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# NMDA receptor expression and activity in osteoarthritic human articular chondrocytes

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## Summary

*Objective*: Classical neuronal signalling molecules such as substance P and glutamate have been identified in cartilage and have roles in regulation of chondrocyte function. This study looks at expression and activity of the ionotropic glutamate NMDA (N-methyl-D-aspartic acid) receptor (NMDAR) in human osteoarthritic (OA) chondrocytes.

*Method*: Chondrocytes were obtained from human knee joint arthroplasty specimens. NMDAR subunits and PSD-95 (postsynaptic density protein 95) expression were analysed by reverse transcription-polymerase chain reaction and Western blotting. Activity of NMDAR was assayed by radioactive calcium$^{45}$ uptake and changes in membrane potential in the presence and absence of NMDA and NMDAR antagonists and blockade of cell membrane ion channels.

*Results*: NMDAR 1, 2A, 2B and PSD-95 were detected in human OA chondrocytes whereas NR2B was absent from normal chondrocytes. NMDA induced calcium flux into OA chondrocytes and cell membrane depolarisation. These responses were blocked by NMDAR antagonists, removal of extracellular calcium, inhibition of nNOS (neuronal nitric oxide synthase) activity and uncoupling of NMDAR from PSD-95. Blockade of sodium channels by tetrodotoxin resulted in NMDA-induced membrane hyperpolarisation which was, in turn inhibited by apamin, a blocker of SK channels. NMDA-induced changes in cell membrane potential were not affected by L-type and stretch activated calcium channel inhibitors.

*Conclusions*: Human OA and normal articular chondrocytes differ in the expression of NMDAR subunits. In OA chondrocytes NMDAR signalling requires extracellular calcium, association with PSD-95, and nNOS activity. Downstream signalling results in activation of tetrodotoxin sensitive sodium channels and SK channels, a response that differs from that of normal chondrocytes suggesting altered activity of NMDAR in OA.


*Key words*: Osteoarthritis, Chondrocyte, NMDA receptor, Cell signalling.

## Introduction

Articular cartilage is a highly specialised connective tissue which provides a frictionless bearing surface while it also functions to absorb and transmit compressive, tensile and shear forces across diarthrodial joints. Mechanical forces are required for maintenance of normal structure and function of articular cartilage but are also involved in the pathogenesis of osteoarthritis. Chondrocytes, the sole cells within cartilage recognise and respond to mechanical forces. It appears likely that changes in the mechanical environment as a result of alterations to the composition and organisation of the cartilage matrix or through excess or decreased physical loading are perceived by articular chondrocytes and modify cellular function leading to tissue remodelling.

*In vitro* studies have begun to provide insight into the mechanotransduction process by which chondrocytes recognise a mechanical stimulus and transduce this stimulus into a biochemical response. Integrins and stretch activated ion channels (SAC) have been shown to be potential mechanoreceptors in human articular chondrocytes[1]. Mechanical stimulation of monolayer cultures of human articular chondrocytes, results in activation of $\alpha5\beta1$ integrin and SAC with stimulation of downstream signal cascades that lead to production of paracrine/autocrine signalling molecules regulating chondrocyte responses. In normal articular chondrocytes (interleukin 4) appears to be the predominant cytokine produced and is necessary for mechanical stimulation induced upregulation of aggrecan gene expression[2]. In contrast, chondrocytes from osteoarthritic (OA) cartilage mechanically stimulated in an identical manner produce the catabolic cytokine IL1$\beta$ and do not show changes in aggrecan gene expression.

A number of molecules traditionally identified and associated with neuronal and neuroepithelial cells are now

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recognised to be more widely distributed and there is increasing evidence that, at least some of, these molecules may have important roles in regulation of chondrocyte function. N-CAM and n-cadherin are important in chondrogenesis and NG2/human melanoma proteoglycan (HMPG) a chondroitin sulphate proteoglycan (CSPG) has been shown to have roles in chondrocyte adhesion to type VI collagen[3]. More recently human articular chondrocytes have been shown to express preprotachykinin, substance P and its receptor NK1[4] and NMDA receptors (NMDARs)[5]; the latter being ligand gated ionotropic glutamate receptors that mediate fast synaptic transmission in the central nervous system. Roles for substance P and NMDAR in chondrocytes remain unclear but evidence is accumulating for involvement in mechanotransduction.

The NMDAR receptor family is unique among ligand gated ion channels in that it requires the binding of both glutamate and a co-agonist, glycine, for its activation[6]. NMDAR consists of three types of subunit; NR1, NR2 and NR3, with NR2 and NR3 having several different subtypes (NR2A–D; NR3A–B)[7]; For NMDAR to be functional both NR1 and NR2 subunits are required, the functional NMDAR consisting of a tetramer of NR1 and NR2 subunits[8,9] although pentamers associated with NR3 subunits have been found to exist. NMDAR activation is enhanced by phosphorylation events and the receptor is involved in a variety of signalling cascades in neuronal cells. NMDAR is serine/threonine phosphorylated by PKA, PKC and CaMKII and tyrosine phosphorylated by Src and Fyn[6]. It is believed that the NMDAR may regulate its own activity in association with intracellular proteins. Intracellular proteins the NMDA receptor is associated with include actin, filamentous proteins, Src and CaMKII. Other signalling proteins are associated with NMDA receptor through binding to PSD-95 which acts as an anchor, clustering important signalling molecules to the NMDA receptor[10].

The current study was undertaken to extend further analysis of NMDAR subunit expression in normal and OA chondrocytes and to examine involvement of PSD-95 and nNOS in NMDAR dependent signalling in OA chondrocytes. In addition we aimed to assess whether NMDAR signalling results in stimulation of either or both the SK channels and tetrodotoxin sensitive sodium channels that are known to be activated in chondrocytes in response to mechanical loading.

## Materials and methods

All materials were obtained from Sigma–Aldrich (Poole, UK) unless otherwise stated.

### ISOLATION AND CULTURE OF CHONDROCYTES

Chondrocytes were isolated by sequential enzymatic digestion from cartilage obtained from human adult knee joints following knee replacement surgery (OA) or resection for peripheral vascular disease (normal) with patient's consent. Isolated cells were seeded in Iscove's modified Dulbecco's medium supplemented with 10% foetal calf serum (first link), 2 mM glutamine, 100 IU/ml penicillin and 100 ug/ml streptomycin (both Invitrogen), at $5 \times 10^4$ cells/ml in 58 mm diameter tissue culture dishes (Nunc) unless otherwise stated. Primary non-confluent, non-passaged cultures of chondrocytes and freshly isolated OA cells (not cultured) were used as indicated in experiments.

### REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted from chondrocytes using a denaturing buffer of 4 M guanidine isothiocyanate, 0.75 M sodium citrate, 10% (w/v) lauryl sarcosine and 7.2 μl/ml β-mercaptoethanol. The quantity of RNA in the samples was determined by absorbance readings at 260 nm on a spectrophotometer (Genequant; Pharmacia). Template cDNA was synthesised using 0.5 μg RNA, Superscript II (Invitrogen), and oligo dT (12–18; Amersham) according to the manufacturers instructions. Exon specific primers used for the PCR reaction were as follows:

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| GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase) | 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′<br>5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′ |
| NR1 | 5′-GTC CTC GGC CAT GTG GTT CTC C-3′<br>5′-GTG GGA CTT GAG GAT GGA CAG GG-3′ |
| NR2A | 5′-GCT TCA GGA GAC AGG TAA CCC AGC C-3′<br>5′-CTG TCC CTG GAA CAG TAC GAT GCC G-3′ |
| NR2B | 5′-TGA CTT CCA CCA TCT CTC A-3′<br>5′-GAA TGA TGG GGC TTT GGA-3′ |
| NR2C | 5′-AAG GTG CTG GAG ATA CGA-3′<br>5′-TCC CAG GTG ACA TTC AGA-3′ |
| NR2D | 5′-TGT CTA CAT GAG GTG CAG-3′<br>5′-TTC ACA GAA GCC TCC TCA-3′ |
| NR3 | 5′-GAC ATC AGT GAG GAC AAC TCC C-3′<br>5′-CAG ATG TCC TCT GGA AAC GGG-3′ |

A typical 20 μl PCR reaction contained 20 mM ammonium sulphate, 75 mM Tris–HCl pH 8.8, 0.01% (v/v) Tween-20, 1 μl each primer, 2 μl cDNA, 100 μM dNTPs, 0.1 (w/v) BSA (bovine serum albumin), 0.25 units Taq polymerase (Biogene). The magnesium chloride concentrations used were 2.5 mM GAPDH and 3 mM NMDAR. Samples were run for 3 min at 94°C and then 35 cycles which consisted of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1.5 min, followed by 10 min at 72°C. PCR products were run on a 1% agarose gel make up in SYBRsafe (Invitrogen). PCR products were visualised under UV light using Versadoc (BioRad).

### PROTEIN EXTRACTION AND WESTERN BLOTTING

Chondrocytes were washed with ice-cold PBS (phosphate buffered saline) containing 100 μM $Na_3VO_4$ (Sigma) and lysed *in situ* with ice-cold lysis buffer containing 1% Igepal (Sigma), 100 μM $Na_3VO_4$, and protease inhibitor cocktail tablet (Roche) at 4°C for 15 min. Supernatants were collected after centrifugation at 13,000 rpm for 15 min. Whole cell extracts were separated on a 6% SDS-PAGE under reducing conditions. Following electrophoresis whole cell lysates were transferred onto polyvinylidene fluoride (PVDF) membranes (BDH). Membranes were blocked overnight at 4°C with TBST (tris buffered saline-Tween) Marvel (12.5 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20, 5% Marvel). After washing with TBST, blots were incubated for 1 h at room temperature with primary antibodies and then HRP (horseradish peroxidase) labelled secondary antibodies. Membranes were rewashed extensively and bands detected using Enhanced Chemiluminescense Plus (Amersham). Detection of NMDAR subunits was undertaken using goat anti-human antibodies (NR1, 2A, 2B; Santa Cruz) diluted 1/250, loading control antibody used was α-tubulin (Abcam) diluted at 1/5000.

### CALCIUM ASSAY

Chondrocytes were seeded at a density of $2 \times 10^4$/ml in 24 well plates (Nunc) and cultured for 1 week in media with 10% foetal calf serum. The day before the experiment culture medium was replaced with serum free media. Chondrocytes treated with 50 μM NMDA plus 20 μM glycine in the absence or presence of NMDAR antagonists were incubated with 30–50 MBq/l $Ca^{45}$ for 10 min at 37°C, 5% $CO_2$. After the 10 min incubation, cells were washed once with fresh medium added with kynurenate (10 mM) and magnesium (100 mM) prior to lysis with 1% Triton-X in PBS. After