

Aus der Arbeitsgruppe "Zellulare Neurowissenschaften" des Max-Delbrück-Centrum für Molekulare Medizin in Berlin-Buch

DISSERTATION

**„MICROGLIAL ACTIVATION IN ALZHEIMER`S PATHOLOGY:  
ROLE OF DISEASE RELEVANT PROTEINS IN A SYNERGISTIC  
STIMULATION CONTEXT“**

zur Erlangung des akademischen Grades

**Doctor rerum medicarum (Dr. rer. medic.)**

vorgelegt der Medizinischen Fakultät  
Charité – Universitätsmedizin Berlin

von

**Sanja Pavlovic Masnikosa**

aus Pristina, Jugoslawien

Gutachter: 1. Prof. Dr. U. Heinemann  
2. Prof. Dr. H.-J. Rommelspacher  
3. Prof. Dr. Rer. Nat. Habil. U.-K. Hanisch

Datum der Promotion: 10. 09. 2007

## Table of contents

I.	List of figures.....	4
II.	List of abbreviations.....	6
<b>1. Introduction.....</b>	<b>8</b>	
1.1.	The neuroglia.....	8
1.2.	Resting vs. activated microglia; Role in CNS homeostasis and pathology .....	8
1.3.	Cytokines and chemokines as the key inflammatory mediators produced by microglia.....	10
1.4.	Alzheimer's disease- potential contribution of inflammatory factors and processes.....	11
1.5.	A $\beta$ , cytokines and other plaque-related proteins as microglia-activating agents.....	13
1.5.1	Amyloid beta peptide.....	13
1.5.2.	Cytokines implicated in Alzheimer's disease.....	15
1.5.2.1	Interleukin-1.....	15
1.5.2.2	Interleukin-18.....	17
1.5.2.3	Interleukin-6.....	18
1.5.3.	Alpha 2 macroglobulin.....	18
1.6	Lypopolysacharide as a model stimulus for microglial activation.....	20
<b>2. Aim of the study.....</b>	<b>23</b>	
<b>3. Material and Methods.....</b>	<b>25</b>	
3.1.	Materials.....	25
3.2.	Cell Culture.....	25
3.2.1.	Medium.....	26
3.2.2.	Staining of microglial cells by Griffonia simplicifolia isolectin B4.....	26
3.3.	Microglia staining in the retinal organotypic culture.....	27
3.4.	Reverse transcriptase polymerase chain reaction (RT-PCR).....	28
3.5.	Preparation of A $\beta$ peptides.....	29
3.5.1.	Size exclusion chromatography.....	30
3.5.2.	Electron microscopy.....	31

3.6.	Chronic stimulation of microglial cells.....	31
3.7.	Cytokine and chemokine quantification in Enzyme-Linked Immunosorbent Assays (ELISAs).....	31
3.8.	Nitric oxide release assay.....	32
3.9.	Total cell protein measurement.....	33
3.10.	Cell proliferation assay.....	33
3.11.	Immunoprecipitation.....	34
3.12.	Fast Performance Liquid Chromatography (FPLC).....	34
3.13.	Statistical analysis.....	34
<b>4.</b>	<b>Results.....</b>	<b>35</b>
4.1.	Release activity as a parameter of microglial activation state.....	35
4.2.	Co- stimulations of microglia with A $\beta$ and cytokines.....	37
4.2.1.	Amyloid beta- a weak inducer of microglial activation.....	37
4.2.1.1.	Preparation of A $\beta$ peptides and aggregates; presence of oligomers and fibrillar forms.....	37
4.2.1.2	Effect of A $\beta$ preparations on microglial release activity; Fresh versus “aged” peptide preparations and A $\beta$ 1-40 / A $\beta$ 1-42 mixtures in various ratios.....	40
4.2.2.	Costimulation with IL-1 $\beta$ , but not IL-18, results in supra-additive cyto/chemokine release.....	42
4.2.3.	Co-stimulation with IL-6 does not enhance A $\beta$ release-inducing potency...46	46
4.2.4.	Consequences for other properties of microglia (NO release).....	47
4.3.	Strong inducing effect of $\alpha$ 2M on microglial release activity.....	48
4.3.1.	$\alpha$ 2M as a potent co-stimulatory partner of A $\beta$ .....	49
4.3.2.	Microglial proliferation rate does not change following exposure to $\alpha$ 2M...51	51
4.3.3.	$\alpha$ 2M itself, and not some bound compound, activates microglia .....	52
4.3.3.1.	High temperature inactivation of $\alpha$ 2M release-inducing potency.....	52
4.3.3.2.	Partial neutralization of $\alpha$ 2M activity upon immunoprecipitation with anti- $\alpha$ 2M.....	54
4.3.3.3.	Protease treatment leads to a decline in $\alpha$ 2M stimulating activity.....	56

4.3.3.4. Fast Performance Low Chromatography (FPLC) of $\alpha$ 2M preparation.....	57
4.3.3.5. High Molecular Weight co-isolates are carriers of $\alpha$ 2M stimulating activity.....	58
<b>5. Discussion.....</b>	<b>59</b>
5.1. Microglia <i>in vitro</i> versus <i>in vivo</i> .....	59
5.2. Activity through production and release of numerous soluble factors; LPS as a model inducer of microglial release activity.....	60
5.3. A $\beta$ as a weak activator of microglial release activity <i>in vitro</i> .....	62
5.3.1 Structure and conformation of A $\beta$ protein influence its potency to induce microglial activation.....	63
5.4. Putative synergistic co-stimuli of A $\beta$ microglial activation in AD.....	65
5.4.1 Interleukin-1 $\beta$ as an AD relevant factor and its role as a co-stimulatory partner of A $\beta$ in microglia mediated chronic inflammation.....	66
5.4.2 $\alpha$ 2M as a microglial activator and an A $\beta$ 's partner in synergistic stimulation of microglia.....	67
5.5. Relevance of factors secreted from activated microglia for AD pathology.....	71
5.5.1. Cytokines (TNF- $\alpha$ , IL-6).....	71
5.5.2. Chemokines (MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$ , KC).....	73
5.6. Inflammatory mediators as therapeutic targets- use of NSAIDs.....	77
<b>6. Summary.....</b>	<b>78</b>
<b>7. References.....</b>	<b>79</b>
Acknowledgements.....	98
Curriculum Vitae.....	99
List of publications.....	100
Poster and oral presentations.....	100
Erklärung.....	102

## I. List of figures

Figure 1. Morphology of “rested” and activated microglia in organotypic tissue culture.....	9
Figure 2. Primary structure of 1-40 and 1-42 amyloid $\beta$ protein.....	14
Figure 3. IL-1: intracellular actions.....	16
Figure 4. Intracellular signaling cascade upon LPS stimulation.....	22
Figure 5. A $\beta$ peptide preparation protocol.....	30
Figure 6. Microglia in primary cell culture.....	35
Figure 7. Cytokine expression in microglia following LPS stimulation as detected with RT-PCR.....	36
Figure 8. LPS-induced release of cyto- and chemokines from microglia <i>in vitro</i> .....	37
Figure 9. Amount of A $\beta$ peptide in monomeric and oligomeric form over increasing incubation time period.....	38
Figure 10. Fibril formation of aged A $\beta$ mixture.....	40
Figure 11. Inducing potency of fresh vs. aged A $\beta$ 1-40/ A $\beta$ 1-42 mixtures.....	41
Figure 12. Cyto/chemokine release from microglia upon exposure to different ratios of aged A $\beta$ 1-40/ A $\beta$ 1-42 mixture.....	42
Figure 13. Chemokine and cytokine release upon A $\beta$ + IL-1 $\beta$ co-stimulation of microglia.....	43
Figure 14. IL-1 $\beta$ enhances A $\beta$ induced cyto/chemokine release.....	44
Figure 15. Cyto/chemokine release upon A $\beta$ + IL-18 co-stimulation of microglia.....	45
Figure 16. IL-6 does not amplify A $\beta$ -potency to evoke cyto/chemokine release from microglia.....	46
Figure 17. NO release following microglial exposure to A $\beta$ alone and in combination with IL-1 $\beta$ , IL-18 or LPS.....	47
Figure 18. $\alpha$ 2M strongly induces cyto-/chemokine release from microglia .....	48
Figure 19. Induction of microglial NO production by $\alpha$ 2M.....	49
Figure 20. Enhanced cyto/ chemokine release upon $\alpha$ 2M + A $\beta$ co-stimulation of microglia.....	50
Figure 21. Synergistic effect of $\alpha$ 2M+A $\beta$ co-stimulation .....	51

Figure 22. $\alpha$ 2M stimulation does not result in microglial proliferation.....	52
Figure 23. Loss of $\alpha$ 2M release-inducing activity upon treatment with high temperatures indicates protein nature of the activator.....	53
Figure 24. Immunoprecipitation of $\alpha$ 2M with anti-human- $\alpha$ 2M antibody results in diminished inducing activity.....	55
Figure 25. $\alpha$ 2M potency to induce microglial cyto-chemokine release attenuates after a treatment by chymotrypsin.....	56
Figure 26. Purity of $\alpha$ 2M as assessed by FPLC.....	57
Figure 27. Microglia stimulation with $\alpha$ 2M filtration fractions confirmed that activity is carried by high molecular weight material.....	58
Figure 28. Inflammatory network in the proximity of amyloid deposits.....	76

## **II. List of abbreviations**

A $\beta$ - amyloid- $\beta$  peptide

AchE- acetylcholinesterase

AD- Alzheimer disease

$\alpha_2$ M- alpha 2-macroglobulin

APC- antigen-presenting cell

AP-1- activating protein-1

ApoE- apolipoprotein E

APP-  $\beta$ -amyloid precursor protein

BDNF- brain-derived neurotrophic factor

BrdU- 5-bromo-2'-deoxyuridine

CD- cluster of differentiation

CNS- central nervous system

CSF- cerebral spinal fluid

CT- chymotrypsin

DNA- deoxyribonucleic acid

e.g.- for example, exempla gratia

ELISA- Enzyme Linked Immunosorbent Assay

ERK 1/2- extracellular signal-regulated kinase p42/44

FPLC- Fast Performance Liquid Chromatography

GPI- glycosylphosphatidylinositol

GRO $\alpha$ /KC- growth regulated oncogene alpha

HMW- high molecular weight

ICE/caspase-1- interleukin 1 $\beta$  converting enzyme

I $\kappa$ B- inhibitor of NF $\kappa$ B

IL- interleukin

IL-1R- interleukin-1 receptor

INF $\gamma$ - interferon-gamma

iNOS- nitric oxide synthase

IP-10- gamma interferon inducible protein-10

IRAKs- interleukin-1 receptor-associated kinases  
JNK- c-Jun N-terminal kinase  
LPB- LPS-binding protein  
LMW- low molecular weight  
LPS- lipopolysaccharide  
LRP- lipoprotein receptor-related protein  
MAPKs- mitogen-activated protein kinases  
MCP-1- monocyte chemoattractant protein-1  
M-CSF- macrophage colony stimulating factor  
MD2- message digest no. 2  
MDC- macrophage derived chemokine  
MHC II- class II major histocompatibility  
MIP- macrophage inflammatory protein  
mRNA- messenger ribonucleic acid  
MyD88- myeloid differentiation primary response gene 88  
NF $\kappa$ B- Nuclear Factor kappa B  
NGF- nerve growth factor  
NO- nitric oxide  
NSAIDs- non-steroidal anti-inflammatory drugs  
PAMPs- pathogen-associated microbial patterns  
PI 3-kinase- phosphoinositide 3-kinase  
PPARs- peroxisome proliferator-activated receptors  
PTX- pertussis toxin  
RANTES- regulated on activation, normal T cell expressed and secreted  
RAP- receptor associated protein  
RT-PCR- reverse transcriptase polymerase chain reaction  
TGF $\beta$ - transforming growth factor beta  
TIR- Toll/IL-1 receptor  
TLR- Toll-Like receptor  
TNF $\alpha$ - tumor necrosis factor alpha  
TRAF- tumor necrosis factor receptor-associated factor

## **Acknowledgements**

I would like to thank all organizers and professors of the Graduate School GRK 238 “Damage cascades in neurological disorders: studies with imaging techniques” for giving me a chance to take part in this graduate program and get scientific and financial support during my doctoral studies.

In particular I wish to thank the head of the Program, Prof. Dr. Uwe Heinemann, for his great help and valuable advices.

I would like to thank Prof. Dr. Helmut Kettenmann for giving me opportunity to work on this particular topic in his laboratory for Cellular Neurosciences, at MDC, Berlin-Buch.

Many thanks to Prof. Dr. Uwe-Karsten Hanisch for his suggestions, advices and critical comments.

I thank Prof. Dr. Michael Bienert and his colleagues from FMP, for a nice collaboration.

I also wish to thank all former and present members and technical stuff of Cellular Neuroscience Department for help, friendship and support. Especially, I would like to thank Birgit Jarchow for all administrative help, and Gerda Müller and Irene Haupt for their excellent technical work.

Much gratitude to my parents and family in Belgrade for support and full understanding during my doctoral work.

Finally, many thanks to my little son Uros, for giving me so much love and motivation.

**„Mein Lebenslauf wird aus Datenschutzgründen in der elektronischen Version meiner Arbeit nicht mit veröffentlicht.“**

## **PUBLICATIONS:**

Karl Georg Häusler, **Sanja Pavlovic**, Katharina Mertsch, Nico van Rooijen, Joerg R. Weber, Helmut Kettenmann, Uwe-Karsten Hanisch. Cytokine and chemokine release regulation in mixed astro/microglial populations: cell type-specific contributions, reciprocal influences and coordinated control by interferon- $\gamma$ . *Submitted*.

Eva M. J. Peters, Ulrike Raap, Sven Hendrix, **Sanja Pavlovic Masnikosa**, Pia Welker, Carlos Pincelli. Neurotrophins act as neuroendocrine regulators of skin homeostasis in health and disease. Review. *Hormone and Metabolic Research. In press*.

**Pavlovic S**, Schulze G, Wernicke C, Bonnet R, Gille G, Badiali L, Kaminska A, Lorenc-Koci E, Ossowska K, Rommelspacher H. 2,9-Dimethyl-beta-carbolinium, a neurotoxin occurring in human brain, is a potent inducer of apoptosis as 1-methyl-4-phenylpyridinium. *Neuroscience*. 2006 Mar 3.

Ragnhild Bonnet, **Sanja Pavlovic**, Jochen Lehmann, and Hans Rommelspacher. The strong inhibition of triosephosphate isomerase by the natural  $\beta$ -carbolines may explain their neurotoxic actions. *Neuroscience*. 127 (2004) 443-453.

Kuhn SA, van Landeghem FK, Zacharias R, Farber K, Rappert A, **Pavlovic S**, Hoffmann A, Nolte C, Kettenmann H. Microglia express GABA(B) receptors to modulate interleukin release. *Mol Cell Neurosci*. 2004 Feb;25(2):312-22.

Boucsein C, Zacharias R, Farber K, **Pavlovic S**, Hanisch UK, Kettenmann H. Purinergic receptors on microglial cells: functional expression in acute brain slices and modulation of microglial activation in vitro. *Eur J Neurosci*. 2003 Jun;17(11):2267-76.

## **POSTER and ORAL PRESENTATIONS:**

**S. Pavlovic Masnikosa**, M. Daniltschenko, S. Blois, B.F. Klapp, E.M. Peters. *Stress modulates the function of antigen presenting cells in the skin*. 2<sup>nd</sup> International Alfrried Krupp Kolleg Symposium; Stress-Behaviour-Immune response, 2006. Greifswald, Germany.

**S. Pavlovic Masnikosa**, A. Orsal, M. Daniltschenko, B.F. Klapp, E.M. Peters. *Sonic stress exposure causes behavioural changes in a mouse model of experimental allergic dermatitis*. 2<sup>nd</sup> International Alfrried Krupp Kolleg Symposium; Stress-Behaviour-Immune response, 2006. Greifswald, Germany.

**S. Pavlovic Masnikosa**, A. Orsal, M. Daniltschenko, B.F. Klapp, E.M. Peters. *Behavioural changes as a response to stress exposure in a mouse model of experimental allergic dermatitis*. XXXIII Jahrestagung der Arbeitsgemeinschaft Dermatologische Forschung- ADF, 2006. Aachen, Germany. Poster presentation.

**Sanja Pavlovic**: *Neurotoxic effect of  $\beta$ -carbolines - putative underlying mechanisms*  
*Final Symposium. GRK 238 "Damage cascades in neurological disorders - studies with imaging techniques"* Berlin, Germany, 2004. Oral presentation

**Sanja Pavlovic**: *2,9-Dimethyl- $\beta$ -carbolinium is a natural neurotoxin equipotent as 1-methyl-4-phenylpyridinium*. Meeting of German-Polish Society for Investigation of pathomechanisms of Parkinson's disease and search for neuroprotective therapies. 2004. Krakow, Polen. Oral presentation

Karl Georg Häusler, **Sanja Pavlovic**, Katharina Mertsch, Nico van Rooijen, Helmut Kettenmann, Uwe-Karsten Hanisch. *Cytokine and chemokine release regulation in mixed astromicroglial populations: cell type-specific contributions, reciprocal influences and coordinated control by interferon- $\gamma$* . 4th Forum of European Neuroscience- FENS. 2004, Lisbon, Portugal. Poster presentation.

**Sanja Pavlovic**, Ragnhild Bonnet, Jochen Lehmann, and Hans Rommelspacher. *The strong inhibition of triosephosphate isomerase by the natural  $\beta$ -carbolines may explain their neurotoxic actions.* Berlin Neuroscience Forum 2004, BNF 2004. Liebenwalde, Germany. Poster presentation.

SA. Kuhn, F. van Landeghem, R. Zacharias, A. Rappert, **S. Pavlovic**, A. Hoffmann, C. Nolte, H.C. Kornau, H. Kettenmann. *Activation of Microglial GABA<sub>A</sub> receptors modifies the immunological response.* 32th Annual Meeting of Society for Neurosciences, Orlando, Florida, USA, 2002. Poster presentation.

**S. Pavlovic**, H. Kettenmann, U.-K. Hanisch. *Purinergic Receptor Activation Attenuates the LPS-induced Cyto- and Chemokine Production in Cultured Microglia Cells.* III Federation of European Neuroscience Society-FENS Meeting, Paris, France. 2002. Poster presentation.

**S. Pavlovic**, U.-K. Hanisch, H. Kettenmann. *Purinergic Receptor Activation Attenuates the LPS-induced Cyto- and Chemokine Production in Cultured Microglia Cells.* Berlin Neuroscience Forum, 2002. Poster Presentation

**S. Pavlovic**, K. Mertsch, H. Kettenmann, U.K. Hanisch. *Organotypic Culture of Mouse Retina- a model for studying microglial activation.* 31th Annual Meeting of Society for Neuroscience, San Diego, California, USA, 2001. Poster Presentation

**S. Pavlovic**, K. Mertsch, J. Schnitzer, H. Kettenmann, U.K. Hanisch. *Organotypic Culture of Mouse Retina- a model for studying microglial activation.* 28<sup>th</sup> Gottingen Neurobiology Conference; 4<sup>th</sup> Meeting of the German Neuroscience Society, 2001. Poster Presentation

**S. Pavlovic**, K. Mertsch, J. Schnitzer, H. Kettenmann, U.K. Hanisch. Organotypic Culture of rodent Retina as a model for studies of microglial activation. DFG-Schwerpunkt Meeting, Role of Microglia Cells in CNS Disease. 2000. Poster Presentation

## **Erklärung**

„Ich, Sanja Pavlovic Masnikosa, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: „Microglial activation in Alzheimer's pathology: Role of disease relevant proteins in a synergistic stimulation context“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Berlin, den 03.12. 2006

Unterschrift