



Expression and Function of Articular Chondrocyte NMDA Receptors

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NMDAR are ionotropic glutamate receptors. NMDAR are extensively expressed in the nervous system where they play a central role in synaptic transmission, learning and memory. Also expressed by non-neuronal cells such as osteoblasts and osteoclasts much of our current knowledge of the physiological and pathological functions of NMDAR is based on the study of neuronal cells. Recently NMDAR have been shown to be expressed by human and rodent articular chondrocytes but roles for NMDAR in regulating chondrocyte function and cartilage integrity are still largely unknown. Physiological levels of NMDAR activity may be important in chondrocyte responses to environmental stimuli such as mechanical loading. Excessive NMDAR stimulation may be detrimental increasing activation of pro-apoptotic pathways or decreasing cellular oxidative defence mechanisms. Increased knowledge of NMDAR function in regulating chondrocyte activity in normal and osteoarthritic cartilage is an important prerequisite to allow translation of new discoveries of altered NMDAR expression and function in osteoarthritis into rational, novel approaches to therapeutic intervention.

Key words: cartilage, NMDA receptors, osteoarthritis

INTRODUCTION

NMDAR (*N*-methyl *D*-aspartate receptors) are ionotropic glutamate receptors. Glutamate receptors are divided into two groups, ionotropic and metabotropic receptors.¹ Ionotropic glutamate receptors form ion channel pores that activate when glutamate binds to the receptor. The second group, metabotropic glutamate receptors, indirectly activate plasma membrane ion-channels by stimulating G proteins and production of the intracellular second messengers cAMP, inositol triphosphate and di-acyl glyceride. NMDA is a selective agonist for NMDAR and does not bind to AMPA or Kainate ionotropic glutamate receptors that have different pharmacological and electrophysiological properties. NMDAR are further unique among the ionotropic glutamate receptors in that they require binding of both glutamate and a co-agonist, glycine, for activation. NMDAR are cation-passing ion channels. Upon activation the channel pore opens allowing flow of Na⁺ and small amounts of Ca²⁺ ions into

the cell and K⁺ out of the cell. NMDAR show a voltage-dependent block by physiological concentrations of Mg²⁺ that prevents ion flow.² This blockade is relieved by membrane depolarisation at the time of glutamate and glycine binding to NMDAR. The calcium flux through NMDAR following stimulation and channel opening is believed to be pivotal for most of the physiological and pathological effects consequent to receptor activation.

NMDAR Structure

The structure of NMDAR is known.^{3,4} Three types of NMDAR subunits have been identified; NR1, NR2 and NR3. There are four NR2 subtypes (NR2A, B, C and D) and two NR3 subtypes (NR3A and B). The NR1 subunit has a molecular size of 120kDa and is smaller than the NR2A and NR2B subunits (170-180 kDa) and the NR2C and NR2D subunits (150 kDa).^{5,6} GRIN genes, that code for the NMDAR subunits, are present on different chromosomes.⁷ A number of subunit isoforms are recognised. GRIN1, the gene for NR1, contains N1, C1 and C2 cassettes that are subject to alternative RNA splicing leading to the generation of eight splice variants.⁸ The NR2C and NR2D subunit genes also undergo alternative splicing with each potentially being expressed as two isoforms.^{9,10}

Functional NMDAR contain both NR1 and NR2 (and/or NR3) subunits. They typically consist of a tetramer of NR1 and NR2 subunits^{11,12} although NR3 subunits can

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substitute for NR2 subunits. Neither NR1 nor NR2 subunit form functional receptors when expressed alone and remain within the endoplasmic reticulum. The structures of the different subunits are similar. Each of the subunits has a large extracellular N-terminal domain followed by four membrane domains and a C-terminal tail. The extracellular component of the subunits contains two modulatory and ligand binding domains. NR1 subunits bind the co-agonist glycine whereas the NR2 subunits bind the neurotransmitter glutamate. The NR1 subunits have important roles in the properties of NMDAR with isoforms influencing agonist affinity and receptor trafficking. The extracellular N1 cassette of NR1 interacts with various pharmacological modulators of NMDAR including zinc, protons and polyamines.¹ The NR1 C-terminal cassettes control cell surface expression of NMDAR. The membrane domain, consisting of three transmembrane segments and a re-entrant loop, contributes to the ion channel pore and is responsible for the high-calcium permeability and voltage-dependent magnesium block. NMDAR show differences in sensitivity to Mg^{2+} block depending on subunit composition. NR2A and NR2B subunit containing NMDAR are more sensitive than the NR2C and NR2D containing receptors.¹³ Unlike other NMDAR NR1/NR3 containing receptors can be activated by glycine in the absence of glutamate.

The C-terminal intracellular domain of NMDAR is not required for channel formation or gating but is necessary for critical associations with intracellular enzymes and scaffold proteins which are important for receptor function. The cytoplasmic tails of the NR2 subunits directly interact with members of the membrane associated guanylate kinase (MAGUK) proteins, such as PSD-95, SAP97, PSD-9310 and SAP102.^{14,15} The basic structure of MAGUK proteins includes three PDZ domains, an SH3 domain, and a guanylate kinase-like domain¹⁶ allowing extensive protein-protein interactions. Through these interactions NMDAR associate with a range of structural and signal transducing molecules such as actin, filamentous proteins, neuronal nitric oxide synthase (n-NOS), Src and Ca^{2+} -dependent enzymes such as protein kinase C (PKC), calcium/calmodulin dependent protein kinase II (CaMKII), phospholipase A_2 (PLA₂), tyrosine kinases and protein phosphatases.¹⁷⁻¹⁹

NMDAR Function

NMDAR are extensively expressed in the nervous system where they play a central role in synaptic transmission, learning and memory. Also expressed by non-

neuronal cells much of our current knowledge of the physiological and pathological functions of NMDAR is based on the study of neuronal cells. Physiological activation of NMDAR promotes cell survival. On the other hand pathological activation of NMDAR is detrimental to cell survival²⁰. Pathological activation of NMDAR, with strong calcium flux, leads to mitochondrial dysfunction, calpain and MAP kinase activation.²⁰ In circumstances where NMDAR blockade does not result in neuronal death, it can render neurons more vulnerable to mechanical trauma. In contrast to pathological activation, physiological stimulation of NMDAR results in pro-survival signalling involving the PI3K/Akt pathway, activation of CREB, down-regulation of pro-apoptotic gene expression and boosting of intrinsic anti-oxidant defences.²⁰

Transgenic mice have provided insight into how NMDAR function in the nervous system but less information on roles in skeletal development. NR1 and NR2B subunit knockout mice are perinatally lethal whereas mice lacking the NR2A, NR2C, and NR2D subunits are viable. None of the transgenic mice appear to show major skeletal abnormalities. Nevertheless it is now increasingly accepted that bone cells, including osteoblasts and osteoclasts express NMDAR. Targeted knock-out of the NR1 subunit in osteoblasts does appear to have effects on the skeleton with mice showing delayed development, thin bone structure and poor mineralisation of the axial and appendicular bones.²¹ These studies and an extensive body of work have provided evidence that NMDAR are have critical roles in regulation of bone turnover.^{21,22}

Expression of NMDAR in Cartilage.

NMDAR were first shown to be expressed in human articular cartilage by Salter et al.²³ The NR1 subunit was shown to be expressed in articular cartilage by immunohistochemistry and the NR2a subunit was identified by reverse transcription-polymerase chain reaction (RT-PCR) in primary chondrocyte culture. The receptors were shown to be functional by addition of either NMDA or glutamate with glycine to primary chondrocytes and changes in membrane potential assessed. Application of NMDA or glutamate with glycine resulted in membrane hyperpolarisation of chondrocytes from normal articular cartilage. Interestingly application of MDL26630, an NMDAR agonist that acts at the spermine site of the receptor induced membrane depolarisation rather than hyperpolarisation. The effects of NMDA on human chondrocyte membrane potential is blocked by MK801 and APV, both NMDAR antagonists, further supporting the

presence of functional NMDAR in these cells.²⁴ In normal human articular cartilage initial studies indicated that only NR2A but not other NR2 subunits were expressed.²⁴ Subsequently we have identified expression of NR2C using different primer pairs (unpublished observations). NMDAR are also expressed in rat chondrocytes and the chondrosarcoma cell line SW1353.²⁵ In these cells NR2D and NR2A in addition to NR1 appear to be the major subunits expressed. The NR3A subunit is expressed in the hypertrophic cells but not the proliferative chondrocytes of rat growth plate.²⁶

NMDAR stimulation has now been shown to have a range of effects on chondrocytes in a number of different *in vitro* models. Following addition of NMDA with glycine to human articular chondrocytes there is influx of calcium into cells that is blocked by NMDAR antagonists. Using NMDAR induced changes in cell membrane potential down-stream transduction pathways are beginning to be identified.²⁴ Chondrocytes express PSD-95 and this molecule appears to be necessary for signal transduction.²⁴ Changes in membrane potential induced by NMDA in chondrocytes from osteoarticular cartilage are inhibited by n-NOS antagonists indicating possible linkage with NMDAR and n-NOS through PSD-95.²⁴

Physiological Functions of Chondrocyte NMDAR

Knowledge of the possible physiological role(s) for NMDAR in cartilage is currently very limited. There is evidence for activation of functional NMDAR in the cellular differentiation of chondrocytes from a hypertrophic state to calcified chondrocytes in rodents.²⁷ Studies from our group suggest that NMDAR signaling is important in human articular chondrocyte mechanotransduction.²³ *In vitro*, $\alpha 5 \beta 1$ integrins, CD47 and stretch activated ion channels have been shown to act as mechanoreceptors and initiate intracellular cell signalling in response to mechanical stimuli.²⁸ Signal transduction following stimulation of these mechanoreceptors leads to production of cytokines such as IL4 that through paracrine / autocrine activity, induce a variety of physiological and molecular responses.²⁹ These include activation of small conductance K_{Ca} (SK) channels and cell membrane hyperpolarisation and regulation of gene expression. When 30 μ M CPP, a competitive NMDA receptor antagonist or 1 μ M MK801, a non-competitive NMDA receptor antagonist are added to normal articular chondrocytes the membrane hyperpolarisation response to 0.33 Hz mechanical stimulation are blocked indicating roles for NMDAR. Neither the AMPA/Kainate receptor antagonist CNQX

nor MCPG, a competitive metabotropic receptor antagonist had effects on the electrophysiological response of chondrocytes to mechanical stimulation.

Specific roles for NMDAR in chondrocyte mechanotransduction remain to be defined. NMDAR responses may be potentiated directly by stretch of the lipid bilayer³⁰ or by phosphorylation of NR2 subunits.^{31,32} We may speculate that, as part of the mechanotransduction pathway, an integrin/CD47 complex may modulate NMDAR by stimulating serine/threonine kinases such as PKC and protein tyrosine kinases including Src leading to NMDAR phosphorylation. PKC,³³ CaMKII³⁴ and Src³⁵ are known to be activated and necessary for integrin dependent responses to mechanical stimulation. Activation of integrins may induce an intracellular signal cascade involving PKC, CaMKII and Src that leads to phosphorylation of NMDAR NR2 subunits³¹ and hence modulate channel properties. With either spontaneous or mechanical stimulation induced release of glutamate from the cell, NMDAR activation will result in a secondary increase in calcium flux. This second wave of calcium signalling may be important for regulation of downstream events in the mechanotransduction cascade such as substance P.³⁶

POTENTIAL ROLES OF NMDAR IN OSTEOARTHRITIS

Responses of neurones to NMDAR activation appear to follow a bell shaped curve with too much or too little NMDAR activity being harmful.²⁰ Excessive or inadequate NMDAR activation may similarly contribute to the pathological changes in osteoarthritis (OA). Glutamate, the natural ligand for NMDAR, is increased in the synovial fluid and presumably articular cartilage in animal models of knee inflammation.^{37,38} Similarly, high concentrations of glutamate are seen in synovial fluid of patients with active arthritis.³⁹ As with neuronal cells, where high levels of glutamate/NMDA result in cell death, similar effects on chondrocyte viability may contribute to increased apoptosis in osteoarthritis. Possible sources for glutamate release in arthritic joints would include afferent and sympathetic nerve terminals, inflammatory cells or subarticular bone. However articular chondrocytes release glutamate and express the glutamate transporter GLAST-1^{25,38} indicating the likelihood of autocrine and paracrine signaling in either a physiological or pathological setting. In osteoarthritis cytokines such as IL1 β may increase peri-cellular glutamate levels in cartilage by inducing glutamate release or inhibiting glutamate trans-

porter expression.²⁵ The chondrotoxicity of quinolone antimicrobial agents⁴⁰ might be based on their ability to activate NMDAR by abolishing the Mg^{2+} block.

Withdrawal of NMDAR stimulation may also be detrimental to cartilage homeostasis. Chondrocytes isolated from costal cartilage of NR1-deficient mice show decreased alkaline phosphatase (ALP) activity in comparison to chondrocytes from wild-type mice.²⁶ In organotypic culture metatarsals from NR1-deficient mice at embryonic day 15.5 showed decreased mineralization but no difference in metatarsal growth.²⁶ Roles for NMDAR in rat chondrocyte proliferation have also been shown by NR1 knock down studies.²⁵

In addition to intensity of stimulation, neuronal cells show difference in NMDAR responses depending on the location, synaptic or extra synaptic, of the receptors. This may be secondary to differences in subunit composition of the receptors or differences in the composition of associated signalling complexes. Differences in NMDAR subunit composition and/or associated signaling complexes may influence chondrocyte responses to oxidative stress and the activation of survival or death signaling pathways. The composition of NMDAR associated complexes in normal and osteoarthritic chondrocytes have yet to be defined but it is clear that NMDAR stimulation of these cells results in different downstream responses. Addition of NMDA to chondrocytes from normal cartilage results in cell membrane hyperpolarisation but under identical conditions chondrocytes from osteoarthritic cartilage show a membrane depolarisation response.^{23,24} Interestingly higher concentrations of the NMDAR antagonist MK801 is required to block the membrane potential response of osteoarthritic chondrocytes (50 μM) to mechanical stimulation than that of normal cells (1 μM) further indicating potential importance of aberrant NMDAR activity in osteoarthritis

CONCLUSION

In vitro studies provide strong evidence that NMDAR are expressed by articular cartilage chondrocytes. Nevertheless the roles NMDAR may have in regulating chondrocyte function and cartilage integrity is still largely unknown. Excessive NMDAR stimulation in cartilage may be detrimental increasing activation of pro-apoptotic pathways or decreasing cellular oxidative defence mechanisms. Recent studies raise the possibility that in OA cell responses to NMDAR activation are modified. Whether this is a result of altered receptor subunit composition or changes in the composition of

NMDAR-associated signalling complexes remains unclear. Chondrocytes express NMDAR and appropriate capability to release and recycle glutamate^{25,38} indicating significant functional importance. NMDAR may become an important new therapeutic target in osteoarthritis, modulation of activity having the potential to influence disease progression. Enhancing our understanding of NMDAR function in the control of chondrocyte activity in normal and osteoarthritic cartilage is an important prerequisite for attempts to translate new knowledge of altered NMDAR expression and function in osteoarthritis into rational, novel approaches to therapeutic intervention.

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