be elevated in RA synovial fluid compared to OA synovial fluid (Yoshihara 2000) however no comparisons to normal synovial fluid could be found in the literature. TIMP1 and TIMP2 were expressed by RA FLS. TIMP3 expression was not observed in any FLS, however previous studies have shown TIMP3 to be expressed by RA FLS (Jeong *et al.* 2004).

The responses of normal and RA FLS to extracellular glutamate with regard to pro-MMP2 production varied between patients and passage. The normal FLS were much less responsive to glutamate than RA FLS; only 550μM (when compared to 60μM glutamate) glutamate increased pro-MMP2 production by normal FLS at passage 6 and no effects were observed at passage 7. Other studies have demonstrated that MMP levels produced by normal FLS are less responsive to cytokines compared to MMP production by RA FLS (Cheon *et al.* 2002) demonstrating that the two cell types respond differently. The difference in pro-MMP2 production between RA and normal FLS suggests that RA FLS have a heightened sensitivity to the elevated glutamate found in the synovial fluid of RA patients.

The effect of glutamate on pro-MMP2 release by RA synoviocytes was variable between patients and cells at different passages from the same patient. FLS from 2 RA patients did not respond to extracellular glutamate at all. The variability observed between RA patients was also observed with regard to IL-6 production (see chapter 4) and may be due to a number of reasons. For example age, sex and treatments that the patients were receiving may all affect how the FLS respond to glutamate. Alternatively, because RA FLS are exposed to higher levels of glutamate *in vivo*, constitutive expression of glutamate receptors and transporters may be increased, therefore increasing these cells responsiveness to glutamate. These experiments therefore need to be repeated on FLS from more patients in order for any firm conclusions to be drawn.

At passage 5 increased extracellular glutamate caused an increase in pro-MMP2 production by FLS from patient 3, however at passage 7, higher concentrations of extracellular glutamate caused a significant decrease in pro-MMP2 release. This is an unusual anomaly and could be because at passage 7 the FLS were starting to lose their phenotype, however previous studies have shown that this does not normally occur until passage 8 (Williams, A. S., personal communication). Alternatively, RA FLS may be regulating extracellular glutamate levels themselves by release or uptake mechanisms, in which case as extracellular glutamate is regulated, in turn; levels of pro-MMP2 are being regulated. It should be noted that a literature search yielded no evidence of previous studies looking at the effect of human FLS passage number on MMP production.

This study demonstrated that inhibiting the activity of glutamate transporters affects pro-MMP2 and TIMP release by RA and normal FLS. The presence of glutamate transporter inhibitors significantly increased pro-MMP2 (patient 2), TIMP1 and TIMP2 expression at all glutamate concentrations. The non-transportable EAAT inhibitor, TBOA had a more significant effect on pro-MMP2 and TIMP production than the transportable inhibitor, *t*PDC (patient 2). *t*PDC is a transportable inhibitor of EAATs 1-4 and has previously been shown to be a less potent glutamate transport inhibitor than the non-transportable inhibitor, TBOA, in astrocytes and neurons (Waagepetersen *et al.* 2001). Alternatively, these differences in effects may be due to the inhibition of EAAT ion channel activity by TBOA (Arriza *et al.* 1997). The effect of glutamate transporter antagonists on pro-MMP2 was also observed in RA FLS from patient 3; TBOA caused an increase in pro-MMP2 release at 4 out of the 6 glutamate concentrations tested. Since more pro-MMP2 is being produced in the presence of glutamate transporter inhibitors, it may be concluded that high levels of extracellular glutamate induce the production of pro-MMP2 in RAFLS by increased activation of glutamate receptors. However this was not consistent with the experiments discussed above (section 6.3.1) indicating that increased glutamate does not consistently increase pro-MMP2 release. Alternatively, this could be explained by the fact that TBOA inhibits the receptor function of glutamate transporters; the transporters may be acting as glutamate-gated ion channels or as receptors; it has been demonstrated previously that glutamate can activate the MEK/ERK pathway in astrocytes via glutamate

transporters (Abe and Saito 2001). Therefore TBOA could be inhibiting the receptor function of the transporters to increase proMMP2 levels as is the action of MK801 on NMDA receptors.

Normal FLS were less responsive than RA FLS to glutamate transporter antagonists. EAAT inhibition caused an increase in RA FLS production of pro-MMP2 but had no effect on normal FLS pro-MMP2 production. This therefore highlights a difference between the response of RA and normal FLS to the inhibition of glutamate transporter function. Differences between RA and normal FLS to transporter antagonists are discussed in chapter 4 because similar differences were also seen in IL-6 production (see section 4.3.3). The most likely conclusion is that since normal and RA FLS express different glutamate transporters and receptors (see section 3.3.7) they are likely to respond differently to glutamate and glutamate receptor and transporter inhibitors. In addition, since the levels of expression of receptors and transporters may also differ between RA and normal FLS and with treatment, a further level of complexity is introduced.

Glutamate transporter inhibition also affected TIMP production. TIMP1 and TIMP2 levels were increased significantly by inhibition of glutamate transporters in RA FLS indicating a compensatory effect; compensating for the increase in pro-MMP2. The presence of EAAT inhibitors induced production of TIMP1 and TIMP2 in the same way they had with pro-MMP2. TBOA had a greater effect than *t*PDC on TIMP1 and 2 release, which was also observed with regard to pro-MMP2 release by FLS and reasons for this difference have already been discussed above. The increase in TIMPs observed in response to glutamate transporter antagonists may be seen as a compensatory response to the increase in pro-MMP2 or they may have another role. For example, the activation of pro-MMP2 by MMP14 requires the presence of TIMP2 (Sommerville 2003) in which case increases in TIMP2 would be associated with increased degradation. However, expression of active MMP2 was not observed in this study. The relative binding affinities of the glutamate transporter antagonists used are discussed in chapter 4 (section 4.2.2).

Extracellular TIMP-1 has been shown to protect neurons against cell death from high concentrations of glutamate (600μM) by blocking glutamate mediated calcium entry, which suggests an interaction of TIMP-1 with glutamate receptor function (Tan *et al.* 2003). Tan *et al.* suggest that the neuroprotective effect of TIMP-1 only occurs when it is bound to MMPs because when neurons are incubated with TIMP-1 and a synthetic MMP inhibitor (MMPI) the neuroprotective effect is lost. How the effect of TIMP-1 on glutamate receptor calcium influx is mediated is unknown. So the induction of TIMP-1 by high levels of extracellular glutamate observed in this study may serve to block glutamate receptors (thus preventing increases observed in pro-MMP2) or to inhibit MMPs directly.

The effects of ionotropic receptor antagonists had variable effects on pro-MMP2 production by FLS. Treatment of RA FLS with NMDA receptor antagonists (MK801) caused an increase in pro-MMP2 production at all glutamate concentrations except 150μM. This suggests that the elevated glutamate in RA synovial fluid may activate NMDA receptors to down-regulate matrix metalloproteinase production. No effect of NMDA receptor antagonists on pro-MMP2 production by normal FLS was observed thus further highlighting differences between RA and normal FLS in how they can respond to glutamate. DAP5 had no effect on pro-MMP2 production by RA and normal FLS. DAP5 is a competitive inhibitor and therefore may not have been used at a high enough concentration. As discussed in chapter 4, it is difficult to determine the correct concentration of competitive inhibitors. Variable factors affecting inhibitors include number of receptors and transporters expressed, receptor subunit expression, Mg^{2+} concentration, receptor and transporter trafficking, the number of receptors needed to be activated to evoke a response in a cell and the binding affinity of the antagonist.

AMPA/KA receptor inhibition had no effect on pro-MMP2 production by RA and normal FLS indicating that these receptors are not important in the regulation of pro-MMP2 production. This concurs with published studies showing that KA induced seizures has

no effect on MMP2 expression in rat brain sections (Szklarczyk *et al.* 2002) and that KA does not increase expression of MMP2 by rat neurons (Jourquin *et al.* 2003).

No effect was observed on TIMP expression by RA FLS. Evidence of neurotransmitter control of TIMPs has been observed in the CNS. TIMP-1 is upregulated in rat neurons after kainate-induced seizures indicating that kainate receptors may regulate TIMP-1 expression (Tan *et al.* 2003). This is not consistent with the data presented here, therefore, in FLS a different pathway is likely to regulate the TIMP expression observed upon inhibition of glutamate transporters. Either the glutamate transporters are directly influencing TIMP production or the metabotropic receptors may be mediating this response. To determine which pathway is involved requires further experiments. For example using TBOA (to prevent glutamate uptake) in conjunction with receptor antagonists to determine which receptors may affect TIMP production.

This study has used MMPs 2 and 9 as markers of a catabolic response to glutamate. However, as discussed in section 1.5.1.3.1, many other MMPs are involved in the pathology of RA. It would be interesting to see if glutamate and glutamate signalling has effects on other MMPs. These could be measured using ELISAs. Alternatively, MMP1, 8 and 13 (the collagenases), levels of which are elevated in RA (Shaw 2000), can be measured using a collagen degradation assay (Cawston and Barrett 1979).

The data presented here clearly indicate that glutamate and antagonists of glutamate transporters and receptors influence pro-MMP2, TIMP1 and TIMP2 release by RA and normal synoviocytes. Evidence presented here indicates that elevated glutamate may induce a catabolic response in FLS. Furthermore, it has been demonstrated that RA and normal FLS respond differently to glutamate and glutamate receptor and transporter antagonists thus demonstrating different glutamate signalling mechanisms occur in the diseased state. MMPs and TIMPs play a large role in RA (as discussed in sections 1.5.1.3.1 and 1.5.2). The effect of glutamate on MMP and TIMP activity in FLS clearly warrants further investigation given the potential importance of these enzymes in pannus formation and synovial joint degradation in RA.

Chapter 7 The effect of glutamate and glutamate receptor activation on intracellular calcium responses in FLS

7.1 Introduction

The studies presented thus far have shown that FLS express glutamate transporters and receptors (chapter 3) and that stimulation of FLS with glutamate or glutamate receptor and transporter inhibitors can alter FLS phenotype (chapters 4 and 6). The RT-PCR studies in chapter 3 demonstrate that RA FLS express both NMDA and kainate receptor subunit mRNA. Moreover, the data in chapters 4 and 6 demonstrate that production of IL-6, pro-MMP2 and TIMPs by FLS were responsive to inhibition of ionotropic glutamate receptors with the non-competitive NMDA receptor inhibitor MK801 and the AMPA/kainate receptor inhibitor NBQX. Therefore the mechanisms by which glutamate may effect these phenotypic changes were explored.

Section 1.6.2.1 in chapter 1 gives an overview of the different types of glutamate receptors and the signalling pathways they activate. The work described in this chapter focuses on the NMDA and kainate ionotropic receptors since inhibitors of these receptors altered FLS phenotype (chapters 4 and 6). The binding of glutamate to NMDA receptors causes the receptor pore to become permeable to Ca^{2+} as well as Na^{+} and K^{+}. Magnesium ions can block NMDA receptor ion channel pores and inhibit calcium ion flow. Kainate receptors are also ionotropic receptors and stimulate calcium influx into cells either directly through the kainate receptor channel, or indirectly via voltage-gated calcium channels.

There is some evidence from CNS studies linking glutamatergic signalling to pathways that may be important in RA pathology (section 1.6.5). For example, in neurons NMDA receptor activation induces nitric oxide production (Okada *et al.* 2004) and inhibition of nitric oxide synthase can prevent glutamate-induced neurotoxicity (Montoliu *et al.* 2001). Since nitric oxide plays a pivotal role in the inflammatory process of RA (see section 1.4.1.3) activation of glutamate receptors in FLS may lead to similar responses. Furthermore, the transcription factor NFκB is known to induce pro-inflammatory

cytokine expression in RA FLS (Barnes *et al.* 1997, Libermann *et al.* 1990) and is activated by kainate in rat striatum (Cruise *et al.* 2000), and NMDA receptor activation in osteoblasts (Merle *et al.* 2003). To determine whether NMDA and kainate receptors expressed by RA FLS are functional, the effect of kainate and NMDA on intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels was explored. Since IL-6 levels are also increased in RA synovial fluid (Desgeorges *et al.* 1997) and this cytokine is known to influence glutamate signalling in the CNS (Peng *et al.* 2005), the effect of IL-6 on glutamate-mediated $[Ca^{2+}]_i$ increases was also investigated.

7.2 Methods

Confluent RA FLS (from patient 6 and patient 9) in petri dishes (cultured as in section 2.5.2) were incubated for 60-90 minutes at 37°C with the fluorescent indicator of Ca^{2+}, fluo-4 AM (5μM). Background activity (the number of fluorescent cells displaying calcium signals) was measured before stimulation with approximately 50μM thimerosal, 500μM glutamate, 200μM TBOA, 500μM NMDA or 500μM kainate (see section 2.10). These concentrations of glutamate, NMDA and kainate were chosen because the largest changes in IL-6 and pro-MMP2 were observed at 500μM glutamate and the average concentration of glutamate in RA synovial fluid is 326μM. The fluorescence intensity was analysed using laser scanning confocal fluorescence imaging (section 2.10.1). Fluorescence values over time for all responsive cells were exported into Excel (Microsoft) for further analysis and ΔF% plot production. ΔF% is the change in fluorescence and was calculated by dividing the fluorescence change by the basal fluorescence and multiplying by 100 for each cell (Parri and Crunelli 2003). Basal fluorescence was taken as fluorescence intensity immediately prior to the addition of stimulant. These data were plotted as ΔF% against time. The mean time RA FLS took to respond to stimulants and the mean time to recover to basal $[Ca^{2+}]_i$ levels is presented (+/-SEM), however the time taken to return to basal levels could not be presented for all data because measurements were not taken for long enough. To ensure that Ca^{2+} responses were not due to mechanical stimulation through pipetting of the test stimulants, buffer alone was added to the RA FLS. No changes in fluorescence were observed upon

addition of buffer alone therefore all responses are due to specific stimulation of the applied agonist and not the result of mechanical stimulation.

RA FLS cultured from patient 6 (see section 2.5.2) were also pre-treated with IL-6 (50ng/ml) and sIL-6r (20ng/ml) prior to glutamate or NMDA stimulation to see if this affected calcium responses via these receptors. IL-6 (50ng/ml) and IL-6sr (20 ng/ml) were added to the culture media and incubated at 37°C for approximately 6 hours prior to stimulation with 500μM glutamate or 500μM NMDA using the method described above.

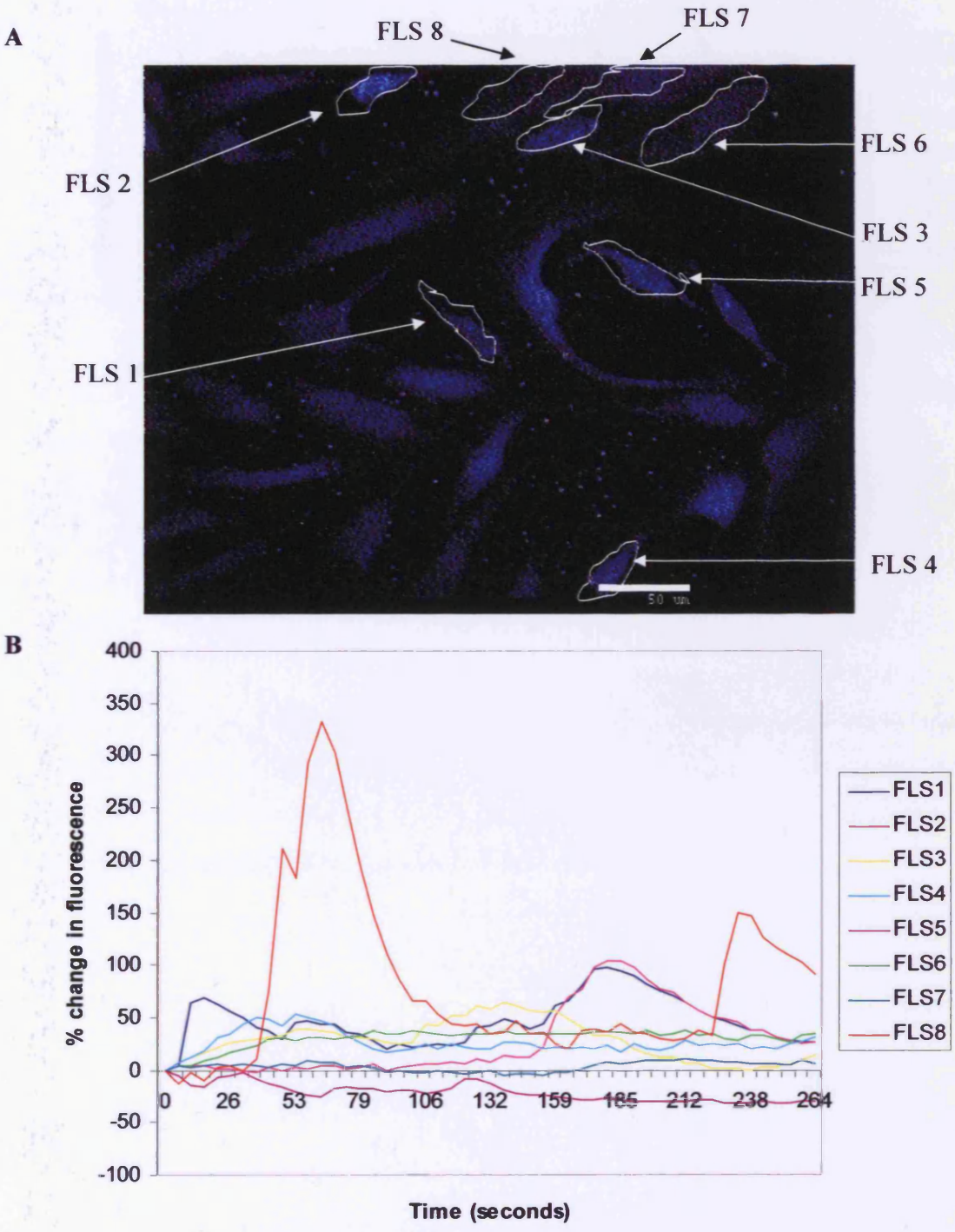
The number of cells observed in each experiment varied from 14 to 33 allowing statistical comparisons on numbers of responding cells using the chi square test (see figure 7.7). Due to the constraints of the chi square test only data sets with all frequencies (number of cells) greater than 5 could be analysed. In some cases enough cells responded (n>2) to allow variation in ΔF%, time to peak $[Ca^{2+}]_i$ and recovery to basal $[Ca^{2+}]_i$ to be assessed (presented as mean +/- SEM).

7.3 Results: The effect of glutamate and glutamate receptor and transporter agonists and antagonists on $[Ca^{2+}]_i$ responses in RA FLS

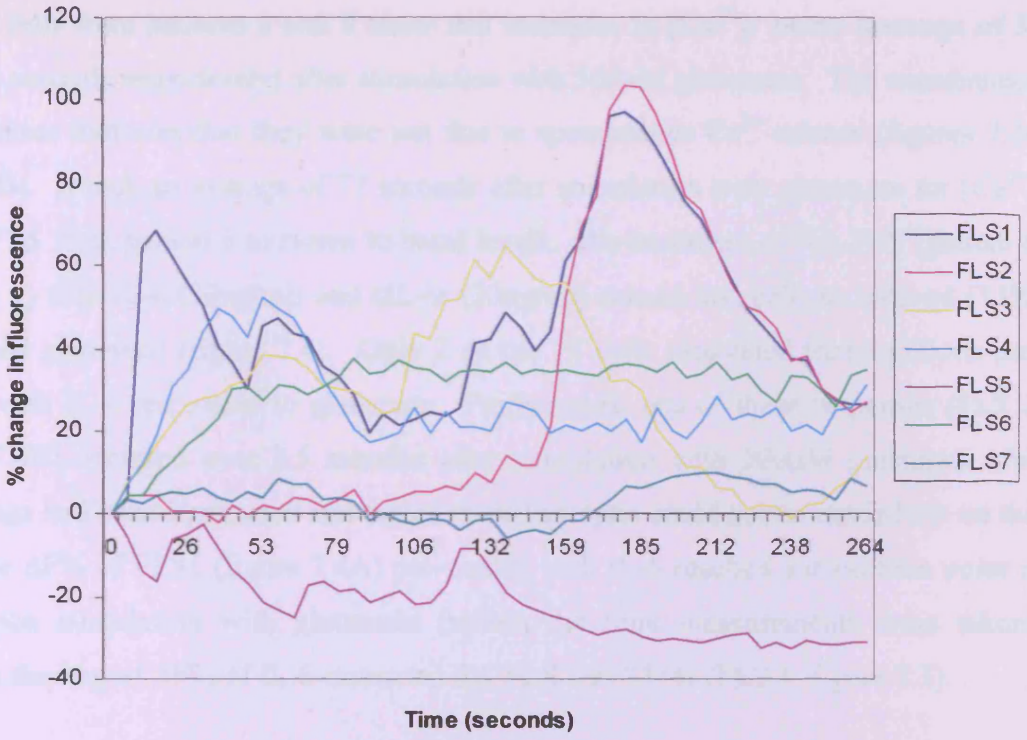
7.3.1 The effect of Thimerosal, an activator of IP_3 on $[Ca^{2+}]_i$ release by RA FLS

Thimerosal is an $[Ca^{2+}]_i$ mobiliser (Elferink 1999) and was used to confirm that the Fluo-4 dye was successfully loaded in the RA FLS cells (patient 6, passage 5) and could respond to increases in $[Ca^{2+}]_i$. 28% of these RA FLS responded to Thimerosal (figure 7.1). The biggest increase in fluorescence induced by Thimerosal in an FLS was 332% (FLS 8, figure 7.1B). Figure 7.1C shows in detail the ΔF% in FLS1 to 7 after removal of the FLS8 plot to allow an increase in the scale of the y axis. All increases in $[Ca^{2+}]_i$ occurred after addition of Thimerosal therefore responses were unlikely to be due to spontaneous $[Ca^{2+}]_i$ release. This data therefore confirms that RA FLS were successfully loaded with fluo-4 and that the $[Ca^{2+}]_i$ changes could be measured using the described methods.

Figure 7.1 The intracellular calcium response of RA FLS (patient 6, passage 5) upon treatment with Thimerosal.
Eight of the 29 cells (28%) in the field of vision (figure A) responded upon stimulation with Thimerosal (approximately 10μM). Changes in fluorescence versus time from RA FLS demonstrating that Thimerosal causes $[Ca^{2+}]_i$ release are shown in figure B (cells 1 to 8) and figure C (cells 1 to 7). Thimerosal addition occurred at time 0.



C


120
100
80
60
40
20
0
-20
-40
% change in fluorescence
0
26
53
79
106
132
159
185
212
238
264
Time (seconds)
FLS1
FLS2
FLS3
FLS4
FLS5
FLS6
FLS7

7.3.2 The effect of glutamate on $[Ca^{2+}]_i$ release by RA FLS

Glutamate caused an increase in $[Ca^{2+}]_i$ in RA FLS from patient 6 and 9 in 24% and 22% of cells respectively (Figures 7.2 and 7.3). Graphs showing changes in fluorescence over time of cells from patients 6 and 9 show that increases in $[Ca^{2+}]_i$ occur (average of 32 and 108 seconds respectively) after stimulation with 500μM glutamate. The coordination in responses indicates that they were not due to spontaneous Ca^{2+} release (figures 7.2C and 7.3B). It took an average of 77 seconds after stimulation with glutamate for $[Ca^{2+}]_i$ in RA FLS from patient 6 to return to basal levels. Pre-treatment of RA FLS (patient 6, passage 5) with IL-6 (50ng/ml) and sIL-6r (20ng/ml) caused less cells to respond (13%) to 500μM glutamate (figure 7.4). Only 2 of the 15 cells visualised that had been pre-treated with IL-6 responded to glutamate. Furthermore, one of these responses (FLS 2, figure 7.4B) occurred over 2.5 minutes after stimulation with 500μM glutamate after recordings had been terminated and therefore full analysis could not be carried out on this cell. The ΔF% of FLS1 (figure 7.4A) pre-treated with IL-6 reached a maximum point of 95% upon stimulation with glutamate (within the time measurements were taken), whereas the largest ΔF% of IL-6-untreated RA FLS was 235% (FLS 1, figure 7.3).

7.3.3 The effect of TBOA on $[Ca^{2+}]_i$ release by RA FLS

The effect of TBOA on release of $[Ca^{2+}]_i$ was investigated to see if RA FLS constitutively released enough glutamate to activate glutamate receptors on the cells surface and thus increase $[Ca^{2+}]_i$. The presence of TBOA would be expected to block glutamate uptake and may activate glutamate receptors if glutamate was being released by RA FLS over the time of recording. Over the course of approx. 300 seconds 200μM TBOA did not induce release of $[Ca^{2+}]_i$ of RA FLS; 0% of cells responded (data not shown, approximately 20 cells visualised in field of view).

Figure 7.2 The intracellular calcium response of RA FLS (patient 6, passage 5) upon treatment with 500μM glutamate.
Figure A shows still images of FLS1 at different time points after stimulation with 500μM glutamate. Five out of the 21 cells (24%) in the field of vision (figure B) responded to treatment with 500μM glutamate. Changes in fluorescence over time from the active FLS show that 500μM glutamate causes $[Ca^{2+}]_i$ release (figure C). Glutamate addition occurred at time zero in figure C.

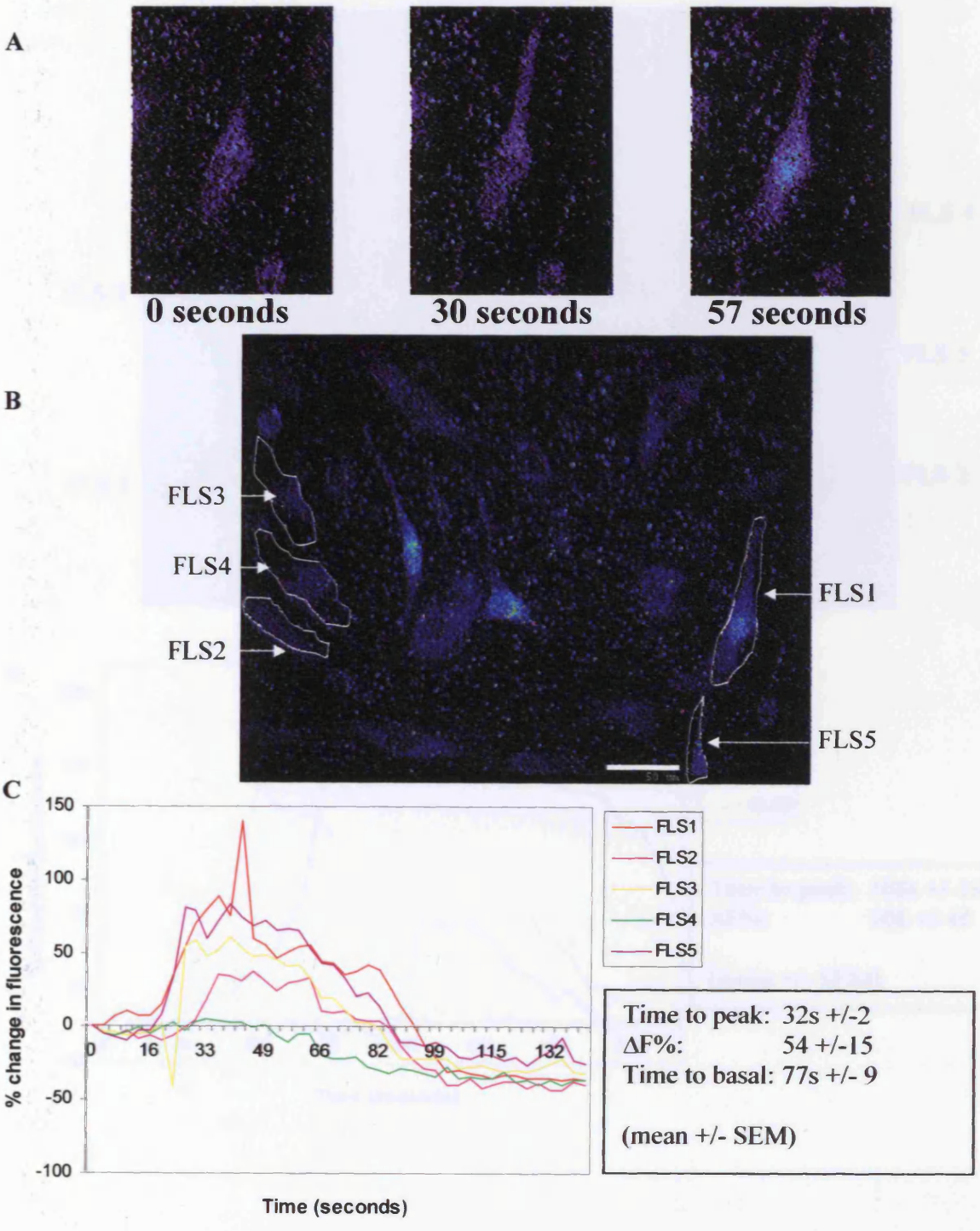
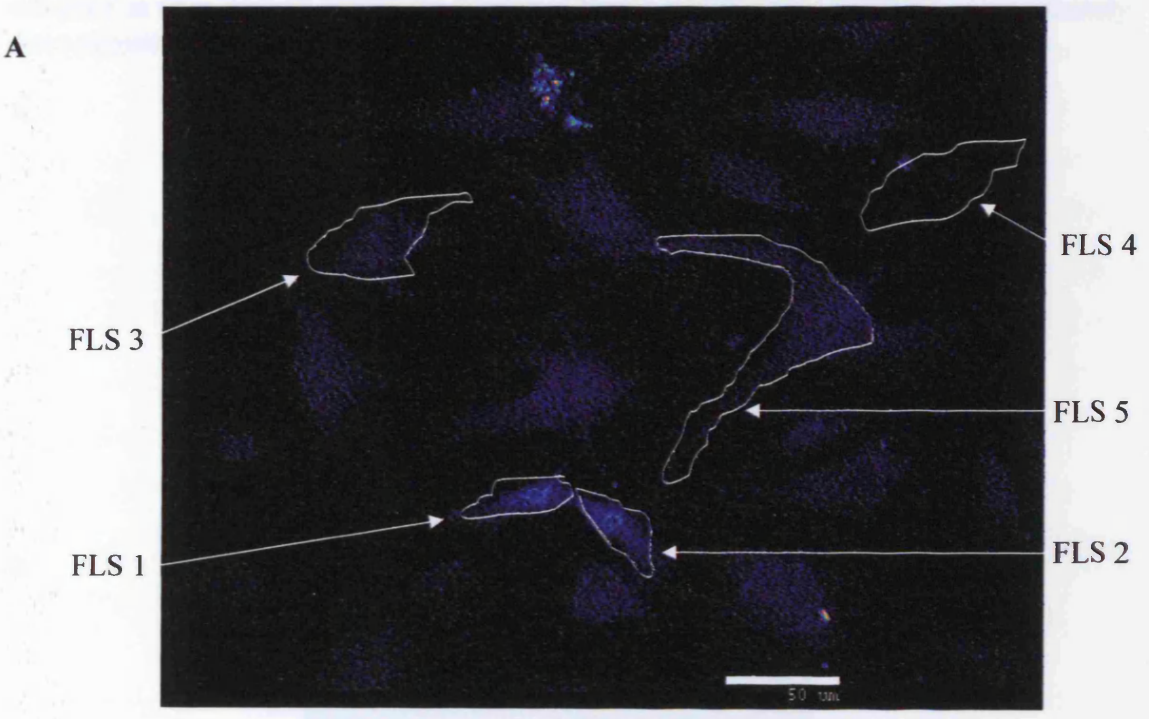


Figure 7.3 The intracellular calcium response of RA FLS (patient 9, passage 7) upon treatment with 500μM glutamate.
Five out of the 23 cells (22%) in the field of vision (figure A) responded to treatment with 500μM glutamate. Changes in fluorescence over time from the active FLS show that 500μM glutamate causes $[Ca^{2+}]_i$ release (figure B). Glutamate addition occurred at time zero in figure B.



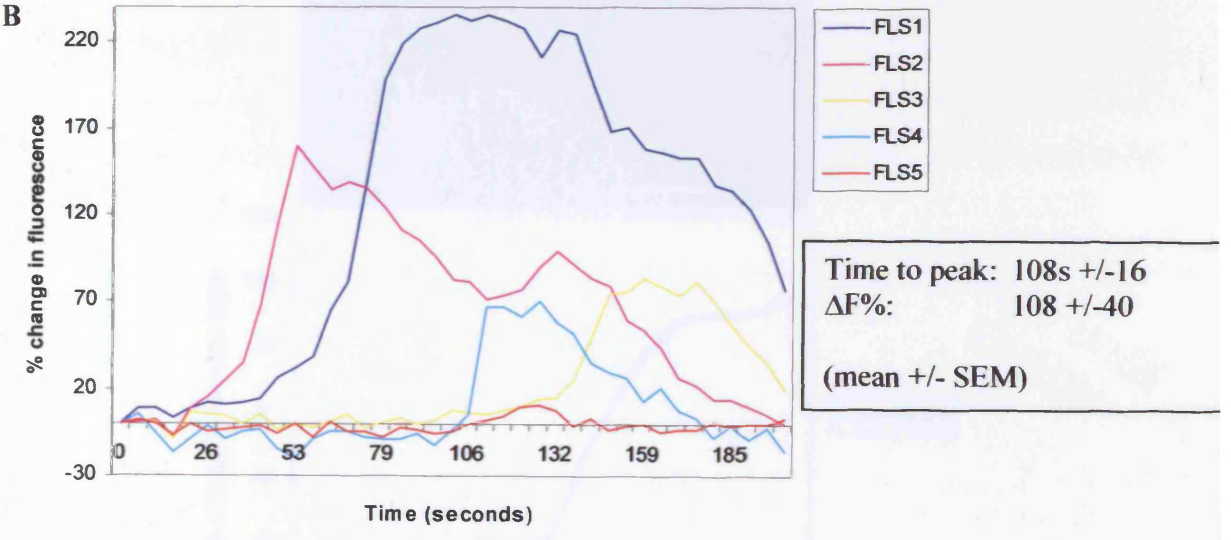
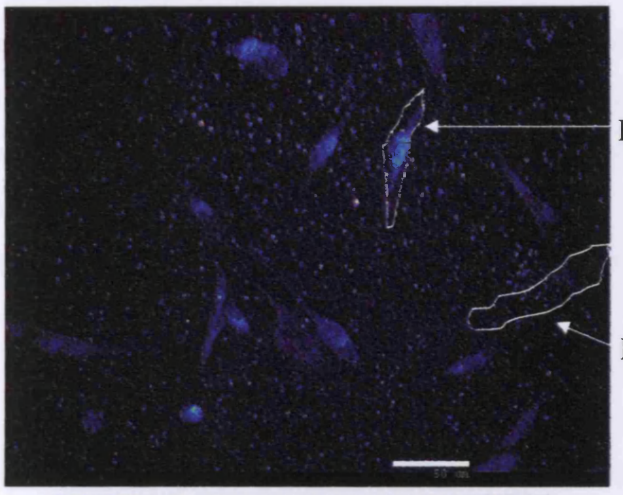


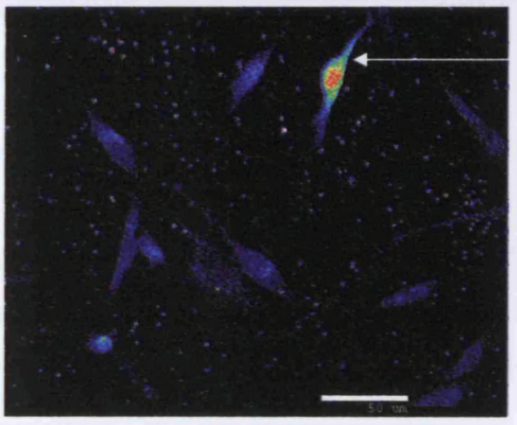
Figure 7.4 The intracellular calcium response of RA FLS (patient 6, passage 5) pretreated with IL-6 (50ng/ml) and IL-6sr (20ng/ml) upon treatment with 500μM glutamate.

Two out of the 15 cells (13%) in the field of vision (figure A) responded to treatment with 500μM glutamate. The change in fluorescence over time in figure C demonstrates that FLS 1 responded within seconds after stimulation, however FLS 2 responded approximately two minutes after recordings had been terminated. Glutamate addition occurred at time zero in figure C. Figure B shows a still image of FLS2 approximately three minutes after stimulation with 500μM glutamate.

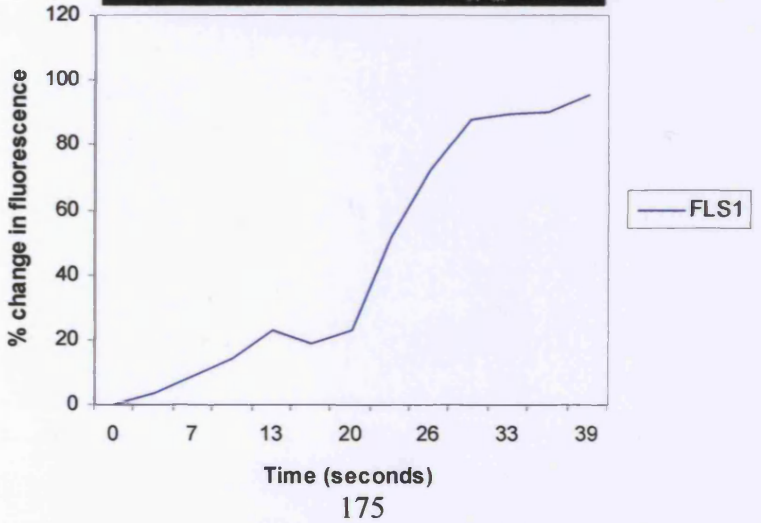
A



B



C

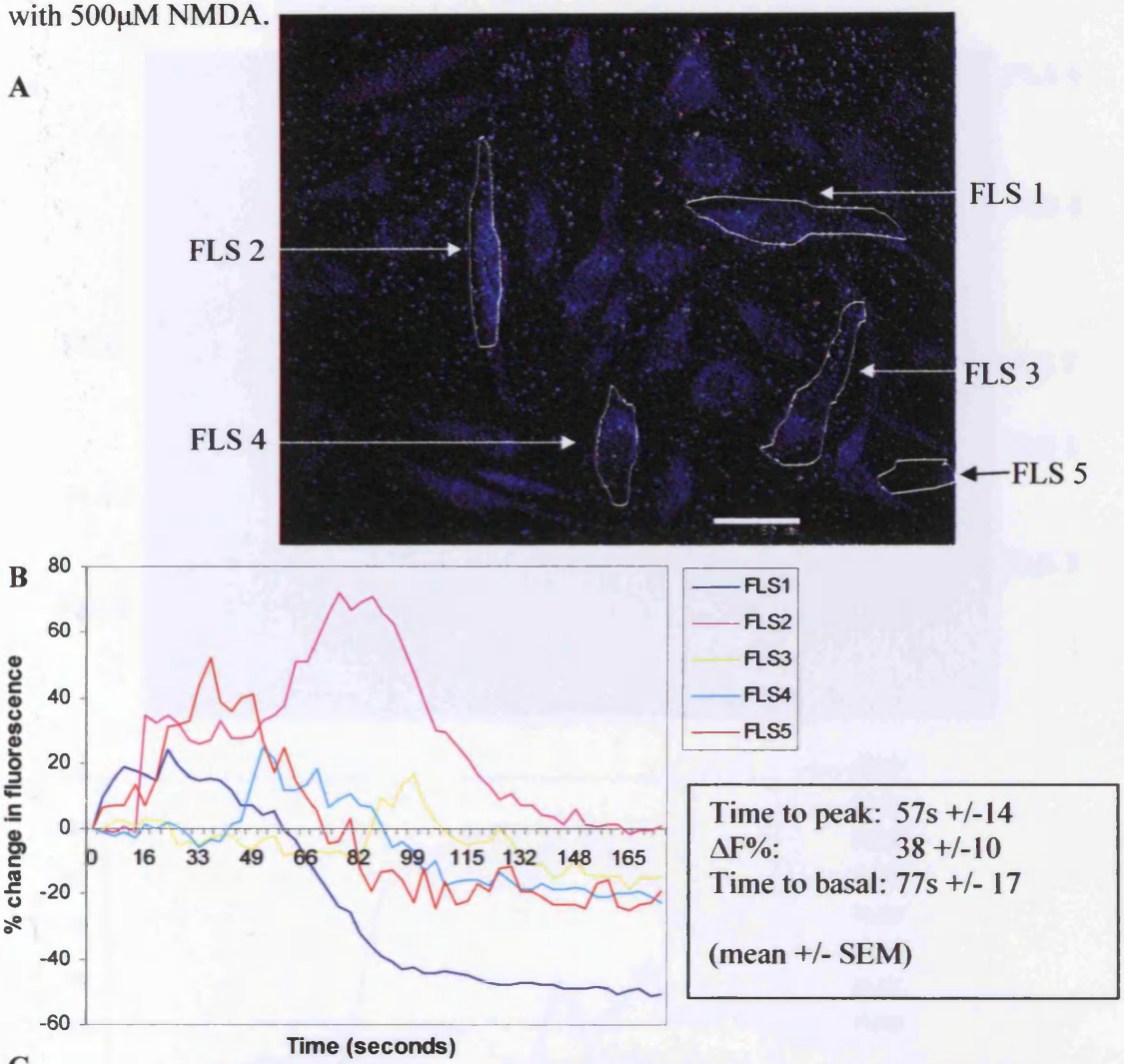


7.3.4 The effect of NMDA on $[Ca^{2+}]_i$ release by RA FLS

To determine which receptors were mediating $[Ca^{2+}]_i$ changes in response to glutamate, the effect of the specific agonist of NMDA receptors, NMDA was tested. NMDA caused an increase in $[Ca^{2+}]_i$ in RA FLS from patient 6 (15% of cells responded an average of 57 seconds after stimulation) (figure 7.5). Changes in fluorescence over time of cells from patients 6 show that increases in $[Ca^{2+}]_i$ occur after stimulation with 500μM NMDA and therefore were not due to spontaneous Ca^{2+} release (figures 7.5B). Figure 7.5C demonstrates the visible change in fluorescence over time following stimulation with NMDA. An increase in the number of RA FLS responding to NMDA occurred in the absence of Mg^{2+} (a mean of 57% of cells responded with a mean of 25 seconds after stimulation) (figure 7.6) consistent with the inhibitory effect of Mg^{2+} on NMDA receptor ion channels. This increase was found to be significant using the chi square test (figure 7.7A). A larger ΔF% occurred in cells stimulated with NMDA in the absence of Mg^{2+} (mean of 50% compared to 38% in the presence of Mg^{2+}). The number of cells responding to NMDA and glutamate was also compared using the chi square test (figure 7.7B). This revealed that no significant difference was observed in the number of cells increasing $[Ca^{2+}]_i$ between treatment with glutamate and NMDA in RA FLS from patient 6 (passage 5).

Fewer RA FLS pre-treated with IL-6 (50ng/ml) and IL-6sr (20ng/ml) (figure 7.8) responded to NMDA (9%) compared to RA FLS that had not been pre-treated with IL-6 (15%). Cells pre-treated with IL-6 took longer to respond (156 seconds compared to 57 seconds in un-treated cells). IL-6 pre-treated and NMDA-stimulated RA FLS demonstrated a greater ΔF% (mean of 171%) compared to RA FLS not pre-treated (mean of 38%).

Figure 7.5 The calcium response of RA FLS (patient 6, passage 5) upon treatment with 500μM NMDA.
Five out of the 33 cells (15%) in the field of vision (figure A) responded to treatment with 500μM NMDA. Changes in fluorescenc over time from the active FLS show that 500μM NMDA causes $[Ca^{2+}]_i$ release (figure B). NMDA addition occurred at zero time in figure B. Figure C shows still images of FLS1 at different time points after stimulation with 500μM NMDA.



Images of FLS 1 after stimulation with 500μM NMDA

Figure 7.7 Chi square analyses

A. χ^2 analysis to show whether absence of Mg^{2+} significantly increases NMDA receptor activation.

Stimulant:	NMDA	NMDA - Mg^{2+}	Total
No. cells responding observed	5	8	**13**
No. cells responding observed	28	6	**34**
Total	**33**	**14**	**47**
No. cells responding expected	9.13	3.87	
No. cells not responding expected	23.87	10.13	

Chi square total: 8.67
Degrees of freedom: 1
Critical chi square value ($p<0.05$): 3.841

Therefore significantly more RA FLS respond to NMDA without Mg^{2+} present by increasing $[Ca^{2+}]_i$ than to when Mg^{2+} is present

B. χ^2 analysis to show whether glutamate and NMDA significantly activate different numbers of cells.

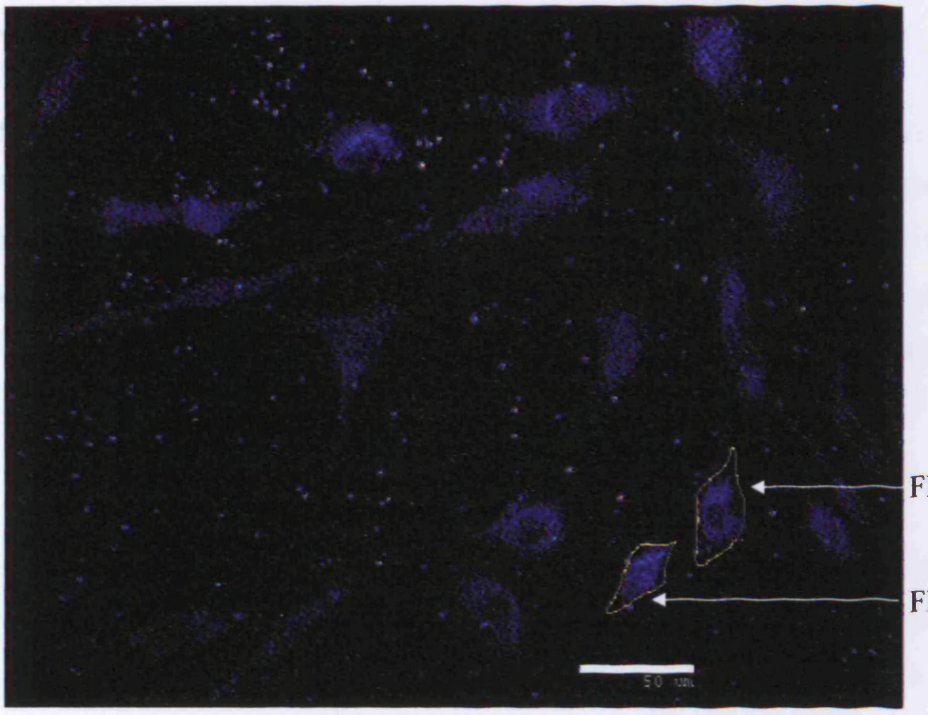
Stimulant:	NMDA	Glutamate	Total
No. cells responding observed	5	5	**10**
No. cells responding observed	28	16	**44**
Total	**33**	**21**	**54**
No. cells responding expected	6.1	3.9	
No. cells not responding expected	26.9	17.1	

Chi square total: 0.62
Degrees of freedom: 1
Critical chi square value ($p<0.05$): 3.841

Therefore there is no significant difference between the number of RA FLS responding to glutamate by increasing $[Ca^{2+}]_i$ than to NMDA.

Figure 7.8 The intracellular calcium response of IL-6-treated RA FLS upon treatment with 500µM NMDA.
Two out of the 22 RA FLS (patient 6, passage 5) cells (9%) in the field of vision (figure A) responded to treatment with 500µM NMDA. Changes in fluorescence over time from the two active RA FLS show that 500µM NMDA causes $[Ca^{2+}]_i$ release (figure B). NMDA addition occurred at time zero in figure B.

A



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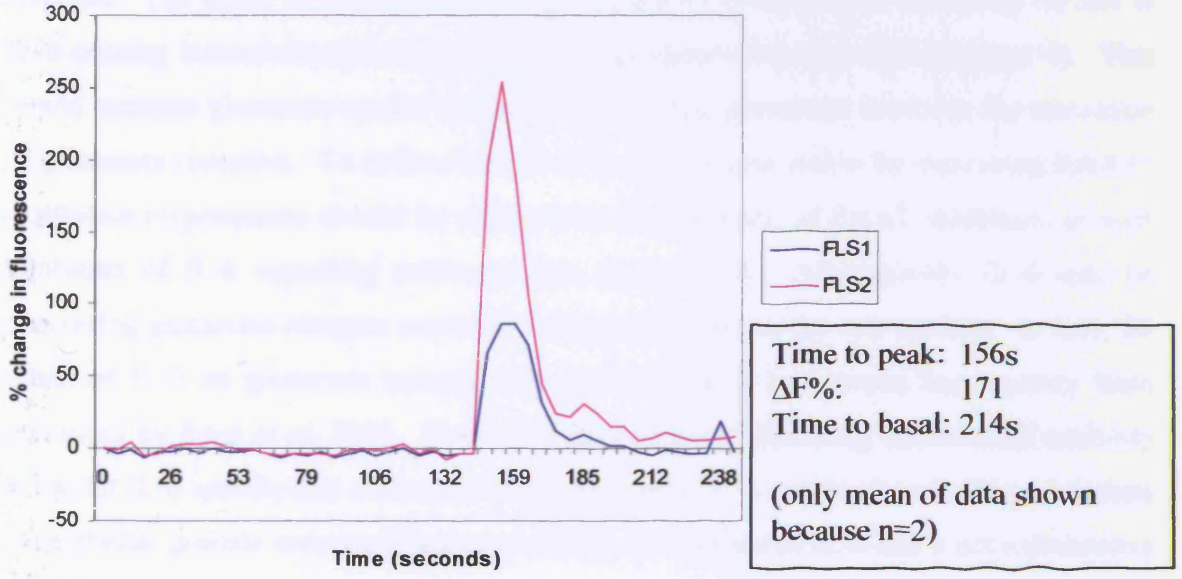


7.3.5 The effect of kainate on $[Ca^{2+}]_i$ release by RA FLS

Kainate caused an increase in $[Ca^{2+}]_i$ in RA FLS (patient 9, passage 7) in 15% of the cells observed (figures 7.9). Changes in fluorescence over time of the cells show that increases in $[Ca^{2+}]_i$ occur after stimulation with 500μM kainate and therefore were not due to spontaneous Ca^{2+} release (figures 7.9B). The average time to peak $[Ca^{2+}]_i$ was 114s, with a maximum average ΔF% of 92 (figure 7.9).

7.4 Discussion

The data presented here demonstrates that RA FLS can be stimulated by glutamate to increase $[Ca^{2+}]_i$. This confirms that RA FLS are responsive to glutamate and highlights possible pathways, by which RA FLS change their phenotype in response to the increases in extracellular glutamate levels observed in RA synovial fluid. Increases in $[Ca^{2+}]_i$ in RA FLS were observed in response to 500μM glutamate (in RA FLS from 2 patients), 500μM NMDA and 500μM kainate thus demonstrating that NMDA, AMPA and kainate ionotropic glutamate receptors are active in RA FLS.

IL-6 pre-treatment decreased the number of cells responding to glutamate and NMDA, however it did not appear to affect the intensity of the $[Ca^{2+}]_i$ increase because the largest ΔF% occurred in IL-6 pre-treated RA FLS stimulated with NMDA. This was only shown in one cell therefore numbers would need to be increased in order to see variability in this response. The effect of IL-6 in decreasing the number of responsive cells may be due to IL-6 causing increased expression of EAAT1, as observed in RA FLS (chapter 4). This would increase glutamate uptake by the cells, reducing glutamate available for activation of glutamate receptors. To deduce how IL-6 is effecting its action by increasing EAAT1 expression experiments should be repeated in the presence of EAAT inhibitors or with inhibitors of IL-6 signalling pathways (see section 9.3). Alternatively, IL-6 may be decreasing glutamate receptor expression or translocation to the cell surface. In fact, the effect of IL-6 on glutamate induced increases in $[Ca^{2+}]_i$ in neurons has recently been published by Peng *et al.* 2005. They used an anti-gp130 blocking monoclonal antibody to inhibit IL-6 activity and measured the changes of $[Ca^{2+}]_i$ overload evoked by glutamate in cerebellar granule neurons (Peng *et al.* 2005). 5-10ng/ml of IL-6 had a neuroprotective

effect (decreased apoptosis) upon stimulation with 10μM glutamate. In addition, glutamate caused an increase in $[Ca^{2+}]_i$ levels in the IL-6-pre-treated neurons but they returned to basal levels faster than neurons not pre-treated with IL-6 (Peng *et al.* 2005). The anti-gp130 monoclonal antibody blocked this effect. They therefore suggest that IL-6 inhibits the glutamate induced $[Ca^{2+}]_i$ overload via the gp-130 pathway, however a mechanism is not described. Results from RA FLS are consistent with this study.

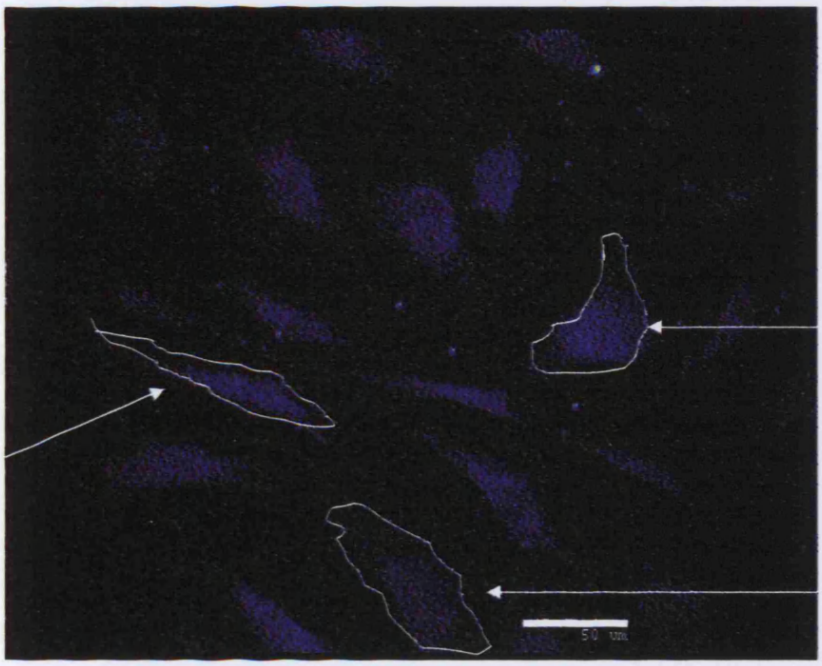
NMDA and kainate both stimulated increases in $[Ca^{2+}]_i$ in RA FLS thus concurring with the expression studies in chapter 3 demonstrating expression of NMDA NR1 and KA1 subunits. Kainate can activate both AMPA and kainate receptors therefore the $[Ca^{2+}]_i$ response may be brought about by activation of either receptor type. Studies in chapter 3 demonstrated that AMPAGluR2 and AMPAGluR3 were not present in normal or RA FLS, therefore the increase in $[Ca^{2+}]_i$ is more likely to have been brought about by kainate receptor activation. Further experiments need to be carried out, either stimulating FLS with AMPA or inhibiting AMPA receptors whilst stimulating with kainate and observing any evoked changes in $[Ca^{2+}]_i$.

NMDA is a specific agonist for NMDA receptors therefore the increase in $[Ca^{2+}]_i$ it evoked clearly indicates the presence of functional NMDA receptors in RA FLS. The buffer in which the $[Ca^{2+}]_i$ measurements were made did not contain glycine and NMDA receptors require glycine as a co-agonist (Kleckner and Dingledine 1988), however the DMEM used for culturing the FLS contained 250μM glycine. Mg^{2+} ions are known to block the NMDA ion channel (Egebjerg *et al.* 2002). The fact that the absence of Mg^{2+} ions significantly increased the number of FLS responding to NMDA supports the assumption that activation of NMDA receptors increases $[Ca^{2+}]_i$. No significant difference was observed between cells stimulated with glutamate and NMDA, this indicates that in patient 6, it is the NMDA receptors that predominantly respond to glutamate with an increase in $[Ca^{2+}]_i$. The effect of kainate on $[Ca^{2+}]_i$ was only tested in patient 9, therefore it is unclear whether kainate would also activate increases in $[Ca^{2+}]_i$ in RA FLS from patient 6.

Figure 7.9 The intracellular calcium response of RA FLS (patient 9, passage 7) upon treatment with 500μM Kainate

Three out of the 20 cells (15%) in the field of vision (figure A) responded to treatment with 500μM kainate. Changes in fluorescence over time from three active FLS show that 500μM kainate causes $[Ca^{2+}]_i$ release (figure B). Kainate addition occurred at time zero in figure B. The three peaks highlighted in figure B (*) were caused by floating fluorescent debris moving across the optical field of the microscope.

A



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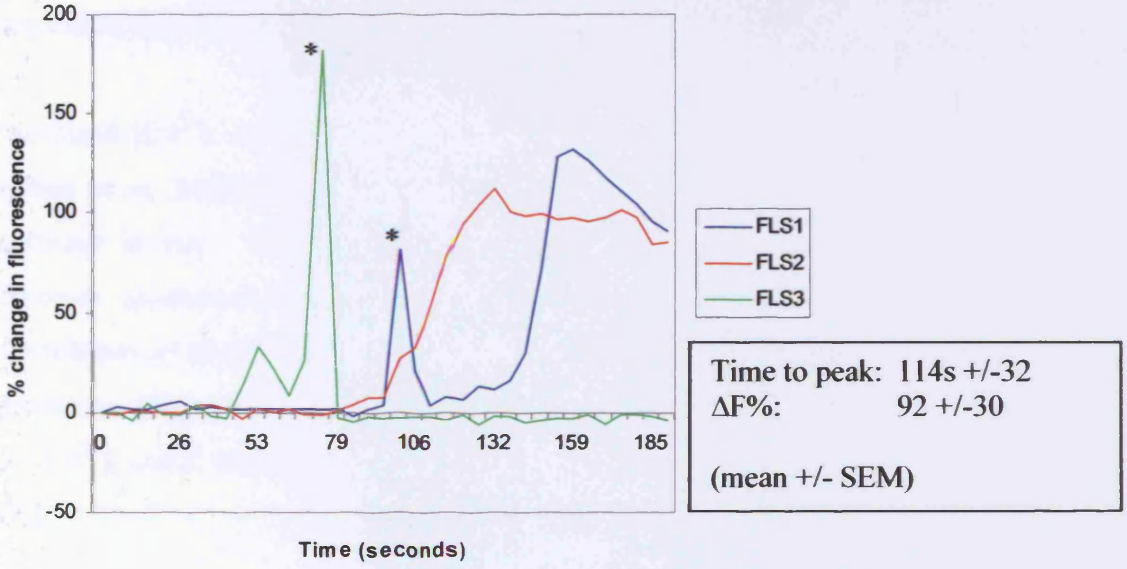


It should be noted that unless stated, the experiments measuring $[Ca^{2+}]_i$ were only carried out once therefore to conclude fully from these results repetitions are necessary. However the fact that spontaneous increases in $[Ca^{2+}]_i$ did not occur in RA FLS and that more than one cell responded upon stimulation strongly indicates that the stimulant effects are real. The experiments described in this chapter should also be repeated in the presence of the appropriate glutamate receptor antagonists to confirm that the observed $[Ca^{2+}]_i$ increases were brought about by specific activation of NMDA and kainate receptors. For example FLS should be stimulated with NMDA alone and the evoked $[Ca^{2+}]_i$ changes compared in the presence of a non-competitive NMDA receptor inhibitor such as MK801.

The evoked $[Ca^{2+}]_i$ increases observed are a possible mechanism for RA FLS to regulate IL-6, pro-MMP2 and TIMP levels in response to extracellular glutamate. The increase in $[Ca^{2+}]_i$ may also be involved in intercellular signalling. D'Andrea *et al.* demonstrated that intercellular calcium signalling occurs between chondrocytes and FLS in co-culture following mechanical stimulation via gap junctions. They hypothesise that this intercellular communication may be involved in pannus formation (D'Andrea *et al.* 1998). Some evidence of intercellular transmission of $[Ca^{2+}]_i$ waves was observed between FLS1 and FLS5 (figure 7.2) since the increase in $[Ca^{2+}]_i$ occurs in FLS1 prior to the adjacent FLS5 following stimulation with 500μM glutamate. This can be seen graphically in figure 7.2C. The increases in $[Ca^{2+}]_i$ brought about by glutamate receptor activation may therefore allow communication between FLS and chondrocytes.

Increased $[Ca^{2+}]_i$ in FLS has been shown to induce expression of VCAM1 and Cox2 (Chen *et al.* 2002). VCAM1 is involved in adhesion of the invasive pannus cells to cartilage in RA. Cox2 is involved in prostaglandin synthesis (see section 1.4.1.2). Protease production has also previously been associated with increases in $[Ca^{2+}]_i$; stimulation of bovine FLS with a calcium ionophore increased production of a 144kDa gelatinase (Howarth *et al.* 1993). Hence NMDA or kainate receptor-mediated increases in $[Ca^{2+}]_i$ could potentially be involved in many important pathological effects of RA FLS.

To conclude, this preliminary study into the effects of glutamate receptor agonists on changes in $[Ca^{2+}]_i$ in RA FLS has demonstrated that NMDA and kainate receptors are functional and the activity of these receptors may be modulated by the presence of IL-6. This therefore represents a possible mechanism for RA FLS to alter their phenotype in response to the elevated glutamate concentrations observed in RA synovial fluid.

Chapter 8 Glutamate transport in RA FLS

8.1 Introduction

Human RA FLS have been shown to express the glutamate transporters EAAT1 and EAAT3 (chapter 3). Furthermore, stimulation of FLS with glutamate or glutamate transporter antagonists can alter FLS phenotype (chapters 4 and 6). The ability of fibroblasts to take up glutamate has been recognised since 1983 when skin-derived human fibroblasts were shown to transport glutamate (Dall'Asta *et al.* 1983). More recently Cooper *et al.* demonstrated expression of EAAT1 and 2 in cultured human embryonic skin fibroblasts (Cooper *et al.* 1998), however glutamate transport has not been investigated in synovial fibroblasts.

This chapter explores whether these transporters are functional in RA FLS by measuring their ability to take up glutamate. To date, glutamate uptake in RA FLS has not been investigated however it has been explored in fibroblasts derived from human adult skin, peripheral nerve tissue, embryonic muscle and embryonic skin (Dall'Asta *et al.* 1982, Cooper *et al.*1998, Balcar *et al.* 1994, Zoia *et al.* 2005). Human skin fibroblasts also express glutamate transporters: EAAT1, 2, 3 and 4 (Cooper *et al.* 1998). Glutamate uptake in fibroblast cell types (see section 1.6.4.3) was shown to occur via a Na^+-dependent mechanism (Balcar *et al.* 1994) although Dall'Asta demonstrated that uptake by RA FLS occurred by more than one mechanism (high affinity Na^+-dependent, low affinity Na^+-dependent and Na^+-independent, see section 1.6.3). Therefore uptake by RA FLS may not be by the Na^+-dependent transporters already demonstrated to be expressed (EAAT1 and EAAT3). However it is clear that specific inhibitors of these transporters (*t*PDC and TBOA) modify FLS phenotype.

Glutamate uptake in RA FLS has been investigated because the ability to uptake glutamate would demonstrate a mechanism for regulation of extracellular glutamate levels that may be of significance to the elevated glutamate levels observed in RA synovial fluid. The effect of IL-6 on glutamate uptake has been studied because IL-6 has been shown to affect EAAT1 mRNA and protein expression (chapter 3). In addition IL-6

has been shown to affect glutamate signalling mechanisms in other cell types (see section 1.6.4). The effect of the EAAT transporter inhibitor *t*PDC (previously used in chapters 4 and 6) was also investigated to explore whether glutamate uptake was occurring via the EAATs.

8.2 Methods

8.2.1 ^{14}C-labelled glutamate uptake assay

To assess glutamate transporter function in RA FLS, a ^{14}C-labelled glutamate uptake assay was designed based on previously published methods in human fibroblasts (Dall'Asta *et al.* 1982, Balcar *et al.* 1994). To optimize glutamate uptake assays for FLS, a preliminary experiment was set up where uptake of 50μM ^{14}C-labelled glutamate was measured in confluent FLS after 0, 5, 10, 20 and 30 minutes (1 well per time point). Uptake of ^{14}C-labelled glutamate was detected by scintillation counting of washed and lysed cells (section 2.11) after 10 minutes incubation. Optimal detection levels were obtained after 30 minutes (see figure 8.1). Therefore initially ^{14}C-labelled glutamate uptake experiments were measured over 30 minutes (detailed methods are in section 2.11.1).

As well as measuring glutamate uptake, the amount of glutamate binding to RA FLS was also investigated. This was done by measuring glutamate bound at zero time (adding ^{14}C-labelled glutamate and immediately aspirating = bound glutamate) and comparing this to total glutamate uptake (= bound glutamate and glutamate taken up). Glutamate uptake was calculated in two ways: i) subtracting the amount of glutamate bound to RA FLS after 0 time from uptake after 30 minutes, or ii) where bound glutamate was not subtracted (section 2.11.4). Data is presented with and without the bound glutamate being taken into account as subtraction of initially bound glutamate is not standard procedure in previously published methods. Bound glutamate measurement data (figure 8.2) was logarithmically transformed prior to statistical analysis (see section 2.13).

Figure 8.1 Data from the preliminary experiment carried out to determine how long uptake of radioactive glutamate should be measured for.

RA FLS were incubated with 50μM C^{14}-labelled glutamate for a range of time durations (1 well per time point). Increases in glutamate uptake were observed after 10 minutes.

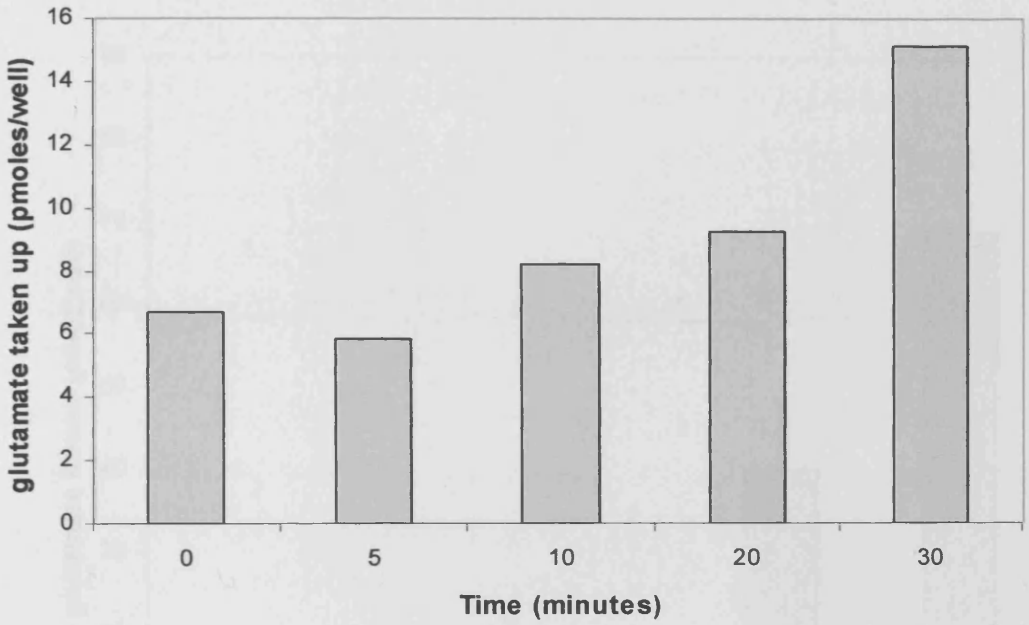
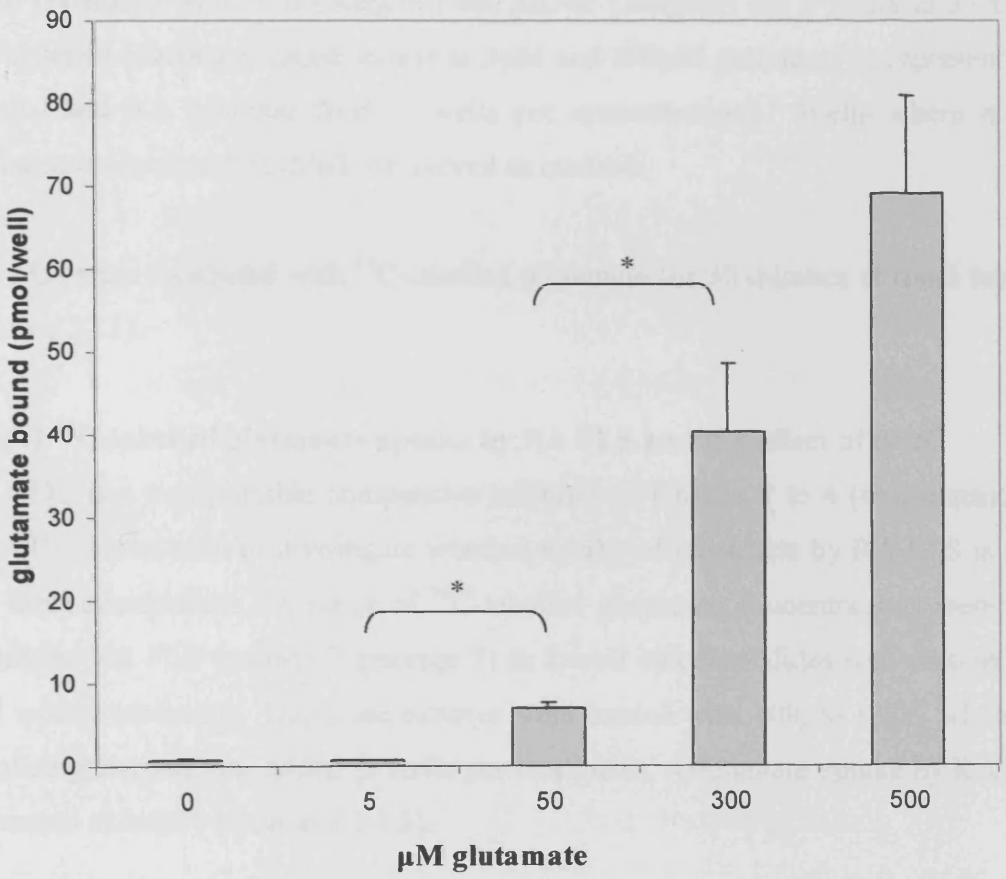


Figure 8.2 The amount of ^{14}C-labelled glutamate bound to RA FLS after 0 time
Glutamate bound to RA FLS was determined by adding ^{14}C-labelled glutamate to RA FLS and immediately removing it and washing the cells with ice cold PBS. The amount of glutamate bound to the RA FLS increased with glutamate concentration. Data was logarithmically transformed prior to paired t-test analysis (SEM error bars, n=3).



The amount of glutamate taken up was determined by scintillation counting (section 2.11.1). Statistical comparisons were made using a paired student t test after testing for normality and equal variance (Minitab).

8.2.1.1 The effect of pre-incubation of RA FLS with IL-6 on glutamate uptake

To investigate whether the cytokine, IL-6, affects glutamate uptake, confluent RA FLS (patient 7, passage 7) in 8-well chamber slides (see section 2.5.2 for cell culture methods) were pretreated with IL-6 (50ng/ml) and sIL-6r (20ng/ml) for 3 hours at 37°C prior to ^{14}C-labelled glutamate uptake assays at 5μM and 300μM glutamate to represent levels in normal and RA synovial fluid (3 wells per concentration). Wells where media was replaced in absence if IL-6/sIL-6r, served as controls

RA FLS were incubated with ^{14}C-labelled glutamate for 30 minutes at room temperature (section 2.11).

8.2.1.2 ^{14}C-labelled glutamate uptake by RA FLS and the effect of *t*PDC

As *t*PDC is a transportable competitive inhibitor of EAATs 1 to 4 (non-transportable to EAAT5) it was used to investigate whether uptake of glutamate by RA FLS is mediated via these transporters. A range of ^{14}C-labelled glutamate concentrations were added to confluent RA FLS (patient 7, passage 7) in 8-well chamber slides (see section 2.5.2 for cell culture methods). Duplicate cultures were treated with 100μM *t*PDC when the ^{14}C-labelled glutamate was added (3 wells per treatment). Glutamate uptake by RA FLS was measured as before (section 8.3.1.1).

8.3 Results

8.3.1 Initial bound glutamate

The amount of glutamate bound, measured by adding ^{14}C-labelled glutamate and aspirating immediately, increased as the concentration of glutamate added to the RA FLS culture media increased (Figure 8.2). However glutamate binding was not significantly increased between 300μM and 500μM glutamate indicating that saturation of glutamate binding sites may be occurring.

8.3.2 Glutamate uptake by RA FLS

Glutamate uptake by RA FLS (patient 7, passage 7) after 30 minutes (figure 8.3A) was greater than the amount of glutamate 'bound' to the RA FLS at 0 time (figure 8.2). The amount of glutamate taken up increased as the concentration of extracellular glutamate increased (figure 8.3 A and B). This demonstrates that RA FLS have the ability to take up glutamate. However as the initial concentration of glutamate increased, the percentage of total glutamate uptake significantly decreased (figure 8.3C) indicating that RA FLS have a limited number of transporters or a limit to the amount of glutamate that these cells can take up over 30 minutes or different transport systems operating at different concentrations.

When bound glutamate was accounted for (figure 8.3A) a significant increase in glutamate uptake by RA FLS (patient 7, passage 7) was only observed between 5μM and 50μM glutamate ($p=0.004$). When bound glutamate was not accounted for (figure 8.3B) a significant increase in glutamate uptake was observed at each increasing concentration increment.

8.3.3 The effect of IL-6 on ^{14}C-labelled glutamate uptake by RA FLS

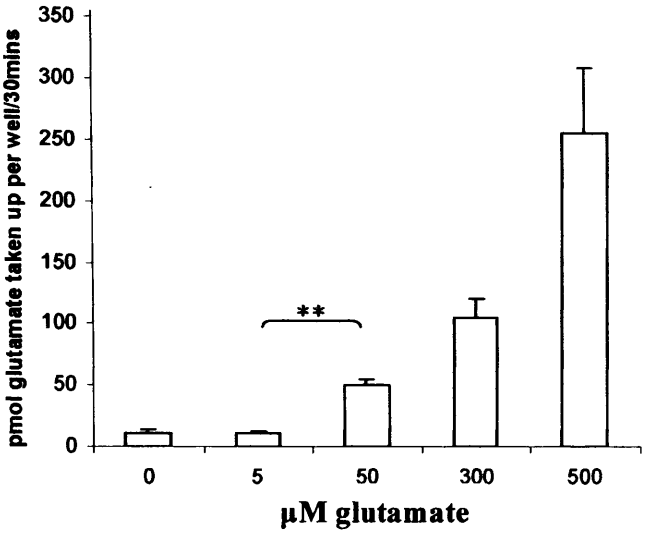
Pre-treatment of RA FLS (patient 7, passage 7) with IL-6 (50ng/ml) and sIL-6r (20ng/ml) for 3 hours significantly decreased uptake of 5μM glutamate compared to the control ($p=0.03$, figure 8.4).

8.3.4 The effect of the EAAT inhibitor *t*PDC on glutamate uptake

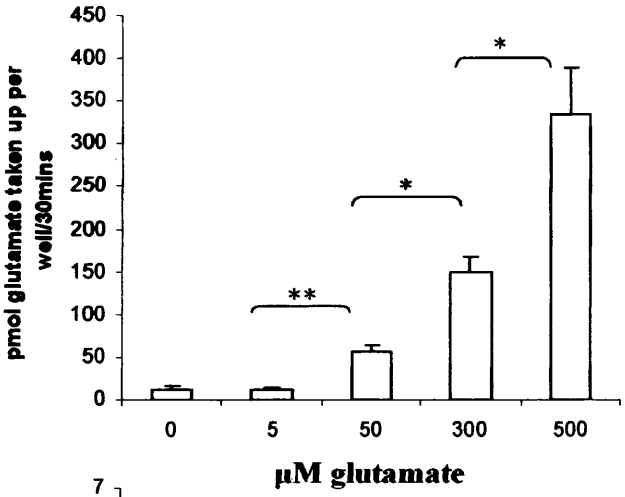
The non-transportable inhibitor of EAATs 1 to 4, *t*PDC, significantly increased glutamate uptake of 300μM glutamate ($p=0.038$) but decreased uptake of 500μM glutamate ($p=0.047$) (figure 8.5). Prior to paired t-test analysis, this data set had to be transformed ($\log_{10}$) in order for compared data sets to have equal variances.

Figure 8.3 Glutamate uptake by RA FLS
All error bars show SEM.

A: The amount of glutamate taken up by RA FLS (patient 7, passage 7) after 30 minutes as measured by a ^{14}C-labelled glutamate uptake assay (glutamate initially bound at zero time has been subtracted). The amount of glutamate taken up increased as the concentration of extracellular glutamate increased. This demonstrates that RA FLS have the ability to uptake glutamate. (Paired t-test, n=3 wells, **:$p<0.005$).



B: The amount of glutamate taken up by RA FLS (patient 7, passage 7) after 30 minutes as measured by a ^{14}C-labelled glutamate uptake assay (glutamate initially bound at zero time has not been subtracted). The amount of glutamate taken up increased as the concentration of extracellular glutamate increased. This demonstrates that RA FLS have the ability to uptake glutamate. (Paired t-test, n=3 wells, *:$p<0.05$, **:$p<0.005$).



C: The amount of glutamate taken up over 30 minutes as a percentage of the total amount of glutamate initially present as measured by a ^{14}C-labelled glutamate uptake assay. The percentage of glutamate taken up by RA FLS significantly decreased as the initial glutamate concentration increased but reached saturation at 500µM glutmate (n=3 wells).

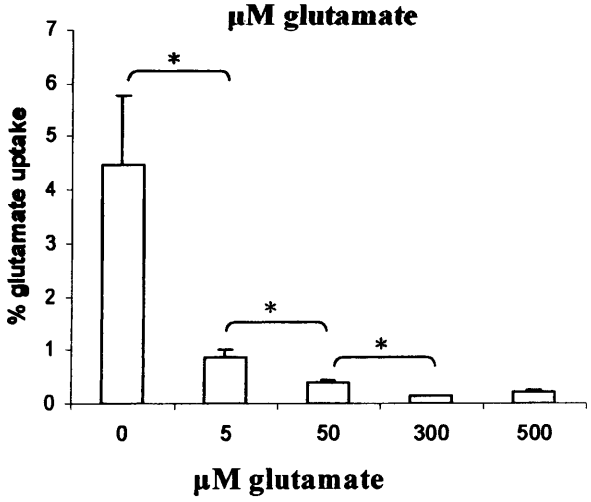


Figure 8.4 The effect of IL-6 on ^{14}C-labelled glutamate uptake by RA FLS

Pre-treatment of RA FLS (patient 7, passage 7) with IL-6 significantly decreased uptake of 5μM glutamate (p=0.03) by RA FLS (patient 7, passage 7) compared to the control. IL-6 had no effect on the uptake of 300μM glutamate by RA FLS (SEM error bars, n=3 wells, *:p<0.05).

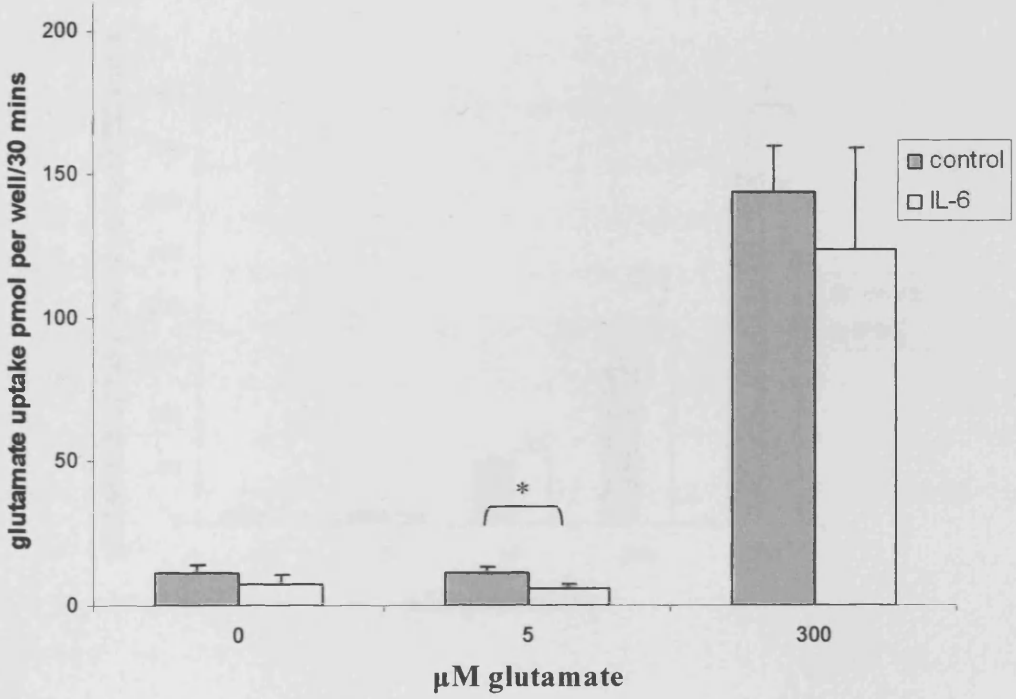
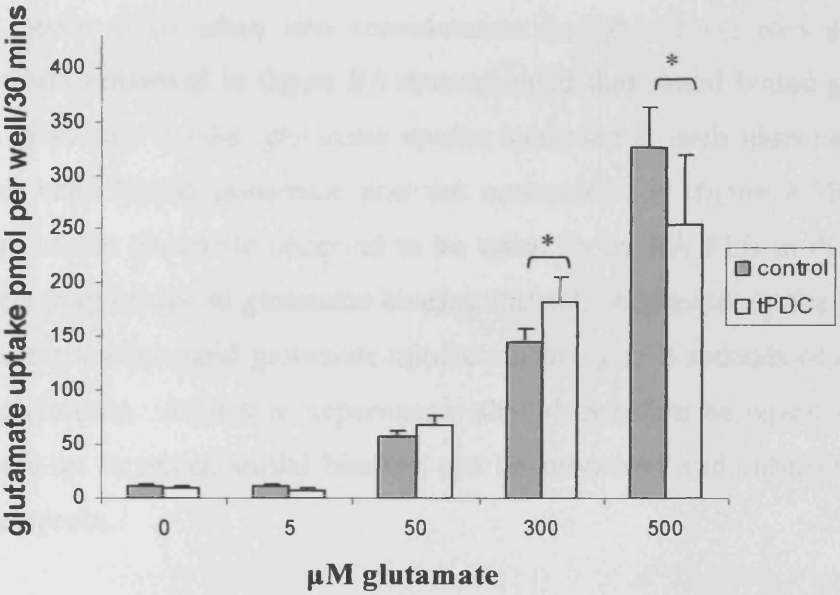


Figure 8.5 The effect of the non-transportable EAAT inhibitor, *t*PDC on ^{14}C-labelled glutamate uptake

The effect of *t*PDC on glutamate uptake by RA FLS (patient 7, passage 7) after 30 minutes as measured by a ^{14}C-labelled glutamate uptake assay. The non-transportable inhibitor significantly increased uptake of 300μM glutamate (p=0.038) however *t*PDC caused a decrease in uptake of 500μM glutamate (p=0.047). (Data was transformed (log_{10}) prior to analysis using a paired t-test, SEM error bars, n=3 wells, *:p<0.05).



8.4 Discussion

The amount of glutamate binding to RA FLS and affecting measurement of glutamate uptake was investigated. It was assumed that binding of glutamate to cell surface receptors and transporters would occur immediately however it is not certain that more glutamate would bind to the cell surface throughout the 30 minutes allowed for glutamate uptake. This may occur if the presence of extracellular glutamate induced translocation of glutamate receptors and transporters to the cell surface. Binding increased as initial extracellular glutamate increased, however no significant increase was observed between binding of 300μM and 500μM glutamate. This therefore suggests that binding was approaching saturation point at 300μM glutamate, or no more binding could occur in the time the RA FLS were exposed to the glutamate (approximately 2 seconds). This therefore needs to be taken into consideration in light of the data presented in this chapter. Data presented in figure 8.3 demonstrated that initial bound glutamate had an effect on glutamate uptake, glutamate uptake increased at each glutamate concentration increment when bound glutamate was not accounted for (figure 8.3B). Therefore a percentage of the glutamate observed to be taken up by RA FLS in the experiments in this chapter may be due to glutamate binding instead. Alternatively the glutamate bound value also represents rapid glutamate uptake occurring in 2 seconds of exposure to ^{14}C-labelled glutamate. All future experiments should therefore be repeated with duplicate cultures set up in which initial binding can be measured and subtracted from overall glutamate uptake.

Glutamate transport has been shown to be functional in RA FLS. Glutamate uptake by RA FLS increased as the initial amount of extracellular glutamate increased. The percentage of glutamate uptake significantly decreased as the initial amount of extracellular glutamate increased suggesting that the RA FLS were limited in the amount of glutamate they could uptake in 30 minutes. This could be due to limited expression or trafficking of glutamate transporters to the cell surface or removal of transporters from the cell surface membrane once a maximal intracellular glutamate concentration is reached. Cytotoxicity data revealed that 2mM extracellular glutamate is not toxic to RA FLS (chapter 4) and therefore there may not be an immediate requirement for these cells

to take up glutamate. The fact that glutamate uptake is occurring though suggests that extracellular glutamate levels are regulated by RA FLS.

The type of glutamate uptake was investigated. Data in chapter 4 and 6 indicated that the EAATs were involved in regulation of IL-6 and pro-MMP2 therefore the effect of the EAAT specific inhibitor *t*PDC on glutamate transport was investigated. The effects of *t*PDC on glutamate uptake have not previously been explored in RA FLS. In skin-derived fibroblasts however, *t*PDC inhibits glutamate uptake (Cooper *et al.* 1998). This study revealed interesting effects of *t*PDC on glutamate uptake by RA FLS. *t*PDC is an inhibitor of EAATs 1 to 5 and therefore would be expected to decrease glutamate uptake if it is mediated by these transporters. However, because *t*PDC is transportable, glutamate uptake still occurs. *t*PDC caused a decrease in the uptake of 500μM glutamate compared to the control (*t*PDC absent). This is consistent with EAAT1 and 3 (demonstrated to be expressed by RA FLS in chapter 3) contributing to glutamate uptake. However, *t*PDC caused an increase in the uptake of 300μM glutamate. The presence of *t*PDC may cause a prolonged increase in local extracellular levels of glutamate by occupying transporters and increasing local extracellular glutamate concentrations. If this effect is large enough an increase in glutamate uptake may be observed through an increased expression of cell surface transporters. Increased levels of extracellular glutamate have been demonstrated to cause trafficking of GLAST to astrocyte cell surface (Duan *et al.*1999). In addition, glutamate has been shown to induce up-regulation of glutamate transport by neurons via EAAT1 and EAAT2 (Munir *et al.* 2000). The glutamate uptake assay experiments described in this chapter were run over 30 minutes. This is enough time for receptors and transporters to be trafficked to the cell membrane from internal stores (Davis *et al.* 1998, Bernstein and Quick 1999, Lissin *et al.* 1999). An increase in uptake of glutamate by *t*PDC was only seen at 300μM glutamate which is close to the pathophysiological levels of glutamate found in RA synovial fluid (326μM). It may be this specific concentration and not 500μM glutamate that increases receptor expression or translocation to the cell surface. Therefore *t*PDC only caused an increase in uptake of pathophysiological levels of glutamate. Other glutamate uptake mechanisms may be important in the presence of 300μM glutamate but transport via the EAATs may

be important at 500μM glutamate. Alternatively, *t*PDC may be blocking uptake of glutamate and the presence of 300μM glutamate (close to RA synovial fluid levels of 326μM) may be optimal to cause an increase in receptor expression or translocation to the cell membrane. This in turn would cause an increase in the amount of glutamate binding to receptors which would be measured as uptake in this assay. Thus the observed increase in glutamate uptake is instead an increase in receptor or transporters available at the cell surface for glutamate binding.

Therefore to summarise, *t*PDC caused a decrease in the uptake of 500μM glutamate whereas an increase in uptake of glutamate by *t*PDC was seen at 300μM glutamate. This may be due to:

i) translocation of glutamate transporters to the cell surface at certain concentrations of glutamate,

ii) certain concentrations of glutamate activating other glutamate uptake mechanisms,

iii) an increase in glutamate binding caused by an increase in cell surface expression of receptors and transporters.

Glutamate bound was not accounted for in the assay exploring the effect of *t*PDC on glutamate uptake. This experiment should therefore be repeated and the amount of bound glutamate bound at zero time measured. Pre-incubation times with *t*PDC should also be increased to ensure transporters are blocked prior to addition of ^{14}C-labelled glutamate.

All high affinity glutamate transporters, the EAATs, are Na^+-dependent (reviewed in Danbolt 2001), therefore the role of Na^+-dependent glutamate transporters could be investigated. This was done by carrying out the ^{14}C-labelled glutamate uptake in Na^+-free conditions; Na^+-free conditions would prevent glutamate uptake via this type of glutamate transporter. However, no uptake was observed in this experiment (data not shown), therefore it should be repeated.

Pre-treatment with IL-6 altered the uptake of glutamate by RA FLS from patient 7; it decreased the uptake of 5μM glutamate compared to the control but had no effect on uptake of 300μM glutamate. This was unexpected due to the fact that IL-6 increases

EAAT1 expression in RA FLS (patients 1, 5 and 7, chapter 5) and therefore would be expected to increase uptake. This may be due to the RA FLS being cultured in different vessels (8-well chamber slides as opposed to flasks). Alternatively, IL-6 may down-regulate the expression of other glutamate transporters; data in chapter 3 demonstrates that EAAT3 is also expressed by RA FLS or inhibit other glutamate transport mechanisms. In fact inhibition of glutamate uptake by cytokines has previously been reported. IL-1β and TNFα decrease glutamate uptake by astrocytes, an effect proposed not to be through alteration of transporter expression levels (Hu *et al.* 2000, Liao and Chen 2001). Therefore, IL-6 may increase expression of EAAT1 but still decrease the overall rate of glutamate uptake.

Further work is needed to determine precisely the type of glutamate uptake that has been observed in this study. The EAAT inhibitor *t*PDC altered glutamate uptake therefore indicating that glutamate uptake via EAATs does occur in RA FLS, the presence of Na^+-independent uptake mechanisms in RA FLS should also be determined. In addition the effect of IL-6 on glutamate uptake should be explored in more patients, and the effect of inhibition of IL-6 signalling with Mab227 could also be investigated.

To conclude, RA FLS demonstrate glutamate uptake, which is altered by the inflammatory cytokine IL-6 and the EAAT inhibitor *t*PDC. Regulation of glutamate uptake *in vitro* appears to be very complicated and is likely to be affected by many other factors *in vivo* such as the type of glutamate transporters and receptors expressed on the RA FLS cell surface, concentration of extracellular glutamate, cytokines present in the synovial fluid and more broadly, the stage of RA development.

Chapter 9 Discussion

9.1 Summary of experimental outcomes seen in FLS

This study has revealed fascinating and novel associations between glutamate and the pro-inflammatory and degradative proteins associated with RA. A summary of all the effects observed in all the FLS used in this study is reviewed in table 9.1. Although some experiments were only performed on one cell line, all were repeated between three and six times. Some correlations in experimental outcomes can be observed between patients and these will now be discussed. As is always the case when using human RA synoviocytes, the effects are not consistent across all patients (Bucala *et al.* 1991).

The most obvious trend is that FLS from the normal patient appeared much less responsive to glutamate than RA FLS. Glutamate had no effect on normal FLS production of IL-6, pro-MMP2 and TIMPs whereas, although variable, effects were seen in all RA FLS patients except patient 2. Furthermore, normal FLS responded differently to RA FLS when incubated with TBOA and MK801. TBOA increased IL-6 production in RA FLS but decreased it in normal FLS (chapter 4). MK801 increased pro-MMP2 production by normal FLS but had no effect on pro-MMP2 production by RA FLS (chapter 6). These differences in response to glutamate and IL-6 could be due to the variable *in vivo* levels of glutamate and IL-6 (McNearney *et al.* 2000, DesGeorges *et al.* 1997) and different expression profiles of glutamate transporters and receptors observed between RA and normal FLS e.g. EAAT2, mGluR4 and KA1 (chapter 3). Since IL-6 and TNFα increased EAAT1 protein expression in RA but not in normal FLS (chapter 5), it is likely that the responsiveness of RA and normal FLS to pro-inflammatory cytokines also varied.

When comparing across RA patients, some RA FLS showed less of a typical RA phenotype (high levels of cytokines and degradative enzymes being expressed) than others. For example, RA FLS from patient 2 demonstrated constitutively low levels of pro-MMP2 and undetectable IL-6, compared to the high levels produced by RA FLS from patient 8. Other studies have shown high production of IL-6 by RA FLS in culture

Table 9.1 A summary of experimental outcomes observed in FLS from the patients used in this study.

Patient (number)	Age	Sex	Passage	Effects observed in FLS from each patient	n=
Normal (4)	64	F	6	-No effect of glutamate on IL-6 production. -Glutamate increased pro-MMP2 production. -TBOA decreased IL-6 production. - No effect of glutamate and TBOA on TIMP production.	6 6 6 4
			7	-No effect of glutamate on IL-6 production. -No effect of glutamate on pro-MMP2 production. -MK801 increased IL-6 production. -No effect of IL-6 and TNFα on EAAT1 protein expression (IHC).	6 6 6 1
RA (2)	56	F	5	-Used for mRNA expression studies in chapter 3.	1
			6	-Constitutive levels of IL-6 too low to be detected. -No effect of glutamate on pro-MMP2 production. -*t*PDC and TBOA increased IL-6 production. -*t*PDC and TBOA increased pro-MMP2 production. -*t*PDC and TBOA increased TIMP1 and TIMP2 production.	6 6 6 6 4
RA (3)	62	F	5	-Glutamate increased IL-6 production. -Glutamate increased pro-MMP2 production. -TBOA increased IL-6 production. -TBOA increased pro-MMP2 production.	6 6 6 6
			7	-Variable effects of glutamate on IL-6 and pro-MMP2 production. -No effect of DAP5 and MK801 on IL-6 production. -MK801 increased pro-MMP2 production. -No effects observed on TIMP production by glutamate, DAP5 or MK801.	6 6 6 4
RA (8)	55	M	6	-Glutamate caused a decrease in IL-6 production. -No effect of glutamate on pro-MMP2 production. -NBQX decreased IL-6 production. - No effect of glutamate, CFM2 and NBQX on TIMP production.	6 6 6 4
RA (1)	?	?	?	- IL-6 increased expression of EAAT1 mRNA	1
RA (5)	67	F	7	-IL-6 and TNFα increased expression of EAAT1 protein (IHC).	2
RA (7)	43	F	7	-IL-6 increased expression of EAAT1 protein (WB). -FLS able to take up glutamate (radioactive assay). -IL-6 caused decrease in glutamate uptake. -Varying effects of *t*PDC on glutamate uptake.	4 6 3 3
RA (6)	64	F	5	-glutamate and NMDA caused increase in $[Ca^{2+}]_i$ and pre-treatment with IL-6 caused less cells to respond to glutamate and NMDA.	2
RA (9)	27	F	7	-Glutamate and KA caused increase in $[Ca^{2+}]_i$.	2

IHC: immunohistochemistry, WB: Western blotting

(8-15ng/ml) (Hirth *et al.* 2001, Okamoto *et al.* 1997) and in RA synovial fluid IL-6 and MMP2 are both elevated (Desgeorges *et al.* 1997, Yoshihara *et al.* 2000). This suggests that patient 2 FLS were not demonstrating a typical RA phenotype. However, cells from this patient did reveal differences in glutamate receptor and transporter mRNA expression from normal FLS and showed some consistency with other RA patient FLS with respect to TBOA and *t*PDC effects on IL-6 and pro-MMP2. It may be the progression of RA disease or the treatments received by patient 2 which make these RA FLS different.

9.2 The role of glutamate in rheumatoid arthritis

9.2.1 IL-6

The effects of glutamate on pro-inflammatory cytokine production within the CNS are not well documented; however stimulation with glutamate is known to increase IL-6 production in cultured astrocytes after 16 hours (Wu *et al.* 1997). In ischemic brain injury, which occurs during stroke, levels of glutamate and IL-6 correlate in patient's plasma and cerebrospinal fluid (Vila *et al.* 2000), and in fact rat brains injected with NMDA (to simulate excitotoxic injury) have increased levels of TNFα and IL-1β mRNA (Szaflarski *et al.* 1995).

This study indicates that glutamate has the potential to play a major role in RA. Increased extracellular glutamate, either by addition of glutamate to the media (patient 3) or inhibiting glutamate transporters (patients 2 and 3), increased IL-6 production. This indicates that the elevated levels of glutamate in the synovial fluid of RA patients could be affecting one of the main mediators of RA pathogenesis. The role of IL-6 in the pathogenesis of RA is clear from current therapeutic strategies in clinical trials that reduce IL-6 activity (section 1.5.4). If glutamate is involved in the regulation or control of IL-6 levels *in vivo* then glutamate transporters and receptors may also serve as therapeutic targets (see section 9.4), especially if glutamate concentrations in synovial fluid increase during RA inflammatory episodes. Furthermore, it is clear that IL-6 and another pro-inflammatory cytokine, TNFα can modulate RA FLS responses to glutamate by increasing EAAT1 mRNA and protein expression (patients 1, 5 and 7). Therefore,

this demonstrates a mechanism for IL-6 to modulate glutamate levels just as glutamate regulates IL-6 levels.

Like the proverbial question of the chicken and the egg it is not clear which may come first in RA, a dysregulation of glutamate or changes in levels of IL-6. However, it is accepted that an autoimmune response triggers the start of RA, therefore IL-6 may pre-empt dysregulation of glutamate and expression of glutamate receptors and transporters. However, in animal models of inflammation glutamate levels have been observed to increase within 10 minutes after induction of inflammation (Lawand *et al.* 2000) whereas increases in IL-6 have been observed after 3 days (Mentzel and Braeuer 1998). In Mentzel's study however, 3 days was the earliest time point measured after induction of arthritis. In another antigen-induced arthritis model, IL-6 levels increased 15 days after inflammation (Magari *et al.* 2003). Taking this evidence into consideration it is likely that in antigen-induced models of RA, an increase in synovial fluid glutamate levels would precede and thus influence increases in IL-6 expression. It is unclear whether this is an accurate indicator of what occurs in the actual onset of the rheumatoid arthritis disease.

A suggested pathway for how glutamate signalling pathways may interact with IL-6 signalling pathways in RA FLS is shown in figure 9.1. Results of chapter 4 indicated that glutamate-induced increases in IL-6 are most likely to be mediated via kainate receptors since treatment with NBQX inhibited IL-6 release whereas CFM-2 did not (patient 8). Although NBQX decreased IL-6 production at all glutamate concentrations, this control is not straightforward because increasing extracellular glutamate caused a decrease in IL-6 production in this patient. Direct application of kainate to RA FLS caused an increase in $[Ca^{2+}]_i$, (chapter 7) demonstrating that KA receptors are functional and thus may mediate this signalling pathway. In microglial cells increases in $[Ca^{2+}]_i$ have been implicated in increasing IL-6 mRNA expression (Sattayaprasert *et al.* 2005). Increases in $[Ca^{2+}]_i$ have also been implicated in IL-6 release by mast cells (Tanaka *et al.* 2005), gingivial fibroblasts (Noguchi *et al.* 2001) and cardiac-derived fibroblasts (Colston *et al.* 2002). It is a standard signalling mechanism that increases in $[Ca^{2+}]_i$ can activate NFκB

via protein kinase C (Alberts *et al.* 1994). Ca^{2+} oscillations have been shown to increase expression of the transcription factor NFκB in T-cells (Dolmetsch *et al.* 1998) and NFκB, in turn, can control expression of IL-6 (Libermann and Baltimore 1990). Furthermore, kainate receptors can activate NFκB via the MAP kinase pathway in neurons (Cruise *et al.* 2000). Therefore increases in $[Ca^{2+}]_i$ induced by glutamate activation of kainate receptors may activate NFκB via protein kinase C and increase IL-6 mRNA expression. This is an example mechanism, glutamate may be activating other signalling pathways such as through the interaction of kainate receptors with PDZ binding domain proteins which can activate JNK (Savinainen 2001). In addition, there are other cis-elements in the complex IL-6 promoter such as AP1, NF-IL-6 binding sites which can affect expression (reviewed in Vanden *et al.* 1999).

It is proposed that IL-6 may operate a feedback mechanism in order to regulate levels of extracellular glutamate (figure 9.1). Treatment of RA FLS with IL-6 caused an increase in EAAT1 mRNA and protein expression (chapter 5). The possible signalling pathways involved in this regulation are discussed in chapter 5. This increase in EAAT1, if trafficked to the plasma membrane, may function to down-regulate extracellular glutamate levels. This in turn may down-regulate IL-6 production. The efficiency of this feedback mechanism may define how FLS from different patients respond to glutamate.

Results presented in chapter 4 indicate that glutamate affects IL-6 production by normal FLS differently to RA FLS. A proposed signalling pathway for regulation of IL-6 by glutamate in normal FLS is shown in figure 9.2. Inhibition of NMDA receptors (patient 4) caused an increase in IL-6 production which suggests that activation of NMDA receptors in normal FLS would decrease IL-6 production. Data presented in chapter 7 demonstrate that NMDA can increase $[Ca^{2+}]_i$ in RA FLS, although normal FLS were not tested. It is unclear how activation of NMDA receptors may increase IL-6 production in the presence of some glutamate concentrations by RA FLS but decrease IL-6 production by normal FLS. One possibility may be alterations in the frequency of Ca^{2+} oscillations, which have previously been shown in T-cells to activate different transcription factors (Dolmetsch *et al.* 1998). Alternatively it may be due to the responsiveness of RA and

Figure 9.1 Proposed mechanisms of IL-6 and glutamate interactions in RA FLS
Activation of kainate receptors leads to an increase in $[Ca^{2+}]_i$ which may increase release of IL-6 by RA FLS. IL-6 may be involved in a feedback mechanism to regulate levels of EAAT1 expression which in turn can modulate the concentration of extracellular glutamate.

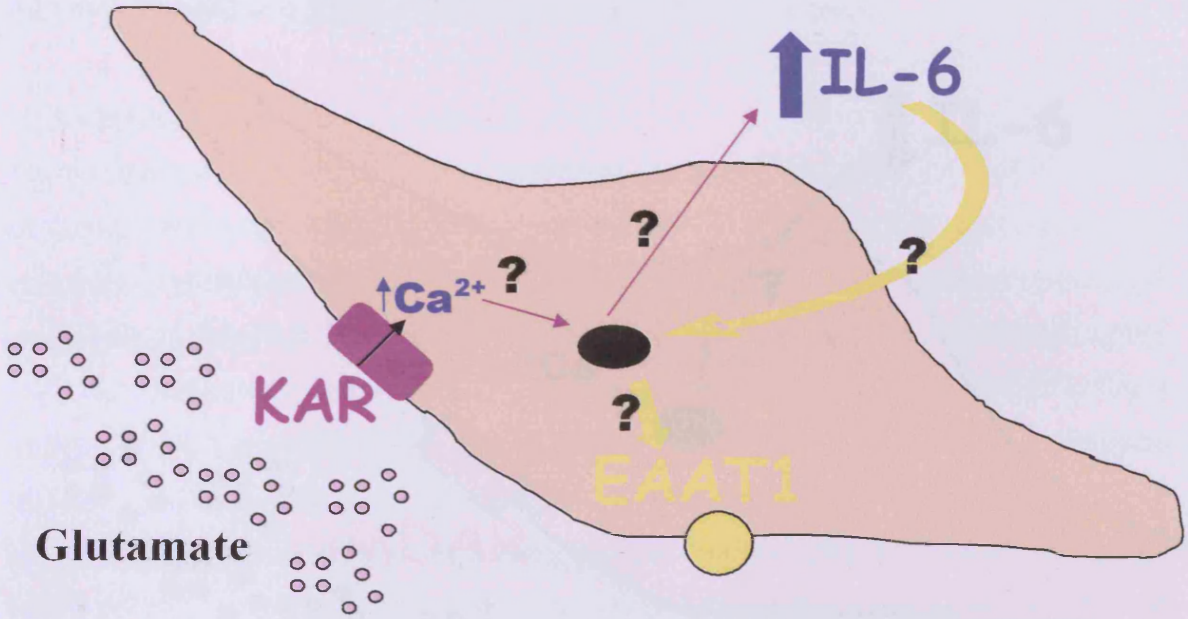
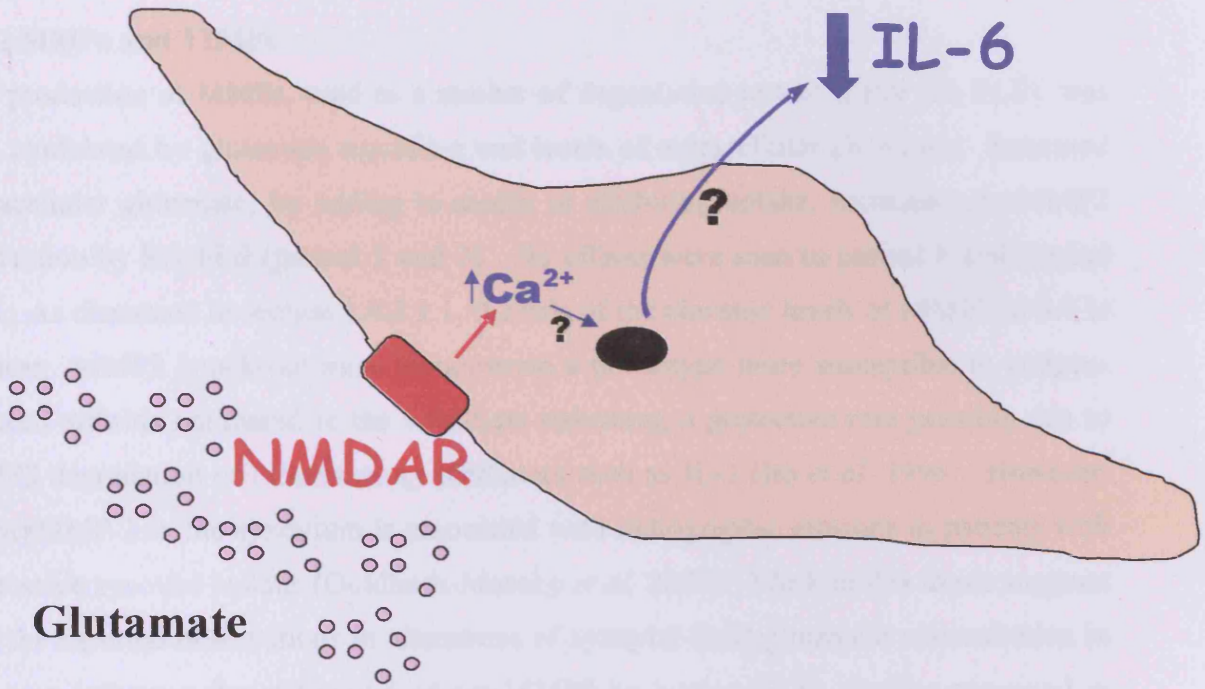


Figure 9.2 Proposed mechanisms of IL-6 and glutamate interactions in normal FLS
Activation of NMDA receptors leads to an increase in $[Ca^{2+}]_i$ which may decrease release of IL-6 by normal FLS. This therefore correlates with the decrease in IL-6 observed in the presence of glutamate transporter inhibitors because glutamate transporter inhibitors are likely to elevate extracellular glutamate levels.



normal FLS to glutamate caused by differences in types and possibly numbers of glutamate receptors expressed.

The fact that activation of NMDA receptors may decrease IL-6 production may explain why increasing extracellular glutamate decreased IL-6 production in patient 8, i.e. glutamate was having a greater effect on NMDA receptors than kainate receptors. This may be due to different levels of expression of glutamate receptors.

9.2.2 MMPs and TIMPs

The production of MMPs, used as a marker of degradative potential (by RA FLS), was also modulated by glutamate signalling and levels of extracellular glutamate. Increased extracellular glutamate, by adding to media or inhibiting uptake, increased pro-MMP2 production by RA FLS (patient 2 and 3). No effects were seen in patient 8 and normal FLS. As discussed in section 1.4.2.1.1, the role of the elevated levels of MMP2 in RA is unclear. MMP2 knock-out mice demonstrate a phenotype more susceptible to antigen-induced arthritis compared to the wild-type indicating a protective role possibly due to MMP2 degradation of inflammatory mediators such as IL-1 (Ito *et al.* 1996). However, active MMP-2 in the synovium is associated with radiographic erosions in patients with aggressive synovial lesions (Goldbach-Mansky *et al.* 2000). Work in this thesis suggests that the reported observations in alterations of synovial fluid glutamate concentration in RA may influence the expression of pro-MMP2 by human FLS. Studies presented in chapter 6 revealed that inhibition of NMDA receptors with MK801 caused an increase in pro-MMP2 production by RA FLS. The demonstration that NMDA receptors can be activated in RA FLS to increase $[Ca^{2+}]_i$ levels (chapter 8) has led to the proposed interaction of glutamate and pro-MMP2 depicted in figure 9.3. Glutamate may activate NMDA receptors which increases $[Ca^{2+}]_i$ levels which in turn may down-regulate pro-MMP2 production. In fact, a Ca^{2+} channel blocker (nifedipine, an inhibitor of voltage-dependent L-type calcium channels) has been shown to increase MMP2 expression by cardiac fibroblasts (Yue *et al.* 2004). Conversely, inhibiting non-voltage gated and voltage gated Ca^{2+} channels (carboxy amido triazole, inhibits receptor-operated calcium influx) in a fibrosarcoma cell line decreased MMP2 levels (Kohn *et al.* 1994). Currently

no literature is published which links NMDA receptor activation to the control of MMP2 expression. Within the synovial joint, FLS are not the only cells producing MMP2. Stromal cells in the sublining synovial layer, macrophages (Hembry *et al.* 1995) and chondrocytes (Stephenson *et al.* 1987) also produce MMP2 although the main source of MMP2 in the RA synovial joint is not currently documented. Therefore unless glutamate affects production by these cells the impact of glutamate on MMP2 levels in the synovial joint remains unclear.

Ultimately it is the balance of MMPs and TIMPs that causes degradation in RA. Results of chapter 6 demonstrated that TBOA and *t*PDC increased TIMP1 and TIMP2 production by RA FLS (patient 2). However, glutamate and glutamate receptor antagonists had no effect on TIMP production by RA and normal FLS. It is unclear how glutamate may interact with TIMP production because no effects were observed when FLS were incubated with ionotropic receptor inhibitors. It is therefore possible that the metabotropic receptors are involved in the increase in TIMP production observed in the presence of high extracellular glutamate (caused by glutamate transporter inhibition).

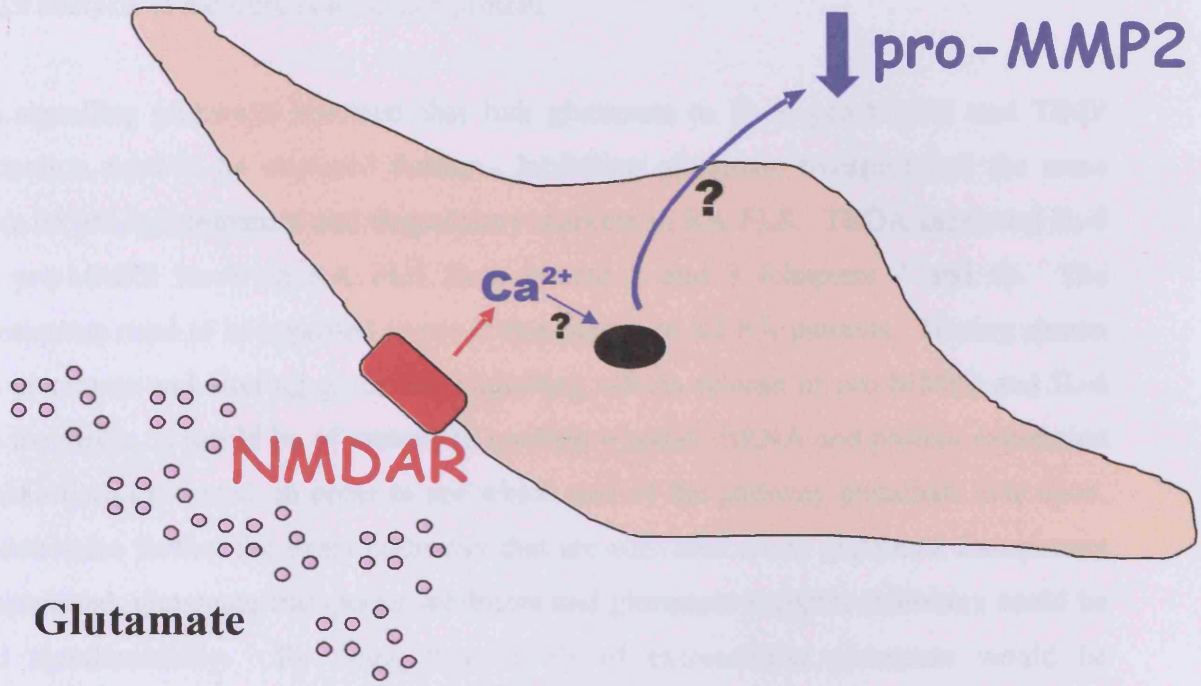
9.3 Future Research

It is clear from the data that even though statistically significant responses were observed with treatments, further work is necessary to characterise the data fully. It would be of interest if all experiments were carried out on cells from the same patient in order to compare results accurately and see interacting effects. Furthermore, in order for accurate trends to be observed, cells from many RA and normal patients need to be tested and data correlated with age, sex, disease progression, treatments received and synovial fluid and serum concentrations of glutamate and IL-6. The results have however provided clear pathways for future research.

mRNA expression of a wide range of glutamate receptors and transporters has been observed in cells and tissues of the synovial joint however only EAAT1 protein expression was tested in FLS. It would be fascinating to see which other glutamate receptor, receptor subunit and transporter protein is expressed by FLS and other cells

Figure 9.3 Proposed mechanisms of pro-MMP2 and glutamate interactions in RA FLS

Activation of NMDA receptors leads to an increase in $[Ca^{2+}]_i$ which may decrease release of pro-MMP2 by RA FLS. Therefore the increase in pro-MMP2 observed in the presence of glutamate transporter inhibitors is likely to be mediated by by mGluR.



present in the synovial joint. In addition, a more complete picture of which glutamate receptors, receptor subunits and transporters are functional or whether expression levels alter at different stages of disease is required to understand fully the responses seen in this study. Immunohistochemistry could be utilised to determine co-localisation of recetor subunits, and the exact location of transporters and receptors expressed in the synovial joint. Furthermore, it would be intriguing to see if expression levels of glutamate receptors and transporters and their splice variants changed upon stimulation with glutamate or IL-6. This could be done using RT-PCR to measure mRNA or by FACS analysis to measure cell surface protein.

The signalling pathways involved that link glutamate to IL-6, pro-MMP2 and TIMP production need to be explored further. Inhibiting glutamate transport had the same effect on pro-inflammatory and degradatory markers in RA FLS. TBOA increased IL-6 and pro-MMP2 levels in RA FLS from patient 2 and 3 (chapters 4 and 6). The experiments need to be repeated to see if this occurs in all RA patients. Having shown that glutamate and altering glutamate signalling affects release of pro-MMP2 and IL-6 into the media, it would be of interest to confirm whether mRNA and protein expression levels are also elevated, in order to see which part of the pathway glutamate acts upon. To determine further the exact pathways that are activated when glutamate transporters are inhibited, glutamate transporter inhibitors and glutamate receptor inhibitors could be used simultaneously. By doing this, levels of extracellular glutamate would be maintained at a higher level which would perhaps enhance the effects of glutamate receptor inhibitors. In addition, the glutamate receptor and transporter inhibitor experiments could be repeated in the presence of intracellular Ca^{2+} chelators, such as 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA; Grant *et al.* 2001), in order to see whether effects are being mediated by alterations in $[Ca^{2+}]_i$. The phosphorylation pathways involved in the hypothesised induction of IL-6 by activation of kainate receptors (figure 9.1) need to be investigated. It should be tested whether kainate (instead of glutamate) can increase IL-6 production by RA FLS and subsequently, the effect of tyrosine kinase inhibitors (e.g. Chelerythrine chloride or Hexadecyl Phosphoholine) and NFκB activation inhibitors (e.g. PDTC) on kainate-mediated

increases in IL-6 could be measured. This would determine whether protein kinase C and NFκB are mediators of this proposed signalling pathway. Antisense techniques could also be used to prevent synthesis of specific signalling proteins. Finally, having shown that glutamate interacts with IL-6 the effects on sIL-6r, IL-6R and gp130 expression also need to be evaluated to determine the overall effects of glutamate on IL-6 signalling.

Further investigation is needed to determine the signalling pathway mediating the effect of IL-6 on EAAT1 expression (chapter 5). As discussed in chapter 5 phosphatidylinositol 3 kinase or STAT proteins could mediate the signalling between IL-6 and EAAT1. Measuring EAAT1 expression after stimulating FLS with IL-6 and inhibiting these pathways with specific inhibitors would determine whether these pathways are involved. The effect of TNFα on EAAT1 expression was also briefly explored (chapter 5). TNFα is a dominant mediator in RA inflammation. Therefore this cytokine also has the potential to affect extracellular glutamate levels in the synovial joint. Synovial fluid levels of TNFα and glutamate are both elevated in RA although these levels were not shown to correlate (McNearney *et al.* 2004). It would be interesting to see if glutamate is involved in the elevation of TNFα by treating cells with a range of glutamate concentrations and measuring TNFα levels as has been done in this study with IL-6.

As mentioned in chapter 6 TIMP1 can protect neurons against cell death induced by high concentrations of glutamate possibly by blocking glutamate mediated calcium entry (Tan *et al.* 2003). Tan *et al.* suggest that the neuroprotective effect of TIMP1 only occurs when it is bound to MMPs because when neurons are incubated with TIMP1 and a synthetic MMP inhibitor (MMPI) the neuroprotective effect is lost. Furthermore, TIMP1 is up-regulated in rat neurons after kainate-induced seizures indicating that action of kainate receptors regulates TIMP1 expression (Tan *et al.* 2003). Studies described in chapter 7 demonstrated that RA FLS respond to kainate with increases in $[Ca^{2+}]_i$. It would be logical therefore, to see if pre-treatment of FLS with TIMP1 can influence levels of $[Ca^{2+}]_i$ following stimulation with kainate. This would demonstrate whether the same signalling pathway between glutamate, TIMP1 and calcium occurs in FLS as it appears to in neurons. How the effect of TIMP1 on glutamate receptor calcium influx is

mediated is currently unknown. The potential for kainate receptor inhibitors in RA therapy is discussed in section 9.4.

Previous studies also indicate that other cells in the synovial joint may be responsive to glutamate (see section 1.6.4). Therefore the scope for future work is great. As discussed in section 1.6.4 macrophages and chondrocytes express glutamate receptors and transporters but no research has investigated how these cells may be influenced by increased glutamate concentrations in RA. If glutamate signalling is important at the onset of RA disease then macrophage-like synoviocytes should be prioritised for investigation for responsiveness to glutamate because along with FLS, they constitute the major cell type of the invasive pannus.

The next step after *in vitro* experiments is to explore effects of glutamate and IL-6 *in vivo*. It would be interesting to see whether levels of glutamate in the synovial joint correlate with the degree of onset of inflammation by measuring synovial fluid levels of IL-6 and glutamate receptor and transporter expression. Glutamate levels can be measured by HPLC in the synovial perfusate of *in vivo* models of inflammatory arthritis; this would confirm studies carried out by Lawand *et al.* (Lawand *et al.* 2000). Furthermore, the effects of injection of glutamate transporter or receptor antagonists into the synovial joint on glutamate, IL-6, TNFα, MMP and TIMP levels and disease progression could be determined to see if changes in these proteins are also mediated by glutamate signalling *in vivo*.

The interaction of IL-6 and glutamate signalling could be investigated in IL-6 knock-out mice. Studies in chapter 5 demonstrated that IL-6 can induce EAAT1 mRNA and protein expression. It would be fascinating to see if glutamate transporter expression and function is altered in IL-6 knock-out mice compared to the wild type phenotype. This could be done by comparing *in vivo* expression of glutamate receptors and transporters using RT-PCR, Western blotting, *in situ* hybridisation and immunohistochemistry on sections of the synovial joints between wild type and knock-out mice. In addition, glutamate regulation could be monitored in IL-6 transgenic mice (over-expressing IL-6)

through measuring synovial fluid levels of glutamate by HPLC and comparing this to wild-type mice. Inducing arthritis in the transgenic mice, injecting inhibitors of glutamate receptors, in particular kainate receptor inhibitors, and glutamate transporters and comparing joint damage to wild type mice would indicate the importance of IL-6 in glutamate regulation.

The interaction of IL-6 and glutamate signalling could also be investigated in glutamate transporter knock-out mice. EAAT1, EAAT2, EAAT3 and EAAT4 and many glutamate receptor and receptor subunit knock-out mice or partial knock-out mice already exist. Having already observed changes in EAAT1 expression in response to pro-inflammatory cytokines, this knock-out mouse should be targeted first. A protection against antigen-induced arthritis, with correlating changes in cytokine production, MMP and TIMP production and synovial fluid levels of glutamate upon induction of AIA would validate an importance for glutamate signalling in the onset of inflammatory arthritis.

It would be intriguing to see whether oestrogen affects glutamate regulation in RA patients. During pregnancy RA symptoms are alleviated and this has been associated with an increase in oestrogen levels (Ostensen *et al.* 1983). Furthermore, onset of RA or worsening of RA symptoms is associated with low oestrogen levels such as after giving birth or the menopause (McHugh 1990). Oestrogen increases glutamate transporter (EAAT1 and EAAT2) mRNA and protein expression in astrocytes and can prevent glutamate-induced cell death of astrocytes (Pawlak *et al.* 2005). Therefore it may be relevant to investigate the effect of oestrogen on glutamate uptake in cells of the synovial joint. Alternatively, the effect of ovariectomy in AIA could also be investigated. It may be that the low levels of oestrogen decrease EAAT1 and EAAT2 expression which in turn may increase glutamate levels in RA patients that make these patients more susceptible to this disease.

To prioritise future work, the main hypotheses that can be drawn from work in this thesis are those represented in figures 9.1 and 9.3. Investigation into the actual mechanisms of these pathways may determine potential therapeutic targets.

9.4 Potential therapies for RA

The most likely therapeutic target identified in this study is the kainate receptor. However, any therapy that targets glutamate signalling machinery needs to be localised to the RA-affected joint or unable to cross the blood brain barrier. Furthermore, due to the role of glutamate identified in other peripheral systems (see section 1.6.4) therapeutics targeting glutamate signalling should be localised to the affected joint. Further work has been suggested to investigate the role of TIMP1 in the mediation of kainate induced increases in $[Ca^{2+}]_i$ (section 9.3). If TIMP1 did decrease the increase in $[Ca^{2+}]_i$ in response to kainate then recombinant TIMP1 or TIMP1 analogues could be considered as therapies for RA.

It is proposed that activation of NMDA receptors decreases pro-MMP2 production by RA FLS. Pro-MMP2 is thought to play an overall degradative role in RA (see section 1.5.1.3) therefore activating this pathway may have beneficial effects in RA patients. However, the impact of enhancing this pathway is not likely to be great because many other degradative enzymes, besides MMP2, are involved in RA joint degradation.

If elevated levels of glutamate are involved in the pathogenesis of RA then a way of lowering synovial fluid levels of glutamate should be devised. Elevating expression of levels of EAATs would enable this. One possibility is through gene therapy, using a vector to deliver DNA encoding the EAAT1 gene to the inflamed synovial joints. In fact, the transfection of glucose transporters has been investigated as a potential way to treat diabetes (Simpson *et al.* 1997).

9.5 Closing comments

The interaction of glutamate with inflammatory and degradatory responses involved in the pathogenesis of RA has been described. Signalling pathways that may mediate these interactions have also been proposed. Furthermore, it has been demonstrated for the first time that human FLS have the ability to take up glutamate and are responsive to glutamate via activation of specific glutamate receptors. Therefore, with further work, new therapeutics targeted to glutamate receptors may be identified that could treat or alleviate symptoms of the debilitating disease that is Rheumatoid arthritis.

APPENDIX 1

2x Sample buffer

to make 50ml:

33.75ml	dH_2O
6.25ml	1M Tris-Cl (pH6.8)
10ml	Glycerol
5g	SDS
25mg	Bromophenol

5x Sample buffer

to make 50ml:

9.37ml	dH_2O
15.63ml	1M Tris-Cl (pH6.8)
25ml	Glycerol
5g	SDS
25mg	Bromophenol

APPENDIX 2

Calculation of K_i values from published IC_{50} values:

The K_i value can be obtained from an IC_{50} value using the Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + L/K_d}$$

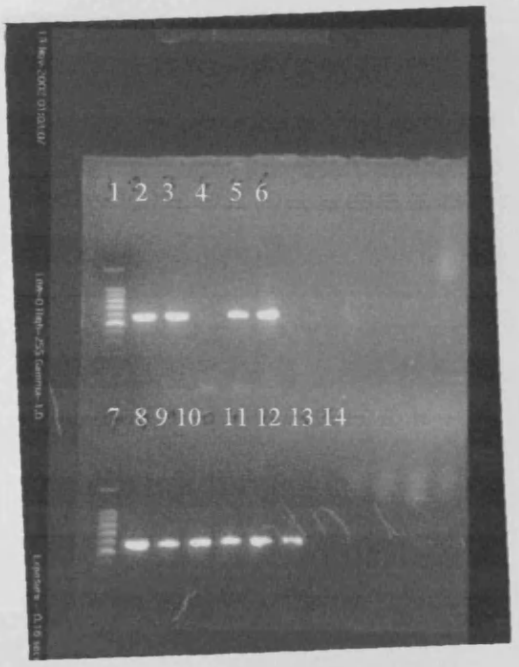
where L is the concentration of radioactive ligand and K_d is the affinity of the ligand (i.e. glutamate) for the receptor (Cheng and Prusoff 1973).

To calculate approximately the binding affinity of DAP5 and MK801 for NMDA receptors, the binding affinity of glutamate (K_d) was taken to be 99nM (Fritz *et al.* 1996). This value does not take into account the different affinities for different receptor subunit compositions.
The IC_{50} values were taken from work published on lymphocytes (Lombardi *et al.* 2001).
IC_{50} for DAP5 = 0.9μM, IC_{50} for MK801 = 0.6μM.
L=1 μM

To calculate approximately the binding affinity of *t*PDC and TBOA the binding affinity of glutamate (K_d) to all glutamate transporters was estimated. The affinity of glutamate for the EAATs varies between 1-100μM (Danbolt 2001), therefore a value of 50μM was used.
The IC_{50} values were taken from work published on astrocytes (Waagepetersen *et al.* 2001).
IC_{50} for *t*PDC = 39μM, IC_{50} for TBOA = 19μM.
L=1 μM

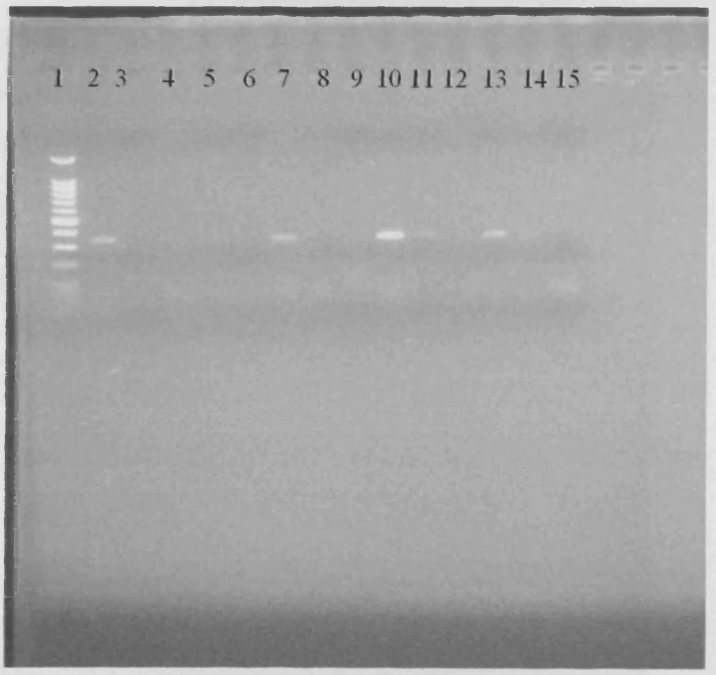
Appendix 3

Key:
β-actin PCR
1: 100bp ladder
2: Rat menisci
3: Female rat menisci
4: Female rat menisci
5: Female rat menisci
6: Female rat menisci
7: 100bp ladder
8: Rat menisci
9: Rat menisci
10: Female rat menisci
11: Rat menisci
12: Rat menisci
13: β-actin plasmid (positive)
14: Water (negative)



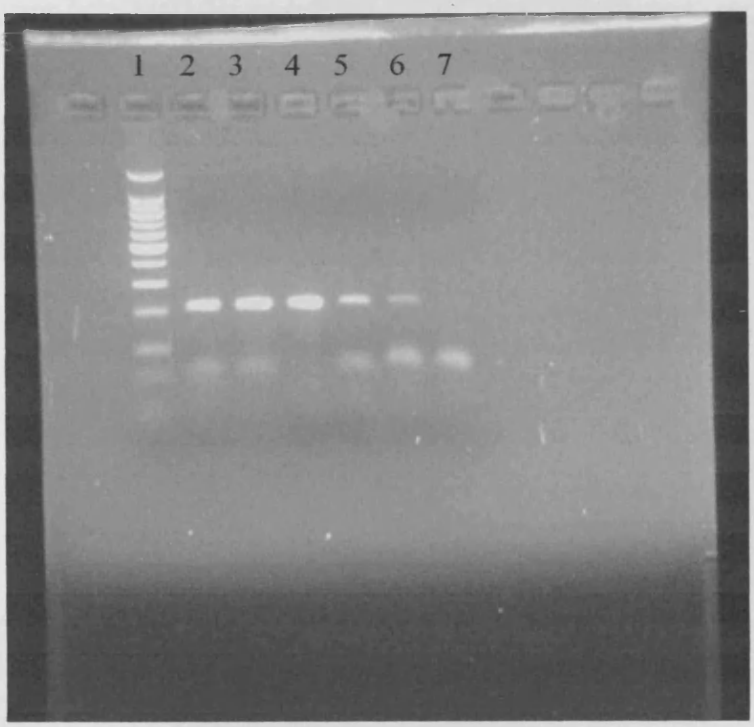
Appendix 4

Key:
GLAST-1/EAAT1 PCR
1: 100bp DNA ladder
2: Rat patella
3: Rat patella
4: Rat menisci
5: Rat menisci
6: RA FLS
7: RA FLS
8: water (negative)
9: NFLS
10 NFLS
11: bovine chondrocyte
12: bovine chondrocyte
13: Rat brain (positive)
14: Rat brain (positive)
15 water (negative)



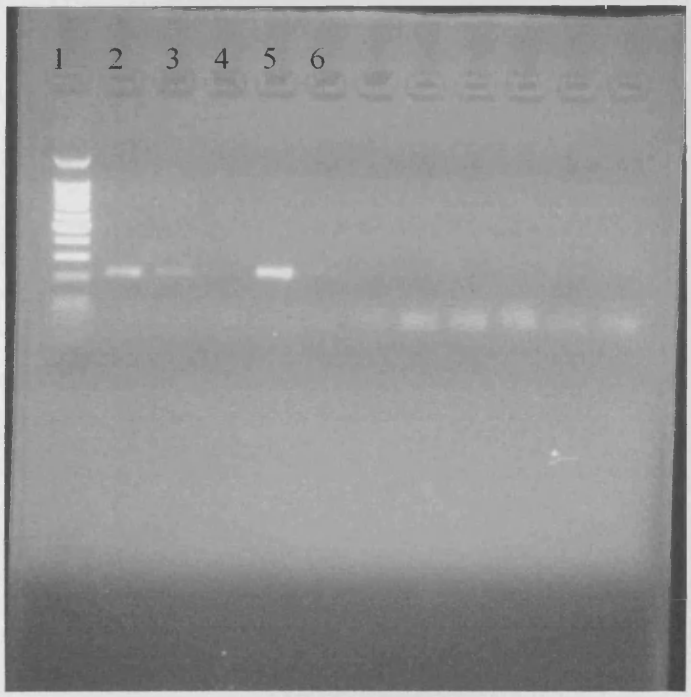
APPENDIX 5

Key:
GLAST1a
1: 100bp ladder
2: Rat patella
3: Rat menisci
4: Rat fat pad
5: Bovine chondrocyte
6: Rat brain (positive)
7: water (negative)



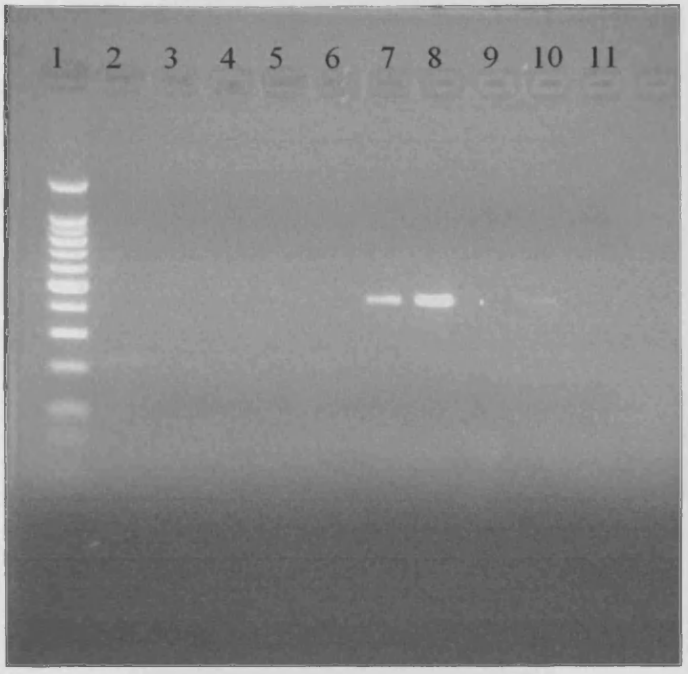
APPENDIX 6

Key:
EAAT2
1: 100bp ladder
2: Rat patella
3: Rat fat pad
4: Rat menisci
5: Rat brain (positive)
6: water (negative)



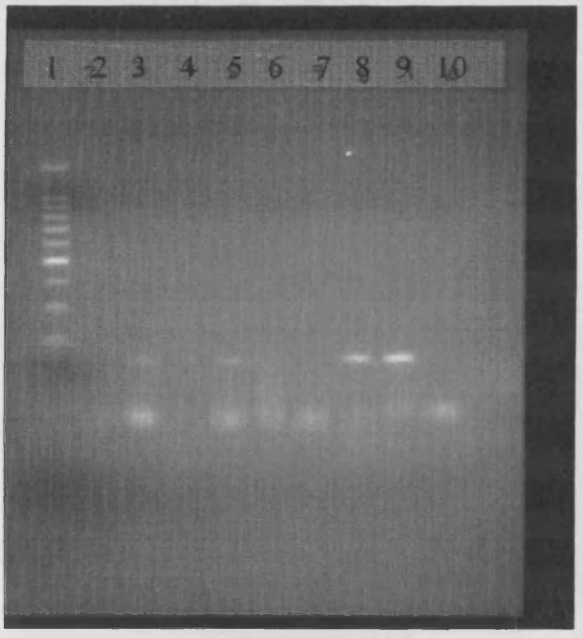
APPENDIX 7

Key:
EAAT2 and EAAT3
1: 100bp ladder
2: EAAT2 rat patella
3: EAAT2 rat fat pad
4: EAAT2 bovine chondrocytes
5: EAAT2 rat brain
6: EAAT2 water
7: EAAT3 rat patella
8: EAAT3 rat fat pad
9: EAAT3 bovine chondrocytes
10: EAAT3 rat brain (positive)
11: EAAT3 water (negative)



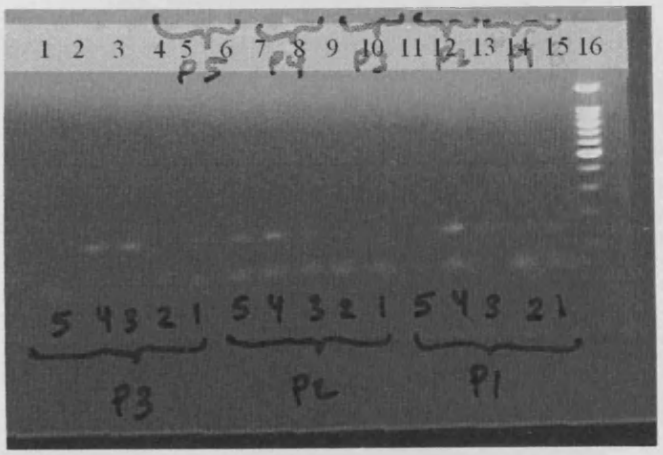
APPENDIX 8

Key:
AMPAGluR3, mGluR4 and NMDA NR1
1: 100bp ladder
2: AMPAGluR3, rat patella
3: AMPA GluR3, rat brain (positive)
4: AMPA GluR3, water (negative)
5: mGluR4, rat patella
6: mGluR4, rat brain (positive)
7: mGluR4, water (negative)
8: NMDA NR1, rat patella
9: NMDA NR1, rat brain (positive)
10: NMDA NR1, water (negative)



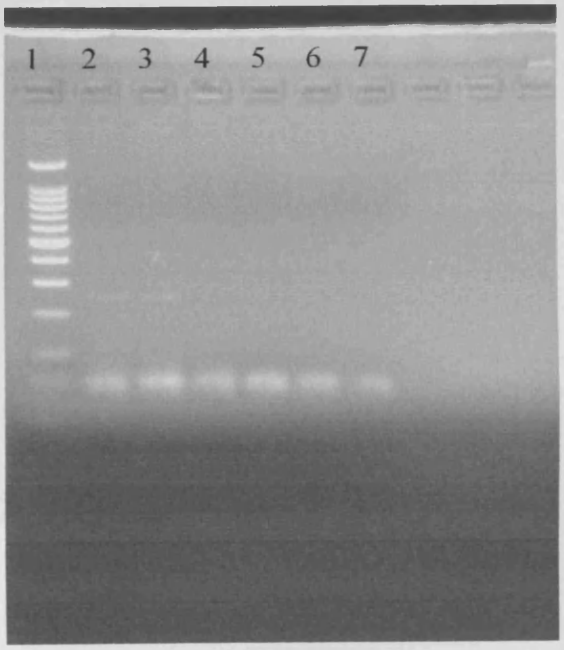
APPENDIX 9

Key:
mGluR4, AMPAGluR3, AMPAGluR2
PCR performed by V. Savanathan
1: mGluR4, water (negative)
2: mGluR4, rat brain (positive)
3: mGluR4, rat patella
4: mGluR4, rat fat pad
5: mGluR4, rat menisci
6: AMPAGluR3, water (negative)
7: AMPAGluR3, rat brain (positive)
8: AMPAGluR3, rat patella
9: AMPAGluR3, rat fat pad
10: AMPAGluR3, rat menisci
11: AMPAGluR2, water (negative)
12: AMPAGluR2, rat brain (positive)
13: AMPAGluR2, rat patella
14: AMPAGluR2, rat fat pad
15: AMPAGluR2, rat menisci



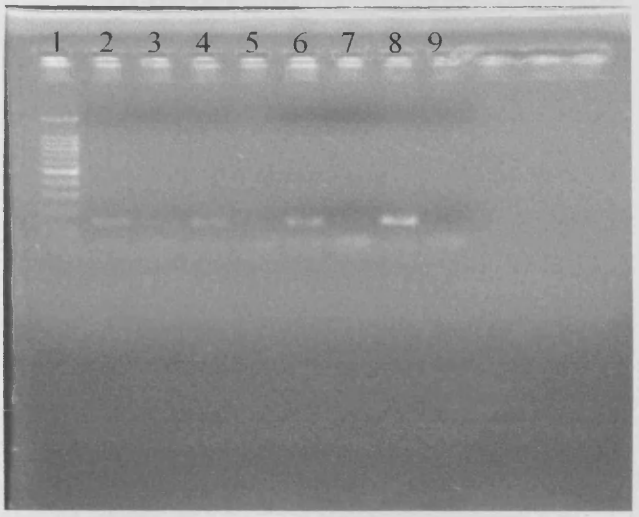
APPENDIX 10

Key:
KA1
1: 100bp ladder
2: Rat patella
3: Rat fat pad
4: Rat menisci
5: Bovine chondrocyte
6: Rat brain (positive)
7: water (negative)



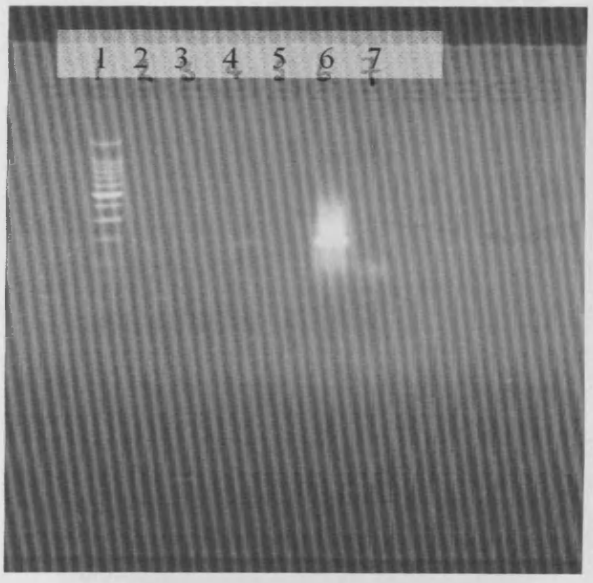
APPENDIX 11

Key:
AMPAGluR2
1: 100bp ladder
2: Rat patella
3: Rat fat pad
4: Rat brain (positive)
5: water (negative)
6: Rat menisci
7: Human OA cartilage
8: Rat brain (positive)
9: water (negative)



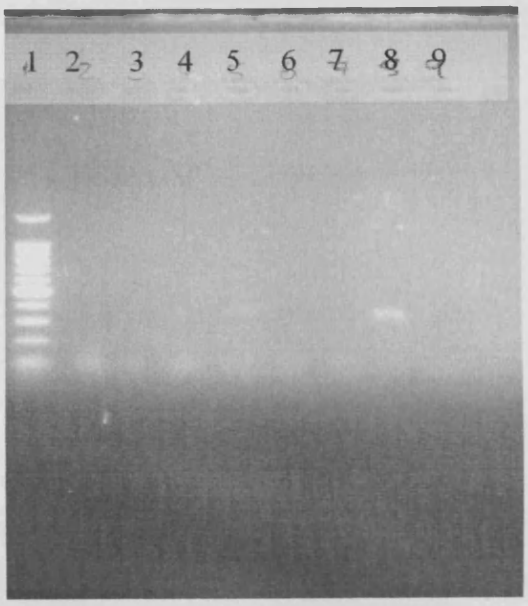
APPENDIX 12

Key:
AMPAGluR3
1: 100bp ladder
2: Rat fat pad
3: Rat menisci
4: Rat patella
5: NFLS
6: Rat brain (positive)
7: water (negative)



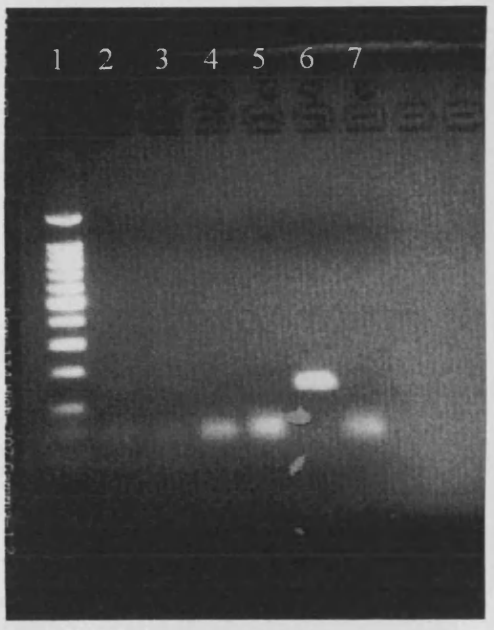
APPENDIX 13

Key:
GLAST-1
1: 100bp ladder
2: Rat menisci
3: Rat menisci
4: Rat menisci
5: Rat menisci
6: Rat menisci
7: Rat menisci
8: GLAST-1 plasmid (positive)
9: water (negative)



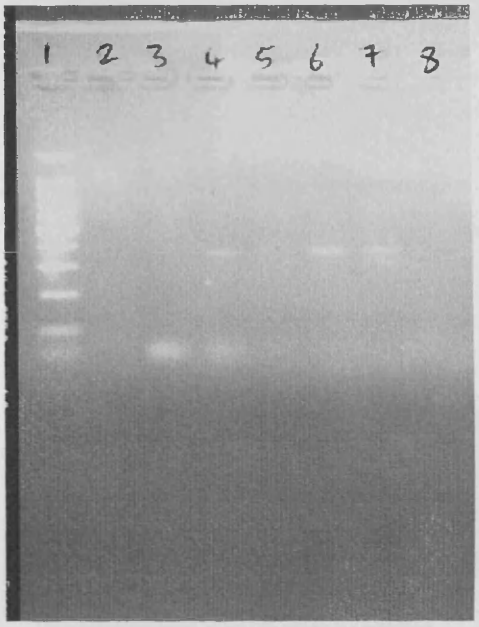
APPENDIX 14

Key:
AMPAGluR3
1: 100bp ladder
2: Rat fat pad
3: Rat menisci
4: NFLS
5: bovine chondrocytes
6: Rat brain (positive)
7: water (negative)



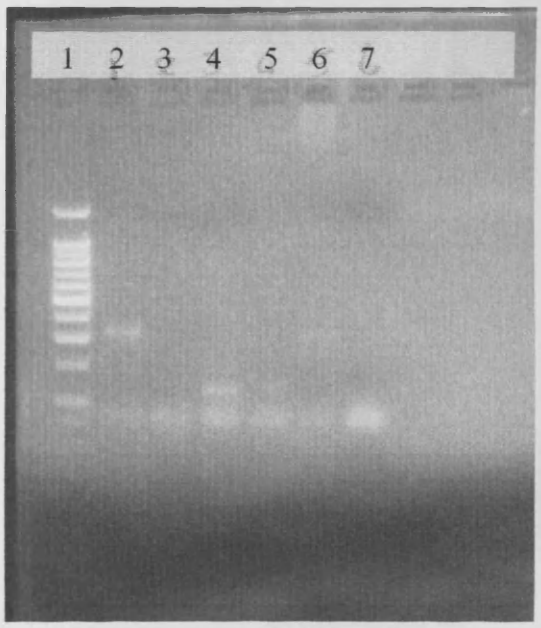
APPENDIX 15

Key:
GLAST-1
1: 100bp ladder
2: one rat fat pad
3: 3 rat fat pads
4: 2 rat fat pads
5: 3 rat fat pads
6: 3 rat fat pads
7: GLAST-1 plasmid (positive)
8: water (negative)



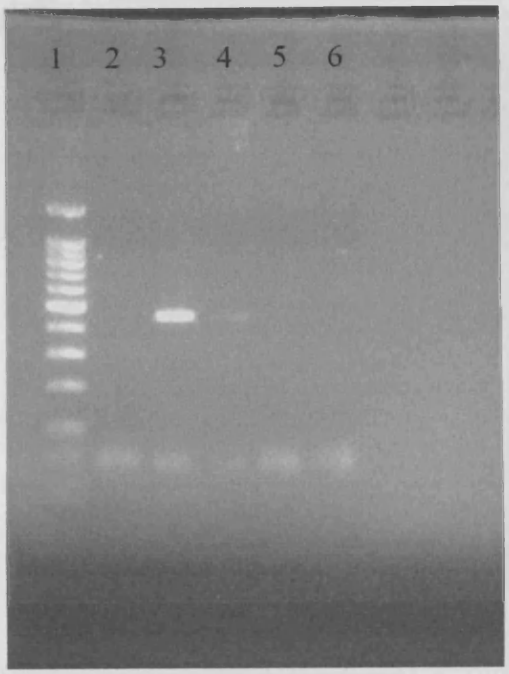
APPENDIX 16

Key:
Human EAAT2 primers
1: 100bp ladder
2: Bovine chondrocytes
3: OA cartilage
4: RA FLS
5: NFLS
6: Chondrocyte library
7: water (negative)



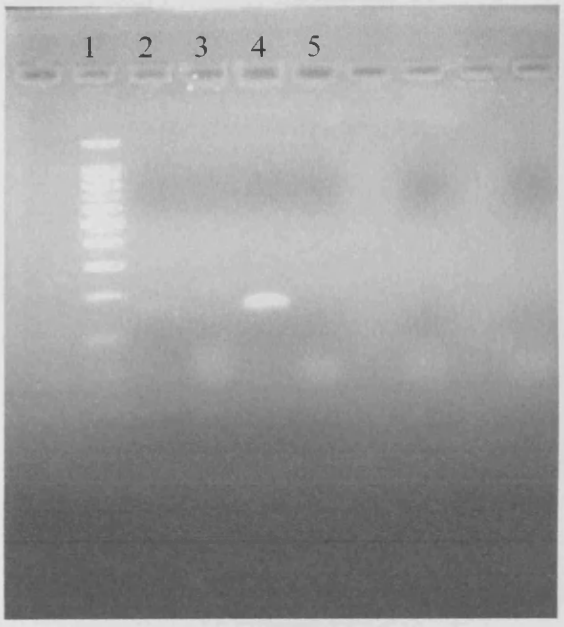
APPENDIX 17

Key:
EAAT3
1: 100bp ladder
2: Bovine chondroyte
3: OA cartilage
4: Chondrocyte library
5: NFLS
6: water (negative)



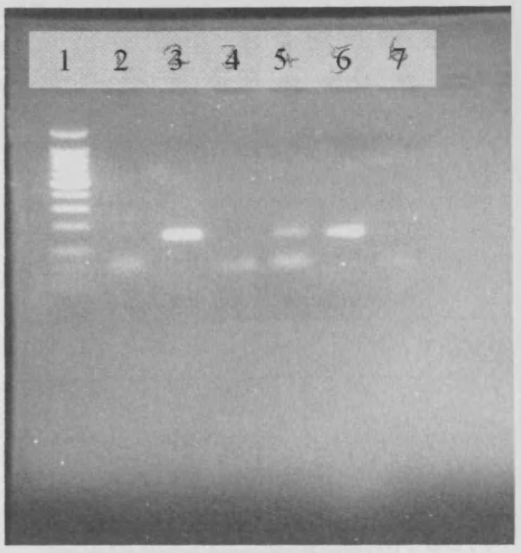
APPENDIX 18

Key:
AMPAGluR2
1: 100bp ladder
2: OA cartilage
3: water (negative)
4: Rat brain (positive)
5: water (negative)



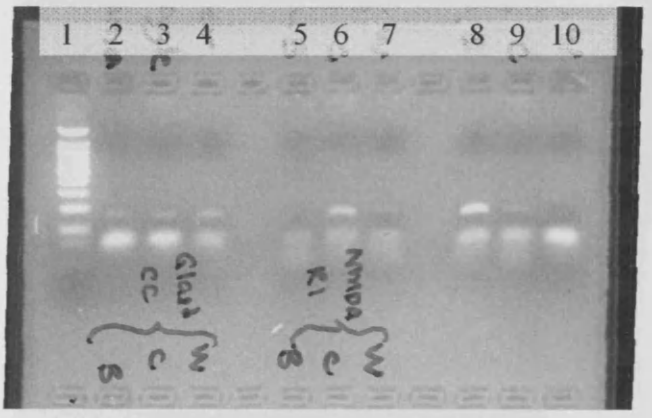
APPENDIX 19

Key:
NMDA NR1 and AMPAGluR3
1: 100bp ladder
2: NMDA NR1, OA cartilage
3: NMDA NR1, rat brain (positive)
4: NMDA NR1, water (negative)
5: AMPAGluR3, OA cartilage
6: AMPAGluR3, rat brain (positive)
7: AMPAGluR3, water (negative)



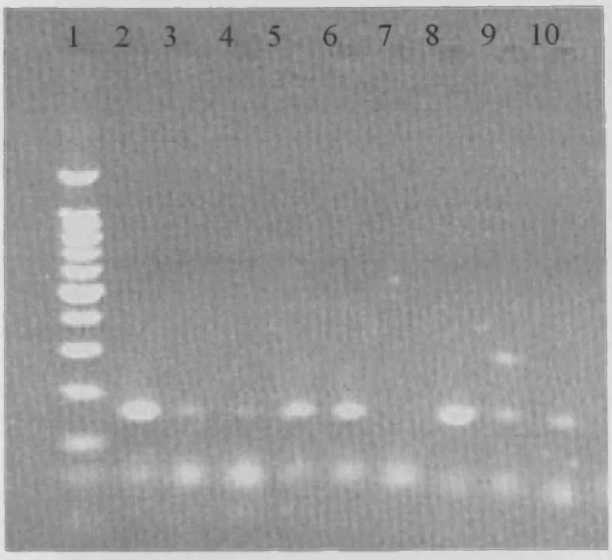
APPENDIX 20

Key:
AMPAGluR2, AMPAGluR3, mGluR4
1: 100bp ladder
2: AMPAGluR2, rat brain (positive)
3: AMPAGluR2, chondrocyte library
4: AMPAGluR2, water (negative)
5: AMPAGluR3, rat brain (positive)
6: AMPAGluR3, chondrocyte library
7: AMPAGluR3. water (negative)
8: mGluR4, rat brain (positive)
9: mGluR4, chondrocyte library
10: mGluR4, water (negative)



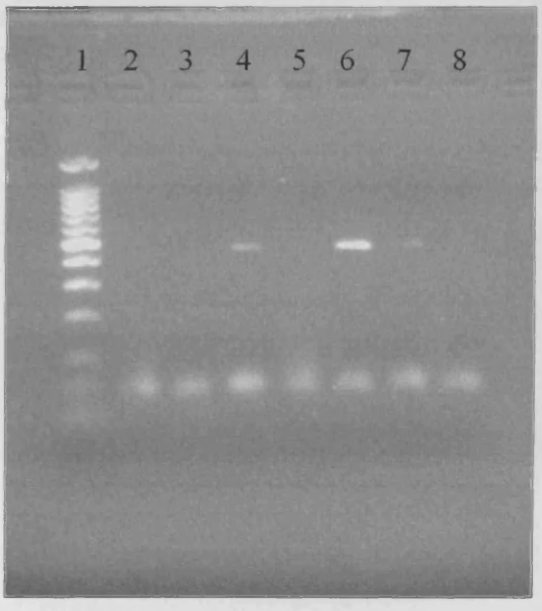
APPENDIX 21

Key:
AMPAGluR2, AMPAGluR3, mGluR4
PCR carried out by V. Savanathan
1: 100bp ladder
2: AMPAGluR2, rat brain (positive)
3: AMPAGluR2, chondrocyte library
4: AMPAGluR2, water (negative)
5: AMPAGluR3, rat brain (positive)
6: AMPAGluR3, chondrocyte library
7: AMPAGluR3, water (negative)
8: mGluR4, rat brain (positive)
9: mGluR4, chondrocyte library
10: mGluR4, water (negative)



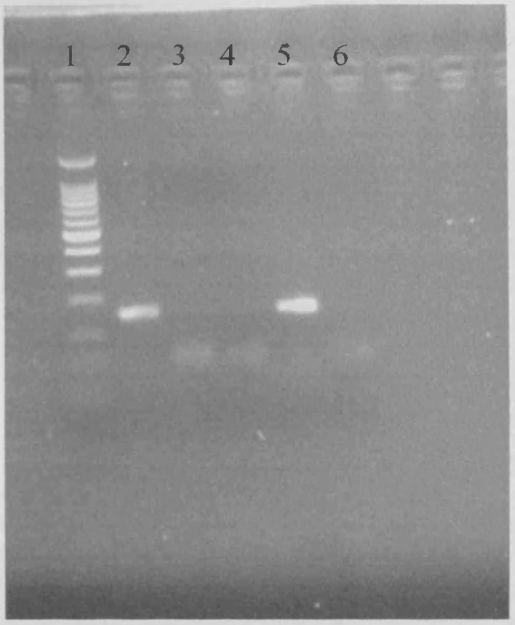
APPENDIX 22

Key:
EAAT3
1: 100bp ladder
2: RA FLS
3: RA FLS
4: RA FLS
5: RA FLS
6: NFLS
7: NFLS
8: water (negative)



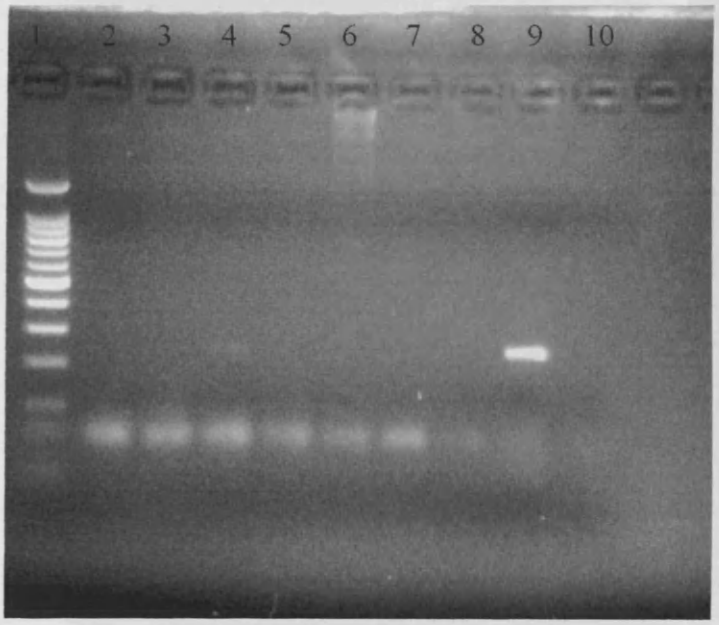
APPENDIX 23

Key:
NMDA NR1
1: 100bp ladder
2: RA FLS
3: RA FLS
4: Bovine chondrocytes
5: Rat brain (positive)
6: water (negative)



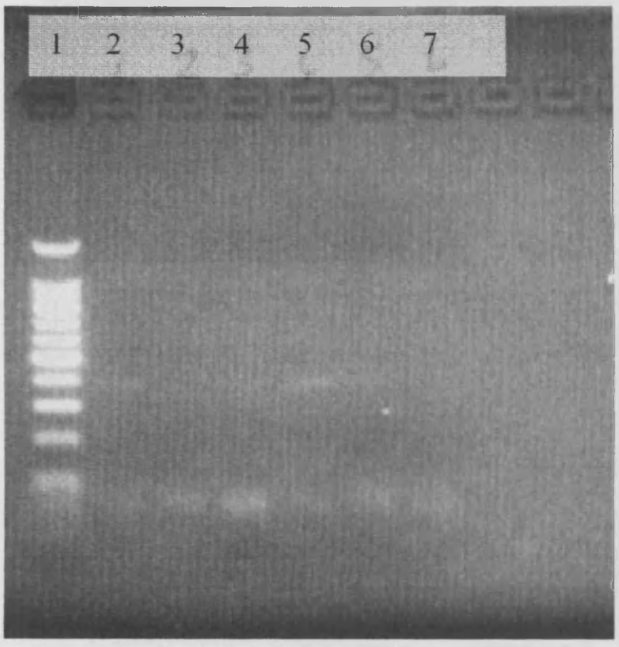
APPENDIX 24

Key:
KA1
1: 100bp ladder
2: Rat fat pad
3: NFLS
4: RA FLS
5: OA Cartilage
6: Chondrocyte library
7: Bovine chondrocytes
8: Rat menisci
9: Rat brain (positive)
10: water (negative)



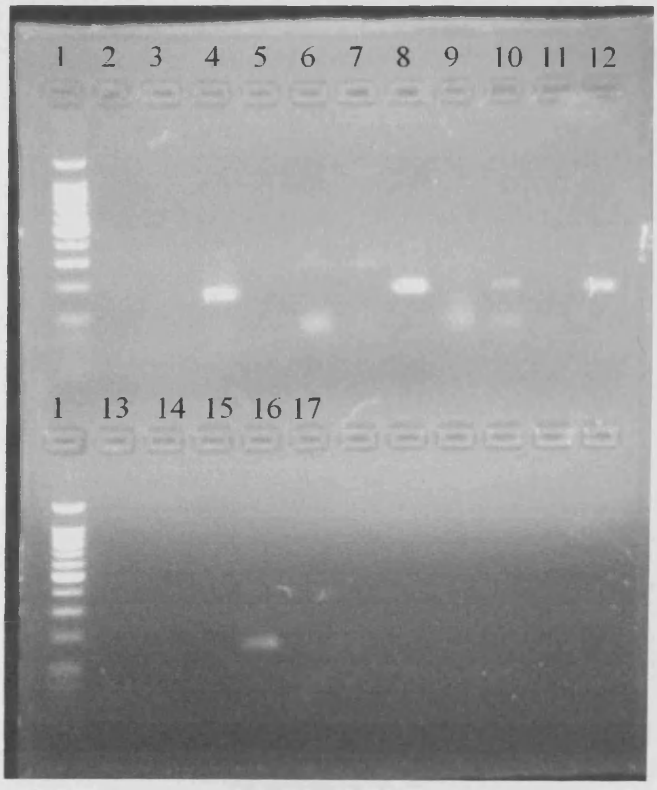
APPENDIX 25

Key:
EAAT2
1: 100bp ladder
2: NFLS, 1.5mM $MgCl_2$
3: NFLS, 2mM $MgCl_2$
4: NFLS, 2.5mM $MgCl_2$
5: NFLS, 3mM $MgCl_2$
6: NFLS, 3.5mM $MgCl_2$
7: water, 2.5mM $MgCl_2$ (negative)



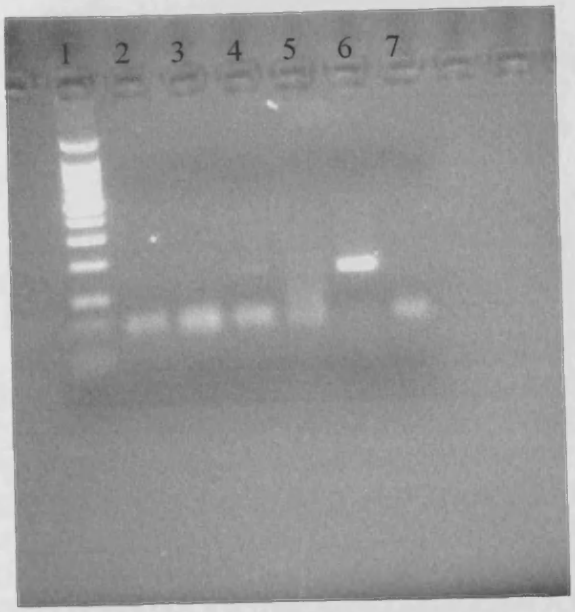
APPENDIX 26

Key:
NMDA NR1
1: 100bp ladder
2: NFLS, 1.5mM $MgCl_2$
3: Chondrocyte library, 1.5mM $MgCl_2$
4: Rat brain (positive), 1.5mM $MgCl_2$
5: water (negative), 1.5mM $MgCl_2$
6: NFLS, 2mM $MgCl_2$
7: Chondrocyte library, 2mM $MgCl_2$
8: Rat brain (positive), 2mM $MgCl_2$
9: water (negative), 2mM $MgCl_2$
10: NFLS, 2.5mM $MgCl_2$
11: Chondrocyte library, 2.5mM $MgCl_2$
12: Rat brain (positive), 2.5mM $MgCl_2$
13: water (negative), 2.5mM $MgCl_2$
14: NFLS, 3mM $MgCl_2$
15: Chondrocyte library, 3mM $MgCl_2$
16: Rat brain (positive), 3mM $MgCl_2$
17: water (negative), 3mM $MgCl_2$



APPENDIX 27

Key:
mGluR4
1: 100bp ladder
2: NFLS
3: NFLS
4: NFLS
5: RA FLS
6: Rat brain (positive)
7: water (negative)



APPENDIX 28

Sequence data of NMDA NR1 amplicon. Forward reaction confirmed 95% homology, reverse primer reaction confirmed 98% homology to rat sequence.
The primer is shown in blue.
The NMDA NR1 sequence is shown in red.

FORWARD:

NAATGCTCCCGGNNGCCATGGCCGCGNGATTCAGGAGCGGGTAAACCCCAGCAACAA
AAAGGAGTGGAANGGAATGATGNGGGAGCTACTCAGTGGCCAAGCGGACATGATTGT
GGCACCACTGACCATGTACAATGAGCGTGCGCAGTACATAGAGTTCTCCAAGCCCTTC
AAGTACCAGGAATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCC
AACGNGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTNAATAGCTTGGCTTAA
TCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCNACACAACAT
ACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCAC
ATTAATTGCGTTGCGCTCACTGCCNGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTG
CATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCG
CTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGC
TCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGGATAACGCAGGAAAGA
ACATGTGAGCAAAAGGNCAGCAAAAGGNCAGGAACCGTAAAAGGCCGCGNTGCTGG
CGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCCAAAAATCNACGCTCAGTTANA
GGNGCGAAACCGANNGNAATATAAGANACAGGCGTTCCCCTGGANCTCCTCGGNGCC
NCTGTTCGACCTGCCCTTACCGAAACTGTCGCCTTTTCCTCGGANGGGNGCTTNNAAC
TNACTGAAGNTTCAATNGGNAAGCCNTCNTCANTGGTGGGGCAACCCCGTNACCGAC
CTGCCTNTCGNAANCCTNGANCACGAAANAATTCCCTGCNNCNTNANGANAAANGGN
TGGGCTAAATTAT

gi|8393483|ref|NM_017010.1| Rattus norvegicus glutamate receptor, ionotropic, N-methyl D-aspartate 1 (Grin1), mRNA
Length=4213

Score = 248 bits (125), Expect = 4e-63
Identities = 144/151 (**95%**), Gaps = 0/151 (0%)
Strand=Plus/Plus

```
Query  1     CAGGAGCGGGTAAACCCCAGCAACAAAAAGGAGTGGAANGGAATGATGNGGGAGCTACTC  60
             ||||||||||||||  ||||||||||||||||||||| ||||||||| | |||||||||
Sbjct  1724  CAGGAGCGGGTAAACAACAGCAACAAAAAGGAGTGGAACGGAATGATGGGCGAGCTACTC  1783

Query  61    AGTGGCCAAGCGGACATGATTGTGGCACCACTGACCATGTACAATGAGCGTGCGCAGTAC  120
             ||||||||||||||||||||||||||||||||||||||  ||||||||||||||||||||
Sbjct  1784  AGTGGCCAAGCGGACATGATTGTGGCACCACTGACCATCAACAATGAGCGTGCGCAGTAC  1843

Query  121   ATAGAGTTCTCCAAGCCCTTCAAGTACCAGG  151
             |||||||||||||||||||||||||||||
Sbjct  1844  ATAGAGTTCTCCAAGCCCTTCAAGTACCAGG  1874
```

REVERSE:
GGCTATGCATCCAACGCGNTGGGAGCTGTCCCATATGGTCGACCTGCAGGCNGACGC
ACTAGTGATTCCTGGTACTTGAAGGGNTTGGAGAACTCTATGTAATGCGCACGCTCAT
TGTTGATGGTCAGTGGTGCCACAATCATGTCCGCTTGGCCACTGAGTAGCTCGCCCAT
CATTCCGTTCCACTCCTTTTTGTTGCTGTTGTTTACCCGCTCCTGAATCCGGCGGCCAT
GGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTACA
ATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACT
TAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGC
ACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGACGCGCCCTGTAG
CGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCC
AGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGG
CTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGNTCCGATTTAGTGCTTTAC
GGCACCTCGACCCCAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCC
TGATAGACGGTTTTTCGCCTTTGACGTTGGANTCACGNTCTTTAATAGTGGACTCTTGT
TCNAACTGGAANACACTCAACCTATCTCGGCTANTCTTTNGATTTTAAGGAATTNGCC
AATTCGNCTATGGGTTAAAATGACTGATTACCAAATTNACGNGAATTTACAAAATACN
CTTAAATTCTGAGNGGAATTTCTTACCACTNGNGGAATCNCNCACAGGGCATTNGGAA
GGCCGACCCATTGTATTTNAAATTCAATGNCCCTGNAANCCGAAAGTNAAA

gi|8393483|ref|NM_017010.1| Rattus norvegicus glutamate receptor, ionotropic, N-methyl D-aspartate 1 (Grin1), mRNA
Length=4213

Score = 285 bits (144), Expect = 2e-74
Identities = 149/151 (98%), Gaps = 0/151 (0%)
Strand=Plus/Minus

```
Query  1     CCTGGTACTTGAAGGGNTTGGAGAACTCTATGTAATGCGCACGCTCATTGTTGATGGTCA  60
             |||||||||||||||| |||||||||||||||| |||||||||||||||||||||||||
Sbjct  1874  CCTGGTACTTGAAGGGCTTGGAGAACTCTATGTACTGCGCACGCTCATTGTTGATGGTCA  1815

Query  61    GTGGTGCCACAATCATGTCCGCTTGGCCACTGAGTAGCTCGCCCATCATTCCGTTCCACT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1814  GTGGTGCCACAATCATGTCCGCTTGGCCACTGAGTAGCTCGCCCATCATTCCGTTCCACT  1755

Query  121   CCTTTTTGTTGCTGTTGTTTACCCGCTCCTG  151
             |||||||||||||||||||||||||||||||
Sbjct  1754  CCTTTTTGTTGCTGTTGTTTACCCGCTCCTG  1724
```

APPENDIX 29

Sequence data of mGluR4 amplicon. Forward and reverse reaction confirmed 100% homology.
The primers are shown in blue.
The mGluR4 sequence is shown in red.

FORWARD:

GCTCCCGGCCGCCATGGCCGCGGGATTAGACCTTCAACGAGGCCAAGCCCATCGGCTT
CACCATGTACACCACCTGCATTGTCTGGCTGGCCTTCATCCCCATCTTTTTTGGCACCT
CACAGTCAGCCGACAAGCTGTACATCCAGACAACCACACTGACGGTCTCCGTGAGTCT
GAGCGAATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGC
GTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATG
GTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGA
GCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTA
ATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATT
AATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTC
CTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCAC
TCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATG
TGAGCAAAAGGCCAGCAAAAGGCCAGGAAACCGTAAAAAGGCCGCGTTGCTGGCGTT
TTTCCATANGCTCCCCCCCCTGACGAGCATCACAAAAATCGACGCTCAGTCANAGTGG
CGAAACCGACAGANTATAAGANACNAGGCGTTTCCCTGGANCTCCTCGNGGCTCTCT
GTTCGACTGCGNTACGAAACTGTCGCTTTTCCTCGGAANNGGGCTTTCNACTCANNNT
AGANCCANTCGGGAGCGTCNCAGTGGTGNGCAACCCGTACCACGTGCTTACGAATTTT
TGTCACGTAANATNCCTGAACTGAAGNAAANGNAGGNAANTAGGCACNCAAAATGTN
CCANCNNAAGTCTCCACNGGTTTNNACAANANTNGCCGAAATTANTTAA

gi|12083594|ref|NM_022666.1| Rattus norvegicus glutamate receptor, metabotropic 4 (Grm4), mRNA
Length=4488

Score = 303 bits (153), Expect = 8e-80
Identities = 153/153 (**100%**), Gaps = 0/153 (0%)
Strand=Plus/Plus

```
Query  1     AGACCTTCAACGAGGCCAAGCCCATCGGCTTCACCATGTACACCACCTGCATTGTCTGGC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3190  AGACCTTCAACGAGGCCAAGCCCATCGGCTTCACCATGTACACCACCTGCATTGTCTGGC  3249

Query  61    TGGCCTTCATCCCCATCTTTTTTGGCACCTCACAGTCAGCCGACAAGCTGTACATCCAGA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3250  TGGCCTTCATCCCCATCTTTTTTGGCACCTCACAGTCAGCCGACAAGCTGTACATCCAGA  3309

Query  121   CAACCACACTGACGGTCTCCGTGAGTCTGAGCG  153
             |||||||||||||||||||||||||||||||
Sbjct  3310  CAACCACACTGACGGTCTCCGTGAGTCTGAGCG  3342
```

REVERSE:
GGNTTCTCTCCCATATGGTCGACCTGCAGGCGGCCGCACTAGTGATTCGCTCAGACTC
ACGGAGACCGTCAGTGTGGTTGTCTGGATGTACAGCTTGTCGGCTGACTGTGAGGTGC
CAAAAAAGATGGGGATGAAGGCCAGCCAGACAATGCAGGTGGTGTACATGGTGAAG
CCGATGGGCTTGGCCTCGTTGAAGGTCTAATCCCGCGGCCATGGCGGCCGGGAGCATG
CGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTT
TTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCAC
ATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCA
ACAGTTGCGCAGCCTGAATGGCGAATGGACGCGCCCTGTAGCGGCGCATTAAGCGCG
GCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCG
CTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTC
TAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAA
AAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTT
CGCCCTTTGAGTTGGAGTCACGTTCTTTAATANTGGACTCTTGTTCAAACTGGAACAC
NTCAACCTATCTCGGCTATCTTTGATTAAAGGATTTGCGATTCGCTATGNTAAAANAC
TGATTACAAATNACNGATTTACAAATAANCTAATTCNAGGGATNCCTACANGGNGAT
TCACNAAGGNNTTNGGAANGCGAACCNTNTNTTAAATCATGTCNCGAAACGAAGTAA
TNANAAAAATATCGCTTCTTGNTTTTTCCACGAATAAGGAGGNAGTAATATCAATAAT
TTGGTTTNACCCNTTNANGAAGAAN

APPENDIX 30

Sequence data of AMPAGluR2 amplicon. Forward and reverse reaction confirmed 100% homology.

The primers are shown in blue.

The AMPAGluR2 sequence is shown in red.

FORWARD:

CCGCCAATGGCCGCGGGATTGGTTGTCACCCTAACTGAGCTCCCATCAGGAAATGACA
CGTCTGGGCTTGAAAACAAAACTGTGGTGGTCACCACAATATTGGAATCTCCATATGT
TATGATGAAGAAAAATCATGAAATGCTTGAAGGGAATGAGCGTTACGAGGGCTACTA
ATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGAT
GCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAG
CTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAA
GCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTT
GCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATC
GGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCA
CTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGC
GGTAATACGGTTATCCACAGAATCAGGGGGATANCGCAGGAAAGAACATGTGAGCAA
AAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTNCCAT
AGGCTCGGCCCCCTGACGAGCATCCCAAAATCGACGCTCAGTCAAAGGTGGCGAANC
NGNCAGGNTNTAAGAACCAGCGTTCCCTGNANCTCCTCGGNGNCTCTGTCNACCTGCG
CTACGAAACTGTCGCNTTTCCTCGGAGGGGGCTTCCTAATCACTGAGATNCATNGGGA
GCGTCCCCAGTGGGNGGGNNACCNNTACANNTGCCTCGAANTNTGTCNCGAACAATN
CCGGCACNTAAAACAAAANGNGCNANNAGGCNCCCAAATTTNCCGACCTAANCTCCA
CCGGNTNAACAAAANTTCGCGNAGNAANCTAAAANAACCCCCCACNCNCCCANN

gi|204381|gb|M85035.1|RATGLUR2A Rat glutamate receptor subunit 2 (GLUR2) non-NMDA mRNA, complete cds
Length=3488

Score = 301 bits (152), Expect = 3e-79
Identities = 152/152 (**100%**), Gaps = 0/152 (0%)
Strand=Plus/Plus

```
Query  1     GGTTGTCACCCTAACTGAGCTCCCATCAGGAAATGACACGTCTGGGCTTGAAAACAAAAC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1500  GGTTGTCACCCTAACTGAGCTCCCATCAGGAAATGACACGTCTGGGCTTGAAAACAAAAC  1559

Query  61    TGTGGTGGTCACCACAATATTGGAATCTCCATATGTTATGATGAAGAAAAATCATGAAAT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1560  TGTGGTGGTCACCACAATATTGGAATCTCCATATGTTATGATGAAGAAAAATCATGAAAT  1619

Query  121   GCTTGAAGGGAATGAGCGTTACGAGGGCTACT  152
             ||||||||||||||||||||||||||||||||
Sbjct  1620  GCTTGAAGGGAATGAGCGTTACGAGGGCTACT  1651
```

REVERSE:
NNTTCAAGCTATGCATCCAACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCG
GCCGCACTAGTGATTAGTAGCCCTCGTAACGCTCATTCCCTTCAAGCATTTCATGATTT
TTCTTCATCATAACATATGGAGATTCCAATATTGTGGTGACCACCACAGTTTTGTTTTC
AAGCCCAGACGTGTCATTTCCTGATGGGAGCTCAGTTAGGGTGACAACCAATCCCGCG
GCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTAT
TACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCC
AACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGC
CCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGACGCGCCCT
GTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACAC
TTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCG
CCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCT
TTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCAT
CGCCCTGATAGACGGTTTTCGCCCTTTGACGTTGGAGTCACGTTCTTTATAGTGGACTC
TTGTTCCAACTGGACAACCTCACCCTATCTCGNCTATCTTTGATTANANGAATTTGCGA
TTCGCCTATGGTAAAANGACTGATTACAAATTACGCGAATTAAAAAATACNTTAANTC
TGAGGGATTTCCTACATGGNGATCACNANAGGGNTTNGGAGGGNGACCATGTTTTNA
ATCAAGTCCNGAAACGAAGTAATGAAAAAANNATCGCCTCTTGNTTTNTTCNAGGAA
AGAATGNNNNAGNNGATATCCATTATTTGGTTNNGCCCTATCCANGANCAATNAATTT
NANAATAACTNGGA

APPENDIX 31

Sequence data of AMPAGluR3 amplicon. Due to sequencing reaction not working correctly, only partial sequence was obtained. However this was 100% homologous to published rat sequence. The primers are shown in blue.
The AMPAGluR3 sequence is shown in red.

REVERSE:

AATCNCTCTCTGTCTCCTCAGGTATCGGAAGGCTTCTGCTATGACCAATATTGCGTCAT
GTGTCAGCGCAGATGTATACTTCAGTGGTGCATTCTTGGCTTCAGGGAATTAATCCCG
CGGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCG
TATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTA
CCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGA
GGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGACGCG
CCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCT
ACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCAC
GTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTA
GTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAAGGTGATGGTTCACGTAGTGG
GCCATCGCCCTGATAGANGGTTTTCGCCNTTGACGTTGANTCACGTTCTTNATAGNGA
NTCTTGTCCAANTGGANACACTCAACCTATCTCGGCTATCTTTGATTAAAGGATTTGCC
AATTCGCTATGGTAAANGANTGATTAAAATTACGATTTANAAAATACCTNATTCGAGN
GATTTCTACATGGGGATCACGACAGGGNTTNGGAGGNGACNATGTTTNAAATANGNC
NNGAAACGAGTAATAAGAANATATCGCCTCTTGTTNTTNCANGNAAGANGGGGNANN
NATATCATATTTGGTTGANCAANNAAAAAATAA

gi|5263284|gb|M85036.2|RATGLUR3A Rattus norvegicus glutamate receptor subunit 3 (GLUR3) non-NMDA
mRNA, complete cds
Length=3083

Score = 208 bits (105), Expect = 2e-51
Identities = 105/105 (**100%**), Gaps = 0/105 (0%)
Strand=Plus/Minus

```
Query  6     CTCTCTGTCTCCTCAGGTATCGGAAGGCTTCTGCTATGACCAATATTGCGTCATGTGTCA  65
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1133  CTCTCTGTCTCCTCAGGTATCGGAAGGCTTCTGCTATGACCAATATTGCGTCATGTGTCA  1074

Query  66    GCGCAGATGTATACTTCAGTGGTGCATTCTTGGCTTCAGGGAATT  110
             |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1073  GCGCAGATGTATACTTCAGTGGTGCATTCTTGGCTTCAGGGAATT  1029
```

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