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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 calcium⁴⁵ 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.

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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 chondrocytes¹. Mechanical stimulation of monolayer cultures of human articular chondrocytes, results in activation of $\alpha 5\beta 1$ 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 expression². In contrast, chondrocytes from osteoarthritic (OA) cartilage mechanically stimulated in an identical manner produce the catabolic cytokine IL1 β 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 collagen³. More recently human articular chondrocytes have been shown to express preprotachykinin, substance P and its receptor NK1⁴ 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 activation⁶. 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 Fyn⁶. 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 receptor¹⁰.

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 5×10^4 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

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

sarcosine and 7.2 μ l/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 μ g 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:

A typical 20 μ l PCR reaction contained 20 mM ammonium sulphate, 75 mM Tris–HCl pH 8.8, 0.01% (v/v) Tween-20, 1 μ l each primer, 2 μ l cDNA, 100 μ M 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 μ M Na_3VO_4 (Sigma) and lysed *in situ* with ice-cold lysis buffer containing 1% Igepal (Sigma), 100 μ M 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 re-washed extensively and bands detected using Enhanced Chemiluminescence 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 2×10^4 /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 μ M NMDA plus 20 μ M 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 kynurenic acid (10 mM) and magnesium (100 mM) prior to lysis with 1% Triton-X in PBS. After

addition of 1 ml of scintillation liquid (VWR), Ca^{45} entry in count per minute (cpm) was quantified using a Beckman LS6500 scintillation counter.

ELECTROPHYSIOLOGICAL RECORDINGS

Membrane potentials of chondrocytes were recorded using a single electrode bridge circuit and calibrator as previously described in detail¹¹. Micro-electrodes having tip resistances between 40 and 60 mega ohms and tip potentials of approximately 3 mV were used to impale the cells. Results from a cell were accepted if on impalement there was a rapid change in membrane voltage potential level in the cell and if this membrane potential remained constant for at least 60 s. In each set of experiments, a control dish of chondrocytes was examined whose culture medium did not contain any agent to be tested. Resting membrane potentials of 5–10 cells were measured and, following addition of the reagent to be tested to the culture medium membrane potentials were assessed in a further 5–10 cells to establish whether the reagent itself had an effect on the resting membrane potential of the cells. Then, following a 10 min period of incubation with 50 μM NMDA plus 20 μM glycine membrane potentials were recorded in a further 5–10 cells. At least three experiments with cells from three separate donors were performed with each reagent.

CHEMICAL REAGENTS

The following NMDAR antagonists were used. MK801 (50 μM ; (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine hydrogen maleate dizocilpine hydrogen maleate) is a non-competitive NMDAR inhibitor. APV (200 μM ; *D*-2-amino-5-phosphonovaleric acid) is a competitive blocker of NMDAR. Ifenprodil, 0.3 μM , selectively blocks NR2B containing NMDAR. The TAT-NR2B9c peptide (5 μM) mimetic of NR2B-PDZ ligand and uncouples NMDAR from nNOS production.

Other reagents used were tetrodotoxin (TTX; 1 μM) a selective sodium channel blocker. Apamin (4.9 μM) was used to block SK channels (small conductance Ca^{2+} dependant K^+ channels). To block L -type calcium channel nifedipine (10 μM) was used whereas, gadolinium hexahydrate (10 μM) was used to block stretch activated channels. EGTA (ethylene glycol tetraacetic acid) (1.5 mM, 86 μM) was used to chelate extracellular calcium and *L*-*N* ω -nitroarginine-2,4-*L*-diamino-butyric amide (*L*-Arg NO₂-*L*-*Dbu*-NH₂ – 2TFA; 10 ng/ml, 100 ng/ml) was used as a highly selective nNOS inhibitor.

STATISTICAL ANALYSIS

All experiments were repeated a minimum of three times each on different days and as appropriate the mean and s.e.m. were calculated. The calcium assay was performed in triplicate each day and the mean value for each was designated $n = 1$. The values for electrophysiological recordings each day were obtained from the mean of five recordings which are designated $n = 1$. Statistical significance was carried out using one-way analysis of variance (ANOVA) and Tukey's *post hoc* test for calcium assay and paired *t* test for electrophysiological recordings. Differences were considered significant when $P < 0.05$.

Results

EXPRESSION OF NMDAR BY OA CHONDROCYTES

NMDAR RNA was detected in both normal and OA chondrocytes (Fig. 1). Following RT-PCR, bands for NR1, 2A and 2B were detectable in OA chondrocytes whereas NR2C, 2D and NR3 were not identified. In contrast in normal chondrocytes NR1 and NR2A but not NR2B was detected.

Western blotting of protein extracts from normal and OA chondrocytes in short term monolayer cultures showed expression of NMDAR NR2A (Fig. 2). NR2B was readily detectable in OA chondrocytes but at best only a faint band was seen in Western blots of protein extracted from normal chondrocytes. NR1 protein was not detected in extracts from either normal or OA chondrocytes. NR1, NR2A and NR2B were each detected in protein extracts from extracted, but not cultured, chondrocytes from OA cartilage. PSD-95, the major protein involved in anchoring NMDAR to intracellular proteins was detectable by Western blot in both normal and OA chondrocytes.

OA CHONDROCYTES EXPRESS FUNCTIONAL NMDAR

To establish whether chondrocytes expressed functional NMDAR cells were incubated with 50 μM NMDA (in the presence of 20 μM glycine) and either influx of radioactive Ca^{45} or changes in cell membrane potential were assessed.

Calcium⁴⁵ uptake (Fig. 3)

NMDA (50 μM) with 20 μM glycine induced calcium uptake by chondrocytes, with cpm of Ca^{45} in cell lysates showing a significant increase by 30–40% over cells at rest. This NMDA-induced influx of calcium into the chondrocytes was inhibited by the competitive antagonist APV (200 μM) in separate experiments with chondrocytes from three donors. The non-competitive NMDAR antagonist MK801 (5 μM) also inhibited calcium flux in NMDA treated OA chondrocytes from three donors but in a fourth experiment no significant change was seen. There was a slight but not statistically significant increase in the calcium uptake in chondrocytes following the addition of NMDAR antagonists alone.

Changes in cell membrane potential (Fig. 4)

Addition of 50 μM NMDA with 20 μM glycine to normal chondrocytes [Fig. 4(A)] for 10 min induced cell membrane hyperpolarisation ($P < 0.01$). This response was inhibited by the presence of MK801 or APV. In the presence of ifenprodil, an NMDAR antagonist that selectively inhibits receptors containing the NR2B subunit, a significant cell membrane hyperpolarisation response to NMDA was seen.

Addition of 50 μM NMDA with 20 μM glycine to OA chondrocytes [Fig. 4(B)] for 10 min resulted in a reproducible, highly significant cell membrane depolarisation ($P < 0.001$). MK801 alone induced a small but statistically significant membrane depolarisation in OA chondrocytes. Addition of NMDA with glycine to MK801 treated cells, however, did not result in further membrane depolarisation. APV had no effect on resting membrane potential of chondrocytes. Addition of NMDA with glycine to APV treated cells induced a small but significant ($P < 0.05$) cell membrane hyperpolarisation. The NR2B selective antagonist, ifenprodil (0.3 μM) when added to cells induced a small, but, not statistically significant membrane depolarisation. Subsequent NMDA treatment resulted in a further cell membrane depolarisation, but this did not reach statistical significance.

SIGNALLING THROUGH NMDAR IN CHONDROCYTES INVOLVES PSD-95 AND nNOS

NMDAR signalling in neuronal cells involves a multimolecular complex in which PSD-95 links NMDAR to nNOS. To investigate whether this molecular complex plays a role in NMDAR signalling in chondrocytes experiments were undertaken with NR2B9c-TAT peptide (mimetic of NR2B-PDZ ligand and uncouples NMDAR from nNOS production) and *L*-Arg NO₂-*L*-*Dbu*-NH₂ – 2TFA a highly selective nNOS inhibitor (Fig. 5). Treatment of chondrocytes with NR2B9c-TAT inhibited the NMDA-induced membrane depolarisation whereas control peptide had no effect (Fig. 6). The nNOS inhibitor was used at two concentrations. At 100 ng/ml *L*-Arg NO₂-*L*-*Dbu*-NH₂ – 2TFA completely inhibited the membrane depolarisation response to NMDA whereas at 10 ng/ml the membrane depolarisation response was attenuated but membrane potentials of NMDA treated cells were significantly lower than untreated cells.

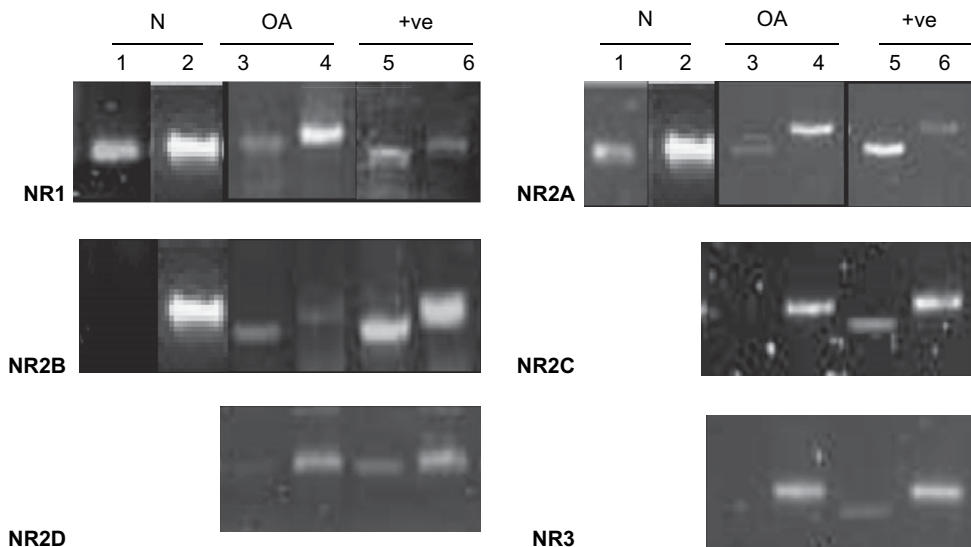


Fig. 1. RT-PCR for NMDA receptors in unstimulated cells. Lanes 1, 3 and 5 are mRNA for NMDARs and, Lanes 2, 4 and 6 are GAPDH. N – normal chondrocytes, OA – chondrocytes from patients with osteoarthritis. Positive control used is rat brain cell lysate. Results shown are representative from four patients.

EFFECTS OF ION CHANNEL BLOCKADE AND CALCIUM CHELATION ON THE ELECTROPHYSIOLOGICAL RESPONSE OF OA CHONDROCYTES TO NMDA

Previous work from our group has suggested the NMDAR may be involved in chondrocyte responses to mechanical stimulation. Mechanical stimulation of OA chondrocytes is calcium and SAC dependent and results in membrane depolarisation due to activation of a tetrodotoxin dependent sodium channel. We assessed whether similar signalling molecules and ion channels are involved the membrane depolarisation induced in OA chondrocytes by NMDA.

EGTA blocked NMDA-induced depolarisation in a dose dependent manner (Fig. 7). EGTA (1.5 mM) inhibited

membrane depolarisation whereas a membrane depolarisation response was seen at lower the concentration of 86 μ M. Treatment with nifedipine, to block L-type calcium channels and gadolinium to block stretch activated ion channels in contrast had no effect on NMDA-induced membrane depolarisation (Fig. 8).

In the presence of tetrodotoxin, NMDA induced a membrane hyperpolarisation response of 46% rather than membrane depolarisation (Fig. 9). As we have previously shown that membrane hyperpolarisation of human chondrocytes may be the result of activation of SK channels we tested apamin, a blocker of SK channels in the current system. In the presence of apamin OA chondrocytes showed

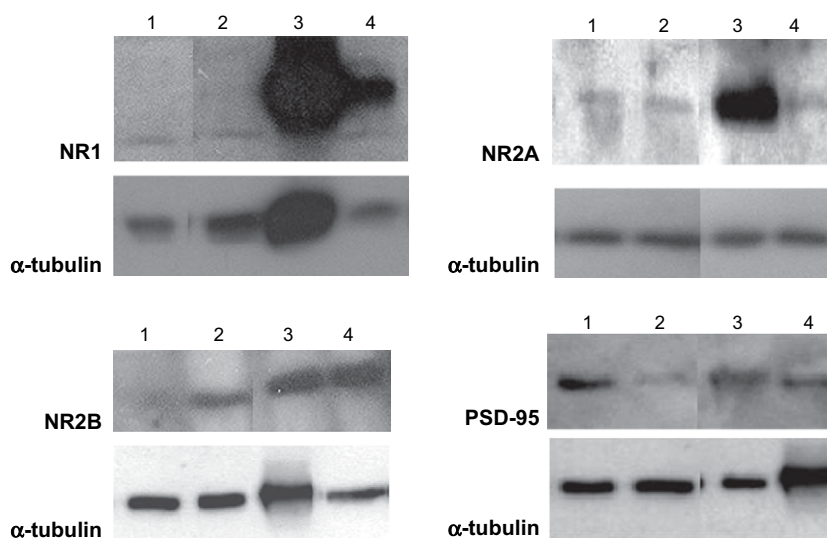


Fig. 2. Western blots for NMDA receptors and PSD-95 in unstimulated cells. Lane 1 is normal and lane 2 is OA cell lysates obtained from primary cell cultures. Positive control used is rat brain cell lysate (lane 3). Lane 4 is uncultured OA cells which were lysed immediately after extraction from cartilage. Results shown are representative from four patients.

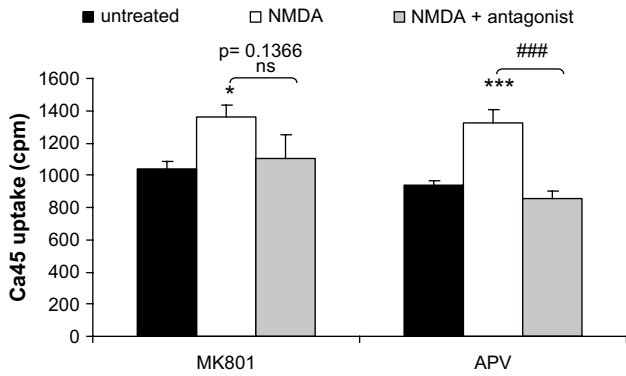


Fig. 3. Ca⁴⁵ uptake assay after treatment with NMDA (50 μ M) and NMDAR antagonists. Concentration of antagonists used was 5 μ M MK801 or 200 μ M APV. Uptake measured as cpm of Ca⁴⁵ in cell lysate. * P < 0.05, *** P < 0.001 compared to untreated cells; ### P < 0.001 compared to APV treated cells. Three of four experiments showed reduction in Ca⁴⁵ uptake in presence of MK801.

a membrane depolarisation response of similar extent to controls. However, when chondrocytes were treated with apamin and tetrodotoxin together the change in membrane potential (membrane depolarisation), with addition of NMDA, was relatively small. Apamin and tetrodotoxin treatment together resulted in a decrease in cell membrane

potential of around 8% compared to 30% observed in the control cells (NMDA treated in the absence of the ion channel blockers).

Discussion

This study investigates the expression of NMDAR in human OA chondrocytes. We have previously reported preliminary data on the expression of NMDAR subunits NR1 and NR2A in normal human articular chondrocytes and suggested that NMDAR may have a role in chondrocyte mechanotransduction⁵. The current work extends these initial observations, characterising in detail, the NMDAR subunits expressed in OA chondrocytes. Like normal chondrocytes OA chondrocytes express NR1 and NR2A but, unlike normal chondrocytes, appear to additionally express the NR2B subunit. By using assays of calcium uptake and changes in cell membrane potential we have identified involvement of PSD-95/nNOS in NMDAR signalling in OA chondrocytes and identified activation of SK and tetrodotoxin sensitive ion channels as downstream responses.

In tissues which express NMDAR, several subtypes of NR2 receptor co-exist. In neurones both NR1 and NR2 subunits are highly expressed. NMDARs are tetramers of two NR1 and two NR2 subunits. The majority of neuronal NMDAR are di-heteromeric and contain pairs of the same subunit. There is evidence, however, of tri-heteromeric receptors containing two different NR2 subunits. The formation of tetramers produces a functional receptor and the

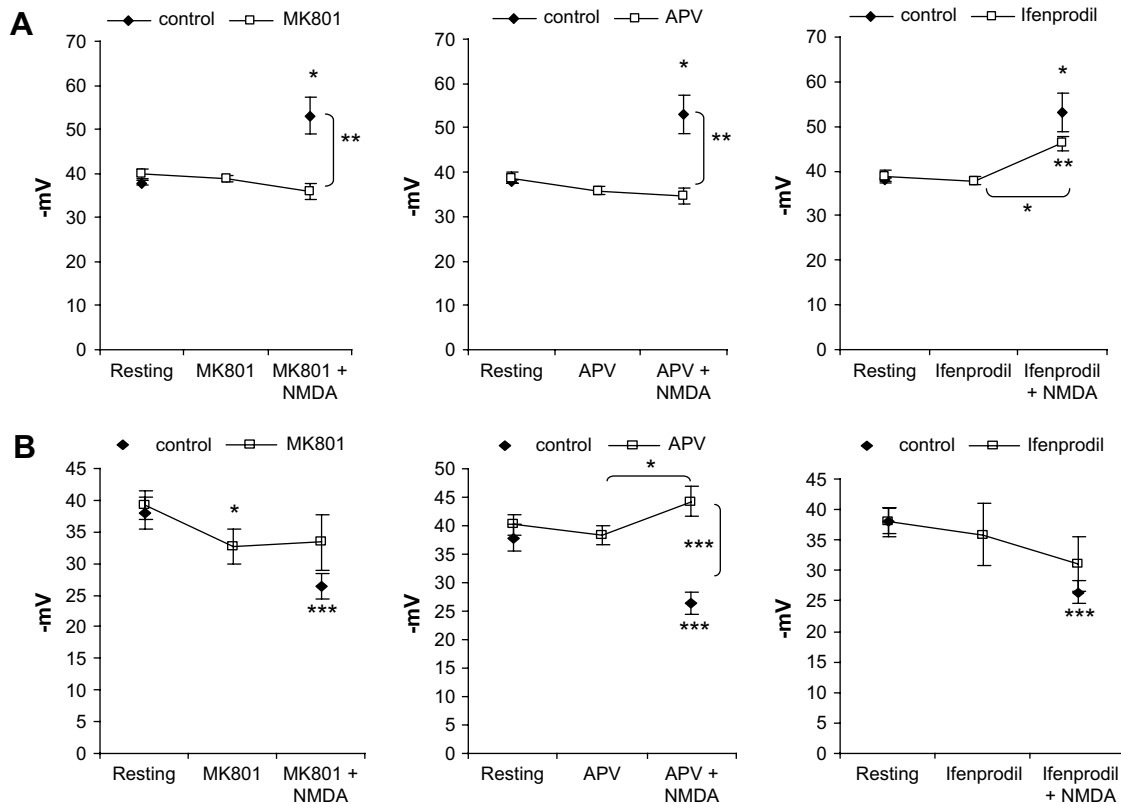


Fig. 4. Electrophysiological response of chondrocytes to 50 μ M NMDA treatment in the presence and absence of NMDAR antagonists (MK801 50 μ M, APV 200 μ M and ifenprodil 0.3 μ M). (A) Normal chondrocytes. (B) OA chondrocytes. Membrane potential is measured in -mV. Readings were taken in resting cells, after 10 min of treatment with antagonist, and after 10 min of NMDA stimulation. Asterisks indicate statistically significant differences between resting and treated cells, or identified cell treatments (* P < 0.05; ** P < 0.01; *** P < 0.001).

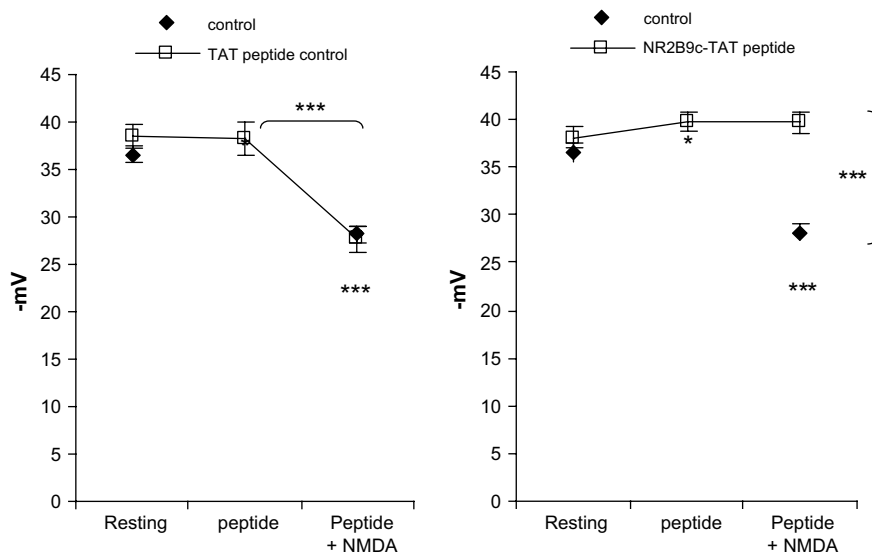


Fig. 5. Electrophysiological response of OA chondrocytes to 50 μ M NMDA treatment in the presence and absence of 5 μ M control peptide or NR2B9c-TAT peptide. Membrane potential is measured in $-mV$. Readings were taken in resting cells, after 10 min of treatment with peptide, and after 10 min of NMDA stimulation. Asterisks indicate statistically significant differences between resting and treated cells, or identified cell treatments ($***P < 0.001$).

properties of the receptor are determined by the subunit composition⁷. Functional NR1/NR2/NR3 tetrameric complexes of receptors have also been identified in neurones¹². The present study demonstrates that OA chondrocytes express NR1, NR2A and NR2B, and the scaffolding protein PSD-95. This is a similar expression profile to that of bone cells^{13,14}. NMDAR is, therefore, likely to exist as either di- or tri-heteromeric tetramers but identification of the exact composition of NMDAR in these cells is outwith the scope of the current study. In cultured normal and OA chondrocytes we could identify NR1 expression by RT-PCR but not by Western blotting. As functional NMDAR requires NR1 and NR2

subunits we believe that our inability to show NR1 protein expression in cells that show responses to NMDA is due to the sensitivity of the Western blotting procedure rather than chondrocytes expressing homomeric NMDAR. The observation that ifenprodil, a preferential NR1/NR2B antagonist did not prevent NMDA-induced cell membrane hyperpolarisation suggests that this electrophysiological response of normal chondrocytes to NMDA is mediated via NR1/NR2A. In OA chondrocytes ifenprodil prevented a statistically significant depolarisation response to NMDA suggesting that this response may, at least in part, be mediated through NR1/NR2B.

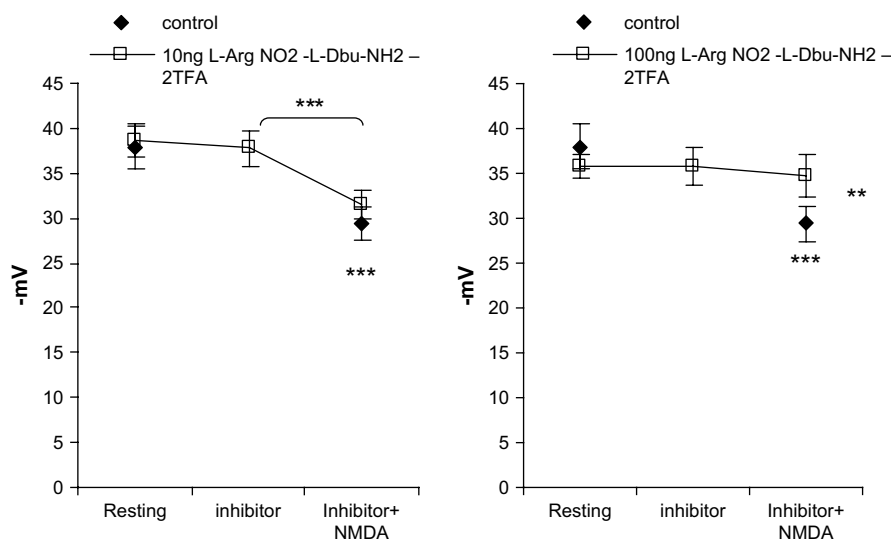


Fig. 6. Electrophysiological response of OA chondrocytes to 50 μ M NMDA treatment in the presence and absence of the nNOS inhibitor L-Arg NO₂-L-Dbu-NH₂ - 2TFA. Membrane potential is measured in $-mV$. Readings were taken in resting cells, after 10 min of treatment with inhibitor, and after 10 min of NMDA stimulation. Asterisks indicate statistically significant differences between resting and treated cells, or identified cell treatments ($***P < 0.001$; $**P < 0.01$).

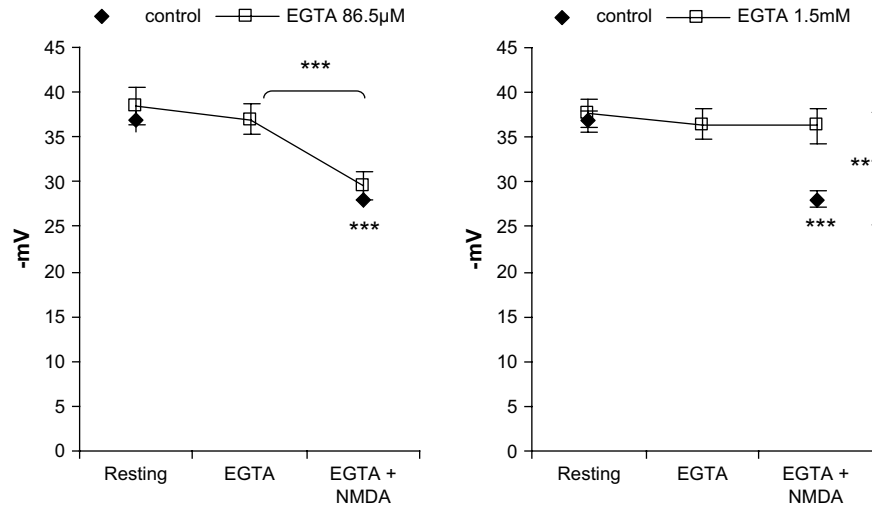


Fig. 7. Electrophysiological response of OA chondrocytes to 50 μM NMDA treatment in the presence and absence of EGTA. Membrane potential is measured in $-\text{mV}$. Readings were taken in resting cells, after 10 min of treatment with EGTA, and after 10 min of NMDA stimulation. Asterisks indicate statistically significant differences between resting and treated cells, or identified cell treatments ($***P < 0.001$).

Glutamate stimulation of neuronal NMDAR arises from α -amino-3-hydroxy-5-isoxazolepropionic acid (AMPA) glutamate induced membrane depolarisation which leads to relief of the voltage dependent channel block of NMDARs by extracellular Mg^{2+} ¹⁵. NMDARs are non-selective cation channels permeable to Na^+ and K^+ but also provide a significant influx pathway for Ca^{2+} . This is accompanied by release of glutamate from the stimulated cell propagating the glutamate signal. The mechanism that links NMDA binding to channel gating is partly known and relies upon subunit dimerisation¹⁶. Functional analysis of the NMDAR receptor carried out in this study measured both calcium influx and changes in cell membrane potential. Stimulation of OA chondrocyte NMDAR by NMDA in the presence of glycine resulted in an increase in calcium influx and membrane depolarisation both of which were blocked by competitive and non-competitive antagonists indicating the presence of functional NMDAR. Activated NMDARs have a high permeability to calcium ions and inhibition of membrane depolarisation by EGTA demonstrates that extracellular calcium is necessary for the response of chondrocytes in our system to NMDA. NMDARs provide a significant influx pathway for Ca^{2+} and the activity of these receptors is, as a consequence, coupled to a variety of Ca^{2+} -dependent intracellular signalling pathways. Neither nifedipine sensitive L-type ion channels nor gadolinium sensitive SAC channels appear to be involved in the production of the NMDA-induced cell membrane depolarisation.

NMDARs are physically linked to a number of intracellular signalling molecules including G proteins, Src, PYK, PKC, calcium calmodulin dependent kinase through the scaffold protein PSD-95. These signalling molecules in turn regulate the activity of NMDAR⁶ whilst calcium influx induces activation of downstream signalling through cascades involving PI3K (phosphoinositide 3-kinase)—Akt, nNOS and MAPK (mitogen-activated protein kinase)^{17,18}. We have not demonstrated a direct physical link between PSD-95 and NMDAR in chondrocytes. The TAT-NR2B9c peptide (a mimetic of NR2B), which is known to dissociate the NMDAR from PSD-95 preventing intracellular signalling via nNOS¹⁹ inhibited NMDA-induced membrane depolarisation

supporting the idea that such linkage exists. Similarly inhibition of NMDA-induced membrane depolarisation by a selective nNOS inhibitor suggests that activation of OA chondrocyte NMDAR requires nNOS activation. In the current study the membrane potential of the chondrocytes is measured 10 min after the application of NMDA and the membrane depolarisation that is seen appears to be the result of downstream activation of tetrodotoxin dependent Na channels. As such, the change in membrane potential is being used as a biological read-out of NMDA-induced effects through NMDAR. The precise roles for nNOS in the NMDAR dependent signalling cascade that results in activation of tetrodotoxin sensitive sodium channel induced membrane depolarisation remains to be defined.

In previous studies we have used changes in chondrocyte cell membrane potential as a highly sensitive and reproducible bio-assay to study chondrocyte mechanotransduction and identify mechanoreceptors and downstream signalling events. Mechanical stimulation of normal human articular chondrocytes results in cell membrane hyperpolarisation that is due to activation of apamin sensitive SK channels (small conductance calcium sensitive potassium channels) whereas similar mechanical stimulation of OA chondrocytes results in a tetrodotoxin sensitive cell membrane depolarisation indicating involvement of sodium channels in the latter response². We used tetrodotoxin in the current study to establish whether NMDA-induced cell membrane depolarisation in OA cells was, like mechanical stimulation induced membrane depolarisation, the result of activation of tetrodotoxin sensitive sodium channels. These experiments unexpectedly resulted in the unmasking of an NMDA-induced cell membrane hyperpolarisation. As a combination of apamin and tetrodotoxin was necessary to block the electrophysiological responses to NMDA it appears that both tetrodotoxin sensitive ion channels and apamin sensitive SK channels are activated downstream of NMDAR stimulation. Interestingly SK channels in neurones can be coupled to NMDARs and are involved in feedback mechanisms²⁰.

In neurones NMDAR may promote cell survival or when excessively activated may stimulate cell death pathways.

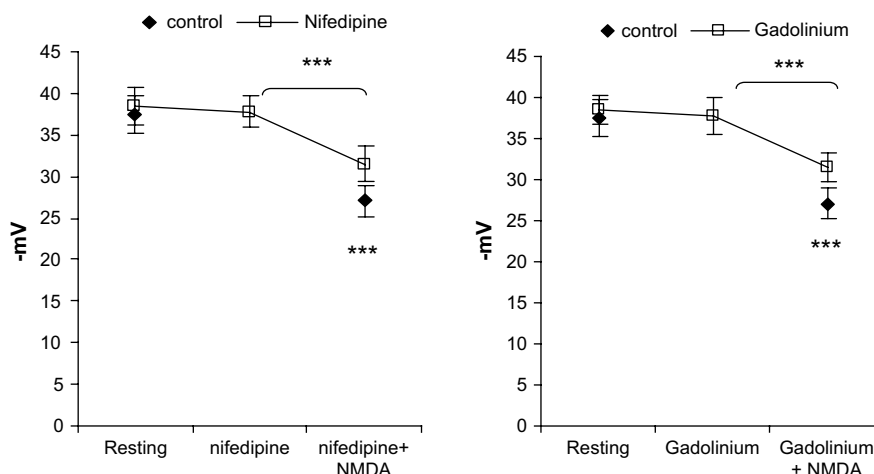


Fig. 8. Electrophysiological response of OA chondrocytes to 50 μ M NMDA treatment in the presence and absence of 10 μ M nifedipine and 10 μ M gadolinium. Membrane potential in response to stimuli is measured in $-mV$. Readings were taken in resting cells, after 10 min of treatment with inhibitor, and after 10 min of NMDA stimulation. Asterisks indicate statistically significant differences between resting and treated cells, or identified cell treatments ($***P < 0.001$).

Roles for NMDAR in chondrocytes remain to be identified but it is possible that functions will be similar to that of other cells such as neurones. NMDAR signalling is likely to have a variety of important functions in cartilage and depending on whether signalling is physiological or pathological, maintains healthy cartilage or contributes to the pathological changes in OA. Glutamate, the natural ligand for NMDAR is increased in the synovial fluid and presumably articular cartilage in animal models of knee inflammation²¹ and high concentrations of glutamate in synovial fluid of patients with active arthritis have been implicated in the pathological process²². Interestingly high levels of glutamate/NMDA result in neuronal cell death. Similar effects on chondrocyte viability may contribute to increased apoptosis seen in the cartilage of arthritic joints whether as a result of production by chondrocytes themselves, possibly *via* mechanical loading, or as a part of a more generalised inflammatory response. Possible sources for glutamate release in arthritic

joints would include afferent and sympathetic nerve terminals, inflammatory cells or subarticular bone.

In conclusion this study categorises NMDAR subunit expression in normal and OA chondrocytes, demonstrating expression of NR2B in OA but not normal chondrocytes. NMDAR function is dependent on association with PSD-95 and nNOS and stimulation results in activation of ion channels that we have previously identified as being activated in chondrocytes secondary to mechanical stimulation. Preliminary studies from our group have suggested roles for NMDAR in chondrocyte mechanotransduction but these roles have yet to be defined in detail. Chondrocyte NMDARs are also likely to be activated by glutamate, produced from several potential sources, in the synovial fluid of joints developing arthritis and this may influence a variety of cellular functions including survival. Whether these receptors are involved in the regulation of anabolic and catabolic activities that are required to maintain healthy

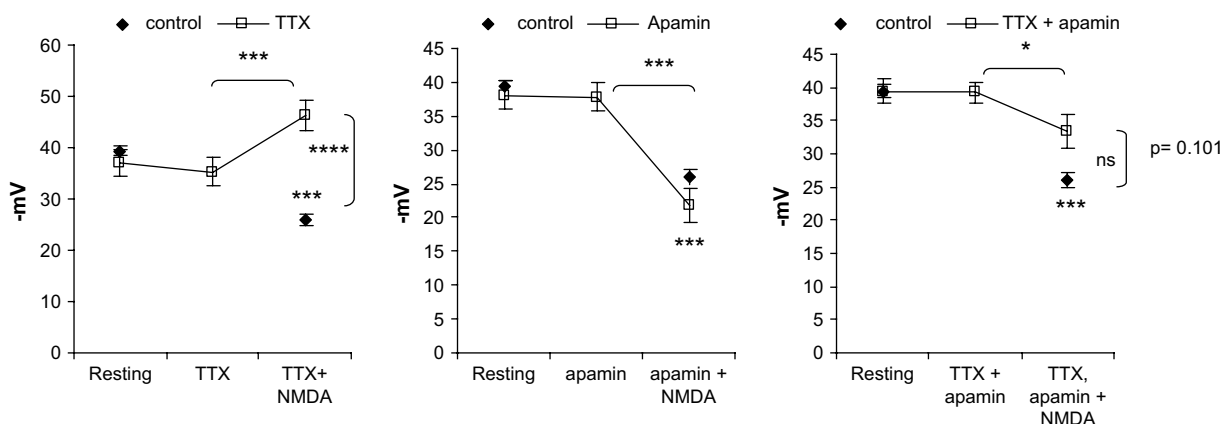


Fig. 9. Electrophysiological response of OA chondrocytes to 50 μ M NMDA treatment in the presence and absence of 1 μ M tetrodotoxin and 4.9 μ M apamin. Membrane potential is measured in $-mV$. Readings were taken in resting cells, after 10 min of treatment with reagent, and after 10 min of NMDA stimulation. Asterisks indicate statistically significant differences between resting and treated cells, or identified cell treatments ($*P < 0.05$; $***P < 0.001$).

cartilage or result in osteoarthritis is as unclear. The differential signalling by NR2B vs NR2A containing NMDARs with regard to their differing roles in synaptic plasticity and cell survival/death is an area of intense interest in neuroscience^{23–26}. Our observations raise the possibility that NR2B vs NR2A containing NMDARs may exert differing responses in chondrocytes.

Conflict of interest

The authors have no conflicts of interest.

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