SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

In vitro examination of *N*-methyl-D-aspartate type glutamate receptors in non-excitable cells

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Head of the **Examination Committee**:	László Virág, MD, PhD, DSc
Members of the Examination Committee:	János Szöllősi, PhD, DSc, member of HAS
	József Tóvári, PhD

The Examination takes place at the Department of Medical Chemistry (3.009-010, library), Faculty of Medicine, University of Debrecen
11 a.m., 1st of April 2022.

Head of the **Defense Committee**:	László Virág, MD, PhD, DSc
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The PhD Defense takes place at the Lecture Hall of the Department of Anatomy, Histology and Embryology, Faculty of Medicine, University of Debrecen
1 p.m., 1st of April 2022.

Introduction

N-methyl-D-aspartate receptors

Ionotropic glutamate receptors (iGluRs) are classified into two groups based on the high specificity of the synthetic iGluR agonist *N*-methyl-D-aspartate (NMDA). According to that, glutamate receptors can be classified as NMDA and non-NMDA type receptors. Although NMDARs function as non-selective cation channels just like the other iGluRs, they are mainly permeable to Ca^{2+}, in contrast to non-NMDARs.

NMDARs are di- or heterotetrameric complexes composed of the combination of three types of subunits (GluN1, GluN2 and GluN3). These subunits exist in a number of splice variants; moreover, there are several isoforms of GluN2 and GluN3 subunits (i.e. GluN2A, GluN2B, GluN2C, GluN2D; GluN3A and GluN3B). Two GluN1 subunits are necessary for a functional ion channel, as these are the pore forming subunits, and they harbour the glycine binding site required for co-activation. The intracellular domain of the GluN1-1a splice variant contains a functionally relevant nuclear localisation signal (NLS). The glutamate (and NMDA) binding site of the receptor is located on the GluN2 subunits. The GluN3 subunit does not bind L-glutamate, but harbours a glycine binding site, similar to GluN1. According to *in vivo* functional studies, the GluN3 subunit forms functional channels together with GluN1 and GluN2 subunits. Such triheterotetrameric complexes are *bona fide* NMDARs, albeit with slightly different features compared to conventional NMDARs.

GluN1-GluN3 diheterotetrameric NMDARs do not respond to L-glutamate or NMDA due to the lack of their binding site. Such NMDARs are glycine-gated excitatory ion channels. Whether GluN1-GluN3 NMDARs, these excitatory, alternative glycine receptors are present and functional *in vivo* is still controversial; however, pharmacological studies may provide clues for their specific identification.

The role of calcium in signal transduction pathways

Since NMDARs are primarily Ca^{2+} permeable ion channels, it is necessary to briefly discuss the multiple roles of Ca^{2+} in general cellular physiology. From an evolutionary perspective, Ca^{2+} is the most ancient secondary messenger, because it does not require enzymes to be synthesised; it can be quickly mobilised and eliminated; and changes in its concentration can be either generalised or local, depending on the actual needs of the cell. In case of long-term

activation and the rapid gating of the ion channels, the Ca^{2+} transients follow each other so quickly that they trigger Ca^{2+} oscillations.

The main advantage of Ca^{2+} is that it offers a virtually endless combination of unique possibilities, which made it indispensable for signal transduction pathways. Since Ca^{2+} binding an alter the function of the majority of cytosolic proteins, the interior of cells is generally characterised by very low Ca^{2+} concentration. Ca^{2+} entering the cytosol upon the opening of Ca^{2+} channels gets involved in almost every cellular physiological process. Of these, its role in controlling and mediating gene expression is worth highlighting, which is attributable to acting on transcription factors translocating to the nucleus upon Ca^{2+} binding.

Outside the nervous system I: Articular cartilage

Cartilage – An overview

Cartilage is one of the basic connective tissue types in vertebrates. The abundant, highly complex cartilage extracellular matrix (ECM), which consists of specific groups of macromolecules, is produced by chondrocytes with a round morphology. The ECM is indispensable for the chondrocyte phenotype.

Cartilage ECM comprises two main components: fibres and ground substance. The fibres are predominantly given by collagens, the vast majority of which is collagen type II in hyaline cartilage. The interfibrillar ground substance is formed of proteoglycans (PG) and multiadhesive, non-collagenous glycoproteins. Aggrecan, which is rather specific to cartilage, is one of the major PGs. An aggrecan monomer consists of high numbers of chondroitin sulphate and keratin sulphate glycosaminoglycan (GAG) side chains attached to a core protein, arranged like a brush. These monomers are linked to hyaluronic acid, forming massive multimolecular aggregates. These aggregates are responsible for the high water content of cartilage ECM.

Chondrocytes in mature cartilage exit the cell cycle and become post-mitotic cells, even in case of tissue injury. In addition, cartilage is avascular, which results in cartilage being characterised by an extremely weak capability for regeneration. This is a key factor in the development of cartilage pathologies. Diseases affecting articular cartilage are often caused by pathological alterations in gene expression or cellular mechanisms associated with chondrogenesis. Therefore, a more detailed understanding of signalling pathways regulating chondrogenic

differentiation may enable the development of novel drugs and therapies targeted at such diseases.

Chondrogenesis

Cartilage is formed during early embryonic life, which makes implementation of *in vivo* experimentation on immature cartilage almost impossible. One of the most widely accepted *in vitro* approaches is based on primary high density (HD) cell cultures established from the developing fore and hind limb buds of early chick embryos (Hamburger and Hamilton developmental stage 22–24). These colonies primarily consist of chondroprogenitor mesenchymal cells.

The main advantage of this model is that the mesenchymal cells differentiate into chondrocytes within 6 culturing days by completing their intrinsic differentiation programme, which takes place automatically due to the initial high cellular density and their state of commitment.

At the beginning of chondrogenesis, chondroprogenitor cells form aggregates, called pre-cartilage nodules, and then differentiate into chondroblasts on culturing days 2–3, which is therefore considered a critical period of the process. Nodule formation is so important in terms of early chondrogenesis that it is often considered an obvious sign of chondrogenic commitment of the progenitor cells.

Due to the formation of cell-cell junctions, the cytoskeletal organisation changes, the formerly elongated, fibroblastoid morphology becomes roundish, and the cells start producing cytokines and growth factors which drive chondrogenic differentiation. Bone morphogenetic protein (BMP), transforming growth factor ß (TGF-ß) and fibroblast growth factor (FGF) signalling pathways upregulate SOX9, a key player in cartilage formation. SOX proteins are responsible for the transcription of cartilage-specific ECM components. SOX9 activity is further increased by phosphorylation, therefore – directly or indirectly – protein kinases (e.g. protein kinase A, protein kinase C) and phosphoprotein phosphatases (e.g. phosphoprotein phosphatase 2A, phosphoprotein phosphatase 2B) also play an important regulatory role in the process.

What we currently know about NMDARs and Ca^{2+} signalling in chondrocytes

It has been almost 20 years ago that NMDAR subunit expression had been reported in chondrocytes. NMDARs detected in mature human articular chondrocytes probably mediate mechanotransduction pathways and may have an important role in the altered behaviour of

osteoarthritic chondrocytes. Despite this, the role of NMDARs in chondrogenic processes has so far been completely unexplored.

NMDARs, irrespective of their subunit composition, are essentially Ca^{2+} channels, which enable Ca^{2+} influx upon activation. Ca^{2+}-dependent signalling pathways have been well documented to be indispensable for the chondrogenic differentiation of mesenchymal cells. Research conducted by members of our team has shown that cytosolic Ca^{2+} levels in the differentiating cells of HD cultures exhibit both long-term (daily) and short-term (rapid oscillatory) changes. We were the first to report on the role of store-operated Ca^{2+} entry (SOCE) in chondrogenic processes.

Outside the nervous system II: healthy and pathological pigment cells

Melanocytes in the epidermis

Localised predominantly in the basal cell layer of the epidermis, melanocytes are pigment-producing cells with an elongated morphology. The main role of pigment cells is the synthesis of melanin pigment, which takes place in cytoplasmic, membrane-bound organelles called melanosomes. Given that melanin can absorb 50–75% of the ultraviolet (UV) radiation, it mainly has a protective role. Pigment-containing melanosomes transported into keratinocytes form an array over the nucleus in the cytoplasm, thus creating some sort of "cap" and protect the nuclear DNA from the mutagenic effects of UV radiation. Obviously, the "pigment array" formed in the epidermis also has a protecting effect for the cells in the dermis.

Cutaneous melanoma

Cutaneous melanoma is a malignant neoplasm of epidermal melanocytes. Although melanoma represents only 4% of skin-related cancers, it is responsible for a considerable proportion of resulting mortality. The constantly increasing incidence and mortality of melanoma makes it one of the most dangerous neoplasms.

Both environmental and genetic risk factors contribute to melanoma formation. One of the most obvious environmental factors is UV radiation from the sun. Certain mutations leading to neoplasm formation (e.g. cyclin dependent kinase inhibitor 2A, BRAF, NRAS) are also important.

Tumour development is a multistep process until it reaches the clinical stage with the most unfavourable outcome, during which a well-defined histological progression takes place. At an

early stage, the tumour is *in situ*, and it does not pass through the basal layer of the epithelium (stage I). However, beyond a certain threshold, tumour cells penetrate the basal membrane and enter the papillary layer of the dermis (stage II). Then, tumour cells spread into the reticular layer of the dermis first (stage III), and then into the subcutaneous layer (stage IV). The next stage of neoplasm progression is characterised by metastatis formation. The main cause of melanoma-related mortality is distant metastases affecting different organs.

What we currently know about NMDARs and Ca^{2+} signalling in human pigment cells

Before entering the epidermis, melanocytes are derived from the neuroectoderm; more specifically, from the neural crest. Therefore, it is not exactly surprising (albeit it is not well documented) that they express NMDAR subunits (GluN2A, GluN2C). However, their specific subunit composition is currently unknown. The GluN2(A) subunit is most frequently associated with melanocytes (and melanoma cells); however, the functions of the GluN1 and GluN3 subunits are largely unknown in this context. Melanocytes are unable to produce glutamate; therefore, it is possible that glutamate-related signalling pathways in melanocytes are linked to their tight functional relations with keratinocytes. Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) are key regulatory factors in the cellular physiology of melanocytes. These involve melanocyte development, their responses to mitogenic stimuli, melanin production, and photoprotective functions.

Research carried out on melanoma-derived tissues confirmed that melanoma cells secrete a considerably higher amount of glutamate via a putative autocrine loop. With regards to NMDARs, the gene coding for the GluN2A subunit (*GRIN2A*) was found to frequently carry mutations, which correlated with a decline in the survival rate of melanoma patients. Furthermore, *GRIN2A* has been identified as a tumour suppressor gene in melanoma, since its declined activity resulted in uncontrolled cell division. Ca^{2+} plays key roles in the cellular processes of malignant pigment cells. Amongst others, Ca^{2+} can generate proliferative signals, or may increase the invasivity of the cells through the process of SOCE.

Aims

The Signal Transduction Research Group at the Department of Anatomy, Histology and Embryology at the University of Debrecen, which conventionally focused on cartilage research, extended its interest to NMDARs in the mid-2000s. Since there was a growing interest to address the presence and role of certain neuronal receptors outside the nervous system after the turn of the millennium, articular cartilage has become a promising subject of our studies.

NMDARs have previously been shown by others in skeletal components. However, their role has not yet been studied in differentiating chondrocytes, especially in terms of those signalling pathways regulating chondrogenesis that are sensitive to alterations of $[Ca^{2+}]_i$.

Therefore, the aims of these experiments were as follows:

1) to study the expression profiles of NMDAR subunits in a chicken chondrogenic model;
2) to determine the subcellular localisation and potential colocalisation of the identified NMDAR subunits;
3) to assess whether chondrogenic cells are capable of producing L-glutamate, and to respond by changing glutamatergic signalling-driven cellular functions;
4) to investigate, using an array of pharmacological compounds, those cellular functions that are mediated by NMDARs, with special emphasis on cartilage-specific ECM production, which is essential for chondrogenesis;
5) to observe whether NMDARs mediate Ca^{2+} transients in differentiating chondrocytes, and whether receptor-driven spontaneous Ca^{2+} events influence chondrogenic differentiation.

Around 2010, we started our experiments with pigment cells, more specifically human melanoma cells, and then cutaneous melanocytes. Melanoma is a malignant neoplasm originating from melanocytes, the healthy pigment cells of the skin. Due to its aggressive nature and its ability to quickly form metastases, it is characterized by poor prognosis and an extremely high mortality rate. Comparison of *in vitro* experimental results obtained on normal and pathological pigment cells may provide useful experience for dermatological and pathological diagnostics.

Studying NMDARs on pigment cells *in vitro* was one of our first promising projects, especially given the scarce literature available on the subject. During the initial pilot experiments performed on melanoma cells, following application of certain NMDAR modulator compounds, elevated Ca^{2+} levels were indicated by the Ca^{2+} sensitive fluorescent dye Fura-2, especially at the central region of the cells, corresponding to the nucleus. This observation has

led to the hypothesis of NMDARs having a putative nuclear localisation, which, taking into account that this observation has been made on melanoma cells, could have been related to the tumour phenotype. This was the beginning of the main topic of my doctoral work in 2013 – the detailed profiling of NMDARs in tumour cells. As my experiments progressed, the project was supplemented by studying NMDARs in melanocytes used as healthy controls.

In the 2000s, knowledge on the role of functionally active receptors in pathological lesions, especially in tumours, gradually expanded.

Therefore, further objectives of my dissertation were as follows:

6) to determine the NMDAR subunit expression profiles of cutaneous melanocytes;
7) based on our preliminary data, to establish the specific expression pattern of NMDAR subunits in different cellular compartments by generating subcellular fractions of melanoma cell lines derived from different stages of the disease;
8) to confirm the subcellular localisation and potential colocalisation of the identified NMDAR subunits using immunocytochemistry followed by confocal microscopy.

The ultimate aim of my doctoral dissertation is to draw conclusions regarding the role of NMDARs in Ca^{2+} dependent processes in these non-excitable cells based on the results of the *in vitro* experiments.

Materials and Methods

Chondrogenic mesenchymal high density cell cultures

To establish HD cultures, chondroprogenitor mesenchymal cells were isolated from the distal parts of the limb buds of 4.5-day-old Ross white hybrid chick embryos. Isolated limb buds were dissociated in 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA), and cells were pelleted by centrifuging the samples.

Cell suspensions with a density of 1.5×10^7 cells/mL were used. After seeding, cells were allowed to attach to the surface for 2 hours in a CO_2 incubator at 37°C, 5% CO_2 and 80% relative humidity. Then, cultures were fed with Ham's F12 medium (N6760, Sigma-Aldrich) supplemented with 10% FBS. The day of setting up the cultures was considered day 0. Differentiating cultures were maintained for 6 or 10 days. Experiments performed on early-stage chick embryos does not require a licence from the Ethics Committee of the University of Debrecen.

Melanocyte and melanoma cell cultures

Normal human epidermal melanocyte (NHEM) cell cultures (PromoCell GmbH, Heidelberg, Germany) were isolated from the epidermis of juvenile skin samples. We also conducted the experiments on 5 different melanoma cell lines (A2058, HT168M1, HT199, M35/01, and WM35). A2058 and WM35 cell lines were obtained from ATCC (ATCC® CRL-1661™, Manassas, VA, USA). HT168M1, HT199, and M35/01 cell lines were kind gifts from Dr Andrea Ladányi (National Institute of Oncology, Budapest, Hungary). Melanocytes were cultured in Melanocyte Medium (PromoCell GmbH), melanoma cells were grown in RPMI-1640 medium (Sigma-Aldrich) at 37°C, 5% CO_2 and 80% humidity.

mRNA expression profiling

RNA isolated from the cell cultures was reverse transcribed to cDNA using the High Capacity RT kit (Applied Biosystems). cDNA was generated during a 2-hour step in a programmable thermal cycler (Labnet MultiGene™ 96-well Gradient Thermal Cycler; Labnet International, Edison, NJ, USA) at 37°C.

The amplification of specific cDNA sequences was carried out by using primer pairs designed based on human or chicken nucleotide sequences available in the GenBank online database (GenBank, National Institutes of Health, Bethesda, MD, USA; available at:

https://www.ncbi.nlm.nih.gov/genbank/). Human primers were designed using the Primer Premier 5.0 software (Premier Biosoft, Palo, Alto, CA, USA); chicken primers were designed using the Primer BLAST service (available at: http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The specificity of primer pairs designed using Primer Premier 5.0 was confirmed *in silico* using Primer BLAST.

Amplification of specific DNA fragments was carried out using a programmable thermal cycler (Labnet International). The resulting polymerase chain reaction (PCR) products were separated using horizontal gel electrophoresis in a 1.5% agarose gel containing ethidium bromide. The bands were visualised using a gel scanner (Fluorchem E, Protein Simple, San Jose, CA, USA). The optical density (OD) values of the bands were determined using ImageJ version 1.8.0_112 (available at: http://rsbweb.nih.gov/ij/).

Protein expression profiling

Protein expression studies using western blot (wb) analyses were performed on total cell lysates and subcellular fractions. First, cells were washed with physiological saline, harvested, centrifuged at 2000 × *g*, and the pellet was resuspended in Radio Immuno Precipitation Assay (RIPA) buffer.

For total cell lysates, cells were lysed using an ultrasound sonicator probe. To isolate cytosolic fractions, samples were sonicated, and then centrifuged at 50,000 × *g* at 4°C for 90 minutes. This was followed by triturating in RIPA buffer containing 1% Triton X-100 (Reanal, Budapest, Hungary). After a second centrifuging, the supernatant containing the membrane fraction was collected. Nuclear fraction was isolated using a sucrose gradient. The original cell pellet was resuspended in buffer A; following the addition of Igepal CA-630 (Sigma-Aldrich) the samples were homogenised using a Dounce homogenisator. Then, samples were centrifuged at 770 × *g* at 4°C for 10 minutes, and the pellet was resuspended in 2.2 mM sucrose-buffer A solution. This was followed by a second centrifuge step at 40,000 × *g* at 4°C for 90 minutes. The resulting pellet was resuspended in 0.25 mM sucrose-buffer A solution.

The protein concentration of the samples was determined using a modified BCA assay (BCA™ Protein Assay Kit, Pierce Biotechnology, Rockford, IL, USA). For subunit detection, samples were separated by electrophoresis in a 7.5% acrylamide gel. Separated proteins were transferred onto a nitrocellulose membrane using a Bio-Rad Trans-Blot Turbo system (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in 5% low-fat milk in PBS, and then incubated with the primary antibodies overnight at 4°C.

Next, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse IgG; Bio-Rad Laboratories) at a dilution of 1:1500 at room temperature for 1 hour. Then, immunoreactive bands were developed using enhanced chemiluminescence. Chemiluminescent signals were documented using a gel imager (DNR Bio-Imaging Systems Ltd, Jerusalem, Israel; and Fluorchem E, Protein Simple, San Jose, CA, USA). Signals were quantifed using ImageJ version 1.8.0_112.

Immunocytochemistry and confocal microscopy

To map the specific subcellular localisation of the NMDAR subunits, immunocytochemical reactions were carried out. On culturing day 3, HD colonies were fixed with Sainte-Marie's fixative (99% ethanol and 1% acetic acid), whereas melanocyte and melanoma cell cultures were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louise, MO, USA) for 1 hour. After washing with PBS, aspecific binding sites were blocked with 1% BSA (bovine serum albumine; Amresco) dissolved in PBS for 30 minutes at 37°C.

Cultures were then incubated overnight at 4°C with the first primary antibody. In case of 3-day-old HD cultures, this was a monoclonal anti-GluN1 (Alomone Labs, Jerusalem, Israel) or a polyclonal anti-GluN2B (Cell Signaling, Danvers, MA, USA) antibody; for pigment cells, these were polyclonal anti-GluN2A (Cell Signaling) or anti-GluN3B (Alomone Labs) antibodies. All antibodies were produced in rabbit and were used at a dilution of 1:50 in PBST.

On day 2, biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) was pipetted onto the samples. Biotinylated antibodies were dissolved at 1:1000 in PBST and incubated for 2 hours at room temperature. Then, a second incubation followed overnight at 4°C with the second primary antibody. For HD cultures, this was either the anti-GluN2B, or the anti-GluN3B antibody. For pigment cells, the second primary antibody, depending on the experimental setup, was as follows: if the first primary antibody was anti-GluN3B, either the anti-GluN1 or the anti-GluN1-1a was used as the second primary antibody. If, however, the first primary antibody was anti-GluN2A, either anti-GluN1 or anti-GluN3B was used as the second primary antibody. All antibodies were produced in rabbit. The second primary antibodies were dissolved in PBST and used at a dilution of 1:50.

Finally, on day 3, following wash steps with PBS, biotinylated goat secondary antibodies were visualised with Streptavidin Alexa Fluor 488 fluorochrome conjugated antibodies (Life Technologies Corporation, Carlsbad, CA, USA), whereas the non-biotinylated, second primary antibodies were visualised with Alexa Fluor 555 fluorochrome conjugated antibodies (Life

Technologies). These fluorochrome-conjugated antibodies were used at a dilution of 1:1000 in PBST. Finally, DAPI-containing Vectashield mounting medium (Vector Laboratories, Peterborough, UK) was applied to the cultures. Immunocytochemical reactions were carried out in three biological replicates. We took fluorescent photomicrographs from 5-5 separate visual fields in each case.

An Olympus FV3000 confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan) was employed to take fluorescent photomicrographs on the reactions. A 60 × PlanApo N oil immersion objective (NA: 1.42) was used for imaging. The excitatory laser wavelengths were 488 and 555 nm. The optical thickness of the serial sections along the z-axis was 1 μm. Images were recorded using the FV31S-SW software (Olympus Corporation).

Determining secreted glutamate concentration

The concentration of glutamate released by chondrogenic cells into the culture medium was measured using the Glutamine/Glutamate Determination Kit (Sigma-Aldrich). On different days of chondrogenic differentiation, 4 × 200 μL culture medium was removed from the colonies and used for the measurements of secreted glutamate. Background values were established using blank samples (sterile water and Ham's F12 medium). Absorbance values were read at 340 nm using a microplate reader (Chameleon, Hidex, Turku, Finland). Measurements were carried out on 3 biological replicates with 4 technical replicates in each case on each day of chondrogenesis.

Pharmacological treatments and functional studies on chondrifying cell cultures

Functions that are possibly linked to NMDARs were studied using various pharmacological compounds. These included the artificial NMDAR agonist NMDA (20 μM; Sigma-Aldrich); glycine, which binds to GluN1 and GluN3 subunits as a coagonist (10 μM; Amresco); DCKA (5,7- dichlorokynurenic acid; 10 μM; Tocris Bioscience, Ellisville, MI, USA), a competitive antagonist of the glycine-binding sites of GluN1 subunits; ifenprodil (20 μM; Sigma-Aldrich), the inhibitor of GluN2B subunits; and strychnine (5 μM; Sigma-Aldrich), the specific antagonist of glycine receptors (GlyR). NMDA, DCKA, ifenprodil and strychnine were continuously applied to chondrogenic cultures from the day of cell isolation (day 0). Glycine treatments either started on day 1, or was only applied on culturing days 2 and 3 for 4-4 hours. To assess the metabolic activity (or viability) of cells, MTT assay was employed. During the assay, a yellow tetrazolium salt, MTT (VWR International, Debrecen, Hungary) was added to

the culture media (MTT stock solution: 5 mg MTT/1 mL of PBS). After that, cells were incubated with the reagent for 2 hours at 37°C. During that time, mitochondrial enzymes converted MTT into purple, insoluble formazan crystals. The crystals were dissolved using 500 µL MTT solubilising solution, which then enables determining the absorbance of the resulting purple solution. Absorbance values were read at 570 nm, and the OD values for each experimental group are shown as percentage changes compared to the control.

The proliferation rate of the cells was determined by measuring the amount of radioactively labelled (tritiated) thymidine (^{3}H-thymidine) into the DNA of dividing cells. For the proliferation assay, chondrogenic cells were seeded into scintillation plates (Wallac, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA). On day 3 or 10 of culturing, cells were given media containing 1 µCi/mL of ^{3}H-thymidine (Amersham Biosciences, Budapest, Hungary) after the treatments with NMDAR agonists or antagonists. Following 16 hours of incubation with ^{3}H-thymidine, proteins were precipitated with ice-cold 5% trichloroacetic acid (Acros Organics, Thermo Fisher, Waltham, MA, USA). Plates were air-dried for two weeks, and then incorporated radioactivity was determined using a microplate reader capable of detecting liquid scintillation (Chameleon, Hidex). The values of each experimental group are shown as percentage changes compared to the control.

Analysis of cartilage ECM production

Qualitative and quantitative ECM production assays were carried out on 6 or 10-day HD cultures (treatment *vs.* control groups) based on metachromatic staining. Some cultures were fixed with a 4:1 mixture of ethanol and formalin, and then stained with 1% dimethyl methyleneblue (DMMB; Aldrich, Germany) dissolved in 3% acetic acid. Photomicrographs of metachromatic cartilaginous nodules were taken using a Spot Advanced camera mounted onto a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan). Some other cultures were fixed with Kahle's fixative (28% ethanol, 4% formalin, 2% acetic acid), and then stained with 0.1% toluidine blue (TB; Reanal) dissolved in glycine–HCl puffer for 5 minutes. The amount of bound dye was determined by measuring the absorbance of the redissolved TB samples at 625 nm; the amount of chief ECM components is proportional to the OD values. The OD of samples were measured in 3 biological replicates, with 3 technical repeates in each case.

Measuring cytosolic Ca^{2+} levels and Ca^{2+} transients

Ca^{2+} transients were measured in Fura-2-loaded HD cultures on culturing days 1, 2 and 3. Fura-2-loaded cells were studied using an inverted fluorescent microscope (Diaphot, Nikon, Kowasaki, Japan). Test solutions (20 μM NMDA in Tyrode's), and Ca^{2+}-free Tyrode's were administered directly onto the cells. Spontaneous Ca^{2+} transients were analysed using an LSM 510 META laser scanning confocal microscope (Zeiss, Oberkochen, Germany) by line scan analysis. 2-day-old cultures were incubated for 30 minutes at 37°C with 10 μM Fluo-4-AM dissolved in F12 medium. Recordings were acquired in standard Tyrode's, and test solutions contained either NMDA or ifenprodil at 20 μM.

Statistical analysis

Data presented are representative of at least three biological replicates, showing the same trends. Values are mean ± standard error of the mean (SEM). For western blots, MTT and proliferation assays, as well as metachromatic cartilage ECM staining, statistical analyses were carried out using Student's unpaired two-tailed *t*-test, followed by Dunnett's test ($*P < 0.05$). The statistical distribution of the length of spontaneous Ca^{2+} events following NMDA treatment was analysed using χ^2 and Mann-Whitney tests.

Results

Analyses of NMDARs in chondrogenic cells

Differentiating chondrocytes express various NMDAR subunits

First, we set out to confirm the following transcripts coding for NMDAR subunits using reverse transcription followed by polymerase chain reaction (RT-PCR) in chicken high density chondrifying cell cultures: GluN1 (*GRIN1*), GluN2A (*GRIN2A*), GluN2B (*GRIN2B*), GluN2C (*GRIN2C*), GluN2D (*GRIN2D*), GluN3A (*GRIN3A*) and GluN3B (*GRIN3B*). Of these, the GluN1, GluN2A, GluN2B, GluN3A and GluN3B NMDAR subunits were detected on each culturing day of chondrogenic mesenchymal cell cultures; however, we could not confirm the expression of GluN2C and GluN2D subunits in the HD cultures.

The protein-level expression of those subunits which we identified at the mRNA level were confirmed using western blots. In total cell lysates, the expression level of the GluN1 subunit showed a steady decline during chondrogenesis, whereas the expression of GluN2A was lost from mature chondrocytes in this model. On the other hand, we detected the highest levels of GluN2B subunit protein expression on days 2 and 3 of culturing, when chondroblasts responsible for cartilage-specific ECM production first appear. GluN3A exhibited stronger chemiluminescent signals from the 3rd culturing day; and GluN3B showed steady expression levels throughout the entire culturing period.

In the membrane fractions of differentiating chondrocytes, we detected the following subunits: GluN1, GluN2B, GluN3A, and GluN3B. GluN1 expression followed a gradually increasing pattern; GluN2B and GluN3B subunits exhibited significantly higher levels of expression on culturing day 2. The GluN3A subunit could only be detected in the membrane fraction on day 6 of culturing, whereas we failed to detect GluN2A on any of the culturing days.

The localisation of subunits implicates functional channel formation on the key day of chondrogenesis

Since critically important processes take place on culturing day 3, we used these cultures for immunocytochemical stainings. We confirmed that the GluN1 subunit colocalises with both GluN2B and GluN3B subunits. Given that due to technical limitations with the antibodies employed, we were unable to perform triple labelling experiments; therefore, we used anti-GluN2B and anti-GluN3B antibodies in the next step. We concluded that the immunofluorescent signals of GluN2B colocalise with those of the GluN3B subunits.

Chondrogenic cells produce and secrete glutamate

Compared to cell-free media, the supernatant of HD cultures contained significantly higher levels of glutamate, which indicates spontaneous endogenous glutamate release. The secreted glutamate concentration in the media was in the realm of 0,2–1,1 nmol/mL, as determined by the colorimetric glutamine/glutamate assay. In relation to this, we also established that differentiating chondrocytes expressed both type 1 and 2 vesicular glutamate transporters (VGLUT1, VGLUT2) at the mRNA and protein levels.

Pharmacological modulation of NMDARs resulted in variable effects

When 20 μM NMDA was applied to chondrogenic cells from the 1st culturing day, we did not detect changes in metachromatic ECM production compared to the control on day 6. Since 20 μM NMDA alone was ineffective on chondrogenesis, we therefore also administered glycine (10 μM) from the 1st culturing day either alone or in combination with NMDA. Whilst glycine alone significantly enhanced metachromatic ECM production, combined treatment with glycine and NMDA did not change cartilage ECM production by day 6 compared to the control. To check whether glycine could activate other receptors in addition to/instead of NMDAR, 5 μM strychnine, an effective GlyR inhibitor, was added to the cells. Strychnine alone significantly reduced cartilage ECM production, but when applied together with glycine, it did not change the amount of metachromatic matrix production compared to the control. Therefore, this probably demonstrates the direct effect of glycine on NMDARs. When DCKA (10 μM), the competitive antagonist of the glycine binding site of the GluN1 subunit was applied from the 1st culturing day, a significant increase in metachromatic cartilage matrix production was detected. In contrast, ifenprodil at 20 μM almost completely blocked chondrogenesis.
Except for DCKA, none of the other compounds caused significant alterations in mitochondrial activity. With regards to proliferation rates, however, ifenprodil and glycine (either alone or in combination with NMDA or strychnine), as well as strychnine alone significantly reduced ^{3}H-thymidine incorporation; in contrast, DCKA significantly enhanced proliferation.

Local administration of NMDA evoked Ca^{2+} transients

Ca^{2+} transients were recorded on various days of culturing following local application of 20 μM NMDA. Whilst administration of 100 mM KCl resulted in depolarisation and a subsequent

rapid increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) on day 1 of culturing, NMDA did not elicit such transients. In contrast, starting from the second day of culturing, local application of NMDA resulted in a clear increase in $[Ca^{2+}]_c$ of differentiating chondrocytes. This response was characterised by a slow onset and was clearly dependent on the availability of extracellular Ca^{2+}, since depletion of extracellular Ca^{2+} ($[Ca^{2+}]_e$) resulted in $[Ca^{2+}]_c$ returning to the baseline levels, whereas restoring the physiological (1.8 mM) $[Ca^{2+}]_e$ once more triggered elevated $[Ca^{2+}]_c$. During this phenomenon, Fura-2 fluorescence levels did not show an aspecific increase. Therefore, local application of NMDA may have influenced ion channels other than NMDARs that mediate Ca^{2+} influx and are characterised by a slow activation and inactivation kinetics. The average amplitude of such $[Ca^{2+}]_c$ transients (Ca^{2+} peaks) elicited by NMDA treatments was 60 nM on day 2 and 3 of culturing. Whilst glycine is indispensable for the function of NMDARs, we failed to detect changes in $[Ca^{2+}]_c$ following local administration of 10 μM glycine on any culturing days.

To further investigate the link between NMDAR activation and spontaneous Ca^{2+} oscillations, we applied 20 μM NMDA and 20 μM ifenprodil during confocal $[Ca^{2+}]$ measurements. Application of NMDA has altered the length of the spontaneous Ca^{2+} events; the frequency of very short (0.5 s) events was diminished, whereas that of longer (1.5–2.5 s) events increased compared to the control (P=0.0002). This was very similar to what has been observed on single cell Ca^{2+} measurements. When NMDARs were blocked with ifenprodil, Ca^{2+} oscillations disappeared altogether, indicating a fundamental role of NMDARs in mediating the spontaneous Ca^{2+} events of chondrogenic cells.

Analyses of NMDARs in human melanocytes and melanoma cells

Pigment cells possess a complete NMDAR subunit transcript profile

The presence of the essential GluN1 (*GRIN1*) subunits, as well as GluN2 (*GRIN2*) and GluN3 (*GRIN3*) subunits and their isoforms were confirmed at the mRNA level in human melanocytes and various melanoma cell lines (A2058, HT169M1, HT199, M35/01, WM35) by RT-PCR.

The protein expressions of NMDAR subunits differ between melanocytes and melanoma cells

Protein expression analyses on total lysates of melanocyte cell cultures confirmed the presence of the GluN2A subunit; the protein levels of GluN1, GluN2B, GluN3A, and GluN3B subunits were below the detection threshold. Although reactions with the anti-GluN1 antibody suitable

for the detection of all known splice variants did not give chemiluminescent bands, we managed to detect signals for the GluN1-1a subunit containing an NLS. We confirmed the presence of GluN1, GluN2A, GluN3A, and GluN3B subunits at the protein level in the cytosolic and membrane fractions of melanoma cells. Furthermore, the NLS-containing GluN1-1a splice variant was also uniformly expressed. We also confirmed the nuclear expression of GluN1, GluN1-1a, and GluN3B subunits; however, there was a complete lack of GluN2A and GluN3A subunits from these fractions. We failed to detect signals for the GluN2B subunit in the subcellular compartments studied, which indicates a complete lack of this subunit, or extremely low protein levels.

NMDAR subunits display a nuclear colocalisation pattern in melanoma cells

Whilst we detected cytosolic expression for the GluN1 subunit in each melanoma cell line, the presence of GluN3B was less diffuse in that compartment. Nevertheless, the immunopositive signals for the GluN1 and GluN3B subunits displayed a clear nuclear colocalization in each melanoma cell line. Although we observed the cytoplasmic expression of GluN1 and GluN3B in melanocytes, their nuclear expression, unlike in melanoma cells, could not be confirmed.
Similar nuclear immunopositivity patterns were detected for the GluN1-1a–GluN3B immunocytochemical reactions: in contrast to GluN1, the NLS-containing GluN1 splice variant was hardly detectable in the cytosol, and its specific antibody gave signals almost exclusively in the nucleus, in colocalisation with the GluN3B subunit. In a similar way to GluN1, GluN1-1a was also found to be expressed in the cytoplasm of melanocytes, but in contrast to GluN1 and GluN3B, the anti-GluN1-1a antibody gave only weak immunopositive signals in the nucleus.
In case of A2058 and WM35 melanoma cell lines, the GluN1–GluN2A reaction showed a cytoplasmic and nuclear distribution of GluN1 which was similar to what we have observed earlier; however, for GluN2A, we observed a distinct pattern around the nucleus (with signals probably localised to the nuclear envelope). The involvement of the nuclear envelope was confirmed by merging the fluorescent images with photomicrographs taken using native transmitted light. Furthermore, whilst GluN2A showed a weak, diffuse cytosolic signal, it was completely absent within the nucleus. Melanocytes also showed the previously observed GluN1 expression pattern; however, GluN2A immunopositivity was less characteristic as in melanoma cells. In particular, GluN2A signals were diffuse and obviously weaker than in melanoma cells; furthermore, immunopositive signals in the nuclear envelope were also hardly visible.

In case of the GluN2A–GluN3B reactions carried out on A2058 and WM35 cells, we confirmed that signals for the GluN3B subunit were dispersed within the cytosol, and were strongly present within the inner parts of the nucleus; however, the immunopositive signals were completely lacking from a distinct nuclear subcompartment which likely corresponds to the nucleolus. When merged with the immunocytochemical results of GluN2A, as well as the native photomicrographs, the spot-like colocalisations of the two subunits were present not only in the nuclear envelope but also in the cytosol. In melanocytes, we observed GluN3B expression in some of the processes only, in addition to the previously detected, very weak cytosolic pattern. Therefore, when merged with the GluN2A fluorescent images, strong colocalization between the two subunits could not be demonstrated.

Discussion

The role of NMDARs in chondrogenic differentiation

NMDAR subunits are expressed in chondrogenic cultures

At the beginning of our experiments, we first analysed the NMDAR subunit expression profile and glutamate-producing capabilility of chondrogenic cells. We confirmed that differentiating chondrocytes expressed GluN1, GluN2B, GluN3A, and GluN3B NMDAR subunits in their membrane fraction; however, GluN2A subunit was only observed in total lysates. Furthermore, we confirmed colocalisations between GluN1–GluN2B, GluN1–GluN3B, and GluN2B–GluN3B subunits in 3-day-old chondrogenic cultures. These results indicate that in addition to diheterotetrameric complexes, triheterotetramers could also be present in differentiating chondrocytes during this critically important period.

We also documented that differentiating chondrocytes secrete glutamate into the medium, and that they also express VGLUT1/2, indicating that chondrogenic cells may possess the toolkit necessary for a cell-autonomous autocrine/paracrine glutamate signalling, possibly during *in vivo* conditions as well.

Pharmacological modulation of NMDARs influence chondrogenesis

Based on our previous results, *in vitro* chondrifying cell cultures are extremely sensitive to manipulation of the precisely set $[Ca^{2+}]_i$. Therefore, we looked at the chondrogenic effects of those NMDAR agonists and antagonists which are unequivocally involved in modulating $[Ca^{2+}]_i$. The applied compounds influenced the amount of metachromatic cartilage matrix production, cell proliferation, and mitochondrial activity.

NMDAR-mediated Ca^{2+} signals in chondrogenic cells

To assess the functionality of NMDARs, live cell Ca^{2+} measurements were carried out. In this model, Ca^{2+} channels directly or indirectly mediated by NMDA only showed a moderate sensitivity, and subsequently, their Ca^{2+} permeability was also rather low (the average amplitude of Ca^{2+} transients was approx. 60 nM). This was especially characteristic on culturing day 1, when NMDA treatment failed to evoke Ca^{2+} transients. In contrast, Ca^{2+} transients upon adenosin triphosphate (ATP) application were characterised by a rather high amplitude (approx. 180 nM) on culturing day 3 in the same model. In contrast to NMDA, glycine application failed

to elicit Ca^{2+} signals in cells of HD cultures. The unique features of GluN3 subunit-containing, probably trihetereotetrameric NMDARs (which are most likely present in chondrogenic cells) may play an important role during the commitment of chondrogenic cells to chondroblasts.

Chondrifying cell cultures are characterised by spontaneous, high-frequency $[Ca^{2+}]_c$ oscillations. The GluN2B specific antagonist ifenprodil almost completely abolished these Ca^{2+} oscillations, which indicates that NMDAR-mediated Ca^{2+} influx was necessary for the spontaneous Ca^{2+} events of chondrogenic cells.

We confirmed that NMDAR subunits expressed by differentiating chondrocytes assemble to form functional channels, which modulate their commitment, proliferation, and viability. These effects are probably attributable to NMDARs functioning as Ca^{2+}-permeable channels, thereby modulating Ca^{2+} oscillations in these cells. Differentiating chondrocytes may activate these channels via a cell-autonomous autocrine/paracrine mechanism.

Involvement of NMDARs in the tumour phenotype of human pigment cells

Healthy and pathological pigment cells are characterised by a different NMDAR subunit expression profile

Our experiments carried out on human pigment cells provide valuable data in terms of the NMDAR subunit expression pattern in cutaneous melanocytes. Moreover, we identified NMDARs with unique characteristics in the nuclei of melanoma cells. Our RT-PCR results revealed that all known NMDAR subunits were expressed at the mRNA level in NHEM and each melanoma cell line investigated. Western blots carried out on total lysates of melanocytes, however, only confirmed the presence of GluN1-1a and GluN2A subunits at the protein level. The analysis performed on the subcellular fractions of melanoma cells revealed that only the GluN1 and GluN3B subunits were universally expressed in all cellular compartment studied (GluN2A and GluN3A were found in the cytosolic and membrane fractions of melanoma cells only). We did not detect GluN2B at the protein level either in melanocytes or in melanoma cells.

NMDARs with unusual composition in the nuclei of melanoma cells

Immunocytochemical reactions revealed the colocalisation of the GluN1 and GluN3B subunits in the nuclei of melanoma cells; this implicates the formation of nuclear heteromers. We did not found evidence on the colocalisation of GluN1 or GluN1-1a subunits with GluN3B in the

nuclei of healthy pigment cells, and their immunofluorescent signals were generally quite weak. Taken all these together, our results regarding the nuclear localisation of NMDARs with a GluN1/1-1a–GluN3B subunit composition suggest a correlation with the malignant pigment cell phenotype.

The immunofluorescent signals of the anti-GluN2A antibody were predominantly localised around the nuclei of melanoma cells; compared to GluN1 and GluN3B, intranuclear signals were almost completely missing. The expression pattern of GluN2A therefore indicates that it localises into the nuclear envelope. It is also noteworthy that by merging the fluorescent images, the signals for GluN2A colocalised with those of GluN1 and GluN3B, which raises the possibility of triheterotetrameric NMDARs in the nuclear envelope. In contrast, significantly fewer and weaker GluN2A signals could be observed in melanocytes, which showed an extremely weak (or a complete lack of) colocalisation with GluN1 and GluN3B compared to what we observed in melanoma cells.

Non-excitable cells from outside of the nervous system in the framework of NMDARs and Ca^{2+} signalling

The results of my doctoral dissertation are intended to demonstrate the presence and potential functions of NMDAR, a classical receptor known from the central nervous system, in cell cultures *in vitro*, thus providing insight into the role of NMDARs in non-excitable cells from outside of the nervous system. NMDARs are non-specific cation channels mainly permeable to Ca^{2+}; therefore, they are primarily considered as Ca^{2+} channels. Ca^{2+} plays a universal role in cellular physiology, given that they are the most ancient and most widely used secondary messengers in signalling pathways. Several results suggest that most non-excitable cells express Ca^{2+} channels as members of the Ca^{2+} signalling toolkit, which provide a means for Ca^{2+} entry from the extracellular space to modulate and fine-tune their cellular processes.

Our results confirmed that NMDAR-mediated signalling plays a key role in the early stages of chondrogenesis, by modulating Ca^{2+} oscillations in differentiating mesenchymal cells. Our results confirm and complement the reports of other research groups on the role of NMDARs in the mechanotransduction pathways of adult chondrocytes, as well as their altered composition and function in inflammatory joint diseases. The fact that the specific composition of NMDARs changes according to the differentiation stage of the chondrogenic cells is one of our key observations in this model; we think that the different subunit composition confers

different properties to the Ca^{2+} currents, thus fine-tuning intracellular signalling pathways during differentiation.

In contrast, our observations regarding NMDARs in human pigment cells raise the possibility of the presence of nuclear Ca^{2+} signalling in melanoma cells which is linked to malignant transformations. Recent *in vitro* reports confirmed the effects of NMDAR (and iGluR) antagonists on carcinoma and melanoma cells. Therapies targeting glutamatergic signalling may therefore open new avenues in melanoma treatment.

The fact that NMDARs are involved in distinct processes in cartilage and pigment cells *in vitro* is rather intriguing. Whilst NMDARs appear in early undifferentiated mesenchymal cells and assist in chondrogenic differentiation, the expression of these receptors in pigment cells appears to be more intense in malignantly transformed cells. Therefore, it is plausible to assume that the heteromeric receptors may be associated with dedifferentiation during neoplastic transformation. Of course, the latter being a cause, or a consequence remains unanswered.

Novel findings

The most important novel results discussed in this thesis are as follows:

1) Chondroprogenitor cells express GluN1, GluN2B, and GluN3B NMDAR subunits on the critically important days of chondrogenic differentiation (days 2 and 3), which colocalise with each other.
2) More mature chondrocytes are characterised by a different assembly of NMDARs (GluN1, GluN3A), with lower expression levels.
3) Chondrogenic cells produce L-glutamate, the natural agonist of NMDARs.
4) Studies involving pharmacological modulators of NMDARs indicate their involvement in optimal cartilage differentiation and cell proliferation.
5) NMDA, the artificial agonist of NMDARs, modified the spontaneous Ca^{2+} oscillation pattern in differentiating chondrocytes, while the NMDAR antagonist ifenprodil abolished these Ca^{2+} events.
6) We were the first to describe the expression of at least one splice variant of the GluN1 subunit in normal human epidermal melanocytes.
7) The detailed analyses of NMDAR subunit protein expression profiles in melanoma cells, involving subcellular fractions, confirmed the cytosolic, membrane and nuclear presence of GluN1, GluN1-1a and GluN3B subunits.
8) The receptor subunits colocalize with each other in the nuclei of melanoma cells.

Summary

The experimental work presented in this thesis was based on studies involving *N*-methyl-D-aspartate type glutamate receptors in chondrogenic cells of chicken high density cultures, as well as in healthy and pathological pigment cells. Given that NMDARs primarily mediate Ca^{2+} currents, we focused on the changes of Ca^{2+} homeostasis during chondrogenesis and tumorigenesis.

Chondroprogenitor cells express GluN1, GluN2B, and GluN3B NMDAR subunits in the early stages of cartilage formation, indicating the presence of heteromeric receptors. L-glutamate secretion by the cells possibly correlates with the course of chondrogenesis. Studies involving pharmacological modulators of NMDARs indicate their involvement in regulating cellular functions of the differentiating chondrocytes. Our experiments proved that NMDARs may have direct and indirect roles in the precisely regulated intracellular Ca^{2+} oscillations of chondroprogenitor cells, and NMDAR-evoked Ca^{2+} signals are closely associated with optimal chondrogenic differentiation.

Normal human epidermal melanocytes have so far only been reported to express GluN2A subunits; we were the first to describe the expression of at least one splice variant of the pore-forming GluN1 subunit. The detailed analyses of NMDAR subunit protein expression profiles in melanoma cells, involving subcellular fractions, not only revealed major differences between healthy and malignant pigment cell phenotypes, but also implicated the putative role of the nucleus in Ca^{2+} dependent signalling processes in melanoma cells. The receptor subunits in the nuclei of melanoma cells, in case of potentially functional receptor formation, raise the possibility of NMDAR involvement in nuclear Ca^{2+} homeostasis, and through that in the neoplastic phenomenon.

We demonstrated *in vitro*, for the first time, in non-excitable cells from outside the nervous system the presence of functional NMDARs (in differentiating cells of chicken limb bud-derived high density cultures), and the nuclear localisation of heteromeric NMDARs (in melanoma cells). The former mediate Ca^{2+} dependent pathways that are indispensable to chondrogenesis, while the latter may have appeared as a result of malignant transformation and might mediate novel aspects of Ca^{2+} signalling.

List of publications

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