# MODULATION OF THE IMMUNE RESPONSE FOLLOWING PERIPHERAL NERVE INJURY

**Ph.D. Thesis**

**Bernát Nógrádi, M.D.**

**Supervisors:**

**László Siklós Ph.D., D.Sc.**

**Roland Patai Ph.D.**

Laboratory of Neuronal Plasticity, Molecular Neurobiology Research Unit, Institute of Biophysics, Biological Research Centre, Szeged, Hungary

Doctoral School of Theoretical Medicine, University of Szeged, Hungary

Department of Neurology, University of Szeged, Hungary

**Szeged**

**2022**

## Introduction

Acute injuries of the peripheral nervous system (PNS) are relatively common, with a yearly incidence of 3.69-13.9 per 100 000, occurring mostly in young individuals. While PNS injuries are mostly not life-threatening conditions, they can result in a significant decline in the quality of life, especially when the reinnervation of muscles is delayed, leading to muscle atrophy and permanent loss of function. The currently accepted clinical therapy for peripheral nerve injury is surgical reconstruction, however, several different factors limit the success rate of reconstructive surgeries. While injury-induced inflammation might have both beneficial and detrimental aspects, overactivation of pro-inflammatory pathways can negatively influence the regenerative outcome. Nucleotide-binding oligomerization domain-, Leucine-rich Repeat- and Pyrin domain-containing protein (NLRP)3 is a pattern recognition receptor, the activation of which leads to the release of pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18. Initially the NLRP3 inflammasome was identified as a potential drug target in autoimmune diseases, but recently the involvement of NLRP3 has also been indicated in the pathomechanism of various disorders ranging from type 2 diabetes to neurodegenerative disorders. Based on this, novel small molecule inhibitors were developed in order to target the NLRP3 inflammasome, and earlier elements of the inflammatory pathway, such as the P2X4 purinergic receptor. Additionally, diazoxide (DZX), a mitochondrial $K_{ATP}$ channel opener, was also shown to reduce NLRP3 activation in ischaemia/reperfusion injury by protecting mitochondria. Based on this, DZX might act as a potential modulator of the inflammasome pathway.

## Aims

In our experiments we aimed at exploring the involvement of the NLRP3 inflammasome pathway following acute nerve injury and identifying potential

therapeutical targets through modulation of the inflammasome response. Our specific aims were the following:

- to examine the possible activation of the NLRP3 inflammasome after peripheral injury
- to evaluate the effect of NLRP3 inflammasome inhibition on the regenerative outcome after sciatic nerve injury,
- to assess the involvement of the ATP-P2X4-NLRP3 following sciatic nerve injury through inhibition of the P2X4 receptor
- to determine whether the neuroprotective effect of diazoxide is linked to the regulation of the inflammasome cascade and repression of microglial activation.

**Results**

**NLRP3 expression in the hypoglossal and oculomotor nucleus following axotomy**

Changes in the NLRP3 expression were assessed in the oculomotor and hypoglossal nucleus 4 days after hypoglossal nerve axotomy or oculomotor target deprivation injury. Under control (*i.e.*, intact) conditions, a faint basal NLRP3 staining was present in both sides of the oculomotor and hypoglossal nuclei in mouse brain sections. Target deprivation in the case of the oculomotor nucleus led to the increase in the area of the NLRP3 staining which was absent on the contralateral side serving as an internal control. Similar change was visible in the hypoglossal nucleus after hypoglossal axotomy; however, the reaction was more intense. The majority of NLRP3-positive cells were NeuN- and ChAT-positive, indicating motoneuronal NLRP3 upregulation. In order to identify further cell types that might respond with the upregulation of NLRP3 to peripheral nerve injury, double staining with Iba1 and GFAP was performed. Interestingly, 4 days after the axotomy,

the majority of the microglia were not stained with the NLRP3 antibody, only a few microglial cells were NLRP3-positive. Similarly, NLRP3 was upregulated only in a small fraction of astrocytes, and astrocytic endfeet were largely excluded as demonstrated by the double staining with the astrocyte endfeet marker aquaporin-4. Quantitative analysis revealed that the NLRP3 increase was significant in both the oculomotor nucleus and the hypoglossal nucleus after the nerve transection, when compared to the contralateral (intact) side. Furthermore, NLRP3 increase was significantly lower in the oculomotor nucleus compared to the hypoglossal nucleus following axotomy.

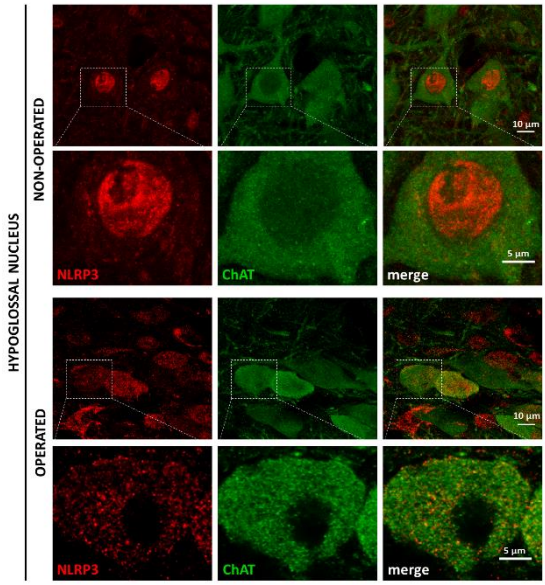


***Figure 1.*** *NLRP3 translocation from motoneuronal nuclei to the cytoplasm in response to hypoglossal nerve axotomy. Top panels (on both the non-operated and operated sides): lower magnification; bottom panels: high magnification.*

In the intact hypoglossal nucleus and on the intact side of the hypoglossal nucleus after axotomy the majority of NLRP3 staining was located in the nuclei of neurons. In response to the transection of the hypoglossal nerve, the staining appeared mainly in the cytoplasm, in parallel to the weakening of the nuclear NLRP3 staining, indicating nucleo-cytoplasmic translocation

(Figure 1). Quantitative analysis revealed that the ratio of neurons showing cytoplasmic NLRP3 was significantly higher on the injured side of the hypoglossal nucleus following axotomy. The activation of inflammasomes involves formation of a multiprotein complex, which includes the binding of NLRP3 with the adaptor molecule ASC. Similarly to NLRP3, ASC was also upregulated after axotomy in the hypoglossal nucleus (Figure 2A). Immunofluorescent staining performed with NLRP3, and ASC showed significant overlap of the staining, mainly in neurons (Figure 2B). Interestingly, the colocalization could be observed in the neuronal nuclei as well. Although NLRP3 could be detected in GFAP-positive cells as well, there was almost no colocalization with ASC (Figure 2C), indicating a neuron-specific inflammasome formation following axotomy.

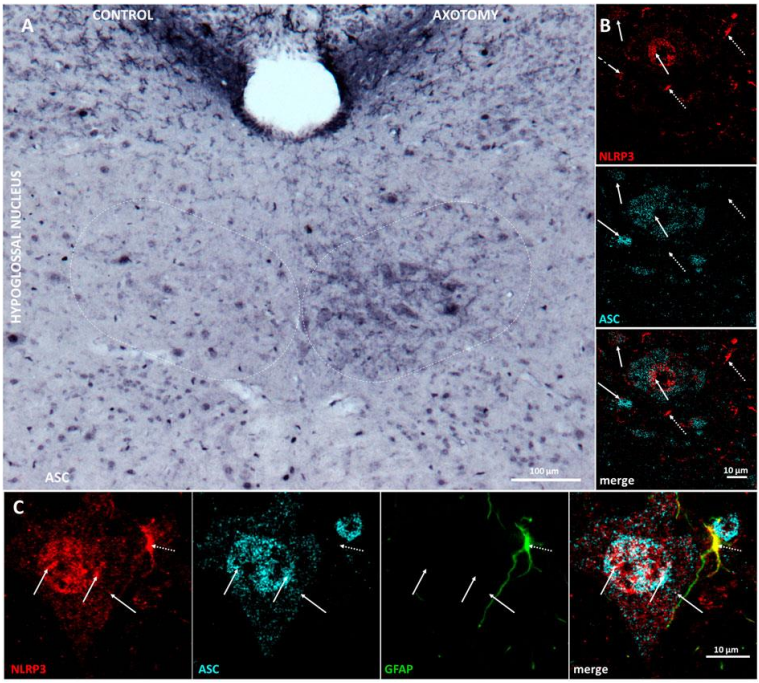

***Figure 2.*** *Colocalization and upregulation of inflammasome components in the hypoglossal nucleus. (A) ASC staining in the hypoglossal nucleus after axotomy (B) NLRP3 and ASC staining in the hypoglossal nucleus following axotomy. Solid arrows indicate the colocalization of NLRP3 and ASC. Dashed arrows indicate NLRP3 without colocalization with ASC. (C) NLRP3, ASC and GFAP staining in the hypoglossal nucleus after axotomy. Solid arrows indicate colocalization of NLRP3 and ASC in the nucleus and cytoplasm of the cells. Dashed arrow indicates colocalization of NLRP3 with GFAP, but not with ASC.*

To further examine NLRP3 inflammasome activation, the protein levels of active IL-1β and IL-18 were quantified with western blot in the hypoglossal nucleus following axotomy. PNI resulted in a 1.507-fold increase of pro-IL-1β levels, indicative of the priming stage of inflammasome activation. In addition, a 1.873-fold increase of the active IL-1β levels was detected in the axotomized hypoglossal nucleus compared to the control side. Similarly, a 1.893-fold increase was observed in the active IL-18 levels after axotomy.

**Effect of DZX on NLRP3 expression and microglial activation after hypoglossal nerve axotomy**

Next, we aimed to investigate the effect of the neuroprotective agent DZX on the axotomy-induced NLRP3 upregulation, thus we treated animals for 3 days post-surgery with DZX. Diazoxide diminished the increase in NLRP3 expression compared to the vehicle-treated group following axotomy in the hypoglossal nucleus. The effect of DZX on microgliosis was examined 7 days post-axotomy in the hypoglossal nucleus. Following axotomy, an elevated microglial reaction was observed on the operated side. In the DZX-treated group, the microglial activation decreased in comparison to the non-treated and DMSO-treated groups, but this change did not reach a significant level. To further clarify the effect of DZX on microglia, their morphology was also assessed in the hypoglossal nucleus. K-means-based Farthest-First cluster

analysis resulted in 2 separate cell clusters with a ramified-like (cluster A) and a bushy-like (cluster B) morphology. 92.5 % of the examined microglial cells were sorted into cluster A on the control side of the hypoglossal nucleus. This dramatically changed on the injured side, where all analyzed microglia were sorted into cluster B. A similar change was observed in the DMSO-treated injured side, where 90 % of the microglia were categorized into cluster B. DZX treatment could prevent this shift to cluster B phenotype.

**Effect of DZX on motoneuronal survival and mitochondrial morphology**

The effect of DZX on motoneuronal survival was also assessed by stereological cell counting in the hypoglossal nucleus to determine the neuroprotective effect of DZX. On day 7 after hypoglossal axotomy, we detected no motoneuronal loss based on the ratio of motoneurons compared to the control side. DMSO or DZX treatment had no significant effect on this ratio. Since DZX is known to modulate mitochondrial function, we conducted ultrastructural analysis of mitochondrial morphology to further examine the effect of DZX 4 days after axotomy. The shape of mitochondria was more circular in the motoneurons on the control side of the hypoglossal nucleus (Figure 3A), but elongated mitochondria with ellipsoid shape and with an increased Feret's diameter could be observed in the motoneurons of the injured side of the hypoglossal nucleus (Figure 3B). DZX treatment restored this altered morphology and the Feret's diameter of these organelles in the hypoglossal motor neurons (Figure 3C). The ratio of the Feret's diameter of the injured motoneuronal mitochondria compared to the control side was increased in the axotomized and in the vehicle (DMSO) control groups, implicating increased oxidative burden. This elevation could be prevented with DZX. The morphology of microglial mitochondria was also examined, but none of the measured parameters showed significant changes.

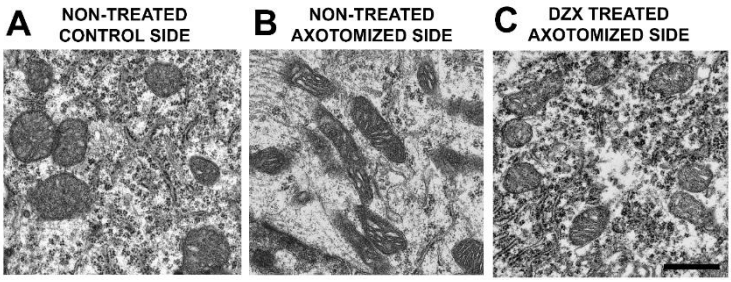


***Figure 3.*** *Changes of the mitochondrial morphology of hypoglossal motoneurons 4 days after axotomy. (A) Electron microscopic images from the control hypoglossal nucleus without any treatment show round-shaped mitochondria with small Feret's diameter. (B) Motoneuronal cytoplasm from the non-treated operated side shows more ellipsoid morphology with increased Feret's diameter. Besides the changes in the shape of these organelles, dilated cristae in the mitochondria could be noted on the operated side. (C) In the DZX-treated group, mitochondria regained their physiological morphology represented by their round shape and small Feret's diameter.*

**NLRP3 expression in the spinal cord and sciatic nerve following PNI**

Next, we examined NLRP3 inflammasome activation following sciatic nerve axotomy. In the L4-L5 spinal cord segments NLRP3 protein expression was upregulated in the injured side at 1, 3 and 7 days post-axotomy. Already on days 1 and 3 after axotomy, NLRP3 was observed in motoneurons, as revealed by ChAT and NLRP3 double staining. The NLRP3 staining was either cytoplasmic or cytoplasmic and nuclear on the injury side, as observed in our previous experiments following hypoglossal axotomy. Notably, NLRP3 showed strong colocalization with the inflammasome adaptor protein ASC in the nuclei and later in the cytoplasm of motoneurons, predicting inflammasome assembly. While NLRP3 was almost exclusively expressed in neurons in the first 3 days, on day 7 post-injury the NLRP3 signal appeared in GFAP- and Iba1-positive cells as well, however, without significant colocalization with ASC. Nevertheless, NLRP3-positive microglia showed an

activated phenotype. Importantly, NLRP3 and ASC were upregulated not only in the spinal cord, but also in Schwann cells and in the microglia/macrophage population at the proximal end of the injured sciatic nerve, 3 days after the injury, accompanied by the increase of p75 and Iba1 expression as well. These results indicate that there is a parallel activation of NLRP3 in both the injured spinal cord and axotomized proximal nerve trunk, however with the involvement of different cell types.

**Effect of NLRP3 inhibition on the microgliosis and astrogliosis in the spinal cord after sciatic nerve axotomy**

Microgliosis became clearly detectable on the injured ventral horn of the L4-L5 spinal cord segments on day 3 post-axotomy, when inflammasomal reaction was still restricted to motoneurons. Strong microglial reaction was present in the vehicle-treated animals, whereas the NLRP3 inhibitor MCC950 diminished the microglial reaction. Microgliosis was even higher on day 7 in the injured ventral horn of the vehicle-treated animals, whereas administration of MCC950 for 3 days significantly decreased microglial reaction 7 days after the injury. Astrogliosis was much weaker than microgliosis both on days 3 and 7 but could be reduced with MCC950. In addition, administration of the NLRP3 inhibitor MCC950 in the first 3 days after axotomy reduced the extent of colocalization of Iba1 and NLRP3 on day 7 post-injury but did not have any diminishing effect on the weak colocalization of GFAP and NLRP3.

**Inhibition of inflammasome activation promotes neural regeneration**

To assess the functional outcome following sciatic nerve injury, nerve stumps were coapted with epineural sutures. We hypothesized that NLRP3 activation is induced, at least partly, through an extracellular ATP – purinergic receptor pathway, thus the effect of P2X4 receptor-specific inhibitor 5-BDBD was evaluated, in addition to the NLRP3 inflammasome inhibitor MCC950. Functional recovery, measured with the sciatic functional index (SFI) method,

was followed for 8 weeks in untreated animals and mice treated with vehicle, 5-BDBD or MCC950 in the first 3 days after axotomy and coaptation. In the untreated and vehicle-treated groups, a slow, but steady increase of the SFI was observed during the 8-week regenerative period. In contrast, in animals treated with 5-BDBD or MCC950, a sharp initial recovery started on day 3 after coaptation, followed by a continuous improvement of SFI values. From week 2 after coaptation, a significant difference was noted between treated and control groups until the end of the observation period. No significant difference was detected within the treated and control groups, *i.e.*, both the 5-BDBD and MCC950-treated groups performed equally well and DMSO treatment had no effect on functional recovery. To quantify the number of motoneurons whose axons were able to grow through the coaptation site and regenerate into the distal sciatic nerve stump, retrograde labeling of regenerating motoneurons was performed. Fast Blue (FB) stained fewer motoneurons in the injured side of the spinal cord of untreated and vehicle-treated animals compared to the intact side, whereas 5-BDBD and MCC950 increased the number of FB-positive motoneurons in the spinal cord on the injured side. As expected from previous studies, less than 80 % of the total sciatic motoneuron pool were able to regenerate their axons into the distal stump in the untreated and vehicle-treated animals, while almost all axons of the sciatic motoneuron population grew beyond the coaptation site in animals treated with 5-BDBD or MCC950. As expected, axotomy only induced a slight reduction in the number of ChAT-positive motoneurons (to 90-95 %). Although the difference was not significant, both 5-BDBD and MCC950 were able to prevent motoneuron loss in the injured motor pool. In order to study the early events of axonal regrowth including growth rate and number of regenerating axons, IF staining was performed using antibodies against neurofilament (NEFM) and the Schwann cell marker p75 in the coaptation region of the sciatic nerve, 5 days after nerve axotomy and coaptation. In

vehicle-treated animals, limited number of axons growing through the coaptation zone (Figure 4A) was observed at this relatively early time point following the axotomy + coaptation. Treatment of the animals with MCC950 increased the length and especially the number of regenerating axons (Figure 4B). These results support our expectations that morphological improvements precede the functional changes. Quantification of the number and length of regrowing axons indicated a more than twofold improvement in regeneration in the MCC950-treated animals compared to vehicle-treated mice. These results show that inhibition of NLRP3 activation in lumbar motoneurons not only hinders microgliosis but also facilitates axonal recovery after sciatic nerve injury.

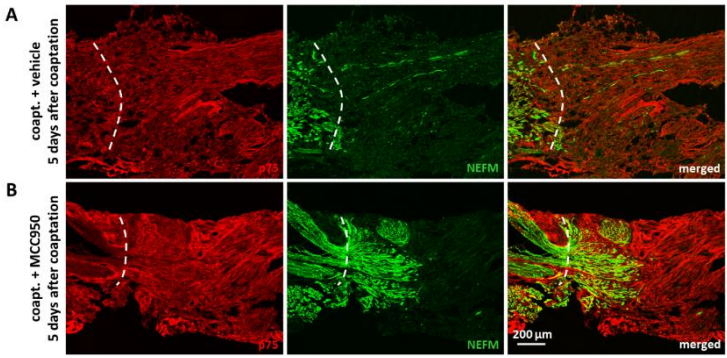


***Figure 4.*** *Effect of MCC950 on axonal regrowth 5 days after sciatic nerve axotomy and coaptation. (A-B) Confocal images of injured sciatic nerves stained with p75 and NEFM. In the vehicle-treated animals (A) a minimal number of short axons growing through the coaptation zone was observed. In comparison, in the MCC950-treated animals (B) a vast number of axons of various lengths have grown through the zone of coaptation. Dashed line indicates the zone of coaptation.*

## Summary

Peripheral nerve injuries are accompanied by inflammatory reactions, over-activation of which may hinder recovery. Among pro-inflammatory

pathways, inflammasomes are one of the most potent, leading to release of active IL-1β. Our aim was to understand how inflammasomes participate in central inflammatory reactions accompanying peripheral nerve injury. Hypoglossal and sciatic nerve axotomy and target deprivation of the oculomotor nerve resulted in the upregulation of NLRP3 in the corresponding brain nuclei and the ventral horn of the lumbar spinal cord. Increased levels of IL-1β and IL-18 were detected in the axotomized hypoglossal nucleus, indicating inflammasome activation. Although glial cells are traditionally viewed as initiators of neuroinflammation, in this acute phase of inflammation, NLRP3 inflammasome activation was found exclusively in affected motoneurons. The upregulation of NLRP3 was also accompanied by its nucleo-cytoplasmic translocation in the injured motoneurons. Although at later time points the NLRP3 protein was upregulated in microglia too, no signs of inflammasome activation were detected in these cells. Inhibition of inflammasome activation with MCC950 in motoneurons in the first 3 days after nerve injury hindered development of microgliosis in the spinal cord. Moreover, P2X4 or inflammasome inhibition in the acute phase significantly enhanced nerve regeneration on both the morphological and the functional levels. DZX treatment reduced upregulation of NLRP3, likely through restoring mitochondrial morphology in motoneurons. Our results indicate that the central reaction initiated by sciatic nerve axotomy starts with inflammasome activation in the injured motoneurons, which triggers a complex inflammatory reaction and activation of microglia. Inhibition of neuronal inflammasome activation not only leads to a significant reduction of microgliosis but has a beneficial effect on the recovery as well.

**List of publications**

*Publications related to the subject of the thesis*

I. Molnár K*, **Nógrádi B***, Kristóf R, Mészáros Á, Pajer K, Siklós L, et al. Motoneuronal inflammasome activation triggers excessive neuroinflammation and impedes regeneration after sciatic nerve injury. J Neuroinflammation. 2022;19(1):268. (IF (2021): 9.594, Journal Ranking: D1)

II. **Nógrádi B***, Nyúl-Tóth Á*, Kozma M, Molnár K, Patai R, Siklós L, et al. Upregulation of nucleotide-binding oligomerization domain-, LRR- and pyrin domain-containing protein 3 in motoneurons following peripheral nerve injury in mice. Front Pharmacol. 2020;11:584184. (IF (2020): 5.811, Journal Ranking: Q1)

III. **Nógrádi B,** Meszlényi V, Patai R, Polgár TF, Spisák K, Kristóf R, at al. Diazoxide blocks or reduces microgliosis when applied prior or subsequent to motor neuron injury in mice. Brain Res. 2020;1741:146875. (IF (2020): 3.252, Journal Ranking: Q2)

*** =** The authors contributed equally and share first authorship.

*Publications not included in the thesis*

I. Polgár TF, Meszlényi V, **Nógrádi B**, Körmöczy L, Spisák K, Tripolszki K, et al. Passive transfer of blood sera from ALS patients with identified mutations results in elevated motoneuronal calcium level and loss of motor neurons in the spinal cord of mice. Int J Mol Sci. 2021;22(18):9994. (IF (2021): 6.208, Journal Ranking: D1)

II. Mészáros Á, Molnár K, **Nógrádi B**, Hernádi Zs, Nyúl-Tóth Á, Wilhelm I, et al. Neurovascular inflammaging in health and disease. Cells. 2020;9(7):1614. (IF (2020): 6.600, Journal Ranking: Q1)

III. Meszlényi V, Patai R, Polgár TF, **Nógrádi B**, Körmöczy L, Kristóf R, et al. Passive transfer of sera from ALS patients with identified mutations evokes an increased synaptic vesicle number and elevation of calcium levels in motor axon terminals, similar to sera from sporadic patients. Int J Mol Sci. 2020;21(15):5566. (IF (2020): 5.924, Journal Ranking: D1)

IV. Obál I, **Nógrádi B**, Meszlényi V, Patai R, Ricken G, Kovacs GG, et al. Experimental motor neuron disease induced in mice with long-term repeated intraperitoneal injections of serum from ALS patients. Int J Mol Sci. 2019;20(10):2573. (IF (2019): 4.556, Journal Ranking: Q1)

V. Meszlényi V, Patai R, **Nógrádi B**, Engelhardt JI, Siklós L. Commentary: Calcium in the pathomechanism of amyotrophic lateral sclerosis – Taking center stage? J Neurol Neuromed. 2017;2(4):1-4. (Journal is not indexed by Clarivate JCR.)

VI. Patai R, **Nógrádi B**, Meszlényi V, Obál I, Engelhardt JI, Siklós L. Az amyotrophiás lateralsclerosis patofiziológiai tényezőinek központi kapcsolóeleme, a kálcium. Ideggyogy Szemle. 2017;70(7-8):247-257. (IF (2017): 0.252, Journal Ranking: Q4)

VII. Patai R, **Nógrádi B**, Engelhardt JI, Siklós L. Calcium in the pathomechanism of amyotrophic lateral sclerosis - Taking center stage? Biochem Biophys Res Commun. 2017;483(4):1031-1039. (IF (2017): 2.559, Journal Ranking: Q1)

**Acknowledgements**

Foremost I would like to express my gratitude to my supervisors, Dr. László Siklós and Dr. Roland Patai (Neuronal Plasticity Research Group, Institute of Biophysics, Biological Research Centre) for the mentorship, support, and guidance throughout my doctoral work. I would like to thank Dr. István Krizbai and Dr. Imola Wilhelm (Neurovascular Unit Research Group, Institute of Biophysics, Biological Research Centre) for their valuable contributions and for enabling our collaborative work. I would like to express my special thanks to Dr. Antal Nógrádi (Laboratory of Neural Regeneration, Department of Anatomy, Histology and Embryology, Faculty of Medicine, University of Szeged) not only for his professional advice, but also for encouraging and motivating me to pursue my passion. I am grateful for the kind support of Dr. Péter Klivényi (Department of Neurology, Albert Szent-Györgyi Health Centre, University of Szeged). I am grateful for all the support and work of the members of the Laboratory of Neuronal Regeneration, Neuronal Plasticity and Neurovascular Unit Research Groups, especially Dr. Kinga Mészáros-Molnár, Rebeka Kristóf, Dr. Ádám Nyúl-Tóth, Dr. Krisztián Pajer and Erika Bánfiné Rácz. Without their professional help, teamwork and the inspiring and friendly atmosphere they created, this dissertation would not have been possible to write. I would like to express my special thanks to my fiancée, family, and friends for their support, tolerance, and love. This accomplishment is just as much theirs as it is mine. This work was financially supported by the GINOP-2.3.2-15-2016-00001, GINOP-2.3.2-15-2016-00034, GINOP-2.3.2-15-2016-00020, FK-124114, K-135425, K-135475, TKP2021-EGA-09, ÚNKP-18-2-I-SZTE-84, ÚNKP-19-2-SZTE-92, ÚNKP-20-2-SZTE-68 and ELKH SA-73/2021 programs and the Szeged Scientists Academy (FEIF/433-4/2020-ITM_SZERZ). Support of UEFISCDI (PN-III-P1-1.1-TE-2019-1302 and PN-III-P4-ID-PCE-2020-1529) is also acknowledged.