Investigation of the signaling events of proteasome inhibitor (MG-132)-induced neurite outgrowth and apoptosis in rat pheochromocytoma (PC12) cells

PhD thesis

Oktávia Tarjányi M.D.

Doctoral school leader: Ferenc Gallyas, Ph.D., D.Sc.

Supervisor and program leader: György Sétáló Jr., M.D., Ph.D.

University of Pécs

Medical School

Department of Medical Biology and Central Electron Microscopic Laboratory

Pécs

2023

Summary

Due to its neuroendocrine origin, the PC12 (rat pheochromocytoma) cell line is a widespread model system for studying neuronal differentiation, survival and apoptosis in cell culture. This cell line is also used for modeling Parkinson's disease or stroke, as well as investigating neurosecretion and neurotoxicity in vitro. These tumor cells originated from the medulla of rat adrenal gland are round-shaped and divide rapidly. After a few days of nerve growth factor (NGF) treatment, however, they stop to proliferate, become polygonal, grow projections and become similar to sympathetic neurons. This process is called the neuronal differentiation of PC12 cells, behind which there are complex signaling changes lying.

The binding of NGF to its high-affinity receptor, TrkA (tropomyosin receptor kinase A), is followed by receptor dimerization, auto-/transphosphorylation, and activation of a monomeric G-protein, Ras (origin of its name: rat sarcoma). Activated Ras initiates a cytoplasmic mitogen-activated protein kinase (MAPK) cascade, consisting of Raf (rapidly accelerated fibrosarcoma, a MAPKKK), MEK (MAPK/ERK kinase, a MAPKK), and ERK1/2 (hereafter referred to as ERK = extracellular signal-regulated kinase, a MAPK). Prolonged phosphorylation and nuclear translocation of ERK are prerequisites for the neuronal differentiation of PC12 cells. During this process, the activation of the Src (origin of its name: sarcoma) tyrosine kinase can also be observed, the level of which is also influenced by the degree of proteasomal degradation of the enzyme. At the same time inhibition of proteasome function can also induce neurite outgrowth in PC12 cells. In our experiments, we used the peptidyl-aldehyde type MG-132 proteasome inhibitor compound to achieve the latter.

Based on our own observations, applying MG-132 at a concentration of 2.5 μM elicits a biphasic response in PC12 cells. First, it initiates the process of neuritogenesis, however, after 24 hours, the morphological changes in the cells increasingly exhibit signs of programmed cell death. Accordingly, our experiments aimed to map the signaling changes occurring first during proteasome inhibitor-induced neuritogenesis and then during the apoptosis of nerve cells. To achieve this, we investigated the TrkA-Ras-ERK pathway, which plays a role in the neuronal differentiation of PC12 cells, and the possible role of Src tyrosine kinase in it, and the activation of several signaling proteins involved in cell survival and apoptosis during proteasome inhibitor treatments.

Based on our results, MG-132 induces TrkA phosphorylation in the first phase of the treatment (in its early stages). This is followed by prolonged ERK activation via the Ras, Raf, MEK pathway, which was considered as a required precondition for neuronal differentiation. In these processes the stabilization of active Src plays an important role, which was achieved by inhibiting its proteasomal degradation. Namely as a result of pretreatment with the Src inhibitor (PP2), the TrkA phosphorylation induced by the proteasome inhibitor failed, the ERK activation turned out to be decreased and ended up earlier, the nuclear translocation of the active ERK was reduced, and the consequent neurite outgrowth was not observed.

At the molecular level, in addition to the activation of ERK and other changes that trigger differentiation, the slow and prolonged activation of Akt and the stress response mediating MAPKs - p38MAPK (hereafter p38) and JNK (c-Jun N-terminal kinase) - as well as the substrate of the latter, c-Jun (ju-nana, 17 in Japanese) can also be observed.

After 24 hours (in the late stage of MG-132 treatment), the phosphorylation of Akt and ERK - which mediate survival - decreased, while stress signaling pathways mediated by p38 and JNK remained markedly active. In addition, activating cleavage of caspase-3 was also detectable at this time, which is an unequivocal sign of apoptosis.

In conclusion, we can declare that proteasome inhibitor treatment with MG-132 induces a biphasic response in PC12 cells. Initially, the TrkA-Ras-ERK pathway is activated, which induces neuritogenesis. For this process an increase in Src activation is essential. After 24 hours, however, the signaling events shift towards the inactivation of survival pathways (Akt and ERK) and the activation of stress pathways (p38 and JNK), which changes - together with the consequent caspase-3 cleavage - promote apoptosis of PC12 cells.

1. Introduction

1. 1. Model system of neuronal differentiation and apoptosis

In recent years and decades, research investigating the processes of neuronal apoptosis, -survival and -differentiation has been increasingly intensive. They contribute to the understanding of neurodegenerative diseases and other conditions associated with neuronal cell death, or even to the development of new therapeutic approaches.

The role of proteasomes, as complexes of regulated protein degradation within the cell, has also emerged in these processes. In the background of various neurodegenerative diseases, the reduced function of proteasomes has been described, which can contribute to the destruction of neurons by the accumulation of unfolded or misfolded proteins within the nerve cells. The successful therapeutic use of the first proteasome inhibitors in multiple myeloma also revealed that one of the serious side effects of these drugs is peripheral neuropathy. At the same time, the fact that some proteasome inhibitors can induce neurite outgrowth has been known for a long time. A study based on a microarray analysis revealed that proteasome inhibition with lactacystin can activate both neuroprotective and pro-apoptotic pathways.

These observations largely correlate with the biphasic morphological changes of PC12 cells treated with MG-132 in our experiments, during which we experienced first the phenomena of neuritogenesis and later those of apoptosis. During our work, we investigated the molecular mechanisms behind these processes. For our experiments, we used cultures of PC12 cells, on the surface of which the TrkA receptor is clearly expressed, which is the high-affinity cell surface binding site of the polypeptide growth factor, NGF. Thanks to this 140 kDa protein, this cell type is a popular model system to study NGF signaling and neuronal differentiation. As a result of several days long NGF treatment, the small round shaped cells stop to divide, grow in size, develop long projections and both their appearance and biological behavior become similar to that of sympathetic neurons. In addition to NGF, other polypeptide growth factors and even other effects (e.g. MG-132 treatment) can trigger the neuronal differentiation of these cells.

In addition PC12 cells are also used for *in vitro* modeling of neuronal apoptosis occurring during embryonic development or induced by neurotoxins or other agents.

1. 2. Mitogen-activated protein kinases (MAPKs)

MAPK cascades regulate the basic processes of cells by integrating and processing intra- and extracellular signals (e.g. mitogenic or stress effects), which can trigger proliferation, differentiation, cell survival or even apoptosis. The response to signals depends on the type of cell, the type and intensity of the signal, the composition of the participating signaling pathways and the dynamics of their activation, as well as the dialogue between them.

MAP-Kinases include extracellular signal-regulated kinases (ERK1/2), p38MAPKs (p38α-δ), and c-Jun N-terminal kinases (JNK1-3). In all three cases, their activation is achieved through the successive phosphorylation of the members of a cascade consisting of kinases. The proximal member of the cascade is a MAPKKK, which is Raf in the case of ERK, and ASK (apoptosis signal-regulating kinase) or MLK (mixed-lineage kinase) in the case of p38 and JNK. The next member of the cascade is MAPKK, which phosphorylates the last members of the cascade, the MAPKs. ERK can be phosphorylated and activated by MEK, p38 by MAPK kinase 3 and 6 (MKK3 and MKK6), and JNK by MAPK kinase 4 and 7 (MKK4 and MKK7). There are also data in the literature on the activation of p38 by MKK4.

1. 3. Signaling of neuronal differentiation (neuritogenesis)

Signal transduction changes in neuronal differentiation can be well investigated during long-term NGF treatment of PC12 cells. Due to the polypeptide nature of the nerve growth factor, it can not pass through the cell membrane, it exerts its effect by binding to its cell surface receptor (TrkA). This results first in receptor dimerization, followed by receptor auto-/transphosphorylation. Cytoplasmic proteins with SH2 (Src homology 2) domains bind to the emerging phosphotyrosine side chains on the surface of the receptor, which afterwards can be phosphorylated by the receptor on their tyrosine amino acid side chains. Such SH2 domain-containing proteins are, for example, Src, PLC-γ (phospholipase C-γ), PI3K (phosphatidylinositol 3-kinase), GAP (GTPase activator protein), and various adapter molecules (e.g. Shc = Src homologous and collagen- like and Grb2 = growth factor receptor-bound protein 2) **(Figure 1)**, the latter of which can bind to additional proteins rich in proline amino acid through their SH3 (Src homology 3) effector domain. An example of this is GEF (guanine nucleotide exchange factor, e.g.: Sos = Son of sevenless). GEF can activate Ras, a monomeric G protein linked to the plasma membrane, by promoting its transformation from the GDP-bound to the GTP-bound form. In order to inactivate Ras at the right time, a GTPase-activating protein (GAP) is required, due to which Ras's own GTPase activity increases and hydrolyzes the bound GTP into GDP. Before its inactivation, Ras initiates a MAPK cascade consisting of protein kinases, whose members pass the signal on by phosphorylating each other. Ras activates Raf, which in turn activates MEK. MEK has dual-specificity protein kinase activity, through which it can phosphorylate its target molecules on threonine and tyrosine side chains. Consequently, as one of the last cytoplasmic members of the cascade, ERK is also phosphorylated. Properly activated ERK enters the cell nucleus, where it regulates the process of gene expression by phosphorylating transcription factors **(Figure 1)**. As a result, cell cycle arrest and neuronal differentiation can be observed in PC12 cells.

In addition to the activation of the ERK pathway, the high-affinity NGF receptor can initiate several other signaling processes **(Figure 1)**. Next to the ERK cascade, the PLC-γ

enzyme is also involved in differentiation, which - by creating two secondary messengers - initiates two parallel signaling pathways. Although the specific role played by PLC-γ in differentiation is not yet fully clarified, it has been proven that it functions independently of Ras.

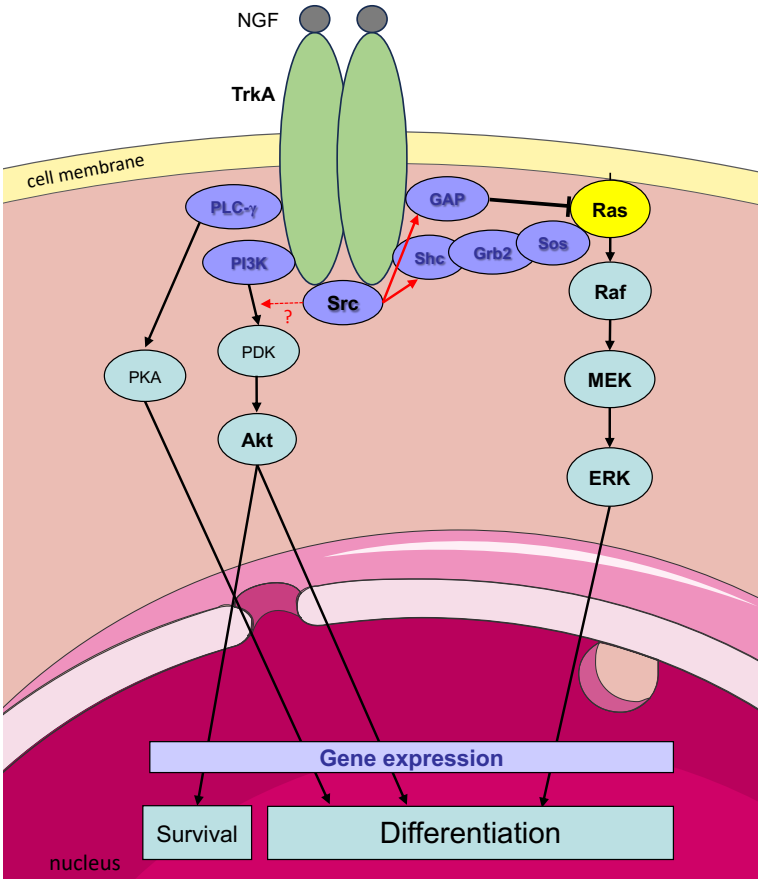

Figure 1. *Signal transduction of NGF-induced neuronal differentiation*

Explanation in the text. Cellular organelles in the figure were made using Servier Medical Art, which is provided by Servier under a "Creative Commons Attribution 3.0 unported license".

Src also plays an important role in NGF-induced neuronal differentiation. The potential importance of Src tyrosine kinase in NGF signaling was previously hypothesized due to the ability of the viral *src* oncogene (*v-src*) to induce neurite outgrowth similar to NGF. Complete Src activation requires activating phosphorylation in the catalytic domain (Tyr 416) and simultaneous removal of the inactivating phosphate group at the C-terminal end of the protein (Tyr 527). Inactivation of Src can occur through several pathways. Inhibitory phosphorylation on the tyrosine side chain at the C-terminus of Src is catalyzed by a specific kinase, Csk (C-terminal Src kinase). However, active Src can also be degraded via the ubiquitin-proteasome pathway. During our work, we investigated the role of Src protein in the signaling processes of PC12 cells by inhibiting this inactivation pathway.

The PI3K pathway primarily mediates cell survival through the activation of Akt. PI3K stimulates the formation of multiple phosphorylated lipid derivatives. These promote cell survival through PDK (3-phosphoinositide-dependent kinase), leading to increased Akt phosphorylation and activity. It is suggested that Src may also play a role in NGF-induced PI3K-dependent neuritogenesis **(Figure 1)**.

1. 4. Signaling of apoptosis

Apoptosis, which essentially means "suicide" of cells, is one of the most extensively studied forms of programmed cell death. This process also occurs physiologically in the human body during the maintenance of the homeostasis of cells and tissues, embryonic development, regulation of the immune response, or aging. If apoptosis is not adequate, it can contribute to the development of diseases. Increased apoptosis can be observed in neurodegenerative-, ischemic- or toxin-induced diseases, or for example during the development of AIDS, while an insufficient level of apoptosis can lead to the development of tumors, autoimmune diseases or viral infections.

Throughout apoptosis, the cell membrane remains intact, and the cell dies without damaging the surrounding tissues or causing inflammation. During the process of programmed cell death, cells undergo numerous morphological and functional changes, such as detachment of apoptotic bodies, condensation of chromatin, and internucleosomal cleavage of DNA by endonucleases, which is followed by the fragmentation of the cell nucleus. An important change in the membrane of these cells is the externalization of phosphatidylserine, which subsequently serves as the so-called "eat me signal" for the surrounding phagocytes, and can easily be detected in laboratory conditions with Annexin V labeling.

In addition, important signaling changes also occur during apoptosis, such as prolonged activation of stress signaling pathways, or proteolytic cleavage of caspase-3, which leads to the activation of the enzyme. Caspase-3 is known as an important, effector protease of apoptosis, because the proteolytic cleavages catalyzed by it lead to the above-mentioned changes characteristic of apoptosis. The enzyme can be activated via 3 main pathways: (1) the extrinsic apoptotic pathway, which starts from cell surface death receptors, (2) the intrinsic or mitochondrial pathway, and (3) the apoptotic pathway induced by endoplasmic reticulum (ER) stress.

1. 4. 1. Extrinsic pathway

During the activation of the extrinsic pathway **(Figure 2, part 1)**, the death ligands mostly secreted by immune cells (e.g. TNF-α = Tumor necrosis factor α, Fas-ligand, TRAIL = TNF-related apoptosis-inducing ligand) connect to the death receptors. TNF-receptor 1 and 2 (TNFR1/2) can bind TNF-α, Fas binds Fas-ligand (FasL), and death receptor-4 and 5 (DR4, DR5) can bind TRAIL as a ligand. Subsequently oligomerization of death receptors occurs, which is accompanied by the binding of adapter proteins (TRADD = TNFR-associated death domain protein or FADD = Fas-associated death domain protein) and the initiator procaspases (procaspase-8 and -10). This creates a signaling complex (DISC = death-inducing signaling complex), in which the proteolytic activation of procaspases takes place with the subsequent activation of the effector procaspase-3, which leads to the aforementioned changes of apoptosis.

1. 4. 2. Intrinsic pathway

The activation of the intrinsic pathway of apoptosis **(Figure 2, part 2)** leads to the permeabilization of the outer membrane of the mitochondria (MOMP = mitochondrial outer membrane permeabilization), which is why this pathway is also called the mitochondrial

pathway. In this case, the underlying cause is inside the cell, such as DNA damage, which activates the tumor suppressor protein p53. Acting as a transcription factor, p53 affects the expression of various genes encoding proteins belonging to the Bcl-2 (B-cell lymphoma-2) family, which play an important role in the regulation of the intrinsic pathway. The Bcl-2 family includes both pro- and anti-apoptotic proteins. The lifetime of the cell depends on the balance of these pro-apoptotic and anti-apoptotic Bcl-2 proteins. Imbalance of these proteins can lead to the development of diseases (e.g. tumors, autoimmune diseases, neurodegenerative disorders).

Members of the Bcl-2 family contain one or more Bcl-2 homology (BH) domains. The anti-apoptotic Bcl-2 family members have 4 BH-domains (BH1-4) and block apoptosis by binding to their pro-apoptotic counterparts and inhibiting their activity. Their best-known representatives are Bcl-2, from which the family itself was named, and Bcl-xL (Bcl-extralarge). Pro-apoptotic Bcl-2 family members can be divided into two groups based on their BH domains. There are pro-apoptotic multi-domain proteins (Bax = Bcl-2-associated X protein, Bak = Bcl-2 homologous antagonist/killer), which have BH1, 2 and 3 domains and are directly involved in causing MOMP. On the other hand, there are pro -apoptotic "BH3-only" proteins, which contain BH3 domain alone and contribute to the function of multi-domain proteins. "BH3-only" proteins can be further divided into activators (Bim = Bcl-2 interacting mediator of cell death, Bid = BH3 interacting-domain death agonist, Puma = p53 upregulated modulator of apoptosis) and sensitizers (Bad = Bcl-2 associated agonist of cell death, Bik = Bcl-2-interacting killer, Noxa). The activators are able to bind directly to the effectors Bax and Bak, which causes their conformation change and oligomerization. These protein complexes then create pores in the outer membrane of the mitochondria. The sensitizers contribute to the process of apoptosis by inhibiting the anti-apoptotic family members involved in the sequestration of Bax and Bak.

The permeabilization of the outer membrane of the mitochondria leads to the release of cytochrome c (among other molecules), which - together with Apaf-1 (apoptotic protease activating factor-1) and procaspase-9 - participates in the assembly of the apoptosomes. Caspase-9, which is activated in the complex, cleaves and activates the effector caspase-3, -6 and -7, which leads to apoptosis within a few minutes.

1. 4. 3. Apoptosis induced by ER stress

ER stress is caused by a malfunction of the endoplasmic reticulum and is primarily characterized by the accumulation of unfolded or misfolded proteins. This condition triggers a so-called "unfolded protein response" (UPR) within the cell to eliminate the problem. At the same time, chronic or unsolved ER stress causes apoptosis of the cell **(Figure 2, part 3)**. The UPR induced by ER stress - which is activated by the imbalance between "unfolded" proteins and chaperones - takes place via three known signaling pathways. First, the pathway mediated by PERK (protein kinase RNA (PKR)-like ER kinase) kinase is activated, which above all inhibits further protein synthesis. This is followed by the activation of the transcription factor ATF6 (activating transcription factor-6) by protease cleavage, which primarily increases the expression of ER chaperone proteins. At last the combined nuclease and kinase IRE1 (inositol-requiring protein-1) is activated, followed by the synthesis of additional chaperones and PERK inhibitors, and initiating protein degradation. The purpose of these processes is to restore the physiological ER function by preventing the synthesis of additional proteins, to promote the folding of proteins that have already been completed, and to degrade the formed protein

aggregates. Great importance is attributed to the latter - in which process the function of the proteasomes is essential - in the development of neurodegenerative diseases.

If the damage is so excessive that it is not possible to restore the proper ER function, the process of apoptosis is started. ER stress induces the down-regulation of the anti-apoptotic Bcl-2, the up-regulation of the pro-apoptotic Bim and Puma, and the activation of Bax and Bim. These changes all contribute to the process of apoptosis. As a result of ER stress, sustained activation of JNK can also be observed. IRE1 can also activate ASK1, which triggers both the JNK and p38 pathways. Activated JNK contributes to apoptosis by the inactivating phosphorylation of Bcl-2 and the activating phosphorylation of Bim. At the same time, p38 activates a pro-apoptotic transcription factor, which increases the expression of genes (e.g. DR5) that contribute to cell death. In addition to these effects, both stress kinases are able to activate p53, which triggers the intrinsic pathway of apoptosis as well.

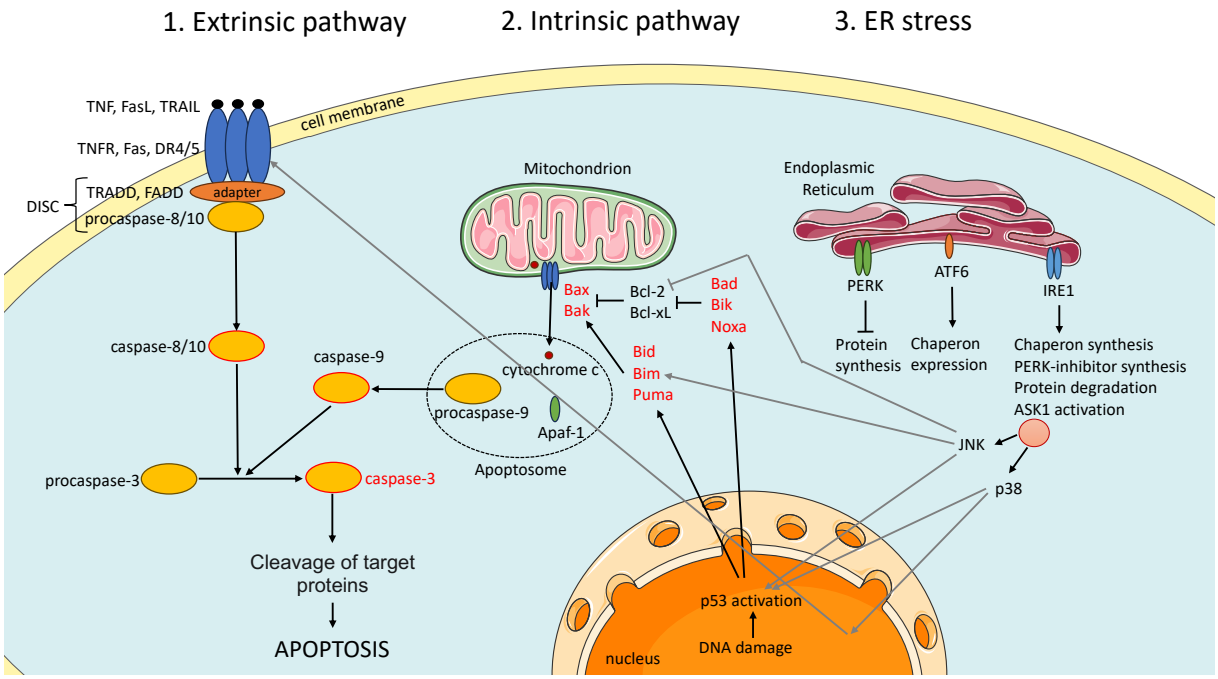

Figure 2. *Signaling of apoptosis*

Explanation in the text. Cellular organelles in the figure were made using Servier Medical Art, which is provided by Servier under a "Creative Commons Attribution 3.0 unported license".

1. 5. The ubiquitin-proteasome system (UPS)

The ubiquitin-proteasome system provides a solution to the precisely controlled breakdown of proteins inside the cells that are no longer needed, misfolded, or damaged in some way. The importance of the system is also indicated by the fact that approximately 80% of intracellular proteins are degraded this mechanism. Poly-ubiquitinated proteins are recognized by the 26S proteasome complex, which degrades them into peptides by its proteolytic activity. During the function of the UPS **(Figure 3)**, as a first step, ubiquitin

molecules are attached to the target proteins by covalent bonds. Ubiquitin is a highly conserved protein containing 76 amino acids, which is linked to the lysine side chains of the protein to be degraded by an enzyme system consisting of 3 enzymes. The members of the enzyme system performing poly-ubiquitination are the ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligase (E3) enzymes.

Proteasomes are large multienzyme complexes located in the nucleus and cytoplasm of cells. The proteolytic core of the complex is the catalytic part known as the 20 S proteasome, with multiple peptidase activities, which creates a cylindrical structure arranged in 4 heptamer rings (α7, β7, β7, α7). The α-subunits form the two outer rings and the β-subunits form the two inner rings. The delivery of the substrate to the active part of the complex is facilitated by the α-subunits, which allow only ubiquitinated polypeptides that have lost their 3D structure to pass through to the catalytic part of the proteasome. The proteolytic activity is bound to the β-subunits. β1subunits have caspase-like, β2s have trypsin-like, while β5 subunits have chymotrypsin-like activity. A so-called 19S cap structure is attached to one or both sides of the 20S central catalytic part, which recognizes the poly-ubiquitin signal and unfolds the proteins before they are transmitted to the catalytic part. Ubiquitin molecules are detached from the proteins to be degraded by deubiquitinating (DUB) enzymes, and the released ubiquitin can be bound to other proteins and thus reused **(Figure 3)**.

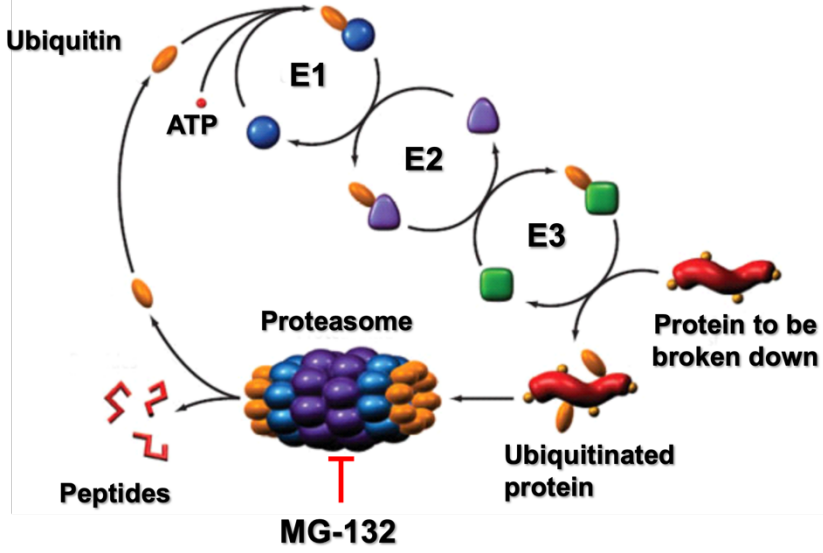

Figure 3. *The function of the ubiquitin-proteasome system*

An enzyme system consisting of 3 enzymes (E1, E2 and E3) uses ATP to attach ubiquitin molecules to the protein to be degraded. Poly-ubiquitinated proteins are broken down into peptides by the proteasome, while the released ubiquitin molecules can be reused by the system. MG-132 can prevent the specific degradation of certain proteins by inhibiting the proteolytic function of the proteasome.

The ubiquitin-proteasome system plays an important role in the degradation of proteins that regulate the cell cycle, proliferation, differentiation, apoptosis and even inflammation, thus influencing the activation state of various signaling pathways. In addition, it also destroys abnormal proteins created by e.g. oxidative stress or mutation, thus preventing the destruction of the homeostasis of the cell. The inadequate function of the UPS can be detected in the background of various diseases, such as tumors, neurodegenerative disorders, or autoimmune/inflammatory diseases.

2. Objectives

Our work was originally inspired by a study published by Hashimoto et al in 2000, in which treatments with a peptidyl-aldehyde type proteasome inhibitor (N-acetyl-Leu-Leu-norleucinal) and lactacystin induced neuronal differentiation of PC12 cells. We searched for the answer to the question, whether the use of another peptidyl-aldehyde type proteasome inhibitor, MG-132, can also induce the neuronal differentiation of PC12 cells and what signaling changes can be found behind this process? During our experiments, however, we found that MG-132 elicited a biphasic response in this cell line. After the initial neuritogenesis, signs of apoptosis dominated during long-term treatments (longer than 24 hours), so we decided to examine the two processes separately.

The previous work of our group has already revealed that MG-132 treatment triggers sustained ERK activation and the nuclear translocation of active ERK (see the PhD thesis of Gergely Berta M.D., University of Pécs, Medical School, 2013/14) , necessary for the neuronal differentiation of PC12 cells.

In the first part of this work, we wanted to investigate during the MG-132-induced neuritogenesis of PC12 cells:

- the role of Src tyrosine kinase,
- the mechanism of the already known ERK activation (the role of MEK and Ras proteins in ERK activation),
- the relationship between the activation of Src and ERK signaling proteins,
- the role of the high-affinity NGF receptor, TrkA, in this process,
- the intracellular localization of active, properly phosphorylated p-Src and p-ERK (with regard to the distribution of p-ERK following Src inhibition),
- the morphology of the cells in the absence and presence of Src inhibitor and
- based on these, we wanted to map the signaling pathways that play a prominent role in the process of neuritogenesis.

In the second part of our work, we wanted to investigate during the apoptosis of PC12 cells induced by long-term MG-132 treatment:

- the rate of apoptosis using different methods,
- the kinetics of activation of some signaling molecules involved in cell survival (Akt) and apoptosis (p38, JNK, c-Jun and caspase-3),
- the intracellular localization of p-p38, p-JNK and p-c-Jun,
- the changes of Akt, p38, JNK and c-Jun activation in the presence of various kinase inhibitors (LY294002, SB203580, SP600125) and in the case of Ras inhibition (M-M17-26 cell line), and
- based on these, we tried to map a signaling network between the studied molecules.

3. Methods

The activation state of the signaling molecules was investigated by Western blot analysis, the intracellular localization of the active proteins was examined by a laser scanning confocal fluorescence microscope, while the morphological changes of the cells were analysed using the aforementioned microscope in phase contrast mode. To determine the percentage of apoptotic cells, in addition to examining the nuclear morphology, flow cytometry and cell viability tests were performed.

4. Results

4. 1. Morphological changes of PC12 cells during long-term MG-132 treatment

Using a long-term proteasome inhibitor treatment with MG-132, the time-dependent changes in the morphology of PC12 cells were examined with a phase contrast microscope. Based on the observed changes in cellular appearance, we concluded that MG-132 treatment induces a biphasic response in PC12 cells. Experiencing these changes, our interest turned to the possible underlying mechanisms within the cells. Thus, we first examined the signaling events underlying the initial neurite outgrowth (related results in the "A. NEURITOGENESIS" section) and then the subsequent cell death (related results in the "B. APOPTOSIS" section) separately.

A. NEURITOGENESIS

4. 2. Kinetics of ERK and Src activation occurring as a result of MG-132 treatment

Since the activity of ERK and Src kinases plays an important role in the neuronal differentiation of PC12 cells induced by NGF, we first wondered whether the activation of these molecules can also be observed in connection with neurite outgrowth caused by MG-132 treatment. Our research group has described it before (see the PhD thesis of Gergely Berta M.D., University of Pécs, Medical School, 2013/14), but in order to understand the phenomena discussed here, it is necessary to mention that MG-132 treatment induces prolonged ERK activation with maximal signal intensity experienced at 6 hours of treatment and the subsequent nuclear translocation of the phosphorylated ERK in PC12 cells. These signaling events are required for the neuronal differentiation of PC12 cells.

The key role of the enzyme Src is also known from the literature in the differentiation of this cell line. On the other hand the mechanism of proteasomal degradation has also been described in the regulation of this tyrosine kinase. Therefore, in the next step, we investigated the time kinetics of Src activation in response to MG-132 treatment in this cell type. Strong Src activity was detectable between 30 min and 9 h during the treatments, and Src phosphorylation remained above the baseline even after 24 h.

Therefore, we detected an increased Src activity in PC12 cells treated with MG-132, which showed a prolonged kinetics, similar to that of ERK.

4. 3. MG-132-induced ERK phosphorylation is a MEK- and Ras-dependent process

Since the activation of ERK as a result of NGF treatment during the neuronal differentiation of PC12 cells occurs through the Ras, Raf, MEK pathway, we were interested whether the functioning of this pathway is also necessary for the ERK activation induced by MG-132.

To determine this, we investigated the role of MEK by pretreatment with a chemical inhibitor of MEK (PD98059), and the function of Ras by using the M-M17-26 PC12 clone, which stably expresses the dominant inhibitory mutant version of H-Ras.

When using a MEK inhibitor compound at a concentration of 20 μM 30 minutes before the proteasome inhibitor treatment, the ERK activation induced by MG-132 failed. The strength of MEK inhibition is shown by the fact that the phospho-ERK signals were below baseline in the presence of PD98059 in both MG-132 treated and untreated samples.

To investigate the function of Ras, time-kinetics experiments were performed using the M-M17-26 cell line. The basal ERK phosphorylation level was initially weaker in this cell line than in the control PC12 cells, and could not be increased at any MG-132 treatment time point.

Based on this, we can conclude that the phosphorylation of ERK is MEK- and Ras-dependent in the MG-132 proteasome inhibitor-treated PC12 cells, as well.

4. 4. The effect of Src inhibition on Src- and ERK phosphorylation

To further investigate the role of Src, we used 10 μM PP2, which is a potent and selective inhibitor of the Src family of tyrosine kinases. PP2 pretreatments always started 30 minutes before the proteasome inhibitor treatments. Experiments were carried out for durations that induced maximal Src phosphorylation in preceding time-course experiments. The Src inhibitor could completely block the stimulating effect of MG-132 on Src phosphorylation.

After making sure that the amount of active Src in the presence of PP2 does not exceed the baseline even after MG-132 treatment, we wanted to investigate the role of Src in the MG-132 induced ERK activation. The presence of PP2 did not completely inhibit the increase in ERK phosphorylation, but reduced its peak intensities elicited by MG-132. Moreover, unlike without Src inhibition, at 24 hours the phosphorylated ERK signal was already below baseline, thus ERK activation has become somewhat more transient in the presence of PP2.

In the presence of the Src-inhibitor compound, the reduced phospho-ERK peak intensities achieved with MG-132 and the more transient nature of ERK phosphorylation also show that ERK activation -at least partially- depends on the intact function of Src.

4. 5. Phosphorylation of TrkA induced by MG-132 and its inhibition by Src inhibitor

In order to investigate the possible relationship between Src and ERK activation in our experimental system, we decided to test the role of the high-affinity NGF receptor (TrkA) in the signaling events induced by MG-132.

MG-132-induced activation of TrkA is a possible starting point for the signal that subsequently activates MEK and ERK via Ras. After Src inhibition with PP2 started 30 minutes before MG-132 treatments, the proteasome inhibitor was no longer able to increase TrkA phosphorylation above basal activity.

The fact that TrkA phosphorylation can be induced in PC12 cells treated with MG-132, but it is prevented by chemical inhibition of Src, supports that Src activity is required for proteasome inhibitor-induced TrkA phosphorylation.

4. 6. Investigation of Src, TrkA and ERK activity in TrkA-inhibited PC12 cells

In order to further investigate the relationship between TrkA and Src during their activation by MG-132, PC12 cells were incubated with the TrkA inhibitor compound, K252a. K252a was added 30 min before the start of the proteasome inhibitor treatment, and it was present in the culturing medium for the entire duration of the experiment. K252a could not prevent MG-132-induced Src phosphorylation, but inhibited TrkA phosphorylation. In the presence of K252a, ERK activation was measurable, but its intensity was lower compared to samples not treated with the TrkA inhibitor. Moreover, the extent of it was comparable to the ERK activation intensities previously measured in the presence of PP2 Src inhibitor.

These data, collectively, suggest that Src is positioned upstream of TrkA during the MG-132-induced activation of the ERK cascade in PC12 cells. They are also supportive of the existence of ERK-activating mechanisms that are independent of pathways originating from TrkA and are mediated by other signaling component(s) stimulated by MG- 132 upstream of Ras and MEK.

4. 7. The intracellular localization of phosphorylated Src and -ERK and the phenotypic changes of PC12 cells

Since the activation of Src and ERK proved to be crucial in the neuronal differentiation of PC12 cells induced by the proteasome inhibitor, as a next step we were interested in the intracellular distribution of the active forms of these proteins and the morphological changes of the cells after MG-132 and/or PP2 treatments.

The intracellular distribution of phosphorylated Src and -ERK was detected by laser scanning confocal microscopy. In the cells of untreated cultures, the phosphorylated Src signal was barely detectable in the cytoplasm of the cells. Between 30 min and 9 h after maximal stimulation with a proteasome inhibitor alone, the intensity of the signal increased, but remained predominantly cytoplasmic. Without treatment, the weak p-ERK signal was mostly cytoplasmic. As a result of incubation with MG-132, the intensity of the p-ERK signal increased and reached its maximum after 6 hours, being detectable in the cytoplasm and in the nucleus, as well.

Inhibition of Src by PP2 alone had no effect on the intracellular distribution and level of the p-ERK signal in PC12 cultures, but attenuated the maximal signal intensity induced by MG-132 treatments. p-ERK immunoreactivity remained predominantly in the cytoplasm of Src-inhibited cells even after 6 h of MG-132 treatment.

Examining the phenotypic changes of the cells, we got the following results: without treatment, the PC12 cells showed a round, sometimes slightly polygonal morphology. During MG-132 treatment, they grew longer and longer extensions until the end of the first day. MG-132-inhibited cells typically grew one or two longer neurites accompanied by a few shorter extensions. Inhibition of Src with PP2 had no effect on the morphology of the cells, however,

after 30 minutes of PP2 pretreatment, the MG-132 induced neuritogenesis was no longer observed even after 24 hours.

B. APOPTOSIS

4. 8. Apoptosis of PC12 cells treated with MG-132 for 24 hours or longer

As mentioned above, the morphology of PC12 cells that started to differentiate during MG-132 treatment changed significantly after 24 hours and the cells tended to show signs of decline. The observed phenomena prompted us to further investigate the processes during long-term proteasome inhibitor treatment of PC12 cells, with particular attention to stress and apoptosis signaling.

First, we tried to determine the rate of apoptotic cells using different methods. As a first step, Hoechst 33342 staining was performed and the nuclear morphology of the cells was analyzed using a laser scanning confocal fluorescence microscope. The nuclei of PC12 cells showed mostly healthy appearances up to 24 hours of MG-132 treatment, however, after 28 or 30 hours chromatin condensation and nuclear fragmentation became more pronounced compared to the untreated control. Seeing chromatin condensation and nuclear fragmentation, we suspected apoptotic changes in the background of the detectable phenomena. In order to quantify the nuclear changes, we counted 100 cells per sample and determined the rate of nuclei with apoptotic morphology, which increased significantly with the increasing treatment time.

We complemented the above morphological analysis with flow cytometric measurements. The combined use of AnnexinV and propidium-iodide (PI) is a reliable way of determining apoptotic cell rate by means of flow cytometry. PC12 cells were stained with Annexin V-FITC/PI after 0, 24, 30 and 48 hours of MG-132 treatments. Annexin V+PI+ double positive cells were considered late apoptotic (not viable) in accordance with the literature. After 24, 30 or 48 hours of MG-132 treatment, the ratio of Annexin V+PI+ double positive cells increased significantly.

Finally, in order to complete the evaluation of apoptotic changes in PC12 cells upon prolonged MG-132 treatment, we performed a cell viability test using the cell proliferation indicator WST-1. In living cells, the WST-1 tetrazolium salt is converted to formazan by mitochondrial dehydrogenases, the amount of which correlates with the number of metabolically active cells in the culture. Optical density (OD) of formazan was measured in samples treated with MG-132 for 0, 24, 30 and 48 h. In accordance with our previous results related to Hoechst and Annexin V/PI staining, and perhaps even more prominently, the proportion of metabolically active cells significantly decreased at 24, 30, and 48 hours of MG-132 treatment.

4. 9. Time kinetics of Akt-, p38-, JNK-, c-Jun phosphorylation and caspase-3 activation in response to MG-132 treatment

Having experienced that an increasing proportion of PC12 cells are affected by apoptosis as a result of MG-132 treatment for more than 24 hours, we also wanted to investigate the molecular background of the observed events. As the proteasome is involved in the regulation of many signaling molecules, we analyzed some key pathways that affect survival, mediate the

stress response of cells, or even lead to their apoptosis. First, we investigated the kinetics of Akt phosphorylation, during which MG-132 induced signal intensification. The maximum of the p-Akt signal was observed after 3 hours of treatment, then its intensity showed a gradually decreasing trend.

Stress signaling pathways also play an important role in the regulation of apoptosis in various cell types, including PC12 cells. Next, we examined the phosphorylation of p38, JNK and its substrate, c-Jun, in response to MG-132 treatment. The increase in p38 phosphorylation was most pronounced after 3-6 hours of MG-132 exposure, and a moderate decrease was observed after 24 hours of treatment. The activation of JNK was already evident after the first 30 min and increased even further up to 24 h of MG-132 treatment. A similar pattern was also detected for c-Jun, the unphosphorylated version of which also showed the same increasing signal kinetics.

The active, cleaved form of caspase-3 was only observed after 24 hours of MG-132 treatment, which coincided with the appearance of other manifestations of incipient apoptosis.

In summary, we can say that as a result of long-term MG-132 treatment in PC12 cells, Akt activation, which mediates survival, gradually decreased after an initial increase, while the stress signaling pathways that trigger apoptosis became more and more active, and finally a strong increase in the amount of cleaved caspase-3 was also observed. All of these changes may contribute to the explanation of the apoptotic changes observed in PC12 cells after long-term MG-132 treatment.

4. 10. Intracellular distribution of stress signaling proteins upon MG-132 treatment

Seeing the proteasome inhibitor-induced phosphorylation changes of the signaling molecules p38, JNK and c-Jun, which play an important role in stress signaling, we were curious about the intracellular localization of the active forms of these proteins after MG-132 treatment and in its absence. Immunofluorescent staining was performed using antibodies that specifically recognize the phosphorylated forms of these proteins. With laser scanning confocal fluorescence microscopy no p-p38 and p-c-Jun signals were detected in the untreated PC12 cells, while minimal phosphorylated JNK immunoreactivity was shown in the cytoplasm, even without treatment. Then MG-132 treatments suitable to induce maximal activation of signaling proteins were used, which corresponded to 6 hours for p38 and 24 hours for JNK and c-Jun. After 6 hours of MG-132 treatment, the phosphorylated p38 signal appeared predominantly in the cytoplasm of PC12 cells and to a lesser extent in the nuclei. After 24 hours of MG-132 treatment, compared to the untreated control, a stronger p-JNK signal was observed both in the cytoplasm and in the nuclei, while in the case of p-c-Jun, the significantly increased immune signal intensity compared to the untreated control showed almost exclusively nuclear localization.

4. 11. The effects of specific kinase inhibitors on Akt-, p38-, JNK- and c-Jun activation induced by MG-132 treatment

In order to better understand the role of the above-examined kinases and to clarify their relationships during proteasome inhibition, PC12 cells were cultured in the presence of specific kinase inhibitors for 1 hour and then treated with MG-132 for 3 hours, during which the kinase

inhibitors were also present the in cultures. We were also curious about the effects of different kinase inhibitors on signaling changes induced by proteasome inhibition.

LY294002 is a highly selective PI3K inhibitor, which indirectly inhibits Akt phosphorylation. SB203580 is a specific inhibitor of p38, while SP600125 is a potent and selective JNK inhibitor. Pretreatment with LY294002 effectively reduced both baseline and MG-132-induced Akt phosphorylation in PC12 cells, while slightly increased (although not statistically significant) p38 phosphorylation. The effect of the drug on the phosphorylation of JNK and c-Jun was negligible. Pretreatment with the p38 inhibitor SB203580 interestingly prevented MG-132-induced Akt phosphorylation and also reduced the basal activity of Akt, but slightly increased the phosphorylation of p38, JNK and c-Jun induced by MG-132 treatment (however the latter two are not statistically significant). Finally, pretreatment with the JNK inhibitor SP600125 prevented MG-132-induced JNK, c-Jun, and Akt phosphorylation and decreased Akt basal activity, but increased (although not statistically significant) p38 basal and MG-132 induced phosphorylation, as well.

Based on these results, we can conclude that the different kinase inhibitors are able to decrease, or in some cases increase, the changes in activity of the tested molecules caused by MG-132.

4. 12. Phosphorylation of Akt, p38, JNK, and c-Jun in wild-type PC12 cells and M-M17-26 mutants after 3 hours of MG-132 treatment

As Ras is a central regulator of signaling in various cell types, including PC12 cells, we also examined the MG-132-induced phosphorylation events in the PC12 cell line expressing a dominant negative H-Ras mutant (M-M17-26). These cells did not show signs of neuronal differentiation as a result of MG-132 treatment, and apoptotic changes could be observed in them after a shorter time than in the case of wild-type PC12 cells. The lack of neuritogenesis in this cell line can be explained -at least in part- by the inhibited ERK phosphorylation of these mutants. As a result of MG-132 treatment, we could not detect a significant difference in the phosphorylation state of the signaling molecules tested with kinase inhibitors (Akt, p38, JNK and c-Jun) between wild-type PC12 and M-M17-26 cells. Although p-Akt levels were elevated in both untreated and MG-132-treated M-M17-26 cultures, p38 and JNK basal activity appeared to be slightly lower in M-M17-26 cells, whereas p38 phosphorylation induced by MG-132 was found to be similar to that in the wild-type PC12 cell line. Interestingly, lack of Ras function reduced JNK, but on the other hand increased c-Jun phosphorylation in response to MG-132 treatment.

Thus, the inhibition of Ras function changes the basal activity of some signal transduction proteins we investigated, in addition to influencing the extent of activity increase induced by MG-132 treatment.

5. Discussion

Summary of our main results:

- Treatment with the proteasome inhibitor MG-132 induces a biphasic response in PC12 cells. Initially (less than 24 h) it induces neuritogenesis, but longer treatments (more than 24 h) lead to the apoptosis of the cells.

Neuritogenesis:

- The prolonged ERK activation induced by MG-132, which is crucial in the differentiation of PC12 cells, is a Ras- and MEK-dependent process.
- MG-132 treatment induces both Src- and TrkA activation in PC12 cells, which are also necessary for proteasome inhibitor-induced neurite outgrowth.
- After pretreatment of PC12 cells with the Src-inhibitor (PP2), the MG-132 induced increase in Src- and TrkA activation was not observable, the level of ERK phosphorylation decreased and became transient, the nuclear translocation of active ERK also decreased, and the consequent neurite outgrowth failed.
- TrkA inhibitor (K252a) pretreatment prevented TrkA activation, did not affect Src phosphorylation, but reduced ERK activation - to an extent comparable to Src inhibitor - in PC12 cells treated with MG-132.
- These results suggest that proper functioning of Src is required for MG-132-induced TrkA phosphorylation, which is followed by Ras- and MEK-dependent, prolonged ERK activation and nuclear translocation of active ERK, which initiates neurite outgrowth of PC12 cells. At the same time, the existence of other ERK-activating mechanisms, which are independent of Src and TrkA, and are mediated by some signaling component(s) stimulated by MG-132 upstream of Ras and MEK (see **Figure 4** later).

Apoptosis:

- During treatment with MG-132 for more than 24 hours, a gradual shift can be observed in signaling from the initial, survival-mediating Akt (and ERK) stimulation to the increasing activation of stress kinases (p38 and JNK) and caspase-3.
- PI3K-, p38-, and JNK inhibitors can modulate MG-132-induced signaling changes.
- The inactivation of the Ras-ERK pathway changes the basic activity of certain signaling proteins as well as their phosphorylation rate after MG-132 treatment.
- Based on our experiments with kinase inhibitors and M-M17-26 cells, expressing dominant negative H-Ras, we found a complex regulatory network in PC12 cells between Akt, p38, JNK and Ras, which is significantly influenced by the activity of the proteosome (see **Figure 5** later).

To study signaling pathways of neuritogenesis and apoptosis can be important in understanding diseases such as Parkinson's or Alzheimer's disease and other neurodegenerative diseases. The most accurate mapping of these signaling pathways can be done in *in vitro* cellular model systems. The role of the proteasome has already been suggested in the regulation of neuronal differentiation and apoptosis signaling. Since proteasome inhibitors are already used in clinical therapies - mainly in the treatment of hematological diseases - it is therefore particularly relevant to find out what other effects these compounds can have. In the present

study, we looked for answers to these questions.In summary, we can draw the conclusion that treatment with the proteasome inhibitor MG-132 induces a biphasic response in PC12 cells: initially it triggers neuritogenesis, but longer treatments lead to apoptosis. In the early phase of the treatment (first 24 hours), MG-132 increases the phosphorylation of TrkA, which was considered a prerequisite for neuronal differentiation, followed by prolonged ERK activation via the Ras, Raf, MEK pathway. The stabilization of active Src plays an important role in these processes, which was achieved by inhibiting its proteasomal degradation. As a result of pretreatment with the Src inhibitor (PP2), the TrkA phosphorylation induced by the proteasome inhibitor failed, the ERK activation proved to be reduced and terminated earlier, the nuclear translocation of the active ERK was decreased, and the consequent neurite outgrowth was not detectable. Similar changes were observed in the case of pretreatment with the TrkA inhibitor, K252a.

Based on the results of our experiments using different kinase inhibitor compounds (PP2, K252a, PD98059) and the PC12 cell line expressing dominant negative Ras (M-M17-26), we composed a signaling model for the mechanism of MG-132-induced neuritogenesis in PC12 cells (**Figure 4**). The essence of it is the Src mediated increase in TrkA phosphorylation, which is followed by Ras- and MEK-dependent sustained ERK activation and the nuclear translocation of active ERK, which ultimately leads to neuritogenesis of the cells (**Figure 4, continuous arrows**). In addition, MG-132 is able to increase ERK phosphorylation at a lower level and with a more transient kinetics by bypassing Src and TrkA, but also through the mediation of Ras and MEK, but in this case, the nuclear translocation of activated ERK is not pronounced and the process of neuritogenesis is not observed (**Figure 4, dashed arrows**).

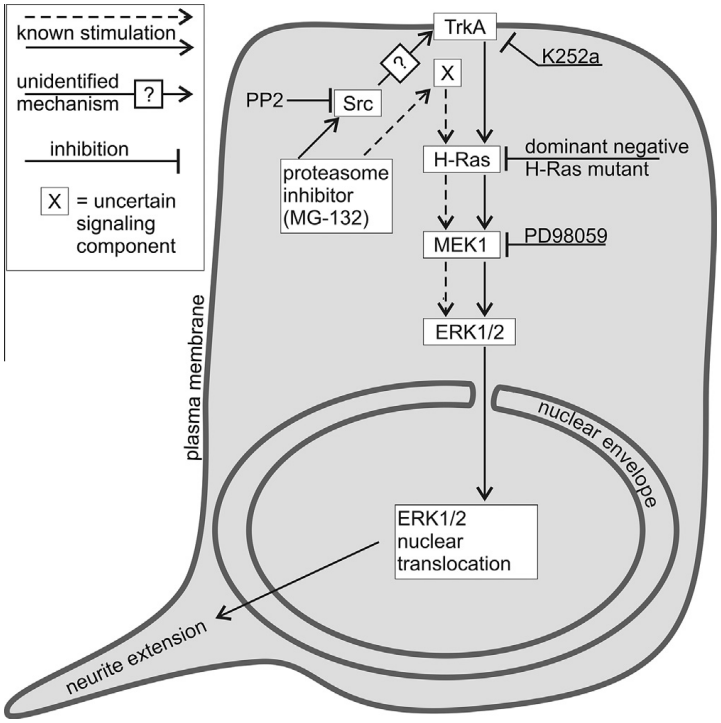

Figure 4. *Our proposed model of MG-132's ERK-activating and neuritogenic effects*

Prolonged activation of ERK by MG-132 is dependent on Ras (inhibited in the dominant negative H-Ras-expressing M-M17-26 variant of PC12 cells) and MEK (chemically blocked by the compound PD98059). Sustained ERK activation, nuclear translocation and neurite extension requires the intact function of Src (chemically inhibited by PP2) and is preceded by the phosphorylation of TrkA.

In the second phase of proteasome inhibitor treatment (after 24 hours), the signs of apoptosis were more dominant. In the background of this the increased activity of stress kinases (JNK and p38) and caspase-3, as well as the decreased activity of the survival-mediating Akt and ERK were observed. By combining proteasome inhibitor and kinase inhibitor (LY294002, SB203580, SP600125) treatments and using the PC12 cell line expressing a dominant negative Ras mutant (M-M17-26), we mapped the details of a complex regulatory network between stress kinases (p38 and JNK) and signaling molecules involved in survival (Akt and ERK). Based on our presented experimental results and additional data taken from the literature, we propose a model of these interactions in **Figure 5**.

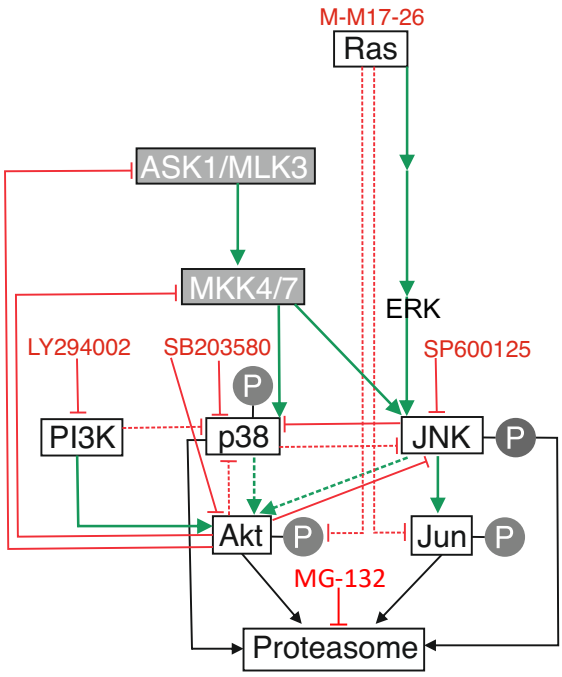

Figure 5. *Proposed summary of proteasome inhibitor- and kinase inhibitor- induced signaling in PC12cells*
Activation is indicated by green arrows, inhibition by red lines. Dashed lines represent proposed connections based on our presented results, continuous lines indicate connections already confirmed in the literature. Signal transduction molecules studied in the apoptosis part of our work are indicated in white boxes with black captions, other components not studied by us are in gray boxes with white captions. ERK was examined in the neuritogenesis part of our experiments, so it is indicated without frame in this version of the figure.

We believe that our experimental data can contribute to a better understanding of the effect of proteasome inhibitors as agents with serious clinical therapeutic potential. Both the differentiation- and apoptosis-inducing effect of these compounds can be beneficial in tumor therapy. At the same time, our results can provide new information regarding the understanding of the molecular background of proteasome inhibitor-induced peripheral neuropathy as well as various neurodegenerative diseases. The expanding knowledge in this field can hopefully contribute to the development of more selective or even novel combination therapies with less side effects in the future.

Acknowledgment

I would like to thank my supervisor, György Sétáló Jr. for supporting my work with his professional knowledge, to József Szeberényi and Hajnalka Ábrahám for allowing me to carry out my experiments at the Department of Medical Biology, to Gergely Berta for the efficient cooperation, to Mónika Vecsernyés for her help in my experiments, and to all my colleagues in the Department of Medical Biology and Central Electron Microscopic Laboratory.

I would also like to thank my loving husband and three wonderful children for their support.

Publications

The thesis is based on the following publications: (IF: 7,259; used in this thesis **IF: 6,087**)

Tarjanyi O., Berta G., Harci A., Bacsa E., Stark B., Pap M., Szeberenyi J., and Setalo G. J. (2013) The role of Src protein in the process formation of PC12 cells induced by the proteasome inhibitor MG-132. Neurochem Int 63:413–422. https://doi.org/10.1016/j.neuint.2013.07.008 IF: 2,659
(IF:1,172 was used in the PhD thesis of Gergely Berta M.D., University of Pécs, Medical School, 2014) Used in this thesis **IF: 1,487**

Tarjányi O., Haerer J., Vecsernyés M., Berta G., Stayer-Harci A., Balogh B., Farkas K., Boldizsár F., Szeberényi J., and Sétáló G. J. (2022) Prolonged treatment with the proteasome inhibitor MG-132 induces apoptosis in PC12 rat pheochromocytoma cells. Sci Rep 12:5808. https://doi.org/10.1038/s41598-022-09763-z **IF: 4,600**

Other publications (IF: 32,984)

Balogh B., Vecsernyés M., Stayer-Harci A., Berta G., Tarjányi O., Sétáló G. J. (2023) Urocortin stimulates the ERK1/2 signaling pathway and the proliferation of HeLa cells via CRF receptor 1. FEBS Open Bio 13:818–832. https://doi.org/10.1002/2211-5463.13602 **IF: 2,600 (2022)**

Balogh B., Vecsernyés M., Veres-Székely A., Berta G., Stayer-Harci A., Tarjányi O., Sétáló G. J. (2022) Urocortin stimulates ERK1/2 phosphorylation and proliferation but reduces ATP production of MCF7 breast cancer cells. Mol Cell Endocrinol 547:111610. https://doi.org/10.1016/j.mce.2022.111610 **IF: 4,100**

Boldizsar F., **Tarjanyi O.**, Olasz K., Hegyi A., Mikecz K., Glant T.T., Rauch T.A. (2016) FTY720 (Gilenya) treatment prevents spontaneous autoimmune myocarditis and dilated cardiomyopathy in transgenic HLA-DQ8-BALB/c mice. Cardiovasc Pathol 25:. https://doi.org/10.1016/j.carpath.2016.05.003 **IF: 2,359**

Hanyecz A., Olasz K., **Tarjanyi O.**, Nemeth P., Mikecz K., Glant T.T., Boldizsar F. (2014) Proteoglycan aggrecan conducting T cell activation and apoptosis in a murine model of rheumatoid arthritis. Biomed Res Int 2014:. https://doi.org/10.1155/2014/942148 **IF: 1,579**

Berta G., Harci A., **Tarjányi O.**, Vecsernyés M., Balogh A., Pap M., Szeberényi J., Sétáló G. J. (2013) Partial rescue of geldanamycin-induced TrkA depletion by a proteasome inhibitor in PC12 cells. Brain Res 1520:. https://doi.org/10.1016/j.brainres.2013.05.015 **IF: 2,828**

Olasz K., Boldizsar F., Kis-Toth K., **Tarjanyi O.**, Hegyi A., van Eden W., Rauch T.A., Mikecz K., Glant T.T. (2012) T cell receptor (TCR) signal strength controls arthritis severity in proteoglycan-specific TCR transgenic mice. Clin Exp Immunol 167:. https://doi.org/10.1111/j.1365-2249.2011.04506.x **IF: 3,409**

Boldizsar F., Kis-Toth K., **Tarjanyi O.**, Olasz K., Hegyi A., Mikecz K., Glant T.T. (2010) Impaired activation-induced cell death promotes spontaneous arthritis in antigen (cartilage proteoglycan)-specific T cell receptor-transgenic mice. Arthritis Rheum 62:. https://doi.org/10.1002/art.27614 **IF: 8,435**

Tarjanyi O., Boldizsar F., Nemeth P., Mikecz K., Glant T.T. (2009) Age-related changes in arthritis susceptibility and severity in a murine model of rheumatoid arthritis. Immun Ageing 6:. https://doi.org/10.1186/1742-4933-6-8 **IF: -**

Boldizsar F., **Tarjanyi O.**, Nemeth P., Mikecz K., Glant T.T. (2009) Th1/Th17 polarization and acquisition of an arthritogenic phenotype in arthritis-susceptible BALB/c, but not in MHC-matched, arthritis-resistant DBA/2 mice. Int Immunol 21:. https://doi.org/10.1093/intimm/dxp018 **IF: 3,403**

Farkas B., Boldizsar F., **Tarjanyi O.**, Laszlo A., Lin S.M., Hutas G., Tryniszewska B., Mangold A., Nagyeri G., Rosenzweig H.L., Finnegan A., Mikecz K., Glant T.T. (2009) BALB/c mice genetically susceptible to proteoglycan-induced arthritis and spondylitis show colony-dependent differences in disease penetrance. Arthritis Res Ther 11:. https://doi.org/10.1186/ar2613 **IF: 4,271**

Poster presentations related to the thesis:

Tarjányi Oktávia, Vecsernyés Mónika, Berta Gergely, Stayer-Harci Alexandra, Balogh Bálint, Boldizsár Ferenc, Szeberényi József, ifj. Sétáló György: Proteaszóma gátló (MG-132) és kináz gátlók hatása patkány feokromocitóma (PC12) sejtek jelátvitelére (51. Membrán-Transzport Konferencia, Sümeg, 2022)

Oktávia Tarjányi, Mónika Vecsernyés, Gergely Berta, Alexandra Stayer-Harci, Bálint Balogh, Ferenc Boldizsár, György Sétáló Jr.: *In vitro treatment with the proteasome inhibitor MG-132 has a biphasic effect in rat pheochromocytoma cells* (11th International Symposium on Cell/Tissue Injury and Cytoprotection/ Organoprotection (ISCTICO) joint meeting of the Hungarian Society for Experimental and Clinical Pharmacology (HUPHAR) and the International Union of Basic and Clinical Pharmacology (IUPHAR), Pécs, 2021)

Oktávia Tarjányi, Gergely Berta, Alexandra Harci, Bálint Balogh, Mónika Vecsernyés, József Szeberényi, György Sétáló Jr.: *Signaling events induced by the proteasome inhibitor MG-132 in PC12 (rat pheochromocytoma) cells* (Federation of European Neuroscience Societies (FENS), Pécs, 2017)

Tarjányi Oktávia, Haerer Julian Tim, Berta Gergely, Harci Alexandra, Balogh Bálint, ifj. Sétáló György: *Patkány feokromocitóma (PC12) sejtvonal proteaszóma gátló (MG-132) által kiváltott apoptózisának jelátviteli változásai* (47. Membrán-Transzport Konferencia, Sümeg, 2017)

Tarjányi Oktávia, Berta Gergely, Harci Alexandra, Bacsa Eszter Bernadett, Stark Borbála, Pap Marianna, Szeberényi József, ifj. Sétáló György: *Proteaszóma gátló (MG-132) által indukált neuritnövekedés jelátvitelének vizsgálata patkány feokromocitóma (PC12) sejtekben* (44. Membrán-Transzport Konferencia, Sümeg, 2014)

Tarjányi Oktávia, Bacsa Eszter Bernadett, Vecsernyés Mónika, Berta Gergely, Galgóci Szilvia, Szabó Klaudia, ifj. Sétáló György és Szeberényi József: *Proteaszóma gátló (MG-132) ERK aktivációra kifejtett hatásainak vizsgálata patkány feokromocitóma (PC12) sejtekben* (41. Membrán-Transzport Konferencia, Sümeg, 2011)

Tarjányi Oktávia, Harci Alexandra, Berta Gergely, Varga Judit, Ifj. Sétáló György és Szeberényi József: *Patkány feokromocitóma (PC12) sejtek jelátvitelének vizsgálata proteaszóma gátló (MG-132) és Src-inhibitorok (PP1, PP2) jelenlétében* (PhD Tudományos Napok, Budapest, 2009)

Harci Alexandra, **Tarjányi Oktávia**, Ifj. Sétáló György, Szeberényi József: *Proteaszómagátló hatása az ERK1/2 és Src kinázok foszforilációjára, illetve intracelluláris elhelyezkedésére PC12 sejtekben* (PhD Tudományos Napok, Budapest, 2009)

Harci Alexandra, **Tarjanyi Oktavia**, Setalo Gyorgy Jr and Szeberenyi Jozsef: *Phosphorylation and intracellular distribution of protein kinases ERK1/2 and Src in proteasome-inhibited PC12 cells* (33rd Federation of European Biochemical Societies (FEBS) and 11th International Union of Biochemistry and Molecular Biology (IUBMB) Conference, Athén, Görögország, 2008)

Berta Gergely, Stark Borbála, **Tarjányi Oktávia**, Harci Alexandra, Ifj. Sétáló György és Szeberényi József: *MG-132 proteaszóma gátló differenciációs és apoptotikus hatása PC12 sejtekben* (Magyar Humángenetikai társaság VII. Kongresszusa, Pécs, 2008)

Tarjányi Oktávia, Stark Borbála, Berta Gergely, Harci Alexandra, Varga Judit, Ifj. Sétáló György és Szeberényi József: *Proteaszóma gátló (MG-132) és Src-inhibitorok (PP1, PP2) neuronális differenciációt befolyásoló hatásainak vizsgálata PC12 sejtekben* (Magyar Biokémiai Egyesület 2006. évi Vándorgyűlése, Pécs, 2006)

Stark Borbála, Berta Gergely, **Tarjányi Oktávia,** Harci Alexandra, Varga Judit, Ifj. Sétáló György és Szeberényi József: *MG-132 jelátviteli és morfológiai hatásai patkány feokromocitóma tenyészetekben* (Magyar Biokémiai Egyesület 2006. évi Vándorgyűlése, Pécs, 2006)

Tarjányi Oktávia, Berta Gergely, Stark Borbála, Harci Alexandra, Ifj. Sétáló György és Szeberényi József: *Proteaszóma gátló (MG-132) és Src-inhibitorok (PP1, PP2) jelátviteli hatásainak vizsgálata patkány feokromocitóma (PC12) sejtekben* (36. Membrán-Transzport Konferencia, Sümeg, 2006)

Stark Borbála, Berta Gergely, **Tarjányi Oktávia,** Harci Alexandra, Ifj. Sétáló György és Szeberényi József: *Proteaszóma gátlás hatása PC12 patkány feokromocitóma sejtek differenciációjára és túlélésére* (36. Membrán-Transzport Konferencia, Sümeg, 2006)

Other poster presentations:

Reka Kugyelka, Katalin Olasz, Zoltan Kohl, Sohn Hee Seung, **Oktavia Tarjanyi**, Peter Nemeth, Timea Berki, Ferenc Boldizsar: *Analysis of partial ZAP-70 deficiency in a murine model of rheumatoid arthritis* (4th European Congress of Immunology (ECI), Bécs, Ausztria, 2015)

Gergely Berta, Alexandra Harci, **Oktávia Tarjányi**, Mónika Vecsernyés, András Balogh, Marianna Pap, József Szeberényi and György Sétáló, Jr.: *Partial rescue of geldanamycin-induced TrkA depletion by a proteasome inhibitor in PC12 cells* (2nd International Doctoral Workshop on Natural Sciences, Pécs, 2013)

Boldizsár Ferenc, **Tarjányi Oktávia**, Olasz Katalin, Kis-Tóth Katalin, Mikecz Katalin, Glant T. Tibor: *A TcR jelátvitel és apoptosis patogenetikai szerepe spontán arthritis modellben* (42. Membrán-Transzport Konferencia, Sümeg, 2012)

Boldizsár Ferenc, **Tarjányi Oktávia**, Olasz Katalin, Hegyi Ákos, Németh Péter, Mikecz Katalin és Glant T. Tibor: *Az interleukin-4 (IL-4) módosítja az in vitro aktiváció indukált sejthalált egér T sejtekben* (39. Membrán-Transzport Konferencia, Sümeg, 2009)

Oktavia Tarjanyi, Ferenc Boldizsar, Katalin Mikecz, Tibor T. Glant *Gene Expression Profile of Secondary Lymphoid Organs in BALB/c Mice with Proteoglycan-Induced Arthritis* (72nd Annual Scientific Meeting of the American College of Rheumatology/43rd Annual Scientific Meeting of the Association of Rheumatology Health Professionals, San Francisco, USA, 2008) Arthritis and Rheumatism, 2008; 58(9) Suppl. S 509.

Ferenc Boldizsar, **Oktavia Tarjanyi**, Balint Farkas, Katalin Mikecz, Tibor T. Glant: *Th1 and Th17 Cells Dominate in the Peritoneal Cavity of BALB/c Mice during the Initiation Phase of Proteoglycan-Induced Arthritis (PGIA)* (72nd Annual Scientific Meeting of the American College of Rheumatology/43rd Annual Scientific Meeting of the Association of Rheumatology Health Professionals, San Francisco, USA, 2008) Arthritis and Rheumatism, 2008; 58(9) Suppl. S 842-843.

Ferenc Boldizsar, **Oktavia Tarjanyi**, Katalin Mikecz, Tibor T. Glant: *Adoptive Transfer of Proteoglycan (PG)-Induced Arthritis (PGIA) to SCID mice with Peritoneal Cells from PG Immunized BALB/c Mice* (72nd Annual Scientific Meeting of the American College of Rheumatology/43rd Annual Scientific Meeting of the Association of Rheumatology Health Professionals, San Francisco, USA, 2008) Arthritis and Rheumatism, 2008; 58(9) Suppl. S 507.

Varga Judit, Bátor Judit, Stark Borbála, Harci Alexandra, **Tarjányi Oktávia**, Szeberényi József: *Nitrogén-oxid szerepe PC12 patkány phaeochromocytoma sejtek apoptózisában* (VII. Magyar Genetikai Kongresszus XIV. Sejt- és Fejlődésbiológiai Napok, Balatonfüred, 2007)

Bátor Judit, Varga Judit, Harci Alexandra, Stark Borbála, **Tarjányi Oktávia,** Szeberényi József: *Nitroziláció hatása PC12 sejtek neuronális differenciációjára* (Magyar Biokémiai Egyesület 2006. évi Vándorgyűlése, Pécs, 2006)

Berta Gergely, Stark Borbála, **Tarjányi Oktávia**, Sétáló György Jr., Szeberényi József: *The effect of proteasome inhibition on PC12 cells* (30th Federation of European Biochemical Societies (FEBS) – 9th International Union of Biochemistry and Molecular Biology (IUBMB) Conference, Budapest, 2005)

Berta Gergely, Stark Borbála, **Tarjányi Oktávia**, Ifj. Sétáló György és Szeberényi József: *Proteaszóma-, és nukleáris export- gátlás hatásának vizsgálata PC12 sejtekben* (VI. Magyar Genetikai Kongresszus XIII. Sejt- és Fejlődésbiológiai Napok, Eger, 2005)

Other oral presentations:

Ferenc Boldizsar, Katalin Olasz, Katalin Kis-Toth, **Oktavia Tarjanyi**, Akos Hegyi, Willem van Eden, Tibor A. Rauch, Katalin Mikecz, Tibor T. Glant: *TcR signal dependent experimental arthritis in proteoglycan-specific TcR transgenic mice* (46th Annual Meeting of the European Society for Clinical Investigation, Budapest, 2012)

Ferenc Boldizsar, Katalin Olasz, Katalin Kis-Toth, **Oktavia Tarjanyi**, Akos Hegyi, Willem van Eden, Tibor A. Rauch, Katalin Mikecz, Tibor T. Glant: *Studying the role of T cell receptor (TcR) signaling in the arthritis of proteoglycan-specific TcR transgenic mice* (IMPULSE, EFIS-EJI Symposium, Visegrád, Magyarország, 2011)

Ferenc Boldizsar, **Oktavia Tarjanyi**, Katalin Kis-Toth, Katalin Olasz, Akos Hegyi, Peter Nemeth, Katalin Mikecz, Tibor T. Glant: *Spontaneous arthritis develops in aged cartilage proteoglycan (PG)- specific TcR transgenic (tg) mice* (7th International Congress on Autoimmunity, Ljubljana, Szlovénia, 2010)

Ferenc Boldizsar, **Oktavia Tarjanyi**, Katalin Olasz, Akos Hegyi, Peter Nemeth, Katalin Mikecz, Tibor T. Glant: *HLA-DQ8 transgenic mice in BALB/c genetic background develop spontaneous myocarditis* (2nd European Congress of Immunology (ECI), Berlin, Németország, 2009)

Varga Judit, Bátor Judit, **Tarjányi Oktávia**, Szeberényi József: *A Ras fehérje szerepe PC12 sejtek nitrogén-oxid indukálta apoptózisában* (PhD Tudományos Napok, Budapest, 2009)

Balint Farkas, Ferenc Boldizsar, **Oktavia Tarjanyi**, Aaron Mangold, Anna Laszlo, Tibor T. Glant: *A New Murine Model of Spondyloarthropathy: Arthritis Resistant DBA/2 Mice Develop Autoimmune Ankylosing Spondylitis* (72nd Annual Scientific Meeting of the American College of Rheumatology/43rd Annual Scientific Meeting of the Association of Rheumatology Health Professionals, San Francisco, USA, 2008) Arthritis and Rheumatism, 2008; 58(9) Suppl. S 945.