# Summary of the doctoral thesis of Agata Ciechanowska

Title:

**"Comparison of immunological changes occurring after injury to the central and peripheral nervous systems and investigation of the influence of pharmacological modulation of selected chemokines on nociceptive transmission in mice"**

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Disorders of the nervous system continue to pose a challenge for doctors and scientists, despite significant advancements in modern medicine and pharmacology. Recent research suggests that damage to both the central and peripheral nervous systems can lead to neuroimmunological changes, which can ultimately result in pain hypersensitivity. It is still unclear which cell populations are involved in the cascade of subsequent molecular changes. It appears that in addition to nerve cells, immune cells and glial cells can also play a significant role in this process. Therefore, identifying similarities and differences arising from the etiology of damage opens up and highlights an interesting area of research. The main objective of this doctoral dissertation was to investigate and compare the involvement of these cells, the complement system factors, and selected chemokines from the CC- and XC- groups in the pathomechanism of damage to the central and peripheral nervous systems. Additionally, the dissertation aimed to examine the influence of pharmacological modulation of selected chemokines and their receptors on pain processes in a mouse model of neuropathic pain. These complex and still not fully understood neuroimmunological mechanisms are responsible for the lack of effective pharmacotherapy for nervous system disorders, highlighting the need for a multidirectional approach in their treatment. The results of the research conducted within this doctoral dissertation were published in **5 original papers.** – *Ciechanowska et al. Immunobiology 2020, Pharmacol Rep 2020, Int J Mol Sci 2021, Front Immunol 2022, Brain Sci 2023.*

**In a traumatic brain injury model,** the involvement of selected cells and immunological factors identified in the literature in the secondary injury development was assessed. This was achieved by examining changes in the mRNA/protein levels of cellular markers, the complement system factors, chemokines from the MIP-1 family (CCL3, CCL4, CCL9) and their receptors (CCR1, CCR5), as well as XCL1 and its receptors (XCR1, ITGA9) at various time points in different brain structures (cortex, thalamus, striatum, and/or hippocampus). Additionally, the cellular source of selected immunological factors and the location of their receptors were determined. The research was conducted in 2018-2021 as part of the ERA-NET NEURONCOFUND /1/LEAP/15/1 grant project. **In a peripheral nerve injury model,** the involvement of the selected cells and immunological factors in neuropathy was determined by examining changes in mRNA/protein levels of cellular markers, chemokines from the MIP-1 family (CCL3, CCL4, CCL9) and their receptors (CCR1, CCR5), as well as chemokine XCL1 and its receptors (XCR1, ITGA9) at various time points in the lumbar spinal cord. Moreover, pharmacological experiments were conducted to investigate the impact of the neutralization of the analysed chemokines and blocking their receptors (CCR1, CCR5, XCR1, ITGA9) on nociception processes in the neuropathic pain model. Owing to the insufficient effectiveness of monotherapy, the objective of the subsequent experiments was to determine whether blocking the aforementioned receptors and their ligands could positively influence analgesic properties of

opioid drugs. The research was conducted in 2020-2023 as part of two grant projects: Opus 2016/21/B/NZ4/00128 followed by Opus 2021/43/B/NZ7/00230.

The *in vivo* experiments were conducted in two stages. The first cycle, carried out in collaboration with a research group led by Prof. M.G. De Simoni, was conducted in a traumatic brain injury model in male C57BL/6J mice (Charles River, Italy). The results were published in 3 original papers – ***Immunobiology 2020, Pharmacol Rep 2020, Int J Mol Sci 2021***. In the second stage, the experiments were conducted in a neuropathic pain model involving loose unilateral sciatic nerve ligation, a procedure long used at the Department of Pain Pharmacology, in male Albino Swiss mice (Charles River, Germany). The results were published in 2 original papers – ***Front Immunol 2022, Brain Sci 2023***. The experiments were conducted at the Instituto di Ricerche Farmacologiche Mario Negri and the Jerzy Maj Institute of Pharmacology, in accordance with the recommendations of the International Association for the Study of Pain, the National Institute of Health, and with the approval of the Ethical Committee for Animal Experiments (Italy – approval numbers 753/2017-PR; Poland – approval numbers 1277/2015, 301/2017, 75/2017, 305/2017, 235/2020, 40/2023, 236/2021, 297/2021, 89/2021, 98/2022). Samples for biochemical analysis (cortex, thalamus, striatum, hippocampus, or spinal cord) were collected at five time points (on day 1, 4, 7, 14, 35) after brain/sciatic nerve injury. Changes in mRNA levels were measured with the RT-qPCR method, whereas changes in protein expression were assessed by means of the ELISA and Western blot methods. Additionally, immunohistochemistry techniques were employed to determine the location of the studied factors. Pharmacological tools were administered either as single or multiple doses, most often intrathecally but also intraperitoneally. Two behavioural tests, von Frey and cold plate, were used to evaluate sensitivity to mechanical and thermal stimuli. Simultaneously, *in vitro* experiments were conducted using primary microglial and astroglial cell cultures obtained from the cerebral cortex of newborn C57BL/6J mice. The results from these experiments were published in 3 papers – ***Immunobiology 2020, Pharmacol Rep 2020, Int J Mol Sci 2021***. The cell cultures were used to determine the cellular source of selected receptors and immunological factors. Data analysis was performed using the GraphPad Prism software, involving a student's t-test, and a one-way and two-way analysis of variance (ANOVA), followed by essential *post hoc* tests.

In the traumatic brain injury model, in the early phase, an increase in the expression of the neutrophil marker was observed not only in the brain cortex, the site of the direct damage, but also in the striatum and hippocampus (***Int J Mol Sci 2021, Immunobiology 2020***). These changes persisted relatively briefly (up to day 7). Further analyses revealed a significant and long-lasting increase in the levels of cellular markers, indicating strong activation of astroglia and microglia/macrophages. Importantly, for the first time, changes were observed in each of the 4 studied brain structures. Based on the obtained data a conclusion was made that depending on the elapsed time and studied brain area, different types of cells played a role after direct brain injury, and the immunological factors secreted by them became the subject of subsequent experiments. The results showed that in the first week after peripheral nerve injury, microglia/macrophages and astroglia were activated, and the levels of the latter remained significantly elevated up to day 35 (***Brain Sci 2023***). Both the results of experiments conducted as part of this work and literature data suggest that an important part of therapy should include drugs that modulate the function of neutrophils, macrophages, microglia, and astroglia in the central nervous system. The search for new, effective substances with such potential is one of the most important tasks in basic research. Activated immune and glial cells play a crucial role in the cascade of changes resulting from damage to the nervous system, especially considering their impact on the biosynthesis of the

complement system factors and numerous, still poorly understood chemokines, which have become the subject of further research.

The results obtained in the subsequent part of the research (*Int J Mol Sci 2021*) suggest that among the initiators of the classical pathway of the complement system activation, the entire C1 complex (C1q, C1r, and C1s), and in the case of the lectin pathway, ficolin A, play an important role after injury to all the studied brain structures (cortex, striatum, thalamus, and hippocampus). Experiments conducted on primary microglial cell cultures suggest that they are largely responsible for the biosynthesis of initiators of the classical pathway, however, their role in the lectin pathway is minor. Selective targeting of C1q and ficolin A may prove to be an effective strategy in treatment of brain injuries. Nevertheless, further research is required to verify this hypothesis.

In the next cycle of experiments, changes in the expression of all chemokines from the MIP-1 family (CCL3, CCL4, CCL9) were detected in the cortex, striatum, and hippocampus after brain injury (*Immunobiology 2020*). However, in the spinal cord after sciatic nerve injury, only CCL3 and CCL9 exhibited changes (*Brain Sci 2023*). Behavioural studies demonstrated that blocking CCL3 and CCL9 with neutralizing antibodies has antinociceptive effects and enhances the analgesic properties of morphine after sciatic nerve ligation. Subsequent experiments focused on the MIP-1 family receptors: CCR1 and CCR5. Their neuronal location suggests an important role in nociceptive transmission, explaining why intrathecal administration of CCL3 and CCL9 has such strong and rapid pronociceptive effects. For the first time, the obtained results indicated that single intrathecal administration of CCR1 antagonists (J113863) and CCR5 antagonists (TAK-220, AZD-5672) reduces pain hypersensitivity developed in tested model in a dose-dependent manner and enhances the effects of morphine (*Brain Sci 2023*). Therefore, pharmacological modulation of selected chemokines from the MIP-1 family and their receptors may represent a completely new approach in the therapy of injury to both central and peripheral nervous systems.

The aim of the last cycle of experiments was to determine and compare the involvement of chemokine XCL1 and its receptors (XCR1, ITGA9) in changes occurring in the brain after injury (*Pharmacol Rep 2020*) and in the spinal cord after sciatic nerve ligation (*Front Immunol 2022*). It was demonstrated for the first time that the levels of XCL1 in the cortex, hippocampus, and thalamus increased in both early and late phases. XCL1 can be considered one of the key factors initiating secondary damage, and among all the receptors for this chemokine, not only the classic XCR1 but also the recently identified ITGA9 may serve as important targets in traumatic brain injury pharmacotherapy (*Pharmacol Rep 2020*). Based on immunohistochemical results, it was shown that XCL1 is produced by astroglia, while both of its receptors are located on neurons (*Front Immunol 2022*). Importantly, also in the spinal cord after nerve injury, a rapid and strong increase in XCL1 expression was observed, persisting for up to 5 weeks (*Front Immunol 2022*). Furthermore, it was demonstrated that intrathecal administration of XCL1 to healthy mice, which induces sensitivity to thermal and mechanical stimuli, was weakened by the administration of both vMIP-II (XCR1 antagonist) and YA4 (ITGA9 neutralizing antibody) (*Front Immunol 2022*). In addition, the research offered initial evidence that after sciatic nerve ligation, blocking XCL1, as well as XCR1 and ITGA9, not only provides pain relief but also enhances the analgesic effects of morphine and/or buprenorphine (*Front Immunol 2022*). These experiments showed for the first time that the XCL1/XCR1 and XCL1/ITGA9 axes are involved in nociceptive transmission. Literature data indicate that neutralization of chemokines and blocking their receptors are

successfully used in the treatment of selected neurodegenerative disorders. Therefore, the proposed modulation of XCL1 signalling can be used to develop more effective pharmacotherapy.

**Summing up, a multidirectional approach in designing new pharmacological tools targeting the modulation of neuroimmune interactions and functions of cells involved in this pathomechanism is a crucial aspect of therapy for nervous system injuries. Treatment based on inhibiting the activation of the complement system initiators (C1q and ficolin A), as well as chemokines (XCL1, CCL3), and blocking their receptors (CCR1, CCR5, XCR1, ITGA9), can significantly improve the quality of life for patients with both brain and peripheral nerve injuries.**

## LIST OF SCIENTIFIC ARTICLES CONSTITUTIONING THE BASIS OF THE DOCTORAL DISSERTATION:

1. **Initiators of classical and lectin complement pathways are differently engaged after traumatic brain injury-time-dependent changes in the cortex, striatum, thalamus and hippocampus in a mouse model. Ciechanowska A.**, Ciapała K., Pawlik K., Oggioni M., Mercurio D., De Simoni M.G., Mika J. *Int J Mol Sci.* 2020; 22(1):45. doi: 10.3390/ijms22010045. **$IF_{2020}$=5,924, $MEN_{2020}$=140**
2. **Changes in macrophage inflammatory protein-1 (MIP-1) family members expression induced by traumatic brain injury in mice. Ciechanowska A.**, Popiolek-Barczyk K., Pawlik K., Ciapała K., Oggioni M., Mercurio D., De Simoni M.G., Mika J. *Immunobiology* 2020; 225(3):151911. doi: 10.1016/j.imbio.2020.151911. **$IF_{2020}$= 3.144, $MEN_{2020}$=100**
3. **Traumatic brain injury in mice induces changes in the expression of the XCL1/XCR1 and XCL1/ITGA9 axes Ciechanowska A.**, Popiolek-Barczyk K., Ciapała K., Pawlik K., Oggioni M., Mercurio D., De Simoni M.G., Mika J. *Pharmacol Rep.* 2020; 72(6):1579-1592. doi: 10.1007/s43440-020-00187-y. **$IF_{2020}$= 3.027, $MEN_{2020}$=100**
4. **Pharmacological modulation of the MIP-1 family and their receptors reduces neuropathic pain symptoms and influences morphine analgesia: evidence from a mouse model Ciechanowska A.**, Pawlik K., Ciapała K., Mika J. *Brain Sci.* 2023; 13(4),579. doi: 10.3390/brainsci13040579. **$IF_{2023}$= 3.300 $MEN_{2023}$=100**
5. **New insights into the analgesic properties of the XCL1/XCR1 and XCL1/ITGA9 axes modulation under neuropathic pain conditions - evidence from animal studies. Ciechanowska A.**, Rojewska E., Piotrowska A., Barut J., Pawlik K., Ciapała K., Kreiner G., Mika J. *Front Immunol.* 2022; 13:1058204. doi: 10.3389/fimmu.2022.1058204. **$IF_{2022}$= 7.300, $MEN_{2022}$=140**

**Total Impact Factor** = 22.695; **Total number of Ministry of Education and Science points** = 580