Microglial cells in Neurodegenerative Diseases. The Role of Galectin-3

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Microglial cells in Neurodegenerative diseases

The role of Galectin-3

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Abstract

Today, dementia such as, Alzheimer's disease (AD), vascular diseases and motor neurodegenerative diseases, such as Parkinson's disease (PD), represent a major public health problem. For instance, PD affect to more than 10 million people worldwide and almost 50 million people are affected by dementia. In fact, every year 9,9 millions new patients are diagnosed. Dementia is one of the major causes of disability among elderly people. The mechanisms affecting the disease progression for PD and AD share some common mechanism, such as the immune responses in the neural tissue. The immune system plays a major role in restoring the balance in our organism after injury and is divided in innate and adaptive immunity. The innate immune response is considered the first mechanism responding to the disease stimulus. The activation of the innate immune response can be triggered by different factors and elicit the activation of several cell types. Among the main cell types involved in the innate immune response, we find: microglial cells, astrocytes and oligodendrocytes. Following the innate immune action, the adaptive immune system is activated and B and T cells are recruited by antigen presenting cells to act on the response. The main task of the inflammatory response is to restore the tissue homeostasis after insult. The nature of the insult can vary, going from pathogens to tissue damage. To resolve the injury and restore the balance in the organism, these cells types can secrete a wide array of molecules, such as pro and anti-inflammatory molecules, growth factors and chemokines, all of them involved in the regulation of the innate immune response. The main cell type involved in the inflammatory response in the brain are microglial cells. They are considered the "macrophages of the brain". Microglial cells can develop different functions such as: phagocytosis, synaptic remodeling or opsonization. Hence, microglial cell activation is essential for the well function of the brain in disease and healthy brain. One of the main receptors involved in microglial activation are the Toll like receptors (TLR's). These receptors can recognize, and be activated, by different molecules derived from injured/damages tissue or pathogen derived molecules. Between the different TLR's, TLR4 is one of the most important due to its capacity to sense bacteria-derived molecules triggering the immune response. Our working hypothesis is focused on the role of the inflammatory response in neurodegenerative diseases with special attention on galectin-3 in the neurodegenerative diseases such as AD and PD. Galectin-3 is a molecule mainly released by microglial cells and involved in different functions including: phagocytosis, microglial activation and cell proliferation.

In the present work, we describe for the first time galectin-3 acting as an endogenous ligand for TLR4 driving the microglial activation towards to a proinflammatory profile. Moreover, the lack of galectin-3 profoundly reduces the microglial activation that might affects to the progression of PD and AD. Furthermore, in our work we found galectin-3 acting as a Triggering Receptor in Myeloid Cells 2 (TREM2) ligand. TREM2 is the main innate immune-related risk factor in Alzheimer's disease and it is involved in microglial activation, phagocytosis and plaque deposition in Alzheimer disease. Moreover, human TREM2 mutations, such as R47H, are related to a higher susceptibility to developed AD. Despite our efforts, further experiments will be necessary to fully elucidate the role of galectin-3 and its interaction with TREM2 in AD.

Despite the before mentioned, when the inflammatory response start is not well known. In our research line, we aimed to study if the inflammatory response is already present before the typical signs of Alzheimer disease pathology appears. To that aim, we studied the microglial proteomic profile in microglial cells before and after the plaque deposits. We used a specific AD mouse model and we discovered an altered innate immune response already present before the plaque deposition.

In summary, during my work, we have been able to identify an inflammatory role of galectin-3 in PD and AD, with special attention on the role of galectin-3 in the inflammatory response in relation with TLR4 and TREM2 signaling. Furthermore, we evaluated the proteomic profile of microglial cells isolated from AD mouse model before and after the amyloid beta plaque deposits and we found important inflammatory pathways and innate immune proteins altered even before the deposition of the first plaques.

We hope our findings will be further investigated and hopefully be useful to find new potential therapeutic targets and elucidate inflammatory-related mechanisms in neurodegenerative diseases.

Key words Microglia, Alzheimer's disease, Parkinson's disease, Galectin-3, neuroinflammation, innate immune system, and neurodegeneration

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Cover picture by Daniel Tornero. Microglial cells expressing galectin-3 (green) surrounding amyloid beta plaques (in red).

Cover photo by Daniel Tornero

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A mi familia.

"Pensamiento crítico es entender
que lo que existe no agota las
posibilidades de la existencia."

Boaventura De Sousa Santos

"Si la razón hace al hombre, el sentimiento lo conduce."

Jean Jacques Rousseau

Table of Content

List of publications

This thesis is based on the following papers, which will be referred to in the text by their roman numerals.

I. *Microglia-Secreted Galectin-3 Acts as a Toll-like Receptor 4 Ligand and Contributes to Microglial Activation.* Miguel Angel Burguillos, Martina Svensson, Tim Schulte, **Antonio Boza-Serrano**, Albert Garcia-Quintanilla, Edel Kavanagh, Martiniano Santiago, Nikenza Viceconte, María Jose Oliva-Martin, Ahmed Mohamed Osman, Emma Salomonsson, Lahouari Amar, Annette Persson, Klas Blomgren, Adnane Achour, Elisabet Englund, Hakon Leffler, Jose Luis Venero, Bertrand Joseph, and Tomas Deierborg. **Cell Reports** 10, 1–13 March 10, 2015. Cell Press

II. *The role of Galectin-3 in α-synuclein-induced microglial activation*. **Antonio Boza-Serrano** A, Reyes JF, Rey NL, Leffler H, Bousset L, Nilsson U, Brundin P, Venero JL, Burguillos MA, Deierborg T. **Acta Neuropathol Commun**. 2014 Nov 12; 2:156.

III. *Innate immune alterations are elicited in microglial cells before the plaque deposition in 5xFAD mice*. **Antonio Boza-Serrano**, Yiyi Yang, Agnes Paulus and Tomas Deierborg. **Scientific Reports (in Publication)**

IV. *Galectin-3 is up regulated in Alzheimer's disease and contributes to the pathology in 5xFAD mice.* **Boza-Serrano, A**; Ruíz, Rocío; Sánchez-Varo, Raquel; Yang, Yiyi; Paulus, Agnes; Vilalta, Anna; Wenstrom, Mallin, Dunning, Christopher; García, Juan; Stegmayr, John; Jiménez, Sebastian; Garrido-Navarro, Victoria; Miguel-Real, Luis; Englund, Elisabeth; Linsen, Sara; Leffler, Hakon; Nilsson, Ulf; Brown, Guy; Gutierrez, Antonia; Vitorica, Javier; Venero, JL; Deierborg, Tomas. (*Manuscript submitted*).

Additional peer-reviewed papers, not included in the thesis

I. *Alpha-Synuclein Expression in the Oligodendrocyte Lineage: an In Vitro and In Vivo Study Using Rodent and Human Models*. Mehdi Djelloul, Staffan Holmqvist, **Antonio Boza-Serrano**, Carla Azevedo, Maggie S. Yeung, Stefano Goldwurm, Jonas Frisen, Tomas Deierborg and Laurent Roybon. **Stem Cell Reports**. 2015 Aug 11;5(2):174-84

II. *Change in autoantibody and cytokine responses during the evolution of neuromyelitis optica in patients with systemic lupus erythematosus: A preliminary study*. Kovacs KT, Kalluri SR, **Boza-Serrano A**, Deierborg T, Csepany T, Simo M, Rokusz L, Miseta A, Alcaraz N, Czirjak L, Berki T, Molnar T, Hemmer B, Illes Z. **Multiple Sclerosis Journal**, 2015

III. *Galectin-3 causes enteric neuronal loss in mice after left sided permanent middle cerebral artery occlusion, a model of stroke*. Xiaowen Cheng, **Antonio Boza-Serrano**, Michelle Foldshak-Turesson, Tomas Deierborg, Eva Ekblad, Ulrikke Voss. **Sci Rep**. 2016 Sep 9;6:32893. doi: 10.1038/srep32893.

IV. *Forced treadmill exercise can induce stress and increase neuronal damage in a mouse model of global cerebral ischemia*. Martina Svensson, Philip Rosvall, **Antonio Boza-Serrano**, Jan Lexell and Tomas Deierborg. **Neurobiology of Stress**, Septembre 2016.

V. *Interleukin-6 is increased in plasma and cerebrospinal fluid of community-dwelling domestic dogs with acute ischaemic stroke*. Gredal, Hanne; Thomsen, Barbara B.; **Boza-Serrano, Antonio**; Garosi, Laurent; Rusbridge, Clare; Anthony, Daniel; Møller, Arne; Finsen, Bente; Deierborg, Tomas; Lambertsen, Kate L.; Berendt, Mette. **NeuroReport**, January, 2017. (doi: 10.1097/WNR.0000000000000728)

VI. *Fumarate decreases edema volume and improves functional outcome after experimental stroke*. Bettina Hjelm Clausen; Louise Lundberg; Minna Yli-Karjanmaa; Nellie Anne Martin; Martina Svensson; Maria Zeiler Alfsen; Simon Bertram Flæng; Kristina Lyngsø; **Antonio Boza-Serrano**; Helle Hvilsted Nielsen; Pernille B Hansen; Bente Finsen; Tomas Deierborg; Zsolt Illes. June 2017, Experimental Neurology.

Additional papers in publication-process

I. *Inflammation affects regulation of microglial extracellular vesicles via TNF pathway*. Yiyi Yang, **Antonio Boza-Serrano**, Christopher J R Dunning, Kate Lykke Lambertsen, Tomas Deierborg. (Manuscript in Publication, Journal of Neuroinflammation).

II. Ulcerative colitis induces the activation of microglia in the ventral mesencephalon along with dopaminergic neuronal death. Rocío M. de Pablos, Ana M. Espinosa-Oliva, **Antonio Boza-Serrano**, Manuel Sarmiento, Rocío Ruiz, Martiniano Santiago, María José Oliva-Martín, María Angustias Roca-Ceballos, Sebastian Serres, Vasiliki Economopoulus, Antonio J. Herrera, Nicola R. Sibson, Alberto Machado, José Luis Venero. (Manuscritp in Publication, Acta Neuropathologica)

Abbreviations

APP	Amyloid precursor protein
ABCA7	ATP binding cassette subfamily A member 7
AD	Alzheimer's disease
ApoE	Apolipoprotein E
Aβ	Amyloid beta
BBB	Blood brain barrier
CD	Cluster of differentiation
CNS	Central nervous system
CRD	Carbohydrate recognition domain
CSF	Cerebrospinal fluid
DAMP's	Danger-associated molecular patterns
DLB	Dementia of Lewy's bodies
DNAJC13	DnaJ homolog subfamily C member 13
GBA	Glucocerebrosidase
GWAS	Genome-wide associated studies
IDE-1	Insulin degradative enzyme 1
IFN-γ	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide syntase
JNK	Jun N-terminal kinase
KO	Knockout
LPS	Lipopolysaccharide
LRRK2	Leucine-rich repeat kinase 2
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NFT	Neurofibrillary tangles

NFκβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	Nod-like receptor protein 3
NOS	Nitric Oxide Synthase
PAMP's	Pathogen-associated Molecular Patterns
PD	Parkinson's disease
PICALM	Phosphatidylinositol binding clathrin assembly protein
PRR's	Pattern recognition receptors
PSEN1, 2	Presenilin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
siRNA	Small interference RNA
SNCA	Alpha synuclein
SNpc	Substansia nigra pars compacta
STORM	Stochastic optical reconstruction microscopy
TEM	Transmission electron microscopy
Thio-S	Thioflavin S
TLR	Toll-like Receptor
TNF-α	Tumor necrosis factor alpha
TREM2	Triggering receptor expressed on myeloid cells 2
VPS35	Vacuolar protein sorted associated 35
WT	Wild type
YLD	Estimated Years of life with Disability

Popular summary

Today, dementia such as, Alzheimer's disease (AD), vascular diseases and motor neurodegenerative diseases, such as Parkinson's disease (PD), represent a major public health problem. For instance, PD affect to more than 10 million people worldwide and almost 50 million people are affected by dementia. In fact, every year 9,9 millions new patients are diagnosed. Dementia is one of the major causes of disability among elderly people. The mechanisms affecting the disease progression for PD and AD share some common mechanism, such as the immune responses in the neural tissue. The immune system plays a major role in restoring the balance in our organism after injures and is divided in innate and adaptive immunity. The innate immune response is considered the first mechanism responding to the disease stimulus. The activation of the innate immune response can be triggered by different factors and elicit the activation of several cell types. Among the main cell types involved in the innate immune response, we find: microglial cells, astrocytes and oligodendrocytes. Following the innate immune action, the adaptive immune system is activated and B and T cells are recruited by antigen presenting cells to act on the response. The main task of the inflammatory response is to restore the tissue homeostasis after insult. The nature of the insult can vary, going from pathogens to tissue damage. To resolve the injury and restore the balance in the organism, these cells types can secrete a wide array of molecules, such as pro and anti-inflammatory molecules, growth factors and chemokines, all of them involved in the regulation of the innate immune response. The main cell type involved in the inflammatory response in the brain are microglial cells. They are considered the "macrophages of the brain". Microglial cells can develop different functions such as: phagocytosis, synaptic remodeling or opsonization. Hence, microglial cell activation is essential for the well function of the brain in disease and healthy brain. One of the main receptors involved in microglial activation are the Toll like receptors (TLR's). These receptors can recognize, and be activated, by different molecules derived from injured/damages tissue or pathogen derived molecules. Between the different TLR's, TLR4 is one of the most important due to its capacity to sense bacteria-derived molecules triggering the immune response. Our working hypothesis is focused on the role of the inflammatory response in neurodegenerative diseases with special attention on galectin-3 in the neurodegenerative diseases such as AD and PD. Galectin-3 is a

molecule mainly released by microglial cells and involved in different functions including: phagocytosis, microglial activation and cell proliferation.

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Resumen

Actualmente, enfermedades relacionadas con la pérdida de las capacidades cognitivas (demencias), como la enfermedad de Alzheimer o el accidente cerebro vascular, y enfermedades neurodegenerativas motoras como la enfermedad de Parkinson suponen un problema de salud pública debido a sus enormes implicaciones sociales y económicas. Para poner en perspectiva el impacto social de estas enfermedades basta valorar las cifras de personas afectadas. Más de 10 millones de personas están afectadas por la enfermedad de Parkinson en todo el mundo, más de 50 millones de personas están afectadas por demencias y cada año casi 10 millones de personas son diagnosticadas por enfermedades relacionadas con el deterioro cognitivo. Dentro de la familia de las demencias, la enfermedad de Alzheimer supone una de las causas principales de discapacidad y dependencia entre las personas ancianas. A pesar de que los mecanismos que influyen en enfermedades como son el Parkinson o el Alzheimer son variados, tienen ciertas similitudes, como por ejemplo, la muerte neuronal, la acumulación de proteínas o la respuesta del sistema inmune.

La principal tarea del sistema inmune es restablecer el funcionamiento del organismo después de sufrir daño. La respuesta del sistema inmune se divide en la respuesta innata y la respuesta adaptativa. En nuestro estudio, nos centramos principalmente en la respuesta inmune innata en el sistema nervioso central. La respuesta inmune innata es la primera acción del sistema inmune frente al daño, ya sea de carácter externo, por ejemplo, un traumatismo, o un daño interno, originado por la acumulación de proteínas malplegadas en enfermedades como el Parkinson o el Alzheimer. La respuesta del sistema inmune tiene su traducción en la activación de los tipos celulares que lo componen. Entre los diferentes tipos celulares que tienen una participación activa en la respuesta del sistema inmune innato encontramos: las células microgliales, los astrocitos y los oligodendrocitos. La acción de estos tipos celulares resuelven las situaciones originadas en el organismos a raíz del daño infringido. Para llevar a cabo dicha tarea disponen de un amplio abanico de herramientas, entre las que se encuentran: citokinas pro y anti inflamatorias, factores de crecimiento, neurotransmisores…todas ellas son capaces de orquestar las respuesta y conducirla hacia una resolución positiva del daño.

La acción que desarrollan las células microgliales en enfermedades neurodegenerativas es una de las cuestiones principales de nuestra investigacion.

Las células microgliales son una suerte de "policía" del sistema nervioso central. Llevan a cabo funciones de vigilancia continua, ayudando a otros tipos celulares a llevar a cabo sus funciones o detectando posibles daños, dando inicio a la respuesta inmune. Entre las funciones más notorias que pueden llevar a cabo podemos destacar su capacidad para: ayudar a la formación de nuevas conexiones neuronales, regular la respuesta inflamatoria, eliminar restos de células muertas y dar soporte a diferentes funciones neuronales. Entender de qué forma las células microgliales se activan en enfermedades neurodegenerativas es uno de los principales motores que guía nuestra investigación. En particular, estamos interesados en la activación de la microglía a través de los denominados receptores Toll (TLRs). De manera particular, nuestro trabajo se centra en el receptor TLR4, implicado en la activación de la forma pro-inflamatoria de la microglial y que se expresa en su membrana cellular externa. Además de TLR4, otra molécula es objeto de estudio en nuestro trabajo, la denominada galectina-3. La galectina-3 se expresa principalmente por las células microglíales, puede ser liberada al exterior celular y desarrolla diferentes funciones, a saber: activación celular, proliferación o fagocitosis.

Dado lo anteriormente descrito, nuestra hipótesis de trabajo se centra en entender como se activa la microglía, a través de TLR4 y galectina-3, en el contexto de una enfermedad neurodegenerativa y el impacto que tendría en el desarrollo de la enfermedad neurodegenerativa modular la respuesta inflamatoría relacionada con la activación de la microglia.

En el presente trabajo, mostramos por primera vez la interacción entre galectina-3 y TLR4, dando lugar dicha interacción a la activación de la microglia en su perfil pro inflamatorio. Durante nuestra investigación descrubimos que cuando inhibimos galectina-3 reducimos drásticamente la activación de la microglía lo que se traduce en una reducción de la respuesta inflamatoria, lo cuál podría implicar una reducción en la progresión de enfermedades neurodegenerativas, tales como, Parkinson o Alzheimer. Además, en nuestro trabajo hemos estudiado la interacción entre galectina-3 y el receptor TREM2. TREM2 se erige en uno de los principales factores de riesgos relacionados con la enfermedad de Alzheimer, en relación con la respuesta del sistema inmune. La actividad de TREM2 está implicada en procesos fagocíticos, de activación de la microglía y en estudios más recientes, en la formación de placas amiloides, muy características de la enfermedad de Alzheimer. No obstante, más experimentos son necesario para dilucidar en qué grado la interacción entre galectina-3 y TREM2 es importante en el desarrollo de la enfermedad de Alzheimer y en la activación de la microglía.

Otro de los aspectos tratados en nuestro trabajo es el relacionado con los mecanismos implicados en la respuesta inflamatoria temprana en la enfermedad de Alzheimer. Actualmente no hay dudas acerca de la implicación de las placas amiloides y de las fibrillas de proteína tau en la progresión de la enfermedad. No obstante, el papel que juega la respuesta inflamatoria genera ciertas preguntas. En esta última parte de nuestro trabajo hemos tratado de determinar si la respuesta inflamatoria está presente en los primeros estadios de la enfermedad, incluso antes de que aparezcan los primeros depósitos de proteína amiloide. Para llevar a cabo este estudio aislamos células microgliales de ratones con la enfermedad de Alzheimer; antes, durante y después de la aparición de las placas de proteína amiloide con el objetivo de estudiar su perfil protéico. Con los experimentos realizados hemos sido capaces de confirmar que la respuesta inmune se encuentra alterada justo antes de que las primera placas aparezcan.

En resumen, en nuestro trabajo hemos identificado la interacción entre galectina-3 y TLR4 en la activación microglial en relación con la respuesta inflamatoria en enfermedades neurodegenerativas. Hemos identificado a la galectina-3 como una proteína fundamental en el desarrollo de la enfermedad de Alzheimer, además de su posible interacción con TREM2, otro factor de riesgo en la enfermedad de Alzheimer.

Abordando la enfermedad de Alzheimer desde otro punto de vista, hemos estudiado el perfil protéico de las células microgliales en relación con la formación de los depósitos de proteína amiloide confirmando la alteración de la respuesta inmune desde los primeros estadios de la enfermedad de Alzheimer.

Los principales hallazgos descritos en nuestro trabajo forman la base sobre la que seguir trabajando para dilucidar nuevos mecanismos relacionados con el origen y el avance de la enfermedad de Alzheimer así como una herramienta a partir de la cual desarrollar terapias que tenga como principal objetivo contrarrestar el avance de la inflamación y sus efectos perjudiciales en enfermedades neurodegenerativas.

Populärvetenskaplig sammanfattning

Neurodegenerativa åkommor, däribland Alzheimers och Parkinsons sjukdom och stroke, är några av våra folksjukdomar och innebär stora belastningar för sjukvården. Globalt är 10 miljoner människor drabbade Parkinsons sjukdom, uppemot 50 miljoner människor lider av olika demenssjukdomar, och ytterligare 9,9 miljoner får demensdiagnoser varje år. Demenssjukdomar är då bland de största orsakerna till handikapp och assistansberoende för våra äldre. Sjukdomsförloppet varierar mellan de olika patologierna, men de har vissa likheter – såsom mekanismer relaterade till immunförsvaret.

Immunsystemet ska återställa den fysiologiska balansen efter skada, och delas in i det ospecifika och det adaptiva immunförsvaret. Det ospecifika immunförsvaret är den första reaktionen mot sjukdomsstimulus, och detta kan leda till, genom triggande signalmolekyler, aktivering av olika celltyper. Mikroglia, astrocyter, oligodendrocyter, B och T-celler är exempel på celltyper som är involverade i det ospecifika immunsystemet i hjärnan. Den huvudsakliga uppgiften för immunförsvaret är att återställa vår homeostas, och för att lyckas med detta så uttrycks bl. a. pro- och antiinflammatoriska molekyler, tillväxtfaktorer och kemokiner av immuncellerna. Den främsta celltypen i hjärnans immunförsvar är mikroglia – som även har andra funktioner funktioner såsom fagocytos, synaptisk ommodellering och opsonisering. Detta innebär att mikroglians aktivitet är vital även för den friska hjärnan.

De Toll-lika receptorerna (TLR) känner igen och kan bli aktiverade av diverse molekyler och är de främsta receptorerna för mikrogliaaktivering. TLR4 är en av de viktigaste pga. dess förmåga att känna igen bakteriella molekyler som triggar immunförsvaret. Vår hypotes är fokuserad på inflammationens roll, med särskilt fokus på den inflammatoriska molekylen Galektin-3, i neurodegenerativa sjukdomar, såsom Alzheimers och Parkinsons sjukdom. I hjärnan släpps Galektin-3 främst ut av mikrogliaceller som fagocyterar, aktiveras eller prolifererar. I denna avhandling så beskriver vi för första gången Galektin-3 som en naturlig ligand för TLR4. Vi visar att Galektin-3, genom TLR4, driver mikrogliaaktivering till en mer

proinflammatorisk profil. Vidare kan vi också demonstrera att frånvaron av Galektin-3 kraftigt reducerar mikrogliaaktiveringen i Parkinsons och Alzheimers sjukdom. Fortsättningsvis kan vi också visa att Galektin-3 potentiellt kan vara en triggande ligand till receptorn TREM2, vilkens roll i mikrogliaaktivering, fagocytos och plack-lagring gör den till oerhört viktig i Alzheimers sjukdom. Mutationer i TREM2 korrelerar till högre mottaglighet att utveckla Alzheimers och mer forskning krävs för att utreda Galektin-3:s interaktion med TREM2 i denna vanliga demenssjukdom. Att klarlägga när den inflammatoriska reaktionen börjar i Alzheimers sjukdom, och att lyckas identifiera de främsta mekanismerna bakom, är de kanske största utmaningarna i detta fält. Den del av sjukdomsförfarandet som innefattar amyloidbeta-plack och Tau-ackumulering i neurofibriller är väl känt sedan tidigare. Dock är det inte känt exakt när den inflammatoriska responsen uppkommer.

I sista delen av vårt arbete försökte vi utforska om den inflammatoriska processen redan är närvarande i de tidigare skedena i Alzheimers sjukdom. Här tyckte vi att det vore viktigt att se den proteomiska profilen i mikroglia före och efter placklagring i mikroglia-cellerna. Vi använde oss av en specifik musmodell med Alzheimers och fann ett förändrat uttrycksmönster i isolerade mikroglia redan innan placken uppstod. Sammanfattningsvis så fann jag under min avhandling en relevant roll för Galektin-3 i Parkinsons och Alzheimers sjukdom, och då Galektin-3:s del i det inflammatoriska svaret i relation till TLR4 och TREM2. Vi upptäckte också att immunförsvaret hade förändrats i möss med Alzheimers redan innan de första plackavlagringarna kunde ses.

De huvudsakliga fynden i vår forskning kommer att ligga till grund för framtida studier om potentiella terapeutiska målmolekyler och inflammations-relaterade mekanismer som kan användas för att motverka progressionen i de vanligaste neurodegenerativa sjukdomarna, såsom Alzheimers och Parkinsons.

Introduction

Neurological disorders

Worldwide, neurological disorders account for more than 6% of the total burden of disease worldwide (WHO Neurological disorders report, Chapter 2, 2015). Among the neurological disorder, there is a wide range of diseases, ranging from the most common form of dementia, Alzheimer's disease, to meningitis. Neurological disorders constitute 12% of the total deaths globally. As a percentage of the total incident in neurological disorders, Alzheimer's disease contributes to 6,28% of total death, PD with 1,55% and cerebrovascular diseases with 85%. (WHO Neurological disorders report, Chapter 2, 2015)

There are different ways to quantify the impact of neurological disorder on the life-quality of patients. One way to do this quantification is based on the "Estimated Years of life with Disability" (YLDs) as results of the disability. The number of YLDs per 100 000 population associated with neurological disorders is projected to decline from 1264 in 2005 to 1109 in 2030 (WHO report, 2015, Section 3, Table 2.9). This reduction is linked to a decrease in YLDs associated with cerebrovascular disease, infections, nutritional deficiencies, neurological injuries and neuropathies. However, YLDs resulting from AD, and other dementias, are projected to increase by 38% until 2030. Moreover, the lost of YLDs in AD patients is higher in high-income countries, where the disease is more present due to the longer life span (Winblad et al., 2016).

Alzheimer's disease

AD is classified as dementia. Dementia is coming from the latin *dementia,* which means "*away from mind*". Dementia encompasses a range of neurological disorders mainly characterized by memory loss and cognitive impairment. Currently, AD is the leading cause of dementia. The primary risk factor for AD is the age, and therefore the prevalence of the disease is increasing dramatically with the ageing populations worldwide. The most common early clinical symptom observed in AD is the difficulty for patients to remember recent events. Over the

progression of the disease other symptom's emerge, such as: mood swings, confusion, sleep disorders, walking problems, disorientation, and struggle in speech. Hence, AD severely affects the daily life quality of the patients and their relatives.

Aging is the main risk factor for AD and for other dementia, and, as the life expectancy increases the number of people with dementia is rising. In 2015, 47 million people worldwide were estimated to be affected by dementia. The projections predicts 75 millions by 2030 and 131 million affected individuals by 2050 (WHO Neurological disorders report, Chapter 2, 2015)

Epidemiology of Alzheimer's disease

AD with onset before 65 years of age (early-onset AD) accounts for up to 5% of all cases. The majority of the early-onset AD cases are considered familial AD. AD patients with early-onset without any family background (sporadic patients) usually have an older onset of the disease than patients with a reliable familial disease history (Joshi et al., 2012). On the other hand, late-onset sporadic AD is the most common form, accounting for 95% of the cases. Different studies on AD mortality suggest that people developing the disease after the age of 65 survive an average of 3 to 9 years after clinical diagnosis (Ganguli et al., 2005; Helzner et al., 2008). Over the progression of the disease, the clinical deterioration in people with dementia can vary. According to WHO, the early stage of the disease vary from 2 to 3 years with symptoms such as language difficulties or mood changes; the moderate stage can vary from 4 to 5 years presenting increasing speech difficulties, more severe memory impairment and need for some help to complete daily tasks. In the late stage of the disease from the fifth year and onwards, patients experience serious memory disturbances and may require full daily assistance (WHO Neurological disorders report, Chapter 2, 2015). Even though women have increased incident of AD, women with dementia used to live longer than men (Brodaty et al., 2012; Rizzuto et al., 2012). They tend to survive longer in the later stage of the disease. Notably, more than 50% of the dementia cases reach the severe stage within 3 years.

Risk and protective factors

AD and dementias are multifactorial disorders, which are mainly determined by the interaction of genetic and environmental factors. Still, aging is the strongest

risk factor for AD and patients who developed the disease before 65 years of age are only a small portion of the total number of patients.

Besides age, most AD cases are partly related to other risk factor such as: genetic, vascular and metabolic diseases, lifestyle, diet and nutrition and other factors (depression, traumatic brain injury, etc)(**Table 1**). However, there are protective factors and life-style habits that are related with the prevention of AD. For instance, genetic factors, psychosocial factors, moderate exercise, diet and nutritional factors (i.e. Mediterranean diet) have been suggested to be protective and even some pharmacological interventions may curb the disease progression. (**Table 2**)

Alzheimer's disease main risk factors
Genetic factors: Familial aggregation, APOE4 Allele or other susceptibility genes
Vascular Risk and metabolic factors: Atherosclerosis, Cerebral macrovascular and microvascular lesions, Cardiovascular diseases, Diabetes mellitus and pre-diabetes, Midlife hypertension, Midlife overweight and obesity and Midlife high serum cholesterol
Life Style factors: Sedentary lifestyle, Smoking, Heavy alcohol consumption
Diet and nutritional factors: Saturated fats, Hyperhomocysteinaemia, Deficiencies in vitamin B6, B12, and folate
Other factors: Depression, Traumatic brain injury, Occupational exposure (eg, heavy metals, extremely-low-frequency electromagnetic fields) and Infectious agents (eg, herpes simplex virus type I, Chlamydophila pneumoniae, spirochetes)

Table 1 – AD main risk factors

Diabetes increases the risk of developing AD by about 50% (Profenno et al., 2010). Vascular and metabolic risk factors such as: hypertension, high cholesterol levels and high BMI, are linked to increase incidence of AD (Qiu et al., 2010). Life-style related studies on cardiovascular risk factors pointed out to that factors, such as: hypertension, hypercholesterolemia, along with bad life-style habits, *i.e.* smoking, in the middle age, with an increased incidence of AD (Qiu, 2012). Hence, multifactorial interventions in the mid-age against these life-style and cardiovascular-related risk factors would probably be more effective to reduce the risk of AD.

Regular physical activity has been reported to reduce the risk of AD by 40% (Blondell et al., 2014). High education level, high work complexity or mentally stimulating activities are considered protective factors reducing the risk of developing AD (Meng and D'Arcy, 2012). The aforementioned factors are

encompassing the so-called “Brain Cognitive Reservoir”, a term used to describe the factors which may compensate the cognitive decline associated to AD. In these individuals, the cognitive decline does not fully correlate with the progression of the pathology. Furthermore, to have a mediterranean diet has been related to reduce risk to develop AD (Scarmeas et al., 2006).

Alzheimer’s disease main protective factors
Genetic factors: Some genes proposed (eg, APP, APOE ε2 allele)
Psychosocial factors: High education and socioeconomic status, High work complexity, Rich social network and social engagement and Mentally stimulating activity
Life Style factors: Physical activity and Light-to-moderate alcohol intake
Diet and nutritional factors: Mediterranean diet, Polyunsaturated fatty acid and fish related fats, Vitamin B6, vitamin B12, and folate, Antioxidant vitamins (A, C, E) and Vitamin D
Drugs: Antihypertensive drugs, Statins, Hormone replacement therapy and Non-steroidal anti-infl ammatory drugs

Table 2 – AD main protective factors

Alzheimer’s disease biology

In 1906, Alois Alzheimer described the brain pathology of the first patient documented with AD. Since that, progress has been made unraveling the mechanisms associated with the pathology. However, the mechanisms triggering and promoting the progression of the pathology are still unknown. Nowadays, the two main pathological hallmarks linked with AD are the formation of small extracellular protein deposits called amyloid plaques and the formation of phosphorylated TAU fibrils called Neurofibrillary Tangles (NFT). Amyloid plaques are mainly formed by amyloid beta protein (Aβ). Aβ is a fragment resulting from the cleavage of the Amyloid Precursor Protein (APP). Depending on the enzymatic activity involved in the cleavage, it will result in different fragments of the protein with different length; Aβ 38, 40 or 42 amino acids are most abundant fragments after APP cleavage (Chen, 2015). Moreover, the cleaved forms of the Aβ can be present in different structural forms (fibrils, oligomers and monomers). Among the different forms of Aβ, Aβ42 is consider the pathogenic form of the Aβ and it is the results of the catalytic activity of two different enzymes, β-secretase and γ-secretase (Ballard et al., 2011). The Aβ42 accumulation is believed to be toxic and its accumulation over the progression of the disease affects to the neuronal function. Indeed, the neurons surrounding the

Aβ deposits present aberrant formations in their somatic projections, known as dystrophic neurites, which is another feature associated with the pathology (Sanchez-Varo et al., 2012; Trujillo-Estrada et al., 2014).

Another hallmark present in the pathology is the intraneuronal deposit of hyperphosphorylated TAU protein called neurofibrillary tangles. Tau is a microtubule-associated protein, which give stability to its structure to maintain the function of cell cytoskeleton. The phosphorylation of TAU reduces its capacity to bind and stabilize the microtubule, inducing its intracellular aggregation into NFT. In pathogenic conditions, the hyperphosphorylation of TAU proteins results in the disruption of the microtubules, affecting cell function and viability (Braithwaite et al., 2012).

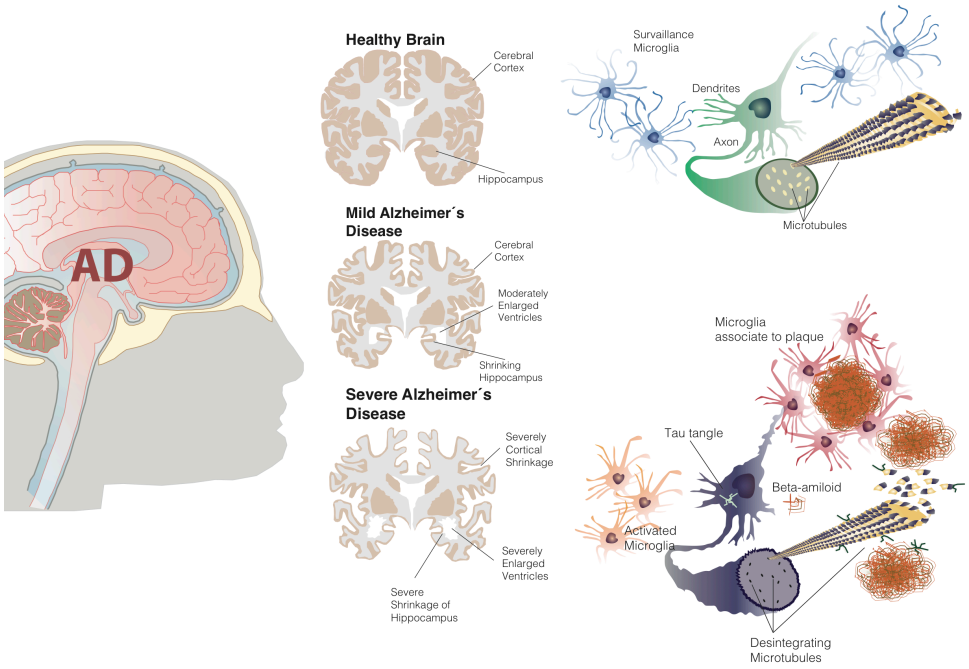
Compelling evidences demonstrate the role of amyloid plaques and NFT in the progression of the disease, both factors are believed to be involved in the neuronal dysfunction leading the neuronal death. To evaluate the levels of NFT formation and Aβ deposition is one of the main clinical tools for AD diagnose and correlates with neuronal death in AD (Andreasen et al., 2001; Hansson et al., 2006; Mattsson et al., 2017). In regards to the progression of AD, tau seems to correlates better with the progressive cognitive decline in AD rather than Aβ deposition (Samgard et al., 2010). This can be linked to the tau and Aβ distribution in the brain, which seems to follow different path, being tau spreding pathways more linked to cognitive-relevant areas of the brain (Hansson et al., 2017).

Aβ plaques and NFT are also present in other neurological disorders such as frontotemporal dementia, where NFT formation is present (Hernandez and Avila, 2007), or cerebral amyloid angiopathy with amyloid deposits in blood vessels (Viswanathan and Greenberg, 2011).

Three different causative mutations have been linked to the early-onset of AD, *i.e.* APP, PSEN1 and PSEN2 genes. These 3 genes are involved in the production of Aβ and their mutations induce the overproduction and accumulation of Aβ. The identification of these genes leads to a hypothesis of the pathogenesis of the disease, which is called, *the amyloid cascade hypothesis*. This hypothesis, which is widely accepted, explains the progression of the disease as a result of the accumulation of Aβ in the brain over decades, having a toxic effect that leads to neuronal death. Several functions of Aβ have been suggested, such as an; antioxidative function (Zou et al., 2002), antimicrobial activity (Soscia et al., 2010), cholesterol transport (Yao and Papadopoulos, 2002) and a role in synaptic plasticity (Parihar and Brewer, 2010). However, the exact detrimental mechanism of amyloid beta accumulation in patients with sporadic AD remains unknown, although it's very likely that the synergic interaction of environmental and genetic risk factors are behind the pathogenesis of the sporadic AD. Despite the link between the Aβ deposition and AD pathology is worth to mention that around

30% of healthy individuals older than 70 years old have Aβ deposits in their brains, in some cases as much Aβ as AD patients (Chetelat et al., 2013). Aside from the amyloid hypothesis, other potential mechanism has been proposed to explain the progression of the disease. For instance, the interaction between the Aβ and NFT formation is considered a potential pathogenic mechanism to explain disease progression. However, amyloid-based AD mouse models do not develop NFT and vice versa, tau mouse models do not develop amyloid plaques, and therefore the link between them remains unclear.

Nowadays, the majority of therapeutic targets tested are mainly focused on these two proteins, Aβ and tau. The goal is to reduce their aggregation or their production in order to reduce their toxic effects. Currently, the main clinical trials and drug therapies are driven by the *amyloid cascade hypothesis*, which might be valid for a small percentage of the total patients affected by the disease (less than 5%) but the vast majority of AD cases are sporadic, which means that there are factors other than the overproduction of amyloid beta, or its aggregation that are involved in the pathogenesis. Therefore, identifying new strategies based on mechanisms apart from the already known is warranted to increase our chance to find effective therapies. To that aim, we must study other potentially instrumental disease mechanism that can be important such as, neuroinflammation, kinases regulation and vascular impairment to understand and counteract the progression of AD.



Main features of AD biology (Ilustration by Itzia Ferrer)

Parkinson's disease

Parkinson's disease is a motor brain disorder characterized by the Lewy body formation and degeneration of dopaminergic neurons in the substancia nigra pars compacta (Kalia and Lang, 2015). Parkinson's disease is a multifactorial neurological disorder described for the first time by John Parkinson in 1817 in a work named "*An Essay on shaking palsy*". The death of dopaminergic neurons result in a deficit in dopamine levels in the basal ganglia which in turn affects the motor behavior of the patients characterized by: tremor, bradykinesia, muscular rigidity or postural instability. Besides motor symptoms, PD patients can present non-motor symptoms such as: constipation, depression, pain, mild-cognitive impairment, dementia and impaired olfaction (Khoo et al., 2013; Postuma et al., 2012). The pre-motor phase, it is the clinical stage before the appereance of the first motor symptons, the pre-motor phase can last longer than 10 years (Siderowf and Lang, 2012) followed by the motor-phase of the disease, which can be longer than 15 years (Kalia and Lang, 2015). Over the progression of the disease the

motor symptoms get worse and symptoms related with long-term treatments can emerge, i.e. complications such as dyskinesia (involuntary movement disorders) or psychosis (Hely et al., 2005; Hely et al., 2008). In late-stage Parkinson's disease, treatment-resistant motor and non-motor symptoms are present and including: postural hypotension, gait freezing, falling, dysphagia and speech dysfunction (Varanese et al., 2011).

Epidemiology of Parkinson's disease

PD is the second most common neurodegenerative disease after AD. Despite the implication of genetic susceptibility in PD, this only applies for a very small percentage of total PD cases. More than 90% of PD cases are not inherited (similar to AD) (Klein and Westenberger, 2012). In fact, there are evidences about the role of environmental and behavioral factors in the progression of PD pathology (Kalia and Lang, 2015).

PD incidence is higher in countries with high income and long life span. Life risk of developing PD is around 2% for men and 1.3% for women. Today, more than 2 million people are living with PD in Europe and the prediction is to reach more than 4 millions by 2050 with the current increase in incident (Bach et al., 2011). PD presents a slow progression with a very high variability between patients. According to *Pagano et al* (2016), the severity of motor and non-motor symptoms in PD increases with the age at on-set. The severity of the motor symptoms was greater in patients with an age at on-set at 70 years old (Pagano et al., 2016).

Risk and protective factors

Similar to AD, risk factors for PD are mainly associated with aging, as well as genetic and environmental components such as: head trauma, rural occupation or pesticide exposure. On the other hand, there are some factors suggested to reduce the risk of developing PD including, non-steroidal anti inflammatory drugs, physical activity or light alcohol consumption (Noyce et al., 2012; Yang et al., 2015).

Genetic mutations are also associated with an increase risk of developing PD. The first gene associated with the familiar version of the disease is *SNCA* (Polymeropoulos et al., 1997). Genetic mutation can be divided into two different categories, dominant and recessive mutations. Within dominant mutations the most important would be: *LRRK2, VPS35 or DNAJC13,* all of them involved in

endosomal pathways (Perrett et al., 2015) , and the previously mentioned, *SNCA*. There are recessive mutations such as *parkin, pink1 or DJ-1* related to PD incidence, most of them involved in mitochondrial viability (Corti et al., 2011). Besides the before mention mutations, β-Glucocerebrosidase (GBA) which is a lysosomal enzyme, confers the highest risk of developing PD among associated-genetic risk factors (Sidransky and Lopez, 2012).

Biology of Parkinson's disease

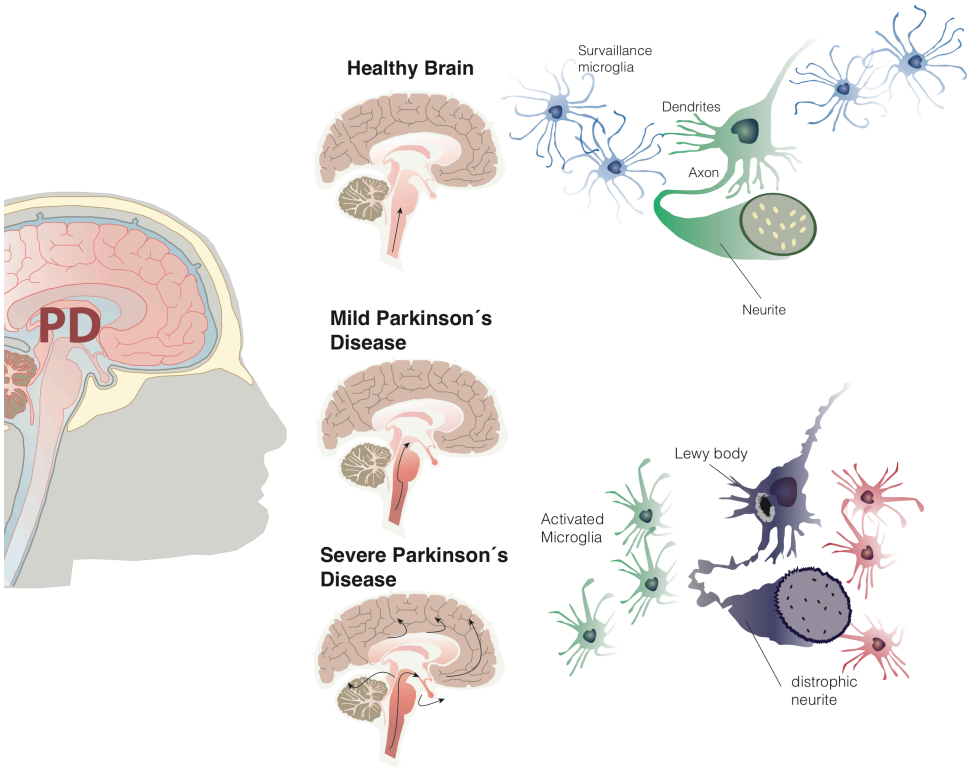
The main pathological feature of PD is the death of dopaminergic neurons in the SNpc. These neurons projects theirs axon from the SNpc to the Putamen and the striatum. The lost of these neuronal connections is probably causing the motor impairment suffered by PD patients. However, the loss of dopaminergic neurons occurs in many other regions such as: locus cereleus, dorsal motor, amygdala or hypothalamus (Dickson, 2012). The aggregation of misfolded α-synuclein in intraneuronal inclusions called Lewis bodies is another significant feature of the pathology. These inclusions can be found in neuronal somas (Lewy's bodies) or in the neuritis (Lewy's Neurites) (Spillantini et al., 1998). Although α-synuclein inclusion is the main pathological hallmark of PD they can be found in other pathologies such as dementia with Lewy bodies (DLB) (McKeith, 2004). Moreover, Lewy's inclusion can be also found in the peripheral nervous system (Beach et al., 2010).

Another important aspect of the biology of PD is the role of α-synuclein in the pathogenesis of the disease. It is known that α-synuclein can be present in different aggregate states, from monomers to small fibrils and it is the main compound of the Lewy bodies. For instance, oligomers of α-synuclein are found to be toxic for neurons (Ingelsson, 2016). Along with PD, some patients also present features typical for other diseases. For example, up to 50% of PD patients is common to also have Aβ plaques or NFT; the occurrence of both features may play a synergic role speeding up the progression of the disease (Irwin et al., 2013). However, some studies show PD patients with no Lewy bodies present, but instead with mutations in *parkin.* This finding launches new possibilities to explain the pathology in a context in PD, where the main pathogenic protein is not α-synuclein (Doherty et al., 2013).

The progression of PD can be described from a clinical and from a pathological point of view. From the clinical point of view, we can divide the progression of the disease in 6 different stages, according to Braak's hypothesis. The stages 1 and 2 would be defined as pre-motor symptomatic stages. In stage 3, the first motor symptoms appear and they are correlated with the dopaminergic deficiency in

basal ganglia. Finally, the stages 4 to 6 are characterized by the non-motor symptoms typical of advanced stages of the disease and long-term motor symptoms treatment (McCann et al., 2016). Moreover, Braak's hypothesis tries to explain the progression of PD based on the spread of α-synuclein pathology, and its effects over the time. α-synuclein production, deposition and spreading within neurons would induce its malfunctions leading to neuronal dysfuntcion and eventually neuronal death. For instance, the aggregation of α-synuclein within cells would affect the mitochondrial activity and would impair cell autophagy (Osellame and Duchen, 2014).

Cell death induces the release of different factors, including α-synuclein and inflammatory factors. According to Braak's hypothesis, the α-synuclein released by dying cells spreads to others neurons, impairing the cell activity of the "infected cells". The mechanism proposed to be involved in this process it is called *prionic* (Brundin et al., 2010). Prionic proteins are misfolded structures with the ability to induce aberrant conformations in other proteins that can become toxic. Prion-like process has been observed in grafted patients and in animal models(Li et al., 2008b; Rey et al., 2013). Along with the above mention, neuronal stress or neuronal death leads to release α-synuclein in different forms and vehicles that can be taken up by surrounding neurons, which will promote the progression of the pathology between cells and regions (Lee et al., 2014). Moreover, neuron-released α-synuclein can induce an innate immune response by activating microglial cells (Kim et al., 2013). This microglial activation leads to an inflammatory response, which may play an important role in the progression of the pathology (Perry, 2012). Braak's hypothesis points to a specific regional spreading pattern for α-synuclein over PD progression (McCann et al., 2016). According to Braak's hypothesis, the propagation of α-synuclein in PD starts from ascending pathway in the brainstem to the telencephalon. In later stages of the pathology, α-synuclein aggregates can be found in the basal forebrain and in the neocortex (Brettschneider et al., 2015). Only in more advanced stages of PD that α-synuclein aggregation causes the loss of dopaminergic neurons in the SNpc (Braak et al., 2003).



Main features of PD Biology (Ilustration by Itzia Ferrer)

Immune system

The immune system activation involves a dynamic reponse, where different cell types are implicated, that carries out the defense of the whole organism. However, the organism protection is not the only task where the immune system is involved: tissue homeostasis and cleaning, cell remodeling, synapsis formation are examples of the most relevant functions of our immune system. In this thesis, we will focus on the role of the immune system in the CNS pathologies, and more specifically the inflammatory response by the innate immune system, which is carried out mainly by microglial cells in the context of neurodegenerative diseases, such as AD and PD.

Innate and adaptive immune system

The immune response is divided in two different stages that are tightly connected and works together. The innate immune response is the first line of defense against internal or external insults and coordinates and activates the adaptive immune system by antigen presenting cells such as dendritic cells (Amor and Woodroofe, 2014). However, the role of the adaptive immune system in AD pathology is not as well described as the innate immune system. Moreover, our study is mainly focus on mechanisms related to the innate immune response. Due to the before mentioned, the focus of my thesis dissertation will be the innate immune system.

Innate immune system

The first line of defense of an organism, the innate immune system, is present in basically every life form (Lemaitre, 2004). Of special importance are the regulation and the activation of the innate immune system in the CNS due to minor involvement of the adaptive immune system in the surveillance and reactivitiy of the CNS (Ransohoff and Brown, 2012). The main components of the innate immune system in the CNS are: the microglial cells, astrocytes, oligodendrocytes, neutrophils and the complement system. Innate immune system related functions go beyond the defense of the organism. The innate immune

system performs different functions in the CNS, such as: synaptic formation and pruning (Parkhurst et al., 2013), phagoptosis (Brown and Neher, 2014), debris clearance (Neumann et al., 2009), provides inputs for the adaptive immune system with antigen presentation to T cells in the lymphatic nodes (Engelhardt et al., 2016; Iwasaki and Medzhitov, 2010), pathogen detection (Kawai and Akira, 2010) and neural protection (Eroglu and Barres, 2010; Nave, 2010).

Microglial cells

The main cell type and the focus of this thesis are microglial cells. Microglial cells are tissue-resident myeloid cells of the CNS that derived from the yolk sac (Ginhoux et al., 2010). Microglial cell survey the brain parenchyma constantly, having the ability to quickly response to disturbances in the brain homeostasis. Microglial are the homologues of the macrophages in the CNS and they share similar functions, such as: debris clearance, neural development, phagocytosis of apoptotic cells and synaptic formation (Kettenmann et al., 2011). The microglial cell surface is equipped with different transporters, channels and receptors; including receptors for molecules such as: neuromodulators, cytokines, chemokines, growth factors, as well as pattern recognition receptors (PRRs).

The molecules triggering the immune system can be classified within two categories, Dangerous-Associated Molecular Patterns (DAMPs) or Pathogen-Associated Molecular Patterns (PAMPs) (Venegas and Heneka, 2017). DAMPs are linked to tissue damage, where endogenous molecules, *i.e.* Aβ or α-synuclein, trigger the inflammatory response. Regarding PAMPs, are molecules associated with virus or bacterias, such as lipopolysaccharides, that are able to trigger the activation of the immune system (Barichello et al., 2015). Also, external situations, like a traumatic brain injury or brain ischemia, can trigger an immune response (Woodcock and Morganti-Kossmann, 2013). In normal conditions, microglial cells have small bodies with long processes that monitor the local microenviroment surrounding the cells (Nimmerjahn et al., 2005). The number and the density of microglial cells in different areas of the CNS can vary (Lawson et al., 1990), as well as the activation ability, which appear to be region dependent. For instance, in the hippocampus and the cortex microglial cells exhibit a more immune vigilant state (Grabert et al., 2016). Differences regarding young and adult microglial have been demonstrated, shedding light on the impact of aging in the microglial ability to carry out different functions (Mosher and Wyss-Coray, 2014). These differences, specially the ones concerning hippocampus, may play a role in the progression of neurodegenerative diseases, such as AD. Moreover, the

genetic profile of microglial cells seems to change with age and make different microglial populations more similar among them (Grabert et al., 2016).

Once microglia detects a molecule associated to an insult, it is able to become active, changing its morphology and genetic profile. The classical view of microglial activation consider 2 different, and opposite, states, the pro and the anti-inflammatory (also called, classical and alternative). This view can be useful to explain different roles of microglial activity but it is insufficient to explain all the possibilities regarding the activation pattern of microglial cells. Despite of the befored mentioned, in some extent, this view is still useful to briefly describe microglial cells activation patterns. For instance, in the M1, or classical phenotype, microglial cells become proinflammatory, reducing their processes and augmenting their cell body. Furthermore, in the M1 phenotype, microglial cells express a wide spectrum of different molecules such as: cytokines, chemokines, reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Tang and Le, 2016). The M1 phenotype is linked to detrimental functions of microglial cell in the brain. A prolong proinflammatory activation of microglial may play a role in neural dysfunction in a context of neurodegenerative diseases (de Pablos et al., 2014; Heneka et al., 2013; Stefanova et al., 2007). On the other hand, the M2 phenotype of microglial cells is related to: debris clearance, inflammatory resolution, phagocytosis, trophic factors release and anti-inflammatory cytokine release (Tang and Le, 2016). The M2, or alternative phenotype, is considered beneficial for the resolution of the damage in the brain.

Astrocytes

Astrocytes play a crucial role in the CNS in neuronal homeostasis, blood-brain barrier integrity and inflammatory response. Astrocytes, along with oligodendrocytes, are considered macroglial cells (Pekny and Nilsson, 2005). As microglial cells, astrocytes display different activation states, depending on the situation, astrocytes activation can be detrimental or beneficial for the CNS (Pekny and Nilsson, 2005). In healthy conditions, astrocytes are involved in glutamate regulation in the CNS (to avoid the toxic consequences of high glutamate levels), scar formation after injury (Hara et al., 2017), and they are also key component of the tight junctions of the blood brain barrier (BBB) (Parpura et al., 2004; Prat et al., 2001). Astrocyte dysfunction is involved in BBB impairment. For instance, in autoimmune encephalomyelitis astrocytes dysfunctions induce BBB malfunction due to Aquaporin 4 misallocation that leads to edema formation (Wolburg-Buchholz et al., 2009). Regarding AD, recent studies suggest that astrocytes dysfunction may play a detrimental role in synaptic connectivity and neuronal

death through increased glutamate excitotoxicity. Moreover, astrocytes participate in the inflammatory response by releasing proinflammatory cytokines such as: TNF-α, IL6 and IL1 (Lau and Yu, 2001) leading to a neuroinflammatory environment that may exert a negative role in neurodegenerative diseases, such as AD or PD (Verkhratsky et al., 2010). Regarding astrocytes and PD, there are different risk associated genes for PD related to the biology of astrocytes. Genes such as *PINK, Parkin 1, and LRRK2*, are known to be implicated in astrocytes functions such as neurotrophic capacity, mitochondrial function, proliferation or autophagy, all of them are related to PD (Booth et al., 2017).

Neuroinflammation

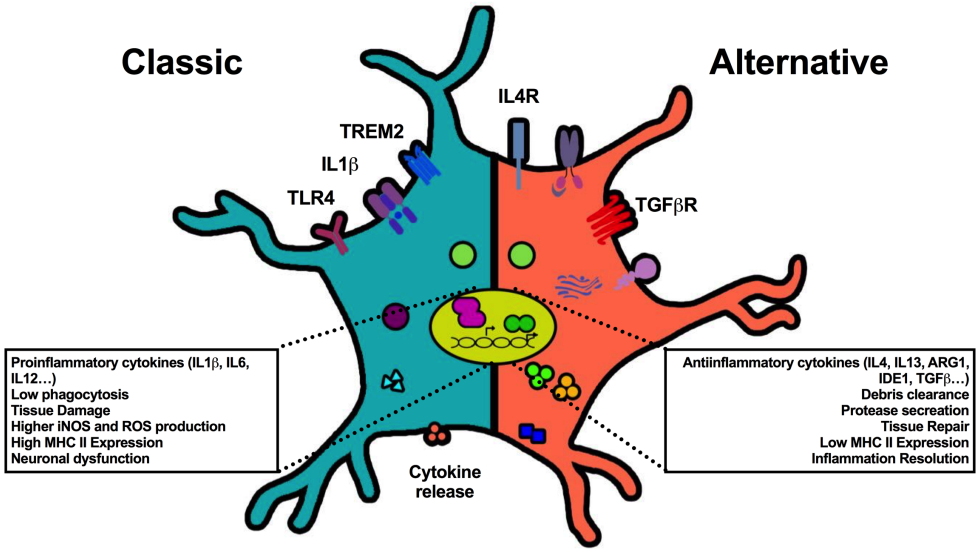
Neuroinflammation is a term used to describe the inflammatory response in the nervous system. Immune responses in the brain are very common, despite the notion of the CNS as an immune privilege site. The concept of the brain as an "immune privileged" site stems from the apparent limited impact of peripheral lymphocytes in the CNS, and homeostasis and the lack of specialized antigen presenting cells despite the presence of cells expressing MHCII, microglial cells. The antigen-presenting mechanism in the brain parenchyma has not been fully demonstrated. However, antigen-presenting events take place in the lymphatic nodules, where antigens from the CNS can be detected and triggers the adaptive immune response. As mentioned befored, the main role of the inflammatory response is to restore the homeostasis after injury. The insults triggering the inflammatory response can vary a lot, from both external and internal sources, such a trauma, to an autoimmune disease.

Over the last decade the role of the inflammatory response in neurodegenerative diseases such as PD or AD has been highlighted. The neuroinflammatory response in the CNS is mainly orchestrated by microglial cells. As mentioned previously, microglial cells express a wide spectrum of molecules to counteract brain damage and restore the tissue homeostasis. However, an uncontrolled and sustained inflammatory response over the time may have detrimental effects on the CNS.

Inflammatory response in neurodegenerative diseases

Aside from the described genetic risk factors, others molecular mechanisms linked to the inflammatory response elicited by microglial cells have been related to the

progression of the neurodegenerative diseases. For instance: TLR regulation, the inflammasome activation and the role of different cytokines have been widely studied in relation to neurodegenerative diseases. Special attention in our work will give to the role of TLR4 in the regulation of the microglial activation.



Classic and Alternative activation state of microglial cells (Ilustration by Antonio and Javier Boza)

Inflammatory response in Alzheimer's disease

AD is a neurodegenerative disease mainly characterized by Aβ plaque formation, intra neuronal NFT formation and neuronal death. However, these 3 factors are not enough to explain the wide phenotype present in AD. Along with the main pathological hallmarks mentioned before, new factors are getting relevance in relation to the pathology, to know, the inflammatory response, environmental factor, social factors or nutritional factors may be contributing to AD. In this work, our main focus will be the inflammatory response in relation to AD.

The inflammatory hypothesis of AD tries to explain the progression of the disease from the point of view of the activation of the innate immune system in the brain. One of the main actors in this hypothesis are the microglial cells. In an AD context, the formation of Aβ plaques and the extracellular Aβ are able to activate the classic microglial profile, triggering the release of different inflammatory factors (Haass and Selkoe, 2007). Theses inflammatory factors will create a stressful environment for neurons, inducing their malfunction, which might be the beginning of Aβ deposition, driven by posttranslational modifications of the protein (Kummer and Heneka, 2014), and tau phosphorylation imbalance that

leads to the NFT formation (Braithwaite et al., 2012). These factors together may contribute to the neuronal dysfunction and the subsequent neuronal death. In order to calibrate to which extent the inflammatory response is important to the progression of the pathology, we need to observe the massive amount of research on this topic published in the last few years. From genetic risk factors to the role of different cytokines in AD, a broad panel of mechanisms trying to underline the role of inflammation has been investigated.

To study how inflammation is involved in AD, one of the most powerful tools we have available nowadays are the *Genetic Wide Associated Studies* (GWAS). GWAS studies are very useful to find genetics risk factors associated to AD in patients with the sporadic form of the disease (Lambert et al., 2013). In relation to inflammatory response, GWAS pointed out different microglial-response related genes as risk factor to develop AD. Few of the main gene related risk factors are associated with the microglial response. Genes such as *TREM2, ApoE, ABCA7, PICALM or CD33* are linked to microglial cell activity and highly associated with the progression of the pathology (Bradshaw et al., 2013; Genin et al., 2011; Guerreiro et al., 2013; Harold et al., 2009; Steinberg et al., 2015). Among all the genetic risk factor, we should highlight *TREM2, CD33 and ApoE*, due to the high risk to develop AD associated to them. For instance, TREM2 R47H mutation has been related to AD progression in different studies (Korvatska et al., 2015). However, the pathogenic mechanisms associated to TREM2 mutations are not fully clear. Recently, Colonna and coauthors demonstrated how TREM2 expressed in microglial cells might be involved in the Aβ plaque compaction by creating a barrier around the plaque, reducing the neurotoxic effects of the plaque deposits in the surrounding neurons (Wang et al., 2016; Yuan et al., 2016). Higher expression of CD33 has been linked with the accumulation of Aβ due to the reduction of the phagocytic capacity of microglial cells *in vitro* and *in vivo* (Bradshaw et al., 2013; Griciuc et al., 2013).

Along with the two previously described genes, ApoE is the most important risk factor for developing AD. ApoE is an apolipoprotein that in the CNS, mainly expressed by astrocytes, where it transports cholesterol to neurons via ApoE receptors (Bu, 2009). There are 4 different forms of ApoE, with the ApoE4 allele being the strongest genetic risk factor associated to late onset AD. Different mechanisms that ApoE4 mutations might involve to AD progression have been described, for example: enhancement of amyloid aggregation, tangle formation, reduction of mitochondrial function or reduced lipid and cholesterol metabolism (Liu et al., 2013). Moreover, the lack of ApoE enhances the inflammatory response and is related with the progression of AD(LaDu et al., 2001).

Different mechanisms related to the cytokine production have been associated with AD progression. For instance, the production of IL1β by the inflammasome

pathways has recently been highlighted by Heneka's lab. The inflammasome is a mechanism related to the detection of DAMP's and the consequent inflammatory response via IL1β (Heneka, 2017). One of the main components of the inflammasome is the so-called; NLR family Pyrin domain containing 3 (NLRP3) that is tightly linked to the microglial activation. Microglial cells have the ability to phagocyte Aβ, however this process may induce the disruption of the lysosome, releasing the content into the cytoplasma and thereby triggering the IL1β production and release via Caspase-1 activation (Bruchard et al., 2013; Lima et al., 2013). Linked with the previously mentioned, it has recently been demonstrated the role of NLRP3 in AD progression. According to Heneka's studies, the lack of NLPR3 reduced AD pathology, microglial activation and presented a better behavioral outcome in AD mouse models (Heneka et al., 2013). The lack of NLRP3 shifted the microglia towards a more anti-inflammatory profile, which may be linked to pathology reduction.

Other cytokines such as IL10, IL12 or IL33 has been also linked to AD pathology. For instance, the lab of Terrence Town and the lab of Todd Golde, have shown in two different studies how IL10 modulation affects the progression of AD pathology and improve the behavioral outcome by affecting the microglial phenotype and its phagocytic ability (Chakrabarty et al., 2015; Guillot-Sestier et al., 2015). A recent study from the lab of Heppner showed that the reduction of the proinflammatory IL12 reduces AD progression in APP/PS1 mice. The lack of IL12/23 seems to enhance the microglial uptake of Aβ, reducing the amyloid burden(Teng et al., 2015). IL33 has also been implicated in AD progression in patients with elevated levels of serum ST2, a decoy receptor for IL33. The administration of IL33 improves the microglial ability to uptake Aβ, reducing the amyloid burden and improving the cognitive outcome in APP/PS1 mice (Fu et al., 2016).

Inflammatory response in Parkinson's disease

Aside from the degeneration of dopaminergic neurons in the SNpc and intraneuronal synuclein deposits, inflammation is an important component in PD pathology. The inflammatory response in PD is mainly carried out by microglial cells and to a minor extent by astrocytes. The activation of both cells type is associated to the areas where the neuronal death is taking place. Especially, microglial cells are associated to the clearance of neuronal death-related debris. Activated microglia can release different inflammatory factor, such as BDNF, proinflammatory cytokines, chemokines etc, along with ROS and RNS. Hence, neuroinflammatory response may contribute to the progression of the pathology.

Microglial activation in PD has been extensively documented. For instance, in 1988 study by McGeer showed HLA receptor reactivity in the SNpc of PD patients (McGeer et al., 1988). Aside from microglial activation, different neuroinflammatory factors have been found in the brain of PD patients. Elevated levels of TNF-α, IL1β and IFN-γ have been detected in striatum and substancia nigra of PD patient compared to healthy controls (Hunot et al., 1999; Mogi et al., 1994a; Mogi et al., 1994b). Regarding CSF, the main protein involved in the progression of the pathology, α-synuclein, has also been proved to be useful as a CSF marker to follow the motor impairment associated to the PD progression (Hall et al., 2015). TNF-α was also found increased in the CSF of PD patients compared to healthy controls (Mogi et al., 1994b). Along with TNF-α, IL6 and IL1β have been detected in CSF of PD patients (Blum-Degen et al., 1995; Lindqvist et al., 2013). Another proinflammatory molecule, Nitric Oxide Synthase (NOS), have been measured in glial cells of PD patients in the vicinity of dopaminergic neurons in the brain (Hunot et al., 1996). The production of nitric oxide by glial cells might be toxic for dopaminergic neurons (Boje and Arora, 1992). In fact, the inhibition of NOS *in vitro* protects neurons in co-culture with LPS activated microglial cells (Boje and Arora, 1992). Different proinflammatory cytokines production might induce a toxic effect in neurons expressing their receptor. For example, TNF-α is highly expressed in dopaminergic neurons of PD patients, which might exert a deleterious effect in an inflammatory context (Mogi et al., 2000).

Similar to AD, there are several known inflammatory-associated risk factors that might increase the probability of developing PD. For instance, TNF-α polymorphism at position 308 in the aminoacid sequence, IL6 polymorphism at position 174 and IL1β at position 511 are more common in PD patients than in healthy controls (Arman et al., 2010; Hakansson et al., 2005; Kruger et al., 2000). Still, it is not fully clear in which way these polymorphisms affect the progression of PD pathology, whether the mutations increase the basal levels of the proinflammatory factors or exacerbate the inflammatory response upon insult.

In order to resemble the main pathological hallmarks of the PD, several animal models have been developed and most of them present microglial activation and inflammatory response along with the progression of the pathology. Up to date, MPTP, 6-hydroxidopamine and LPS are the main PD models used, due to their ability to induce cell death and consequent microglial activation (Barcia et al., 2004; Castano et al., 1998; Rodriguez-Pallares et al., 2007). The link between the inflammatory response and the neuronal death has been assessed in different studies, giving a positive correlation between both factors. For instance, the MPTP models provides robust neuronal death and microglial activation in the SNpc. Inducible Nitric Oxide Synthase (iNOS) KO mice show a reduction of neuronal death upon MPTP injections compared to WT mice (Liberatore et al., 1999). As

mentioned previously, iNOS is an enzyme involved in the production of nitric oxide, a potent proinflammatory molecule. Moreover, single injections of LPS in rats is able to selectively induce dopaminergic cell death in the SNpc more efficiently than in the striatum (Herrera et al., 2000). Removing microglial cells have been successfully tested in animal models. For instance, Venero et al, injected minocycline, a compound used to inhibit microglial cells, in an animal model of intranigral injection of LPS. As a result of the microglial depletion by minocycline, the levels of TNF-α, IL1α and protein peroxynitration were reduced and the dopaminergic neurons were protected compared to minocycline non-injected mice (Tomas-Camardiel et al., 2004).

Moreover, *in vivo* models of α-synuclein overexpression also display microglial activation. For instance, overexpression of a mutant form of α-synuclein induces microglial activation in a transgenic mouse model leading to early proinflammatory activity (Su et al., 2009). Furthermore, upon LPS injection, mice expressing a mutant version of α-synuclein (A53T) provoke selective dopaminergic cell death in the SNpc along with formation of α-synuclein aggregates within neurons, a classical hallmark of PD pathology. Interestingly, inclusions contained α-synuclein nitrated residues, which can be linked with the inflammatory response elicited upon LPS injection (Gao et al., 2008). Additionally, *in vitro* models consisting of α-synuclein overexpression display the classical features of microglial activation, as well as the release of different proinflammatory factors (Su et al., 2008).

Regarding TLR's, TLR4 has been shown to be able to bind to α-synuclein in MSA models where the overexpression of α-synuclein increase motor impairment as well as dopaminergic degeneration in the SNpc. α-synuclein binding to TLR4 enhances microglial phagocytic capacity as well as the production of proinflammatory factors, such as TNF-α (Stefanova et al., 2011).

The main pathways involved in inflammatory process in PD are very similar to the one elicited in AD. JNK, MAPK or NFκβ pathways are mainly involved in the inflammatory response in PD. For instance, MPTP mouse models triggers the activation of Cyclooxygenase 2 (COX2) by JNK2 and JNK3 related to JNK pathways. Indeed, JNK2 and JNK3 deficiency reduces dopaminergic cell death (Hunot et al., 2004). The activation of MAPK p38 pathways in MPTP models has also been shown in dopaminergic neurons in the SNpc (Karunakaran et al., 2008). Finally, NFκβ pathway has been found upregulated in microglia and astrocytes of PD patients intoxicated with MPTP. NFκβ inhibition reduces microglial activation and mRNA levels of TNF-α, IL1β and iNOS in the SN, protects the neurons located in the nigra-striatum pathway and improved motor behavior in mice exposed to MPTP (Ghosh et al., 2007).

The cells of the innate immune system are able to express a wide array of called PRR's that detect pathogens and endogenous molecules in order to initiate the immune response. Among these receptor's we have the TLR family. From an evolutionary point of view TLR's are well-conserved transmembrane proteins named by their homologous in *drosophila*. TLR's are expressed in both, immune and non-immune cells and their activation is triggered by molecules derived from pathogens (PAMP's) and endogenous molecules associated with internal damage (DAMP's). TLR antigens trigger the innate immune response and organize the antigen presentation to initiate the adaptive immune response (Pasare and Medzhitov, 2004). TLR receptors can be classified depending on cellular location and their specific ligands. For instance: TLR2, TLR4, TLR5 or TLR6 are integrated in the outer cell membrane and recognize extracellular motifs, such as, proteins or lipids. Whereas, TLR3, 7, 8 and 9 are localized in the endosomal compartment and are involved in nuclear acid-related motif recognition (Hanke and Kielian, 2011). TLR receptors localized intracellulary have been linked to signaling amplification in relation to membrane bound TLR's (Hanke and Kielian, 2011). TLR activation is mainly linked to the production of inflammatory molecules such as cytokines, ROS or RNS via MYD88 depending and MYD88 independent pathways to NFκβ-dependent gene expression. Depending on the adaptor molecules the activation varies, for instance, MyD88 dependent activation triggers the expression of a broad array of proinflammatory cytokines and MyD88 independent activation induce IFN-related gene expression (Akira and Takeda, 2004). Activation of TLR's expands the inflammatory response, recruiting different immune cells and its regulation is essential for the organism to prevent severe damage. Uncontrolled, or exaggerated activation of TLR's has been associated with different pathological conditions (Hanke and Kielian, 2011). In this thesis, we are particularly interested in the effects and the regulation of microglial TLR4 in brain diseases.

Our particular interest is related to the role of TLR's in microglial activation and its relation with the inflammatory process in neurodegenerative diseases. As we mentioned before, microglial cells are able to sense the surrounding microenvironment through different PRR's, including TLR's. Indeed, microglial cells express all TLR's currently known to date, being able to combine them and tailor the response depending on the challenge. Between the different TLR's, we have paid particular focus on TLR4 and its role in microglial activation. TLR4 is one of the main receptors involved in the detection and response to LPS, a potent proinflammatory molecule found in the membrane of gram-negative bacteria that has been used to induce neuronal degeneration upon a strong inflammatory stimulus (Machado et al., 2011).

The discovery of this interaction has been very helpful to unravel the TLR4-related activation pathways in microglial cells. In relation to the inflammatory response, TLR4 has been linked to the production of different inflammatory cytokines such as IL1β (Facci et al., 2014). TLR4, and its association with neurodegenerative diseases, has been shown to increase the amyloid burden and worsened cognitive outcome in an AD mouse model with a TLR4 mutation that reduces its response to proinflammatory stimuli such us LPS. The same mutation in TLR4 has been linked to lower levels of different proinflammatory cytokines such as: TNF-α, IL1β or IL10 in AD mouse model, which come to demonstrate the role of TLR4 in proinflammatory activation of microglial cells (Jin et al., 2008). These results showed an increase in AD pathology in TLR4 mutants in spite of a reduction of the proinflammatory response. This is contrary to the general view in the field where the reduction of the inflammatory response halts the disease progression, but highlight the highly dynamic, and context-dependent response of TLRs.

Regarding TLR4 and Aβ interaction, it has been shown that Aβ is able to trigger microglial activation by CD36-TLR4-TLR6 complex, leading to a "sterile" inflammatory response (Stewart et al., 2010). Moreover, the lack of TLR4 impairs the inflammatory response upon fibrillar Aβ stimulation of microglial cells, resulting in a reduction of their phagocytic ability and ROS production (Reed-Geaghan et al., 2009). Indeed, TLR4 activation by Aβ along with lipids has been shown to be involved in neuronal apoptosis.

One of the main objectives of our work is to elucidate the role of TLR4 in microglial activation. Notably, our group was the first one finding a new endogenous ligand of TLR4, galectin-3, involved in microglial activation (Tang et al., 2008). In line with that finding, our studies have been focused on the role of galectin-3 in the inflammatory response in neurodegenerative diseases.

Galectins

Galectins is a family of β-galactosidase binding proteins sharing a common Carbohydrate-Recognition Domain (CRD). So far, 15 members have been described and they are classified in 3 different families depending on their structure. The first family it is called proto-type (Galectin 1, 2, 5, 7, 10, 11, 13, 14 and 15), having only 1 CRD and can be found as a monomers or homodimers; the second family presents 2 different CRD (Galectin-4, 6, 8, 9 and 12), it is called tandem type, they can be found forming heterodimers; the third family, chimera type, just one member, galetin-3, present one CRD and one n-terminal tail, can be found forming pentamers (Liu and Rabinovich, 2010).

Galectins are express in different cell types and tissues and their affinities depend on glycan-residues combinations. Galectins are involved in a wide array of functions, such as apoptosis, migration, neurogenesis, inflammation, phagocytosis and can be found in the extracellular or in intracellular compartments, where they can be associated to intracellular vesicles (Liu and Rabinovich, 2010).

Our work is focused on the role of galectin-3 in the regulation of the neuroinflammatory response in microglial cells. Therefore, I will breifly present the different roles of galectin-3 in the innate immune system with regards to different cells types.

In neutrophils, galectin-3 has been shown to induce oxidative burst (Almkvist et al., 2001). Moreover, recombinant galectin-3 plays a role in activating neutrophils, augmenting their phagocytic ability and life span (Farnworth et al., 2008). Galectin-3 in neutrophils has been linked to degranulation and release of inflammatory factor via MAPK pathway (Fernandez et al., 2005). The same study linked galectin-3 with reduced neutrophils life span due to apoptotic mechanisms triggered along with the activation. More recently, galectin-3 has been related to reduce ROS production in neutrophils exposed to *Candida albicans* (Wu et al., 2017).

In monocytes, galectin-3 has been associated with enhancement of phagocytosis due to its ability to function as an opsonin. Adding galectin-3 to human leukocytes and macrophages in co-culture increased the ability of macrophages to uptake apoptotic leukocytes. The effect was blocked by adding lactose, a galectin-3 inhibitor, which suggested the role of galectin-3 as an opsonin involved in macrophage phagocytosis (Karlsson et al., 2009). Indeed, galectin-3 may play a role in upregulation the phagocytic ability of macrophages stabilizing the KRas-GTP complex that promotes the PI3K activity in relation to molecules such as myelin, demonstrated in *in vivo* models of Wallerian degeneration (Mietto et al., 2013). Related to galectin-3 and its effect on the inflammatory profile of

macrophages/monocytes, it has been described that the lack of galectin-3 *in vivo* shifts the profile towards proinflammatory reducing IL4 levels in comparison to wild-type macrophages. However, the authors claimed that this reduction in IL4 production in galectin-3 KO does not affect the classical activation pattern in macrophages (MacKinnon et al., 2008). In another study, using LPS to activate macrophages, it was shown that blocking galectin-3 increased the ability of LPS stimulation to shift macrophages towards the proinflammatory profile. However, the authors stated that intracellular galectin-3 was very likely the one carrying the effects (Li et al., 2008c). In regards to the neuroinflammatory response in BV2 cells, galectin-3 increased the expression of JAK-STAT signaling pathway related proteins and triggered the production of proinflammatory cytokines such as: IL1β, TNF-α, IL6 and IL12 (Jeon et al., 2010).

The role of galectin-3 and microglial cells in neurodegenerative disease has been deeply investigated. Special attention in this paragraph will be designated to the role of galectin-3 in different brain diseases. In the CNS, microglial cells are the main source of galectin-3. However, in healthy conditions, the expression of galectin-3 is very minor compared to expression levels in disease (Kim et al., 2007). Galectin-3 plays a role in different neurodegenerative diseases. For instance, microglial cells expressing galectin-3 enhance the angiogenesis and the neurogenesis, which helps in tissue remodeling in ischemia-reperfusion damage in a mouse model of experimental stroke (Yan et al., 2009). Moreover, IL6 and SOCS3 levels were upregulated, suggesting a potential activation of JAK-STAT pathways, which is related to the proinflammatory response (Yan et al., 2009). The expression of galectin-3 has been linked to higher production of vascular endothelial growth factor in *in vitro* models of glucose and oxygen deprivation of BV2 cells. Moreover, galectin-3 also increases the migratory capacity of BV2 microglial cells *in vitro* via Integrin Kinase signaling (Wesley et al., 2013). Galectin-3 is also involved in the endocytic process in macrophages by carbohydrate dependent and independent pathways. In M1 macrophages, galectin-3 endocytosis is CRD-domain independent, whereas in M2 macrophages, endocytosis can be inhibited using CRD blockers, such as lactose. All together highlight the potential role as a disease-driving molecule in neurodegenerative diseases with a strong inflammatory component.

General materials and methods

Animals

5xFAD-Gal3 KO transgenic mice were generated by first crossing heterozygous 5xFAD (+/-) with homozygous Gal3 KO (-/-) mice to get 5xFAD (+/-)/Gal3 (+/-). Subsequent crossings between animals showing this genotype allowed the generation of 5xFAD (+/-)-Gal3 (-/-), hence referred to as 5xFAD-Gal3 KO, and 5xFAD (+/-)-Gal3 (+/+), hence referred to as 5xFAD. All animal experiments were performed in accordance with the animal research regulations (RD53/2013 and 2010/63/UE) in Spain and European Union, and with the approval of the Committee of Animal Research at the University of Seville (Spain).

For primary microglial cultures, Gal3 null mice with a pure C57BL/6 background were obtained from Dr. K. Sävman at Gothenburg University. All procedures were carried in accordance with the international guidelines on experimental animal research and were approved by the Malmö-Lund Ethical Committee for Animal Research in Sweden (M250-11).

Genotyping

The genotypes of Gal3−/− and Gal3+/+ mice were determined using an integrated extraction and amplification kit (Extract-N-Amp™, Sigma-Aldrich). First, the samples were incubated at 94 °C for 5 min, followed by 40 cycles with denaturation at 94 °C for 45 sec, annealing at 55 °C for 30 sec, and elongation at 72 °C for 1.5 min. The following primers (CyberGene, Solna, Sweden) were used: Gal3 common 5-CAC GAA CGT CTT TTG CTC TCT GG-3′), Gal3−/− 5-GCT TTT CTG GAT TCA TCG ACT GTG G-3′ (single band of 384 bp) and Gal3+/+ 5-TGA AAT ACT TAC CGA AAA GCT GTC TGC-3′ (single band of 300 bp) (Doverhag et al., 2010). For the 5xFAD the primers (5′to 3′) used are listed below: APP Forward AGGACTGACCACTCGACCAG, APP Reverse CGGGGGTCTAGTTCTGCAT, PSN1 Forward AATAGAGAACGGCAGGAGCA, PSN1 Reverse GCCATGAGGGCACTAATCAT, WT APP Forward CTAGGCCACAGAATTGAAAGATCT, WTT APP Reverse GTAGGTGGAAATTCTAGCATCATCC, RD1, RD2 and RD3 AAGCTAGCTGCAGTAACGCCATTT ACCTGCATGTGAACCCAGTATTCTATC, CTACAGCCCCTCTCCAAGGTTTATAG. The PCR products were separated by gel

electrophoresis labeled with SYBR® Green (Sigma Aldrich) and visualized using a CCD camera (SONY, Tokyo, Japan).

Protein preparation

Amyloid Beta preparation (Paper IV)

Aβ42 monomer was isolated using gel filtration (Superdex 75) with 20 mM sodium phosphate, 0.2 mM EDTA, pH 8.0 as running buffer, collected on ice in a low-binding tube (Genuine Axygen Quality, Microtubes, MCT-200-L-C, Union City, CA) and the concentration was determined by absorbance at 280 nm using ε280 = 1440 M-1cm-1. The solutions was diluted with buffer and supplemented with concentrated NaCl and Thioflavin T (ThT) stocks to achieve 10 μM monomer, 6 μM ThT and 150 mM NaCl. The solution was placed in wells of a PEGylated polystyrene plate (Corning 3881), and sealed with a plastic film to avoid evaporation. Plates were incubated at 37 °C for 24 h in a FLUOstar Omega plate reader under quiescent conditions (BMG Labtech, Offenburg, Germany) at 37°C for 24h to follow the fibrillation of amyloid-beta through the bottom of the plate, and the samples were collected after reaching the plateau of the sigmoidal transition.

α-synuclein prepation (Paper II)

Briefly, human α-synuclein was purified using the heat treatment, ion exchange, and gel filtration chromatography as previously described(Boza-Serrano et al., 2014). α-synuclein monomers were placed on an orbital shaker at 250 rpm, shaking the monomers for 5 days at 37°C in sterile PBS. After 5 days of incubation, the protein aggregates were sonicated using a Branson Sonifier 250 (All-Spec, Willington, US) with the following conditions: 3/9 output and 30/100 Duty Cycle. We tested the composition of our aggregates and monomers using Western Blot analysis and transmission electron microscopy (TEM) (FEI, Einhofen Holland). We performed negative stain of monomeric and sonicated aggregated forms of α-synuclein by using 2% uranyl acetate in water. The concentration of endotoxin was measured in our protein preparations using the Limulus amebocyte lysate assay (Chromogenic Endotoxin Quantification Kit, Thermo Scientific, US). We detected very low levels of endotoxin (0.14 ng of LPS/ml) that was unable to influence on the microglial activation (data not shown).

Cryogenic Transmission Electron Microscopy (cryo-TEM) *(Paper II and IV)*

Specimens for electron microscopy were prepared in a controlled environment vitrification system (CEVS) to ensure stable temperature and to avoid loss of solution during sample preparation. The specimens were prepared as thin liquid films, <300 nm thick, on lacey carbon filmed copper grids and plunged into liquid ethane at -180°C. This leads to vitrified specimens, avoiding component segmentation and rearrangement, and water crystallization, thereby preserving original microstructures. The vitrified specimens were stored under liquid nitrogen until measured. An Oxford CT3500 cryoholder and its workstation were used to transfer the specimen into the electron microscope (Philips CM120 BioTWIN Cryo) equipped with a post-column energy filter (Gatan GIF100). The acceleration voltage was 120kV. The images were recorded digitally with a CCD camera under low electron dose conditions. The node-to-node distance was measured using the Digital Graph software (Gatan Inc.).

Endotoxin test *(Paper I, II, IV)*

To further evaluate the properties of our protein preparation we performed an endotoxin assay to discard a potential BV2 microglial cell activation due to the presence of endotoxins such as LPS. Fibrils preparations used for *in vitro and in vivo* experiments were tested for endotoxins using Pierce® LAL Chromogenic Endotoxin Quantitation KIT, (ThermoScientific) according to manufacturers instructions.

XTT (Cell Viability) Assay *(Paper II and IV)*

XTT assays were performed to measure mitochondrial activity (mitochondrial dehydrogenase) in living cells using XTT (2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide salt) (Sigma-Aldrich, Sweden). The assay was performed following manufacturer's protocol on a 96-well plate (Biochrom Asys Expert 96 micro plate reader, Cambridge, UK).

In Vitro Experiments. Cell lines and primary cultures. *(Paper I, II and IV)*

Primary microglia cultures from wild-type (WT) (C57BL/6) or galectin-3 knockout (KO) mice, cells were prepared from postnatal day 1-3 and cultured as

previously described by Deierborg et al, 2013(Deierborg, 2013). Briefly, the cerebral cortex were dissociated in ice cold Hank's Balance Salt Solution without bivalent ions (HBSS, Invitrogen), Trypsin (0.1%) (Invitroge n) and DNase (0.05%) (Sigma-Aldrich). The cells were then plated in T75 flask with 10 ml/flask of Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% Fetal Bovine Serum (Invitrogen) with 100 U/ml Penicillin and 100 U/ml Streptomycin (Invitrogen) in 5% CO_2 atmosphere at 37°C. After 14 days, cells were harvested in the medium by smacking the flask 10-20 times and plated in 96 wells plates at a density of 2×10^4 cells/well. The primary cultures were then treated with α-synuclein or amyloid beta at different concentrations.

Cell line transfection *(Paper I and II)*

Transfection of BV2 cells was carried out using Lipofectamine 2000 following the manufacturer's recommendation (Life Technologies). Non-targeting control and galectin-3 siRNAs were obtained from Dharmacon. (SMART pool) siRNA sequence used: siLGal3S3(1) J-041097-09 GAGAGAUACCCAUCGCUUU, siLGal3S3(2) J-041097-10 ACUUCAAGGUUGCGGUCAA, siLGal3S3(3) J-041097-11 ACAGUGAAACCCAACGCAA, siLGal3S3(4) J-041097-12 GGAUGAAGAACCUCCGGGA.

Olfactory bulb recombinant α-synuclein injections *(Paper II)*

We analyzed brain sections from mice injected into the olfactory bulb with different α-synuclein species (monomeric, oligomeric and fibrillar α-synuclein) as previously described. Briefly, α-synuclein was produced in Escherichia coli and purified and filtered as described previously(Rey et al., 2013). Oligomers were obtained by incubating soluble α-synuclein at 4 degrees for 7 days without shaking, in 50 mM Tris-HCl, and then separated from monomers by size exclusion chromatography. Fibrils were obtained from incubation of monomers under continuous shaking at 37°C, and samples were assessed by electron microscopy. α-synuclein was then tagged with ATTO-550 as described previously. We injected α-synuclein monomers, oligomer and fibrils (1 mg/mL; 0.8 uL) stereotactically into the olfactory bulb of mice (coordinates AP: +5.4 mm, L: -0.75 mm, DV: -1 mm relative to bregma and dural surface). After injection, 12 h and 72 h, we perfused the mice transcardially with saline solution, followed by 4% paraformaldehyde (PFA) in phosphate buffer. We dissected the brains and post-fixed them for 2 h in PFA 4% followed by saturation in 30% sucrose solution. We then cut brains into 30 µm free-floating coronal sections, as shown previously(Rey et al., 2013).

Sequential Protein extraction *(Paper II and IV)*

Soluble and insoluble protein fractions were obtained from the whole cortex (mice) and temporal cortex (human) using sequential protein extraction. Fractions were obtained by disruption of the cortex with a dounce homogenizer in the presence of PBS (1 mL/100 ug of tissue) and the soluble fraction. Supernatants, S1 fractions, were aliquoted and stored at −80 °C S1) after centrifugation for 1h at 40.000 rpm in special tubes for high-speed centrifugation from Beckman-Coulter. The pellets were extracted in RIPA buffer (Sigma-Aldrich, Germany) ultracentrifuged at 30.000 rpm and supernatants, S2 fractions (intracellular particulate proteins), were aliquoted and stored. Pellets were re-extracted in buffered-SDS (2 % SDS in 20 mM Tris–HCl, pH 7.4, 140 mM NaCl), centrifuged as above and supernatants, S3 (SDS releasable proteins) were stored. Finally, the remaining pellets (P3) were extracted in SDS-urea (20 mM Tris–HCl, pH 7.4, 4 % SDS and 8 M urea). PBS and RIPA solution were prepared using a protein inhibitor (Protein Inhibitor Cocktail, ThermoScientific) to prevent protein degradation and to inhibit the enzymatic activity of phosphatases (PhosphoStop, Roche).

Western Blot *(All Papers)*

Proteins were extracted from: brain homogenates, cell cultures and cortex using PBS or RIPA buffer (Sigma-Aldrich, Germany) with proteinase and phosphatase inhibitors. Protein extracts were then separated by SDS-PAGE using pre-cast gels (4-20% from Bio-Rad) in TGS buffer (Bio-Rad, Sweden). The proteins were transferred to nitrocellulose membranes (Bio-Rad, Sweden) using the TransBlot turbo system from BioRad. The membranes were subsequently blocked for 1h with caseine at 3 % (w/v) in PBS, then washed 3x10 mins in PBS supplemented with 0.1 % (w/v) Tween 20 (PBS-T), Blots were then exposed to relevant primary antibodies before washing in PBS-T as above with subsequent incubation with secondary antibodies. After a final wash with PBS-T, blots were developed using ECL Clarity (Bio-Rad) according to the manufacturer´s protocol and ChemiBlot XRS+ system from Bio-Rad.

ELISA Plates *(All Papers)*

MSD (MesoScale) plates were use to evaluate the cytokine (Proinflammatory panels for IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 and TNF-α) levels in culture medium, blood and brain soluble fraction form both Human and mice samples. To measure the cytokines in mice and human soluble fractions, we

pulled together 50 ug of the Soluble 1 and Soluble 2 fraction. MSD plates were also used to measure the levels of Aβ38/40/42, phosphorylated Tau and Total Tau in the soluble and insoluble fraction of the brain extraction from WT, Gal3 KO, 5xFAD and 5xFAD Gal3 KO. Serial dilution of the soluble and the insoluble fraction were tested in order to obtain an accurate measure of the protein levels. 1 µg from the soluble fraction was diluted to evaluate Aβ 38/40/42, and 0,3 ug from the insoluble fraction was diluted to evaluate Aβ38/40/42. The plates were developed using the 4x reading buffer dilute 2x time with distilled water and the plates were read using the QuickPlex Q120 reader from Mesoscale.

Immunohistochemistry *(Paper IV)*

Mice were transcardially perfused under deep anesthesia with 4% paraformaldehyde and PBS, pH 7.4. The brains were removed, cryoprotected in sucrose and frozen in isopentane at −15°C, after which were cut at a 40 µm thickness in the coronal plane on a freezing microtome and serially collected in wells containing cold PBS and 0.02% sodium azide. 5xFAD and 5xFAD-Gal3KO free-floating sections were first treated with 3% H_2O_2/ 10% methanol in PBS, pH 7.4 for 20 min to inhibit endogenous peroxidases, and with avidin-biotin Blocking Kit (Vector Labs, Burlingame, CA, USA) for 30 min to block endogenous avidin, biotin and biotin-binding proteins. For single immunolabeling, sections were immunoreacted with one of the primary antibodies: anti-Aβ42 rabbit polyclonal (1:5000 dilution; Abcam); anti-amyloid precursor protein (APP) rabbit polyclonal (1:20000 dilution; Sigma), over 24 or 48 hours at room temperature. The tissue-bound primary antibody was detected by incubating for 1 h with the corresponding biotinylated secondary antibody (1:500 dilution, Vector Laboratories), and then followed by incubating for 90 min with streptavidin-conjugated horseradish peroxidase (Sigma–Aldrich) diluted 1:2000. The peroxidase reaction was visualized with 0.05% 3-3-diaminobenzidine tetrahydrochloride (DAB, Sigma–Aldrich), 0.03% nickel ammonium sulphate and 0.01% hydrogen peroxide in PBS. After DAB, sections immunolabeled for APP, CD45 or Gal3 were incubated 3 mins in a solution of 0.2% of Congo red. Sections were mounted on gelatine-coated slides, air-dried, dehydrated in graded ethanol, cleared in xylene and coverslipped with DPX (BDH) mounting medium. Specificity of the immune reactions was controlled by omitting the primary antisera.

For visualizing fibrillary plaques, sections were incubated for 5 mins with 0.015% Thioflavin-S (Sigma Aldrich) in 50% ethanol, and washed in 50% ethanol, in PBS, and mounted onto gelatine-coated slides.

Immunofluorescense *(Paper II, III and IV)*

For double CD45/Gal3 (anti-CD45 rat monoclonal (clon IBL-3/16, 1:500 dilution, AbD Serotec and anti-Galectin3 (Gal3) goat polyclonal, 1:3000 dilution, R&D) or Iba1/Gal3 immunofluorescence free-floating sections were first incubated with the primary antibodies followed by the corresponding Alexa 488/568 secondary antibodies (1:1000 dilution; Invitrogen). Sections were embedded in autofluorescence eliminator reagent (Merck Millipore), following the manufacturer's recommendations, to eliminate fluorescence emitted by intracellular lipofuscin accumulation. Finally, sections were coverslipped with 0.01M PBS containing 50% glycerin and 3% triethylenediamine and examined under a confocal laser microscope (Leica SP5 II) or Olympus BX-61 epifluorescent microscope.

For Aβ (1:1000, Sigma-aldrich), Gal-3 (1:1000, R&D Systems) and Iba 1 (1:500, WAKO) tissue was incubated for 24h and the following day, the brain section were rinsed for 1 h in PBS containing 0.1 % Triton X-100. After incubating for 1 h with the corresponding secondary antibodies (1:500; Alexa antibodies, Invitrogen), and rinsing again with PBS containing 0.1 % Triton X-100 for 60 min, the brain section were mounted in Glycerol 50 % for visualization. Fixed tissue was examined in an inverted ZEISS LSM 7 DUO confocal laser-scanning microscope using a 20x air objective with a numerical aperture of 0.5. All Images were obtained under similar conditions (laser intensities and photomultiplier voltages), and usually on the same day. Morphometric analysis of the fluorescently labeled structures was performed offline with Fiji ImageJ (W. Rasband, National Institutes of Health). Areas for the specific antibodies were determined automatically by defining outline masks based on brightness thresholds from maximal projected confocal images.

Plaque/dystrophic loading quantification and morphology *(Paper IV)*

Plaque loading was defined as the percentage of hippocampal CA1 region or dentate gyrus area stained for Aβ (with anti Aβ42 or Thioflavin-S), and the same applied to dystrophic loading when stained for APP. Quantification of extracellular Aβ content/APP-positive dystrophic neurites content was done as previously described (Sanchez-Varo et al., 2012; Trujillo-Estrada et al., 2014). Images were acquired with a Nikon DS-5M high-resolution digital camera connected to a Nikon Eclipse 80i microscope. The camera settings were adjusted at the start of the experiment and maintained for uniformity. Digital 4x (plaques) or 10x (dystrophies) images from 6 and 18 month-old 5xFAD and

5xFAD/Gal3KO mice (4 sections/mouse; n = 6-7 /age/genotype) were analyzed using Visilog 6.3 analysis program (Noesis, France). The hippocampal area in each image was manually outlined, leaving out pyramidal and granular layers in the case of APP quantification. Then, plaque/dystrophic areas within the hippocampal regions were identified by level threshold that was maintained throughout the experiment for uniformity. The color images were converted to binary images with plaques. The loading (%) for each transgenic mouse was estimated and defined as (sum plaque or dystrophic area measured/sum hippocampal area analyzed) x 100. The sums were taken over all slides sampled and a single burden was computed for each mouse. The mean and standard deviation (SD) of the loadings were determined using all the available data. Quantitative comparisons were carried out on sections processed at the same time with same batches of solutions.

Plaques and dystrophic neurites form were also evaluated. First, plaques were evaluated using ThioS staining on 5xFAD and 5xFAD-Gal3 KO brain slices. 3 different animals and 3 brains sections of each animal were used. A total of 182 plaques analyzed for each genotype and time point. 3 different parameters were taken into consideration for our analysis: area of the plaque, perimeter of the plaque and circularity. Morphometric analysis of the fluorescently labeled structures was performed offline with Fiji ImageJ (W. Rasband, National Institutes of Health). Areas for the specific antibodies were determined automatically by defining outline masks based on brightness thresholds from maximal projected confocal images

Regarding the dystrophic neurites LAMP1 (Rat anti-mouse, 1:1000, DSHB, USA) staining was performed along with 6E10 and ThioS to truly identify the dystrophic neutires. 4 different mice and 3 different sections were used of each genotype and time point. A total of 211 neurites from 5xFAD and 233 neurites from 5xFAD-Gal3KO were analyzed at 6 months. The parameters taken into consideration: Area of the dystrophic neurite, circulatory and shape factor. Pictures were taken at 20x using Confocal Microscope Nikon Eclipse Ti (Nikon, Japan) and NIS elements software (Nikon, Japan). Dystrophic analysis was performed using in-house software NIS Elements by Nikon. Quantitative comparisons were carried out on sections processed at the same time with same batches of solutions.

STORM Microscopy

The samples were the same that we used for the confocal images (see the protocol in immunofluorescence staining section in material and methods) changing the buffer and the detection device. Images were acquired as previously describe by Van der Zwaag et al. (van der Zwaag et al., 2016). Briefly, to acquire the images it

is used a Nikon N-STORM system configured for total internal reflection fluorescence (TIRF) imaging. STORM buffer contains 10 mM Tris pH 8; 50 mM NaCl; oxygen scavenging system (0.5 mg/mL glucose oxidase (Sigma), 34 μg/mL catalase (Sigma), 5% (w/v) glucose, and 100 mM cysteamine (Sigma). Excitation inclination was tuned to adjust focus and to maximize the signal-to-noise ratio. Fluorophores were excited illuminating the sample with the 647 nm (~125 mW), and 488 nm (~50 mW) laser lines built into the microscope. Fluorescence was collected by means of a Nikon APO TIRF 100x/1.49 Oil W.D. 0.12 mm. Images were recorded onto a 256 × 256 pixel region of EMCCD camera (Andor Ixon3 897). Single molecules localization movies were analyzed with NIS element Nikon software.

Immunofluorescense in Human Sections *(Paper IV)*

Endogenous peroxidases were deactivated by incubation in peroxidase block for 15 mins with gentle agitation. The sections were then washed (3 x 15 min) in 0.1M KPBS, after which they were incubated in blocking buffer (5% Goat Serum blocking with 0,1M KPBS 0.025% Triton-X) for at least 1h with gentle agitation. The sections were then washed (3 x 15 min) in 0.1M KPBS, and the primary antibody added (1:300). The sections were then incubate at 4°C overnight with gentle agitation. The sections were washed (3 x 15 min) in 0.1M KPBS, after which poly-HRP secondary antibody was added. The sections were then incubated for 1h at room temperature. For triple Iba1/Gal3/Amyloid-beta immunofluorescence sections were first incubated with the primary antibodies followed by the corresponding Alexa 647/488/555 secondary antibodies (1:1000 dilution; Alexafluor, Life Technologies). Sections were embedded in 0.6 g Sudden Black (sigma) dissolved in 70% ethanol. The sections (after mounted and dried on slide) are incubated in the sudden black solution for 5 mins. Thereafter, the sections were washed in PBS and mounted with mounting medium. The camera settings were adjusted at the start of the experiment and maintained for uniformity. Confocal Microscope Nikon Eclipse Ti (Nikon, Japan) and NIS elements software (Nikon, Japan) were used to take 20x magnification pictures and for the final collage.

Fluorescent Anisotropy

Production of Recombinant Galectins

Recombinant human galectins (*i.e.* galectin-3 wild type and the R186S mutant) were produced in *E. Coli* BL21 Star (DE3) cells and purified by affinity

chromatography on lactosyl-sepharose columns, which has been previously described by Salomonsson *et al* (Salomonsson et al., 2010).

Establishment of the affinity between galectins and TREM2

A fluorescence anisotropy (FA) assay was used to determine the affinity of recombinant TREM2 and wild type or mutant galectin-3 CRD in solution. Sörme et al. have previously described the method in detail for saccharides and synthetic small-molecule galectin inhibitors (Sorme et al., 2004). In short, increasing concentrations of galectins were first titered against a fixed concentration (0.02 μM) of saccharide probe, when this is done the anisotropy value increases from a value for probe free in solution (A_0) to a value where all probe molecules are bound to galectin CRDs (A_{max}). To establish the dissociation constant (K_d) values between TREM2 and the galectin-3 wild type or mutant CRD a competitive variant of the FA assay was used, in which increasing concentrations of TREM2 were titered against fixed concentrations of galectin and probe (see below for details). By obtaining the anisotropy values for the different TREM2 concentrations, together with the values for A_{max} and A_0, the K_d values could be calculated according to the equations presented in Sörme *et al* (Sorme et al., 2004).

The fluorescence anisotropy of the fluorescein-conjugated probes was measured using a PheraStarFS plate reader and PHERAstar Mars version 2.10 R3 software (BMG, Offenburg, Germany). The excitation wavelength used was 485 nm and the emission was read at 520 nm. All experiments were performed in PBS at room temperature (RT, ~20 °C). The anisotropy values for each data point were read in duplicate wells of 386-well plates (at a total volume of 20 μl), galectin-3 and TREM2 where used at RT (to allow for steady-state). K_d values were calculated as weighted mean values from concentrations of TREM2 that generated between 20-80 % inhibition (where inhibition values of approximately 50 % had the highest impact on the mean value).

Galectin-3 (wild type) affinities: experiments were performed with galectin-3 at a concentration of 0.30 μM and the fluorescent probe 3,3'-dideoxy-3-[4-(fluorescein-5-yl-carbonylaminomethyl)-1H-1,2,3-triazol-1-yl]-3'-(3,5-dimethoxybenzamido)-1,1'-sulfanediyl-di-β-d-galactopyranoside at 0.02 μM (Salomonsson et al., 2010).

Galectin-3 (R186S mutant) affinities: experiments were performed with galectin-3 R186S at a concentration of 2 μM and the fluorescent probe 2-(fluorescein-5/6-yl-carbonyl) aminoethyl-2-acetamido-2-deoxy-α-d-galactopyranosyl-(1–3)-[α-l-

fucopyranosyl-(1–2)]-β-d-galactopyranosyl-(1–4)-β-d-glucopyranoside at 0.02 μM (Carlsson et al., 2007).

Flow cytometry *(Paper III)*

Microglia isolated by CD11b microbeads (Miltenyi Biotec) were analyzed for microglial cell surface antigens by flow cytometry. Briefly cells were incubated with anti-CD45/CD11b antibody (1:100)(BD Bioscience) to block Fc receptors. Samples were then incubated with anti-CD11b-APC (Biolegend; 1:800) and anti-CD45-PE (BD Bioscience; 1:400) to confirm purity of the microglial population. Gating was determined by proper negative isotype stained controls and compensation was made with single stainings. A viability staining, 7-aminoactinomycin D (7AAD)(BD Bioscience) was used to exclude dead cells. Flow cytometry was perfomed in a FACSArial III cytometer (BD Biosciences) and FACS Diva software (BD Biosciences). Ten thousand events were recorded and microglia were identified by CD11b+ and CD45+ expression. Data analysis was made using FlowJo 10.3 software (Three Star, Inc).

Total RNA extraction and pPCR *(Paper IV)*

Total RNA and proteins were extracted using TriPure Isolation Reagent (Roche). RNA integrity (RIN) was determined by RNA Nano 6000 (Agilent). The RIN was 8.5 ± 0.5. RNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Fischer, Spain).

Retrotranscription and quantitative real-time RT-PCR. Retrotranscription (RT) (4 μg of total RNA) was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems). For real-time qPCR, 40 ng of cDNA were mixed with 2× Taqman Universal Master Mix (Applied Biosystems) and 20× Taqman Gene Expression assay probes (Applied Biosystems, supplemental Table 1). Quantitative PCR reactions (qPCR) were done using an ABI Prism 7900HT (Applied Biosystems). The cDNA levels were determined using GAPDH as housekeeper. Results were expressed using the comparative double-delta Ct method (2-ΔΔCt). ΔCt values represent GAPDH normalized expression levels. ΔΔCt was calculated using 6-month-old WT mice samples.

Primers

Iba1 (Ref. Mm00479862_g1), CD45 (Ref. Mm01293577_m1), CD68 (Ref. Mm03047343_m1), TREM2 (Ref. Mm04209424_g1), Cx3Cr1 (Ref. Mm02620111_s1), GAPDH (Ref. Mm99999915_g1), IL-6 (Ref.

Mm00446190_m1), TNFa (Ref. Mm00443258_m1), GFAP (Ref. Mm01253033_m1).

Gene Array *(Paper IV)*

Hippocampal samples from 5xFAD, 5xFAD-Gal3KO, WT and Gal3KO out mice at 6 and 18 months were collected and snap frozen in dry ice to perform the mRNA evaluation. mRNA was extracted using the RNAeasy Mini Kit (Qiagen) according to manufacturer's protocol. The extraction was performed automatically using the QIAcube device from Qiagen. RNA concentration was subsequently quantified using the NanoDrop 2000C. Samples with a RIN value under 5 were excluded. cDNA synthesis was performed using Superscript Vilo cDNA Synthesis (ThermoScientific) according to the manufacturer's protocol. TaqMan® OpenArray® Mouse Inflammation, TaqMan® OpenArray® Real-Time PCR Master and TaqMan® OpenArray® Real-Time PCR were used to performed the qPCR. Real-Time PCR Open Array from Applied Biosystems was used to read the Open Array 384 well plate used to perform the qPCR.

Gene Array Analysis

Differently expressed genes are represented using the data from the HTqPCR assay assessed in the Openarray platform (Qiagen). The statistical analysis was performed using the software DataAssist v3.01. The maximum CT permitted was 35. First, we sort the data based on the Gene Fold Change and then we convert the data to Log2FC. We compared the 5xFAD at 6 and 18 months to WT mice and 5xFAD with 5xFAD Gal3KO at 6 and 18 months as well. We selected genes with a Log2FC value between ±2 at 6 months and ±4 at 18 months. 95 genes out of 629 were selected at 6 months and 106 genes at 18 months. For the analysis of the main pathways affected by the lack of galectin-3 at 6 and 18 months in our 5xAFD mouse model we used Reactome database. Cluster-diagram and tables were generated with the same databases.

Behavioral tests *(Paper IV)*

Open Field: The open field test was used to assess both exploratory behavior and locomotor activity. The mice were placed for 5 mins in an open field (45x45x45cm3). Monitoring was done by an automated tracking system (SMART 2.5, Panlab). The behavioral parameters registered during 15-mins sessions were the total travel distance (cm) and the resting time (s) (Bachiller et al., 2015).

Rotarod: To habituate mice to the rotarod (Rota-Rod/RS- Panlab-Harvard Apparatus), the animals were placed on the roller at a speed of 4 rpm until they could remain on it for three mins without falling off. To assay motor coordination, animals were tested during three consecutive days. The protocol consisted in, after three trials of 60 seconds at low velocity separated between them for 10 mins, the rotarod automatically start the acceleration from 4 to 40 rpm during 5 mins (ramp: 1rpm/8s) The rotarod parameter analyzed was the latency to fall during the third day of test (Bachiller et al., 2015; Cubillos-Rojas et al., 2016).

Elevated plus maze: The elevated plus maze has the shape of a "+" with two alternate open and two alternate closed arms extending from a central platform. The whole maze is raised 45 cm above the floor. During the test, the mouse is placed onto the center field and is allowed to explore the maze for 5 min (Jawhar et al., 2012). The percentage of the time spent in the each arms ant the total travel distance (cm) was measured using an automatic video tracking system (SMART 2.5, Panlab).

*Clasping test***:** to test clasping behavior, the tail for 30 sec to initiate the clasping phenotype suspended mice. A score was given on a scale from 0 to three, where 0 represented no clasping, 1 = forepaws clasping, 2 = forepaws and one hind paw clasping, and 3 = all paws clasping (Miller et al., 2008). Unpaired t-test was used to compare the clasping score between the 6 mo old 5XFAD mice and WT.

Morris Water maze test: to test spatial acquisition memory test was conducted in a pool consisting of a circular tank (180 cm diameter) filled with opaque water at 20° C +/- 1° C). A platform (15 cm diameter) was submerged 10 mm under the water surface. A white curtain with specific distal visual cues surrounded the water maze. White noise was produced from a radio centrally positioned above the pool to avoid the use of auditory cues for navigation. Spatial learning sessions were conducted on the following 10 consecutive days with four trials per day. Each trial was started by introducing the mouse, facing the pool wall, at one of four starting points in a quasi-random fashion way to prevent strategy learning. If a mouse did not find the platform within 60 s, it was gently guided to the platform. Each mouse remained on the platform for 30 s before transfer to a heated waiting cage. During all acquisition trails, the platform remained in the same position. On the day following the last learning trial, a 60 s probe test was conducted, where the platform had been removed from the pool. All mouse movements were recorded b a computerized tracking system that calculated distances moved and latencies required for reaching the platform (Anymaze)

Genetic association analysis *(Paper IV)*

Datasets

Genotypic datasets from four GWAS were used in this study: a) The Murcia study (Antunez et al., 2011); b) The Alzheimer's Disease Neuroimaging Initiative (ADNI) study (Mueller et al., 2005); c) The GenADA study (Li et al., 2008a); and d) The NIA study(Wijsman et al., 2011). (For GWAS dataset details see supplementary information). Researchers from our group previously performed the Murcia study (Antunez et al., 2011). Datasets from ADNI, GenADA, and NIA, studies were obtained from dbGAP (http://www.ncbi.nlm.nih.gov/gap), Coriell Biorepositories (http://www.coriell.org/), or ADNI (http://adni.loni.ucla.edu/). Prior to the genetic association analysis, each dataset (Murcia, ADNI, GenADA, NIA, and TGEN) was subjected to both an extensive quality control analysis and a principal component analysis. In addition, since different platforms were used in the five GWAS analyzed, we imputed genotypes using HapMap phase 2 CEU founders (n = 60) as the reference panel. These approaches have been previously described (Antunez et al., 2011; Boada et al., 2014; Martinez-Mir et al., 2013). Overall, a total of 2252 cases and 2538 controls were included in the meta-analysis.

SNP selection

To select single nucleotide polymorphisms (SNPs) within LGALS3 gene including 1000 pb upstream and downstream of that genetic region we used the UCSC Table Browser data retrieval tool (Karolchik et al., 2004), release genome assembly: Mar. 2006 (NCBI36/hg18), from the UCSC Genome Browser database (http://genome.ucsc.edu/) (Hinrichs et al., 2006). Selected SNPs were extracted from GWAS datasets using Plink v1.06 software (Purcell et al., 2007).

Linkage disequilibrium blocks

Linkage disequilibrium (LD) blocks were determined along the genomic regions studied using Haploview software and genotyping data from the largest dataset used (NIA dataset) (Barrett et al., 2005).

SNP Association analyses *(Paper IV)*

Unadjusted single-locus allelic (1 df) association analysis within each independent GWAS sample was carried out using Plink software. We combined data from these four GWAS datasets using the meta-analysis tool in Plink selecting only those markers common to, at least, three studies. For all, single locus meta-analyses, fixed effects models were employed when no evidence of heterogeneity was found. Otherwise random effects models were employed.

Multiple-testing correction was applied taking into account the number of different LD blocks detected. Thus, the p-value threshold was established by the following formula: p = 0.05/number of LD regions in the meta-analysis.

Proteomic analysis *(Paper III)*

Protein concentration was measured for each sample using Pierce™ BCA Protein Assay (Thermo Scientific, Rockford, USA) and the Benchmark Plus microplate reader (Bio-Rad Laboratories, Hercules, USA) with BSA solutions as standards. Aliquots of 1 or 4 µg of each protein extract sample were mixed into the pooled reference sample. Aliquots containing 10 µg of each experimental sample or reference sample were digested with trypsin using the filter-aided sample preparation (FASP) method 7. Briefly, protein samples were reduced with 100 mM dithiothreitol at 60°C for 30 min, transferred on 30 kDa MWCO Nanosep centrifugal filters (Pall Life Sciences, Ann Arbor, USA), washed with 8M urea solution and alkylated with 10 mM methyl methanethiosulfonate in 50 mM TEAB and 1% sodium deoxycholate. Digestion was performed in 50 mM TEAB, 1% sodium deoxycholate at 37°C in two stages: the samples were incubated with 100 ng of Pierce MS-grade trypsin (Thermo Scientific, Rockford, USA) for 3h, then 100 ng more of trypsin was added and the digestion was performed overnight. The peptides were collected by centrifugation labelled using TMT 10-plex isobaric mass tagging reagents (Thermo Scientific) according to the manufacturer instructions. The labelled samples from brain were mixed into corresponding sets, sodium deoxycholate was removed by acidification with 10% TFA.

The mixed labeled samples were subjected to the reversed-phase high pH fractionation on the AKTA chromatography system (GE Healthcare Life Sciences, Sweden) using the XBridge C18 3.5 µm, 3.0x150 mm column (Waters Corporation, Milford, USA) and 25 min gradient from 7% to 40% solvent B at the flow rate of 0.4 ml/min; solvent A was 10 mM ammonium formate in water at pH 10.00, solvent B was 90% acetonitrile, 10% 10 mM ammonium formate in water at pH 10.00. The initial 31 fraction was combined into 15 pooled fractions in the order 2+17, 3+18, 4+19 etc. The pooled fractions were dried on Speedvac and reconstituted in 20 µl of 3% acetonitrile, 0.1% formic acid for analysis.

LC-MS/MS Analysis *(Paper III)*

Each fraction was analyzed on Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) interfaced with Thermo Easy-nLC 1200 nanoflow liquid chromatography system (Thermo Fisher Scientific, Odense, Denmark). Peptides were trapped on the C18 trap column (100 µm X 3 cm, particle size 3

μm) separated on the home-packed C18 analytical column (75 μm X 30 cm) packed with 3 μm Reprosil-Pur C18-AQ particles (Dr. Maisch, Germany) using the gradient from 6% to 32% B in 70 min, from 32% to 50% B in 5 min, from 50% to 100% B in 5 min; solvent A was 0.2% formic acid and solvent B was 80% acetonitrile, 0.2% formic acid. Precursor ion mass spectra were recorded at 70 000 resolution. The 10 most intense precursor ions were selected with the isolation window of 1.6, fragmented using HCD at stepped collision energy of 27, 35 and 47 and the MS2 spectra were recorded at a resolution 35 000. Charge states 2 to 6 were selected for fragmentation, dynamic exclusion was set to 30 s.

Proteomic profile evaluation *(Paper III)*

Data analysis was performed using Proteome Discoverer version 1.4 (Thermo Fisher Scientific, Waltham, USA). The protein database for Mus musculus (February 2017, 16854 sequences) was downloaded from Swissprot. Mascot 2.5.1 (Matrix Science) was used as a search engine with precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da, one missed tryptic cleavage was accepted. Mono-oxidation on methionine was set as a variable modification, methylthiolation on cysteine and TMT-6 reagent modification on lysine and peptide N-terminus were set as a fixed modification. Percolator was used for the validation of identificated results; target false discovery rate of 1% was used as a threshold to filter confident peptide identifications.

Reporter ion intensities were quantified in MS3 spectra using Proteome Discoverer 1.4 at 0.003 Da mass tolerances with reporter absolute intensity threshold of 2000. The resulting ratios were normalized on the median protein value of 1.0 in each sample.

Protein bioinformatics analysis

Proteins profile similarities were analyzed by using Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/index.html) among the different analyzed groups. Then, Gene Ontology (GO) analysis and Panther pathways analysis database were used to analyse the data from 2 different approaches. Individual groups were analyzed followed with group comparison. The first 200 of the most abundant proteins form each individual group (5xFAD 2, 6 and 10 weeks and WT 2 and 10 weeks) were used to evaluate the main pathways affected by EnrichR (Panther data base). Then, GO analysis was performed using STRAP software. After the GO analysis, the resulted top 20 upregulated and downregulated proteins selected from group comparison (5xFAD 2w vs 5xFAD 6w, 5xFAD 10w vs 5xFAD 6w, 5xFAD 10w vs WT12w and 5xFAD 2w vs WT 2w) were used to further analyze the main pathways involved. Briefly, to select the proteins for the

GO analysis we did the next: 1. For each protein, calculate group means, fold changes and perform t-tests to get p-values, filter on fold change > 0 (to picks only up-regulated proteins) and sort on p-values. To get downregulated proteins we only change step 2 to: filter on fold change < 0. The pathway analysis was performed using EnrichR (Panther database for pathways). EnrichR implements four scores to report enrichment results: p-value, q-value, rank (Z-score), and combined score.

The p-value is computed using a standard statistical method used by most enrichment analysis tools: Fisher's exact test or the hypergeometric test. This is a binomial proportion test that assumes a binomial distribution and independence for probability of any gene belonging to any set.

The q-value is an adjusted p-value using the Benjamini-Hochberg method for correction for multiple hypotheses testing.

The rank score or z-score is computed using a modification to Fisher's exact test in which we compute a z-score for deviation from an expected rank.

Finally, the combined score is a combination of the p-value and z-score calculated by multiplying the two scores as follows:

$$1)\ c = log(p) * z$$

Where "*c"* is the combined score, p is the p-value computed using Fisher's exact test, and z is the z-score computed to assess the deviation from the expected rank.

PCA (principal analysis component) analysis was performed using Ingenuity Pathway Analysis (IPA) from Qiagen. PCA is a quality controls analysis and it was performed in order to detect outliers and to evaluate the variability within each group.

Antibodies

Antibodies used for this study; Anti-rabbit iNOS primary Antibody (1:5000, Santa Cruz), Anti-rat Galectin-3 Antibody, (1:3000, M38 clone from Hakon Leffler's lab, (in-house antibody) (Add references), Anti-Goat Galectin-3 Antibody (1:1000, R&D Systems) Anti-mouse Actin antibody 1:10000 (Sigma-Aldrich), Anti-human Aβ antibody (1:5000, Covance), Anti-Rabbit Iba-1 antibody (1:500, Wako), Anti-mouse TLR4 Antibody (1:1000, Santa Cruz), Anti-mouse NLRP3 Antibody (1:5000, Adipogen), Anti-rabbit C83 antibody (369) (1:1000, Gunnar Gouras Laboratory, BMC, Lund, Sweden). Anti-rabbit IDE-1 antibody (1:1000, Calbiochem), Anti-Rabbit p-Tau (pTau181, Santa Cruz), Anti-mouse Aβ (1:1000, Sigma-Aldrich). Anti-CD45 rat monoclonal (clon IBL-3/16, 1:500 dilution, AbD Serotec) LAMP-1 (Rat anti-mouse, 1:1000, DSHB, USA). Secondary antibodies

used for western blot were: anti-rabbit, anti-mouse, anti-goat and anti-rat from Vector Labs. Secondary antibodies used for immunofluorescense raised in donkey were: anti-rabbit, anti-goat, anti-mouse and anti-rat from Life Technology (AlexaFluor).

Papers Highlights and Results

Paper I

Microglia-Secreted Galectin-3 Acts as a Toll-like Receptor 4 Ligand and Contributes to Microglial Activation

- Galectin-3 acts as an endogenous TLR4 ligand with a Kd value around 1 mM.
- Galectin-3 can initiate TLR4-dependent inflammatory response in microglia.
- Galectin-3 is required for complete TLR4 activation upon LPS treatment.
- Galectin-3-TLR4 interaction is confirmed *in vivo* in experimental models of Parkinson's disease and in human stroke brains.
- Galectin-3 deficiency results in reduced inflammation and neuronal death, and improved cognitive behavior in mice presenting brain ischemia.

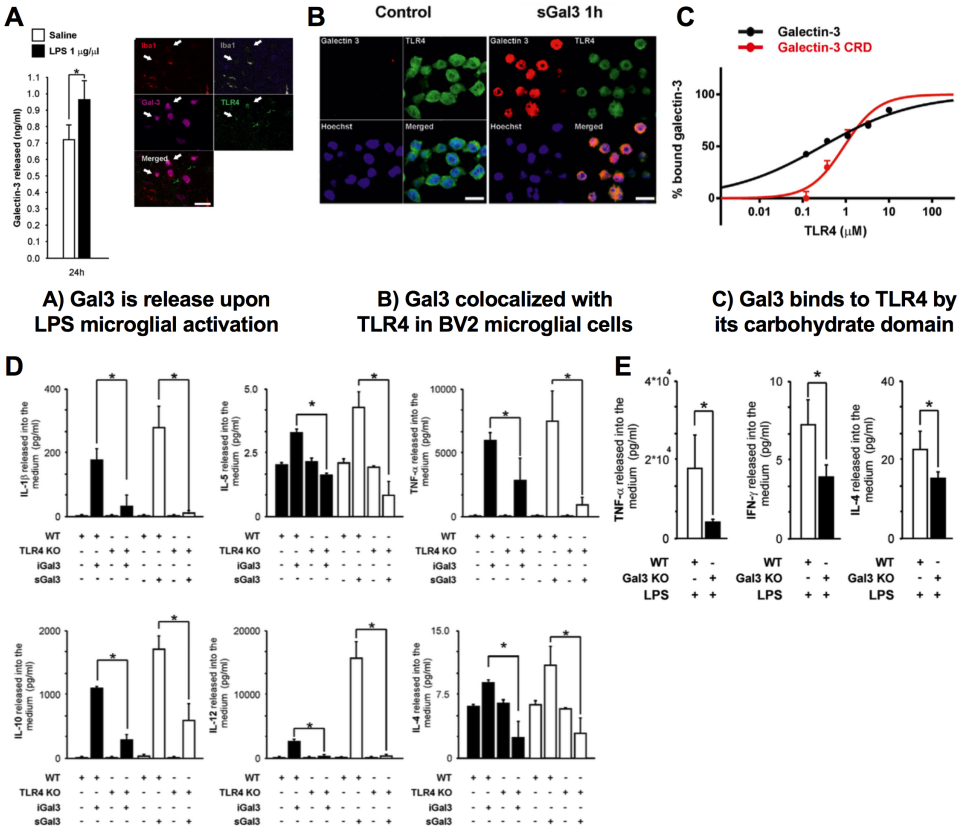
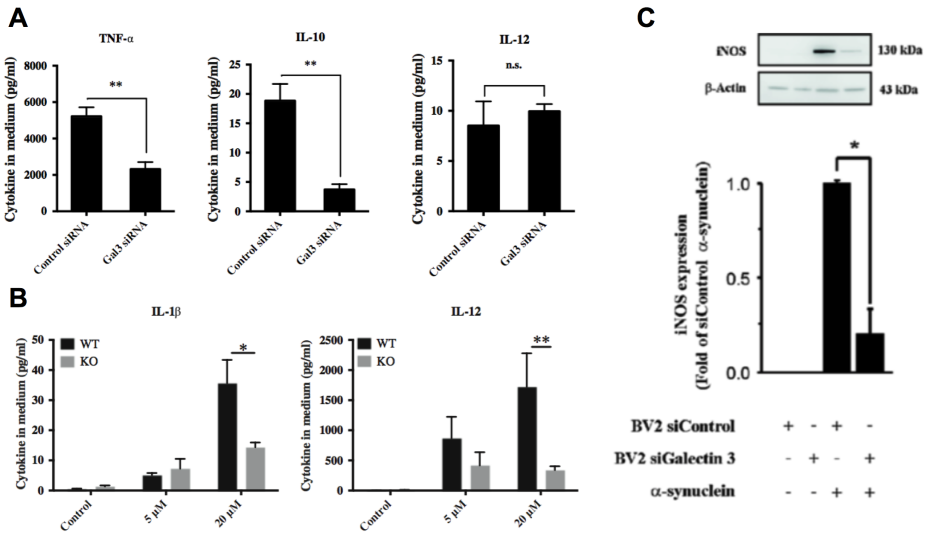


Figure 1. Paper I, "Microglia-Secreted Galectin-3 acts as a Toll-like Receptor 4 Ligand and Contributes to Microglial Activation", main findings.

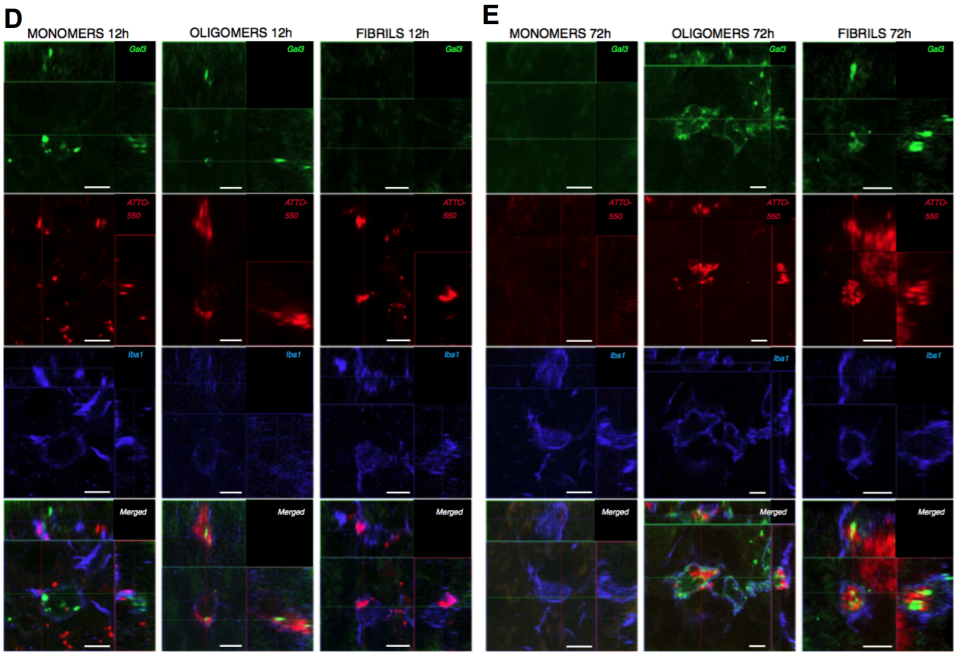
Paper II

The role of Galectin-3 in α-synuclein-induced microglial activation

- Galectin-3 plays a significant role in microglia activation induced by α-synuclein.
- Genetic downregulation or pharmacological inhibition of galectin-3 reduces microglial activation in BV2 and primary microglial cultures stimulated with α-synuclein.
- α-synuclein monomers, oligomers and fibrils injected into the olfactory bulb are taken up by microglial cells that will express galectin-3.



A-C) The lack of gal3 reduces the proinflammatory activation of BV2 microglial cells or primary microglial cells stimulated with α-synuclein



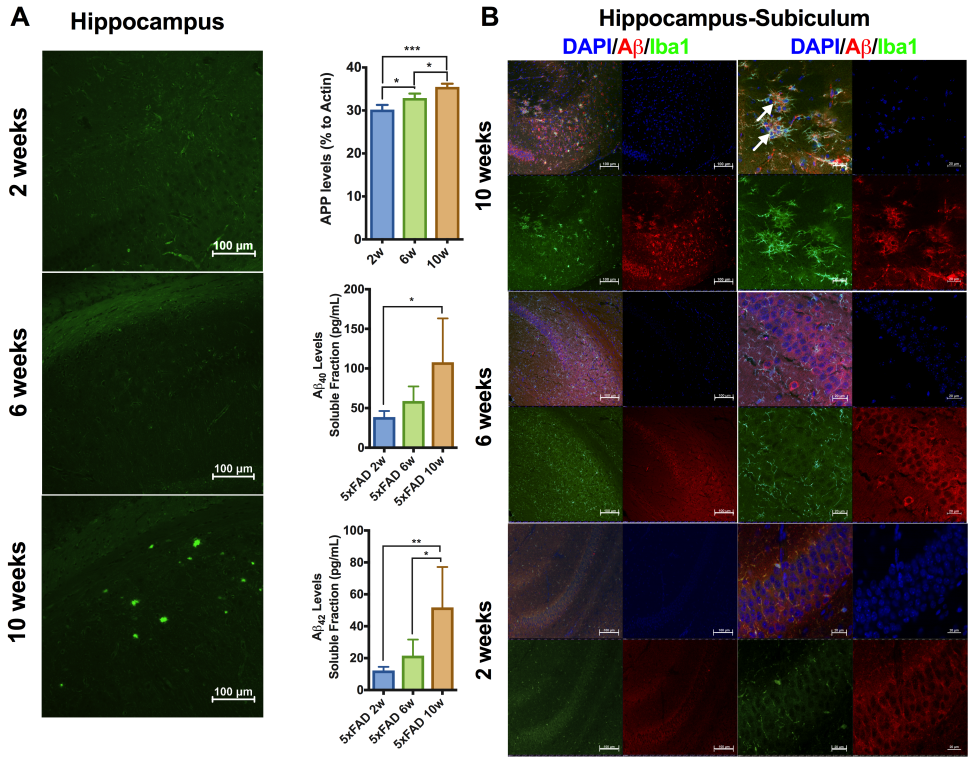
D-E) Brain injection of monomers, oligomers and fibrils of α-synuclein are taken up by microglial cells inducing gal3 expression

Figure 2. Paper II, "The role of Galectin-3 in α-synuclein-induced microglial activation", main findings

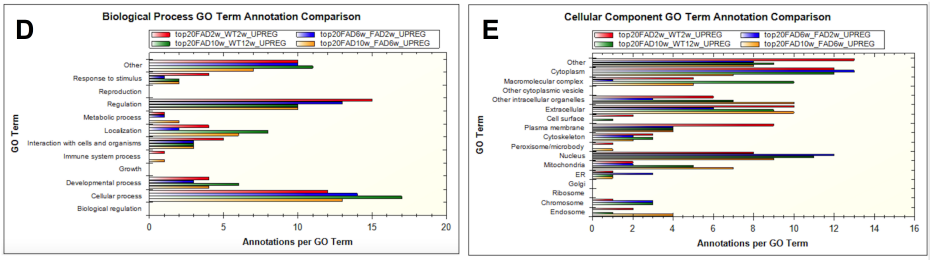
Paper III

Innate immune alterations are elicited in microglial cells before the plaque deposition in 5xFAD mice

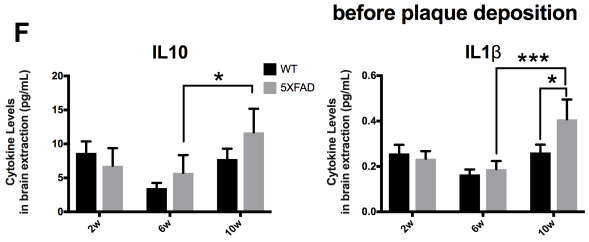
- Significant increase in IL4 in the brain of 5xFAD mice at 2, 6 and 10 weeks. IL12 was found increased at 6 and 10 weeks in the 5xFAD mice.
- Interestingly, IL1β and IL10 were elevated after the formation of Aβ plaque at 10 weeks only.
- JAK/STAT, p38 MAPK and Interleukin pathways were affected in microglial cells already before plaque deposition at 6 weeks.
- In 5xFAD at 10 weeks, a number of different innate-immune related pathways were highlighted including: interferon-gamma regulation pathways, interleukins-related pathways and B and T cells activation pathways.



A-B) 5xFAD mice present Aβ plaque deposit in the subiculum at 10 weeks. Aβ40 and 42 soluble fraction levels were upregualted in a time-dependent fashion.



D-E) Gene Ontology analysis revealed innate immune related pathways affected in 5xFAD mice before plaque deposition



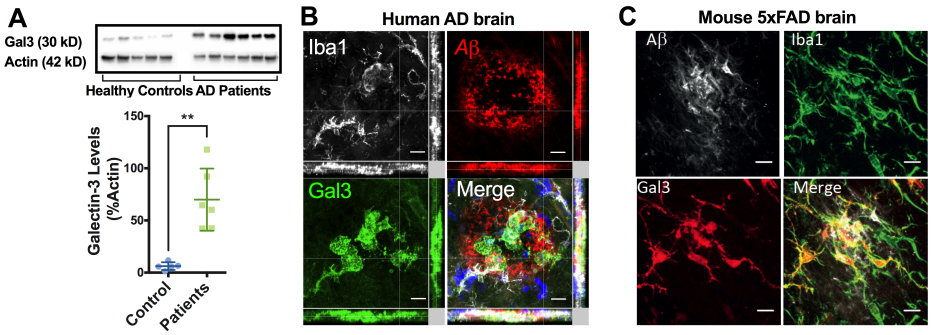
F) Proinflammatory pathways such as IL10 or IL1β were altered over the progression of AD pathology in 5xFAD mice

Figure 3. Paper III, "Innate immune alterations are elicited in microglial cells before the plaque deposition in 5xFAD mice", main findings.

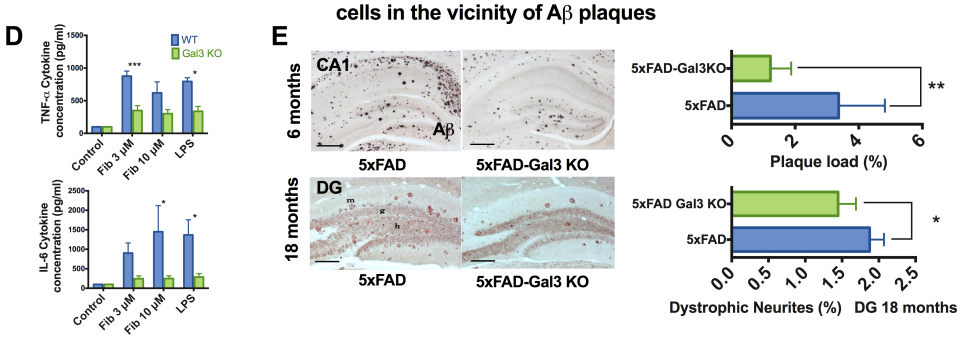
Paper IV

Galectin-3 is up regulated in Alzheimer's disease and contributes to the pathology in 5xFAD mice

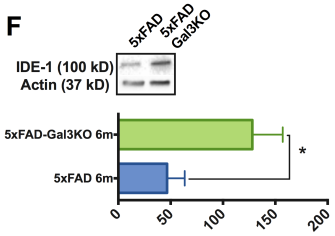
- Galectin-3 is robustly up regulated in the human AD brain and transgenic mice,
- Galectin-3 expression is mostly confined to reactive microglia/macrophages associated to Aβ plaques in human and transgenic mice,
- Galectin-3 gene deletion strongly decreased Aβ plaque burden in adult 5xFAD and associated brain inflammatory response in adult and aged 5xFAD,
- Galectin-3 was found to interact with TREM2 in fluorescent anystropy test and being co-expressed in microglial cells expressing galectin-3 using confocal and STROM microscopy
- Galectin-3 is a critical microglial immune mediator involved in amplifying fAβ-induced proinflammatory response,
- GWAS analysis demonstrated a significant involvement of galectin-3 in AD incidence and
- Galectin-3 gene deletion overall improved aged 5xFAD mice health status, exerting neuroprection in hippocampal and cortical areas, improved cognitive and motor behavior in adult mice.



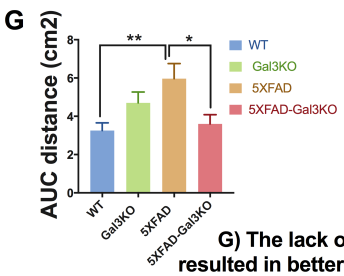
A-C) Gal3 was upregulated in the brain of AD patients and was highly expressed in microglial cells in the vicinity of Aβ plaques



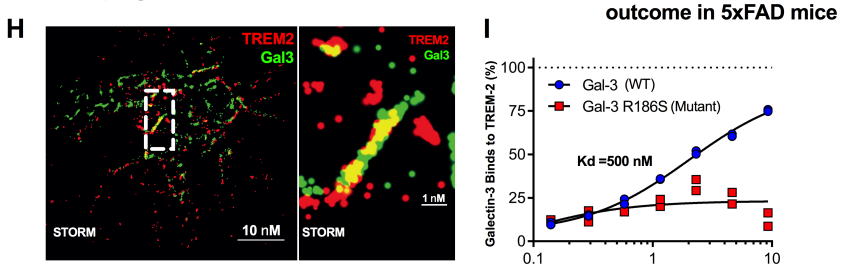
D-E) The lack of gal3 reduces microglial activation in microglial primary cultures and AD pathology in 5xFAD-Gal3KO mice compared to 5xFAD control mice.



F) The lack of gal3 upregulates the levels of IDE-1



G) The lack of gal3 resulted in better cognitive outcome in 5xFAD mice



H-I) Gal3 colocalized with TREM2 in microglial cells from 5xFAD and binds to TREM2 by its carbohydrate domain

Figure 4. Paper IV, "Galectin-3 is up regulated in Alzheimer's disease and contributes to the pathology in 5xFAD mice", main findings.

Main Results

The neuroinflammatory response is one of the main components associated with neurodegenerative diseases such as AD, PD and even Stroke (Gao et al., 2008; Heneka et al., 2015; Jin et al., 2010). Still, we don't fully understand the mechanisms regulating the inflammatory response. Different components of the immune system elicit the inflammatory response. The theme in this thesis is the innate immune system, with microglia being the main focus.

During the development of my thesis the main working hypothesis suggest the detrimental role of galectin-3 in the neuroinflammatory response excerted by the microglial cells in different brain pathologies

Paper I. Microglia-Secreted Galectin-3 Acts as a Toll-like Receptor 4 Ligand and Contributes to Microglial Activation

Genetic profile of BV2 microglial cells activated with Galectin-3 or LPS.

In our first study, "*Microglia-Secreted Galectin-3 Acts as a Toll-like Receptor 4 Ligand and Contributes to Microglial Activation",* we established the ground of the thesis by finding a clear connection between galectin-3 and TLR4 in regards to microglial activation and proinflammatory response. We evaluated the gene profile of BV2 cells activated with LPS or galectin-3, using either soluble or immobilized galectin-3 (the plate was coated with galectin-3 to avoid galectin-3 internalization, only enabling an extracellular effect of galectin-3 on microglial cells) and we found differences between free and immobilized galectin-3 compared to LPS in BV2 gene profile. Notably, some of the genes upregulated by LPS were also affected by Galectin-3, albeit to a minor extent.

Galectin-3 binding to TLR4 analysis.

Next, we did analyzed the binding ability of galectin-3 to TLR4 using two different techniques, Microscale thermophoresis and Fluorescent Anisotropy. To that aim, we used mutant galectin-3 lacking of CRD domain and

WT galectin-3. We found a clear interaction between galectin-3 and TLR4 that was absent when using the galectin-3 CRD mutant, or when adding a lactose, which acts as a competitive inhibitor binding the CRD domain of galectin-3 blocking the ligation with TLR4.

The lack of TLR4 or Galectin-3 reduces the microglial activation capacity.

Later, we evaluated the activation ability of BV2 microglial cells after knocking down either TLR4 or galectin-3 using siRNA. TLR4 siRNA reduced TLR4 levels and impaired the microglial activation stimulated by galectin-3 compared to control BV2 cells. iNOS levels were significantly reduced in TLR4 knockdown BV2 cells compared to control BV2 using both, immobilized and soluble galectin-3. Moreover, the release of proinflammatory cytokines to the medium was also significantly reduced in BV2 cells lines treated with siTLR4. Proinflammatory cytokines, including: IL12, IL10, IL4, IL5, TNF-α and IL1β were significantly downregulated compared to control cells. Furthermore, using galectin-3 siRNA in BV2 stimulated with LPS resulted in a significant downregulation of iNOS expression along with IL4, TNF-α and IFN-γ release by BV2 cells treated with galectin-3 siRNA compared to control BV2 cells.

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The lack of Galectin-3 protects TH positive neurons after LPS injection.

Next, we injected LPS in the SN of WT mice to assess the interaction of TLR4 and galectin-3. Notably, using FRET to study physical interaction between proteins, we were able to detect TLR4-galectin-3 interaction in the SN 24h-post injection. Next, we did inject WT and galectin-3 KO model with LPS in the SN and we evaluated the number of TH positive neurons and iba1 levels. Notably, the number of TH positive neurons was higher in the galectin-3 KO mice compared to WT after LPS injection 7 days after injection. Furthermore, the number of Iba-1 positive cells was lower in the KO compared to the WT. mRNA expression of IL6 and IL1β were also measured and we found a significant reduction of both cytokines in the galectin-3 KO mice compared to WT animals.

Paper II. The role of Galectin-3 in α-synuclein-induced microglial activation

α-synuclein activates BV2 in a concentration dependent manner.

The next project, "*The role of Galectin-3 in α-synuclein-induced microglial activation"*, is our first approach to the role of galectin-3 in the context of PD. In this case, we combined *in vitro* and *in vivo* experiments to study the role of galectin-3 in microglial cells stimulated with α-synuclein. First, BV2 microglial cells were stimulated with α-synuclein to test that our protein preparation was able to activate our cells. iNOS and different proinflammatory cytokines (IL2, IL12 and TNF-α) were markedly up regulated in BV2 cells stimulated cells with our protein preparation.

The lack of Galectin-3 reduces microglial cell activation ability.

To test the role of galectin-3 in microglial cells stimulated with α-synuclein, we used a specific galectin-3 inhibitor in BV2 microglial cells or microglial cells from WT and galectin-3 KO primary cultures. Notably, the inhibition of galectin-3 reduced microglial activation upon stiumulation with α-synuclein. iNOS expression was significantly reduced in a concentration dependent-manner in BV2 cells treated with galectin-3 inhibitors along with α-synuclein. Moreover, IL6, IL12 and TNF-α were reduced in BV2 treated with galectin-3 inhibitors along with α-synuclein compared to BV2 cells from the control group. Similar results were achieved using galectin-3 KO microglial cells from primary cultures, where IL12 and IL1β levels were reduced.

α-synuclein injections elicited Galectin-3 in microglial cells.

Finally, we injected α-synuclein in the olfactory bulb to evaluate microglial galectin-3 expression in the presence of α-synuclein. We injected α-synuclein monomers, oligomers and fibrils in WT mice. After 12 and 72h, we analyzed galectin-3 in areas close to the injection site and we found a markedly upregulation of galectin-3 only in microglial cells containing α-synuclein inclusions. Our data demonstrated that galectin-3 plays an important role in microglial activation by α-synuclein, and microglial expression of galectin-3 was linked to the phagocytosis of α-synuclein.

Paper III. Innate immune alterations are elicited in microglial cells before the plaque deposition in 5xFAD mice

In our third study, "*Innate immune alterations are elicited in microglial cells before the plaque deposition in 5xFAD mice"*, we aimed to describe the inflammatory response at very early stage of AD pathology. To that aim, we used 5xFAD AD mice at different ages, from 2 to 10 weeks. First, we evaluated the disease progression. It was first at 10 weeks that we found clear Aβ deposit in the subiculum. Before that, at age 2 and 6 weeks, we couldn't find any plaque in the whole brain. Microglial cell activation was also evaluated in 5xFAD at different time-points based on galectin-3 expression by microglial cells. We found microglial cells expressing galectin-3 around Aβ plaques in the subiculum. Moreover, at 6 weeks, before plaque deposition was detectable, we found microglial cells expressing galectin-3 around APP expressing neurons in the molecular layer of CA1 hippocampus.

Notably, we found a clear upregulation in the soluble levels of Aβ40 and 42 over the progression of the pathology. The increase in the levels was already presented from 2 to 6 weeks, before the Aβ plaques at the subiculum appeared. At 10 weeks, Aβ40 and 42 soluble levels were upregualted compared to the previous time-points. Cytokines such as IL10, IL12, IL1β and IL4 were significantly affected in 5xFAD over the progression of the pathology. It is important to mention that the cytokine alteration is of particular interest as the plaque deposits were confined in the subiculum but microglial cells where isolated from the whole cortex and hippocampus, so that, the alteration of cytokines levels are very likely due to both, plaque deposition and Aβ soluble fraction in the extracellular space.

Microglial cells proteomic profile present a proinflammatory alteration in 5xFAD before plaque deposits.

Next, we isolated microglial cells from 5xFAD and WT mice to analyse their proteomic profile. To isolate microglial cells we used CD11b magnetic beads and we confirmed the isolation by flow cytometry using CD45 marker. The bioinformatics analysis revealed a clear immune response in microglial cells at time points earlier than 10 weeks, before the first plaques were present. For example, JAK/STAT, p38 MAPK and Interleukin pathways were up regulated at 6 weeks in isolated microglial cells. Remarkably, at the age of 10 weeks we found altered synaptic vesicle trafficking as the most altered pathway in our analysis. We did not find the previous proinflammatory pathways upregulated

among the main pathways affected at 10 weeks compared to the pathways described at 6 weeks. However, individual proinflammatory proteins found at 6 weeks were ranked higher at 10 weeks. The higher prevalence of vesicle trafficking pathways compared to proinflammatory pathways at 10 weeks is very likely related with the first plaque deposits found in the subiculum. Indeed, at 10 weeks, the inflammatory pathways highlighted were linked to vesicles metabolism, which recently has been related to amyloid toxicity and plaque formation (Joshi et al., 2014).

Paper IV. Galectin-3 is up regulated in Alzheimer's disease and contributes to the pathology in 5xFAD mice

Our last study, "*Galectin-3 is up regulated in Alzheimer's disease and contributes to the pathology in 5xFAD mice*." This study is the result of a collaborative effort between different labs to elucidate the role of galectin-3 in the context of AD. The aim of the project was to investigate how the lack of galectin-3 affects AD progression from an inflammatory perspective.

Amyloid beta fibrils activates BV2 microglial cells.

First, we evaluated if our protein preparation was able to activate BV2 microglial cells stimulated with monomers or fibrils of Aβ. Monomers failed to induce BV2 activation. However, Aβ fibrils clearly activated BV2 cells, increasing the expression of iNOS, NLRP3 and a number of different proinflammatory cytokines (TNF-α, IL12, IL10 and IL1β) in a concentration dependent fashion. Interestingly, IDE1, an enzyme involved in Aβ degradation (Farris et al., 2003; Kummer et al., 2012), was downregulated in BV2 cells stimulated with Aβ fibrils in a concentration dependent-fashion.

The lack of Galectin-3 reduces microglial activation.

Next, we tested how the inhibition of galectin-3 affected to microglial activation. First, we stimulated BV2 cells with Aβ fibrils along with galectin-3 inhibitor in a concentration dependent fashion. Then, we analyzed the cytokine release to the medium. Proinflammatory cytokines (IL6, IL8, IL12 and TNF-α) released by BV2 were downregulated by galectin-3 inhibitor in a concentration dependent fashion. Regarding iNOS, galectin-3 inhibition in BV2

cells significantly reduced the levels of iNOS in a concentration dependent-manner compared to non-treated cells. Regarding IDE-1, we found significant increased levels of IDE-1 in BV2 cells treated with galectin-3 inhibitor along with Aβ fibrils. Furthermore, we used WT and galectin-3 KO primary microglial cultures to measure cytokine release. We stimulated the cells with an increasing concentration of Aβ fibrils and found a significant downregulation of TNF-α, IL6 and IL8 in galectin-3 KO primary microglial cultures compared to WT controls.

Plaque-associated microglial cells express Galectin-3 in 5xFAD mice.

After testing the importance of Galectin-3 in microglial activation *in vitro,* we aimed to study the role of galectin-3 in AD using a 5xFAD AD mouse model. First, we described where and when galectin-3 was expressed. Galectin-3 expression was tightly associated to microglial cells in the vicinity or in contact to Aβ plaques in 5xFAD mice. Moreover, galectin-3 was found heavily upregulated in 5xFAD mice from 6 to 18 months, along with an increasing deposition of Aβ. Moreover, the coverage of Iba1 positive microglial cells associated to plaque deposits were significantly lower in 5xFAD-Gal3KO mice compared to 5xFAD animals. The percentage of Iba1 positive microglial cells expressing galectin-3 in 5xFAD was higher than 30% compared to the total amount of microglial cells analyzed.

The lack of Galectin-3 reduces microglial proinflammatory response in vivo.

Next, we evaluated the role of galectin-3 in the inflammatory response in the 5xFAD mice compared to 5xFAD-Gal3KO mice. We measured the expression of different proinflammatory molecules with western blot and qPCR. First, with western blot, we found a clear downregulation of iNOS at 6 months in 5xFAD-Gal3KO compared to 5xFAD mice. TLR4 was also downregulated in 5xFAD-Gal3KO mice at 6 and 18 months. Interestingly, IDE1 was upregulated in 5xFAD-Gal3KO mice at 6 months compared to 5xFAD control mice. The mRNA levels of different inflammatory and cell factors were tested by qPCR. TNF-α, TREM2, CD68 and CD45 were downregulated in 5xFAD-Gal3KO mice at 6 months compared to 5xFAD animals. CD45 labelings confirmed the reduction of CD45 positive cells around Aβ plaques in 5xFAD-Gal3KO mice compared to 5xFAD control mice.

To further analyze the impact of galectin-3 in the inflammatory response, we performed a gene array of the hippocampus from 5xFAD and 5xFAD-Gal3KO at 6 and 18 months. Notably, the proinflammatory profile of 5xFAD-Gal3KO was markedly downregulated compared to 5xFAD mice. Specifically, TLR pathways

were significantly affected along with IL1 signaling pathways or chemokine's signaling. Moreover, DAP12 pathways were also significantly affected in 5xFAD-Gal3KO compared to 5xFAD mice at 18 months. Notably, in the 5xFAD-Gal3KO mice, some genes involved in the microglial response, such *ascsf1, stat4, p2rx and clec7a* were clearly downregulated.

The amyloid beta burden was lower in 5xFAD-Gal3KO mice.

We next analyzed the plaque burden in our mouse models. First, we analyzed the Aβ burden in the hippocampus and frontal cortex. Notably, 5xFAD-Gal3KO mice presented a significantly reduced Aβ burden at 6 months compared to 5xFAD mice in the hippocampus. The Aβ plaque burden at 18 months was evaluated using ThioS instead of 6E10 antibody due to the extremely high Aβ burden present at 18 months. We found a similar trend at 18 months in 5xFAD-Gal3KO animals, displaying a reduction in amyloid plaque burden. In the frontal cortex, at 6 months, the plaque burden appeared lower in the 5xFAD-Gal3KO compared to 5xFAD, albeit not significant. However, at 18 months, we found a significant reduction in the plaque burden in 5xFAD-Gal3KO mice compared to 5xFAD animals.

Next, we evaluated the amount of Aβ40 and 42 in the soluble fraction using samples from the cortex. Notably, Aβ40 was significantly reduced at 6 and 18 months in 5xFAD-Gal3KO mice compared to control animals. Aβ42 levels in 5xFAD-Gal3KO mice were lower compared to 5xFAD animals, albeit not significant. Then, we analyzed the levels of APP, C83 and C99. APP levels did not differ in regards to the genotype or the time points. However, C83 was significantly reduced at 6 months in the 5xFAD-Gal3KO group compared to 5xFAD mice, and C99 displayed the same trend at 18 months. Aside from the histological analysis, we collected CSF from both genotypes to measure the levels of different Aβ fractions. Interestingly, Aβ42 levels were upregulated in the CSF of 5xFAD-Gal3KO mice compared to WT animals. Aβ40 presented a similar trend in 5xFAD-Gal3KO but was not significantly altered.

Amyloid beta plaques displays a different morphology in 5xFAD-Gal3KO mouse.

The analysis of the plaque morphology in our mouse models reveal differences between genotypes. Interestingly, 5xFAD-Gal3 KO mice showed a clear reduction in the area of the plaque and the perimeter of the plaque at 18 months compared to 5xFAD mice. Moreover, the circularity of the plaque was increased in the 5xFAD-Gal3KO mice compared to the control animals.

The lack of Galectin-3 reduces dystrophic neurites formation in vivo.

In order to address how the lack of galectin-3 is affecting AD pathology in 5xFAD mice, we analyzed another important pathological feature, the dystrophic neurite formation. First, we counted the number of dystrophic neurites in our mouse models. Using anti-APP antibody, we evaluated the amount of dystrophic neurites. Notably, at 18 months the number of dystrophic neurites was significantly lower in 5xFAD-Gal3KO mice compared to 5xFAD controls. Furthermore, we analyzed the morphology of the dystrophic neurites using LAMP-1 staining along with Aβ and ThioS. The formation of dystrophic neurites correlates with the accumulation of vesicles in the synaptic neurites, and, LAMP-1 is therefore a suitable marker to evaluate the morphology of the dystrophic neuritis (Yuan et al., 2016). We considered 3 different factors for the morphological analysis of the dystrophic neurites: area, circularity and shape factor. Notably, all of them were significantly affected at 6 months n 5xFAD-Gal3KO mice compared to 5xFAD. First, the area of the dystrophic neurites was significantly reduced in 5xFAD-Gal3KO animals compared to control mice. Then, the analysis of the circularity and the shape factor displayed a significant reduction in 5xFAD-Gal3KO compared to 5xFAD mice. We failed to identify size differences in dystrophic neurites at 18 months in 5xFAD-Gal3KO mice compared to aged match controls.

Galectin-3 interacts with TREM2.

To further elucidate the role of galectin-3 in AD, we tested if galectin-3 interacts with TREM2. As we have shown before, galectin-3 is expressed in microglial cells in the vicinity of amyloid plaque deposits. TREM2 has being extensively studied in AD and shared some common features with galectin-3. It is mainly expressed in microglial cells in proximity to Aβ plaques, it is related to microglial activation, tplaque formation and phagocytic processes in AD. Moreover, microglial cells release galectin-3; so, it would be plausible to think on a potential interaction between released galectin-3 and TREM2. Using a molecular interaction technology, fluorescent anisotropy, we evaluated the potential interaction between TREM2 and galectin-3. Interestingly, the interaction between TREM2 and galectin-3 was even stronger than our discovered interaction between TLR4 and Galecin-3.

Next, using confocal microscopy and STORM microscopy, we confirmed the co-localization of galectin-3 and TREM2 in Iba1 positive microglial cells around Aβ plaques from 5xFAD brain slides. Moreover, mRNA levels of TREM2 were downregulated at 6 months in 5xFAD-Gal3KO mice compared to age-matched 5xFAD animals.

5xFAD-Gal3 KO mice display a better cognitive and motor behavior compared to 5xFAD mice.

The most important clinical aspect of AD pathology is the cognitive decline over the progression of the disease due to the neuronal death in the hippocampus of AD patients. Consequently, cognitive parameters are one of the main aspects evaluated in AD research. Hence, evaluation of cognitive and motor impairment was made in our mice.

First, we evaluated the behavioral outcome in 5xFAD and 5xFAD-Gal3KO mice. Both motor and cognitive behavior displayed a better outcome in 5xFAD-Gal3KO mice. First, cognitive behavior was evaluated at 6 months using Morris water maze. The mice lacking of galectin-3 mice displayed a better cognitive outcome compared to 5xFAD mice after 10 days of training in Morris water test. Next, we did analyze the motor behavior using the clasping test. Again, 5xFAD-Gal3KO demonstrated a better motor behavior in compared to 5xFAD mice. Later, at 18 months, we performed different behavioral tests motivated by the evident motor problems in our mice due to the aggressive mouse model we are working with and the age of the mice. In this case, we performed an open-array test to evaluate the impact of the cognitive decline in our mouse model.

At 18 months, 5xFAD-Gal3KO mice displayed a cognitive behavior similar to WT and galectin-3 KO mice regarding the total running distance and resting time. Next, elevated plus maze was assessed and we found a significant time spent in the center region, similar to WT and galectin-3 KO mice, compared to 5xFAD mice. Finally, rotarod test was performed and 5xFAD-Gal3KO mice displayed reduced failing behavior compared to 5xFAD mice, similar to WT and galectin-3 KO animals performed. Altogether, our data demonstrated the detrimental role of galectin-3 in the progression of AD pathology in the 5xFAD mouse model.

Galectin-3 expression is upregulated in the brain of human AD patients

Finally, we studied the expression of galectin-3 in human brains. First, we measured the levels of galectin-3 in the temporal cortex of AD patients. Notably, the level of galectin-3 in the cortex of AD patients was markedly upregulated compared to healthy controls. Moreover, galectin-3 expression was found in Iba1 positive microglial cells associated to Aβ plaque deposits in brains of AD patients. Furthermore, we measured the levels of different proinflammatory cytokines in the brain of AD patients and healthy control subjects. We found elevated levels of IL6 in AD patients compared to healthy controls in both soluble fractions. Next, we measured the area, the circularity and the shape of amyloid plaque containing microglial cells expressing galectin-3 and Aβ plaque without

any microglial cells expressing galectin-3 in the vicinity. Notably, galectin-3 positive plaques were significantly smaller compared to non-galectin-3 positive plaques. Furthermore, the shape factor revealed a higher symmetry in galectin-3 negative plaques compared to galectin-3 positive plaques. Finally the plaque density displayed a significantly higher in galectin-3 positive plaques compared to the negative ones.

Discussion

During my project, we were able to address few questions in regards to microglial activation, the role of galectin-3 in that process and the impact of the inflammatory response in neurodegenerative diseases. I will center the discussion in the 4 most important findings of my thesis: i) the relation of galectin-3 with the inflammatory response trigger by microglial cells, ii) the role of galectin-3 in the progression of AD pathology, iii) galectin-3 as a TLR4 and TREM2 ligand and iv) the inflammatory response in early stages of AD pathology.

Galectin-3 and the inflammatory response

Elucidating the mechanisms behind various inflammatory responses has been one of the biggest challenges for immunologists. Currently, understanding and counteracting the action of the inflammatory response can be a major therapeutic approach for different pathologies, including neurodegenerative diseases. Our findings prove that galectin-3 plays a significant role in the inflammatory response triggered by microglial cells. We first described the capacity of galectin-3 to bind to TLR4 receptor in the membrane of microglial cells, triggering the inflammatory response. The role of TLR4 in the inflammatory response was revealed by the capacity of LPS to binds TLR4 (Chow et al., 1999). However, the brain is considered an "immune privilege site", so LPS is not a common endogenous ligand for TLR4, and is mainly found in the outer membrane of gram-negative bacteria. The inflammatory response described in our thesis is the so-called sterile inflammation where the triggers are endogenous molecule known as DAMPs (Rock et al., 2010). As a result of our studies, galectin-3, alone or with LPS, is able to increase the inflammatory response via TLR4 (Paper I). Furthermore, the inhibition of galectin-3 by specific inhibitors, or by genetic downregulation, was able to drastically reduce the capacity of microglial cells to respond to LPS. The reduction of the inflammatory response protects TH positive neurons in galectin-3 KO mice injected with LPS in the SN (Paper I). In fact, the injection of LPS generates an inflammatory response that previously has been used to induce neuronal death mainly affecting to dopaminergic neurons in the SN (Herrera et al., 2000). So that, the role of the inflammatory response in neuronal

death has been widely studied. For instance, LPS modulation of TNF-α has been shown to be involved in neuronal apoptosis in neonatal cerebral cortex (Nimmervoll et al., 2013). Besides TNF-α modulation, MAPK inflammatory pathways have been linked to neuronal death. For instance, p38α MAPK protein activation due to LPS and TNF-α release is involved in neuronal death (Xing et al., 2011). Indeed, p38α KO produce less TNF-α and protects against neuronal dysfunction and neuronal death (Xing et al., 2011). In our study, the lack of galectin-3 drastically reduced TNF-α production and release (Papers I, II and IV). The reduction of the inflammatory response protected TH positive neurons in galectin-3 KO mice injected with LPS in the SN (Paper I).

To address the inflammatory response in AD or PD, we first performed *in vitro* experiments where we stimulated BV2 with α-synuclein or Aβ along with galectin-3 inhibitor. Notably, in both cases, galectin-3 inhibition resulted in a significant reduction of different proinflammatory cytokines (Paper II and IV). Aside from the inhibitor, we used primary microglial cultures from WT and galectin-3 KO animals to evaluate the inflammatory response upon stimulation with α-synuclein or Aβ. This approach corroborates our previous finding with BV2 cells as the proinflammatory cytokined released were significantly reduced in galectin-3 KO primary cultures compared to WT controls.

The last point is of special importance as it has been demonstrated that proinflammatory cytokines can play an important role in the progression of neurodegenerative diseases (Heneka et al., 2015; Heneka et al., 2014; Ransohoff and Brown, 2012). For instance, high levels of IL12 has been linked to AD pathology. Indeed, IL12 production by microglial cells was found particularly higher in APP-PS1 mice along with high levels of p40, a signaling molecule involved in IL12 pathway that can be found in CSF of AD patients (Teng et al., 2015). Furthermore, IL10 has also been linked to AD progression. The lab of Golde has demonstrated how over expression of IL10 in APP-PS1 mice accelerates the progression of AD pathology, increasing the plaque burden and worsening the cognitive outcome (Chakrabarty et al., 2015). In a similar way, the lack of IL10 improves AD pathology in APP-PS1 mice, shown by the lab of Terrence Town (Guillot-Sestier et al., 2015). Another interleukin involved in AD pathology is IL33. Levels of ST2, a receptor related to IL33, are elevated in the serum of AD patients. The injection of IL33 reduces the plaque burden and improves the cognitive outcome in APP-PS1 mice. Moreover, the microglial state shifts towards an anti-inflammatory profile upon IL33 injection, reducing the levels of IL1β, IL6, and NLRP3 (Fu et al., 2016). These findings reaffirm the importance of the inflammation in the progression of AD.

To fully understand the implication of galectin-3 in the inflammatory response, we performed a gene array using the hippocampus of 5xFAD and 5xFAD-Gal3KO

mice and it turns out that TLR, DAP12 and Interleukin pathways were the main affected by the lack of galectin-3. These results reinforce the findings of our first paper, where galectin-3 played a role as a TLR4 ligand and the lack of galectin-3 reduced the expression of different Interleukins factors. The occurrences of DAP12 among the main pathways affected by the lack of galectin-3 is a crucial aspect for our study has one of our findings is the potential interaction of TREM2 and galectin-3 in AD context and *in vitro*.

In neurodegenerative diseases such as PD or AD the main proteins involved in the pathology, α-synuclein and Aβ, are consider DAMPs, being able to activated microglial cells enhancing their inflammatory response (Venegas and Heneka, 2017). In our study, we wanted to evaluate if the specific activation elicited by these proteins was linked to the role of galectin-3 in microglial activation. Indeed, in both cases, the lack of galectin-3 reduced the inflammatory activation of microglial cells stimulated with α-synuclein or Aβ. In our second study, we used α-synuclein to activate microglial cells along with galectin-3 inhibitors or we used galectin-3 KO primary cultures. In both cases, the reduction in the inflammatory response was evident. Regarding the ability of Aβ to activate microglial cells, in our fourth study, we used Aβ fibrils to stimulate BV2 microglial cells or primary microglial cultures, inducing strong proinflammatory activation. Indeed, iNOS, TNF-α, NLPR3, IL12 or IL10 were among the proteins highly upregulated compared to the control samples. Similar to what we did in our second study, we treated BV2 microglial with Aβ fibrils along with galectin-3 inhibitor, which reduce significantly the ability of Aβ fibrils to activate microglial cells. The same was found when we treated the primary microglial cells with Aβ fibrils. In this case, galectin-3 KO primary microglial cells showed less activation capacity compared to WT primary microglial cells based on the readout of different proinflamatory proteins and released cytokines measured.

The ability of these proteins to elicit inflammatory responses has been addressed many times before. For example, Aβ oligomers has been linked with a higher activation capacity in microglial Aβ oligomers are able to trigger the IL-1β release due to NLRP3 pathway activation (Parajuli et al., 2013). Regarding α-synuclein, its ability to activate microglial cells has been demonstrated previously. For instance, Freeman et al, demonstrated the ability of α-synuclein to activate NLPR3 in microglial cells in a mechanism where α-synuclein is engulfed by microglial cells, disrupting the lysosome, and releasing proteins with the ability to activate NLRP3 pathway leading to IL-1β production (Freeman et al., 2013). Moreover, α-synuclein release by neurons is able to activate microglial cells via TLR2 and TLR4 (Fellner et al., 2013; Kim et al., 2013). Further, intranigral injection of α-synuclein triggers the activation of microglial cells in the SN (Couch et al., 2011). Moreover, glial reaction associated to Aβ has been linked to neuronal death in APP mutant mice (Luccarini et al., 2012). The same was shown regarding α-

synuclein and neuronal death. LPS injection in mice expressing a mutant version of α-synuclein leads to microglial activation and the release of proinflammatory factors that modifies α-synuclein (nitration or oxidation), enhancing its toxicity leading to neuronal death (Gao et al., 2008). The inhibition of proinflammatory pathways, such as JAK-STAT or Nfκβ is a promising therapeutic approach to protect dopaminergic neurons (Hoenen et al., 2016; Qin et al., 2016). The proinflammatory response and the production of iNOS by microglial cells has also been linked to post translational modification of Aβ, making the proteins more prone to aggregate leading to Aβ plaque deposits formation in APP-PS1 mice (Kummer and Heneka, 2014).

Galectin-3 in neurodegenerative diseases

Along with the role of galectin-3 in microglial inflammatory response, other questions emerged in our work regarding the role of galectin-3 in neurodegenerative diseases. For instance, in our second study (Paper II), we performed α-synuclein injections of monomer, oligomers and fibrils in the olfactory bulb of WT mice. We found that only microglial cells with α-synuclein inclusions expressed galectin-3. Interestingly, galectin-3 expression disappeared upon degradation of α-synuclein inclusions, *i.e* injections of monomers elicited the galectin-3 expression at 12h. However, at 72h, when microglial cells degraded the α-synuclein monomers inclusions, galectin-3 was not expressed. For oligomers and fibrils, galectin-3 expression was present from 12 to 72h, along with the presence of α-synuclein inclusions in the cells. Hence, we can speculate that the presence of α-synuclein inclusions and the expression of galectin-3 are coupled with proinflammatory activation of microglial cells in the brain. In fact, microglial cells taking up α-synuclein become active, increasing the expression of ROS (Freeman et al., 2013). Another key observation in our second study, in line with the before mentioned, was the delayed expression of galectin-3 in microglial cells activated with fibrils. That delay in the expression of galectin-3 can be related with the digestion of the proteins within the cells up to a point where the conformation of the proteins induce stronger microglial activation. This observation helps us to speculate with the nature of the protein aggregation that is more prone to activate microglial cells once the protein has been internalized. Future experiments would be necessary to address this latest point in our work.

Regarding galectin-3 and AD, the lack of galectin-3 not only affects the inflammatory response but also the plaque burden, the dystrophic neurites and plaque formation. In our study (Paper IV), we found a clear up regulation of galectin-3 in a time dependent manner in 5xFAD mice. However, among the most notorious findings in our work, we have to highlight the presence of microglial cells expressing galectin-3 only in contact with Aβ plaque deposits in both, 5xFAD mouse and human AD brain. Furthermore, galectin-3 may be involved in the plaque formation. In our mouse model, the lack of galectin-3 significantly reduces the Aβ burden compared to the control.

In line with the before mentioned, AD pathology is also associated with the formation of dystrophic neurites due to Aβ plaque formation (Sadleir et al., 2016). Dystrophic neurites are the results of vesicle accumulation within terminals, usually due to impaired lysosomal function or transport, which might be linked to impairment function of motor proteins involved (Sanchez-Varo et al., 2012). The lack of galectin-3 in 5xFAD mice results in the reduction of APP positive dystrophic neurites at 18 months. Moreover, the morphology of the plaque was also affected in the galectin-3 KO mice, making the plaque smaller and more circular at 6 months. As well as the size, the morphology of the dystrophic neurites in the mice lacking galectin-3 was more irregular. The number of microglial cells around amyloid plaque was also significantly reduced in 5xFAD-Gal3KO animals, similar to what Colonna recently shown in a TREM2 study (Wang et al., 2016). Taken together, these data made us to consider galectin-3 as an important factor in the plaque and the dystrophic neurites formation.

Similar to our galectin-3 findings was described by the lab of Colonna lab in different studies where TREM2 plays a key role in the progression of AD pathology and was linked to the formation of Aβ deposits and dystrophic neurites. In 2 different studies, Colonna describe how the lack of TREM2 in Trem2-, and DAP12,-haplodeficient (a TREM2 adaptor) mice, and also in humans with R47H TREM2 mutations, reduces microglial ability to envelop plaque deposits, leading to reduced plaque compaction making the plaques more diffuse, resulting in greater surface exposure to adjacent neuritis (Wang et al., 2016; Yuan et al., 2016). Moreover, the lab of Ransohoff demonstrated in APP-PS1 TREM2KO CD45(hi)-Ly6C(+) macrophages were virtually eliminated, resulting in a diminished inflammatory response and amyloid burden compared to APP-PS1 mice (Jay et al., 2015). More recently, it has been proved that the soluble fraction of TREM2 (sTREM2) has the ability to activate microglial cells inducing their inflammatory response and changes in the morphology upon injection in the hippocampus of WT and TREM2 KO mice (Zhong et al., 2017).

All these findings related to TREM2 and our results about galectin-3 made us to seek for a potential relation between them. Presently, we know that microglial cells can release galectin-3 and that TREM2 is a membrane receptor in microglial cells. Both are involved in AD pathology in a similar way, so the question would be, is there any interaction between them? Surprisingly, we found a clear co-localization between galectin-3 and TREM2 around plaque deposits in 5xFAD brain slices. This finding was confirmed using both, confocal a STORM imaging technique. Furthermore, we wanted to study is there was any interaction, so we performed a Fluorescent Anisotropy assay and we found a strong interaction between TREM2 and galectin-3, greater than previously described in our first paper between TLR4 and galectin-3 (Burguillos et al., 2015). TREM2 mRNA levels were also tested in our 5xFAD-Gal3KO model and we found them significantly reduced compared to 5xFAD mice. The lower levels of TREM2 in our galectin-3 KO would partially give an explanation to the differences in plaque and dystrophies found in our model compared to the 5xFAD mice. However, more experiments are necessary to fully address the nature of this interaction.

Inflammatory response in early stages of AD pathology

In this thesis summary, I have written about the nature and the potential mechanisms behind the inflammatory responses in AD. However, there are still many questions that remain unanswered. One of the main questions is when is the inflammatory response initiated? In order to address this question, we have to consider few components of the inflammatory response and how they are connected. First, the inflammatory response in the CNS is mainly driven by the innate immune system. We have previously described the main cells involved in the inflammatory response, which belong to the innate immune system, are astrocytes and microglial cells. Then, the main protein involved in AD pathology, Aβ, which is considered a DAMP in AD etiology and has the ability to activate microglial cells. The Aβ plaque formation is the result of the aggregation of the Aβ in the extracellular space. On that basis, it would be plausible to imagine a potential interaction between microglial cells in the brain parenchyma and the extracellular fraction of Aβ leading to microglial activation and the subsequent inflammatory response. In fact, it has been shown that different forms of Aβ, especially oligomers and fibrils, are able to activate microglial cells (Haass and Selkoe, 2007; Parajuli et al., 2013). Both, the soluble Aβ and microglial cells were the main focus of our third paper.

In this work, we wanted to unravel the mechanism behind the inflammatory response in early stages of AD in 5xFAD mice. With regards to this topic, other studies have been published about the microglial activation in early stages of the pathology but to our knowledge none has used the 5xFAD model. For instance, López-González et al demonstrated an altered inflammatory response in APP-PS1 mice in an elegant study where gene expression evaluation in the cortex was conducted (Lopez-Gonzalez et al., 2015). Other studies are focused on the role of microglial activation in early stages covering different topics, from autophagy, to the complement system and synapse loss (Hong et al., 2016; Pomilio et al., 2016). All of them highlighted the importance of the innate immune system and microglial cells in the progression of AD pathology.

5xFAD mice display strong microglial activation, being an ideal model for our purpose (Landel et al., 2014). To this aim, we isolated microglial cells from 5xFAD mice before and after plaque deposition. Our idea was to map the protein profile of microglial cells to investigate if the inflammatory pathways were affected over the time in 5xFAD mice. First, we evaluated the progression of the pathology. We measured the levels of the soluble fraction, Aβ40 and Aβ42, in our 5xFAD mice. As we thought, there was an increase in the levels of Aβ conformations, 40 and 42, in a time dependent-manner before the first plaque deposits appear in the subiculum. It is important to emphasize that the plaques were only present in the subiculum at 10 weeks. The importance of the cytokines in AD pathology has been discussed thoroughly in this thesis. We found a clear alteration of the proinflammatory cytokines in 5xFAD from 6 to 10 weeks. The levels of IL10 and IL1β were clearly affected during the disease progression, and it is helpful to mention that the levels were higher in at 10 weeks compared to 6 weeks despite few numbers of plaques just localized in the subiculum. The proteomic profile of the isolated microglial cells confirms our finding. From 6 weeks, MAPK and Interleukins pathways were markedly affected in 5xFAD compared to WT mice. As we described previously, differente cytokines have been linked to AD progression. The same applies to the MAPK pathway, which has been highlighted in our study. MAPK pathway and their role in triggering the inflammatory activity in microglial cells have previously been studied in an AD context (Munoz and Ammit, 2010). For instance, p38 is one of the most studied proteins in regards to MAPK pathways in AD. In fact, p38 has been shown to regulate the proinflammatory response via TLR's (Bachstetter et al., 2011). Moreover, MAPK pathways have been linked to IL1β production in microglial cells (Kim et al., 2004). Indeed, in our study p38 is one of the main proteins highlighted in the proteomic profile obtained from microglial cells. At 10 weeks, vesicle-related pathways were highlighted in our study in regards to microglial cells in 5xFAD. These pathways has recently been linked to Aβ modification and microglial activation. Indeed, at 10 weeks, specific metabolic-related pathways

such as adrenergic, glutamatergic or acetylcholine pathways were affected. Recently, the role of the metabolic pathways on microglial activation has been study by the lab of Colonna, underlining their importance in microglial response in AD pathology (Ulland et al., 2017).

It is important to mention that there are some promising studies showing early inflammatory events in mild cognitive impairment, an early stage of AD pathology, where S100A9 correlated with levels of Aβ42 and the progression of the pathology in the CSF of AD patients. Moreover, IL6, IL8 and IL10 have been found elevated in peripheral blood mononuclear cells of MCI patients compared to controls, highlighting the role of the inflammation in the early stage of the pathology (Magaki et al., 2007). Another interleukin cited before, IL23 subunit p40, was decreased in patients with cognitive impairment, and correlates with markers of AD disease status (Johansson et al., 2017). IL1β, along with IL6, has been linked to AD progression due to their increasing in mood swing episodes in patients that were followed during 10 years (Honma et al., 2013). However, we are facing various technical and study limitations on cytokines evaluation in AD. In 2014, Heneka and colleges published an extensive review on the main limitations on cytokines measurement in AD. The main problem come from the variability introduced by the different ELISA kits system used to measure cytokines. The epitope specificity of the antibodies is another major point to improve to get reliable measures as well as the variations in how to collect and handle samples. So, it is important to establish a standard procedure for the samples handling and storage (Brosseron et al., 2014). Among the main cytokines analysed in several studies, IL-1β, IL-6, and TNF-α seems to correlate better with AD progression in CSF samples despite the smalls changes often displayed, rendering them not entirely reliable as a biomarkers.

Other molecules tested to evaluate AD progression are C-Reactive proteins or MCP-1. C-Reactive proteins were measured in serum and used as markers in a study where the patients were followed over 25 years. Subjects with higher levels of C-Reactive proteins had higher risk of developing AD and vascular dementia (Schmidt et al., 2002). Regarding MCP-1, serum samples were evaluated and the ones revealing higher levels were associated with patients with MCI and mild AD, but not in severe AD patients as compared with controls (Galimberti et al., 2006). Furthermore, MCI patients diagnosed with AD one year after in follow up study, displayed lower levels of MCP-1 compared to healthy patients. Upregulation of MCP-1 probably occurs in early stages of AD pathology (Galimberti et al., 2006).

Despite all the efforts on understanding the inflammatory responses in AD, the early stages of the pathology are not fully characterized and more research is necessary to answer the questions that remain to be answered.

Conclusions

Collectively the results presented in this thesis suggest the following:

I. Galectin-3 plays a crucial role in microglial activation due to its binding capacity to TLR4. Indeed, galectin-3 binding to TLR4 triggers microglial cells proinflammatory activation

II. The lack of galectin-3 reduces the expression and the release of different proinflammatory molecules, such as: iNOS and cytokines. The lack of galectin-3 protects TH positive neurons in galectin-3 KO mice injected with LPS compared to WT mice.

III. Galectin-3 plays a role in α-synuclein induced microglial activation. Indeed, the lack of galectin-3 reduces the microglial activation in cells stimulated with α-synuclein

IV. α-synuclein triggers the expression of galectin-3 in microglial cells with α-synuclein inclusion from mice injected with α-synuclein monomers, oligomers and fibrils in the olfactory bulb.

V. The inflammatory activation of microglial cells is triggered in Alzheimer's disease at very early stages when the plaque burden is very minor or even non-existant.

VI. In Alzheimer's disease, galectin-3 expression by microglial cell plays a role in the progression of the pathology. Importantly, the lack of galectin-3 reduces AD pathology in 5xFAD mice.

VII. The expression of galectin-3 in human AD samples is tightly associated with microglial cells in the vicinity of the amyloid beta plaques.

Future Perspectives

Over the last decade, the role of the inflammatory response in neurodegenerative diseases has been widely studied but still there are many questions that remain to be answered. Of particular interest is to elucidate when the inflammatory response is taking place, which are the particular pathways involved depending on the stimulus and the mechanisms affecting the progression of the disease linked to the inflammatory stimuli.

Neuroinflammation in neurodegenerative diseases, the compass needle points towards microglial cells.

Some of the aspects we need to cover in the coming years are related to the impact of the inflammatory response in neurodegenerative disease. One of the main aspects we need to determine is the timing of the inflammatory response. Elucidating the timing of the inflammatory response is key to develop efficient therapies. To that aim, it would be important to find the main mechanisms affected in very early stages of the pathology, even before the neurodegenerative processes takes place.

Another question would be related to the capacity of the inflammatory response to impair the neuronal function inducing neuronal dysfunction and cell death. So that, study the specific molecular mechanisms increasing the neuron susceptibility in the inflammatory context, it will be essential to find new molecular targets suitable for novel future therapies. Moreover, the inflammatory response has been linked with protein aggregation dynamics in several studies (Kummer and Heneka, 2014). Understanding alterations in aggregation dynamics due to inflammatory processes can be essential to fully comprehend the plaque formation process and to design new therapeutic approaches related to protein aggregation.

Many of the previous questions are related to the activation of the innate immune response, as it is the main system involved in the inflammatory response in the CNS. Among the different actors playing a role in that response there is no doubt

about the importance of microglial cells. Hence, most of the effort should be driven to understand the biology of these cells in different conditions. Many of the questions that remain unanswered are related to the nature of the microglial response in relation to the events taking place in the brain when patients are developing brain diseases. However, there are many factors affecting the activation of microglial cells such as the nature of the insults, the area of the brain and age. All of them need to be taken into consideration in order to unravel the mechanism where microglial cells are involved in the inflammatory response.

Finally I would highlight the importance of the plaque deposition in AD pathology. To find the exact mechanism leading to Aβ plaque deposits and being able to predict where the plaque will be sitting in the parenquima might be crucial to counteract AD progression.

Galectin-3 may has something to say

In this thesis, the role of galectin-3 in the inflammatory response has been thoroughly addressed with regards to neurodegenerative conditions. From the TLR4 interaction to the role in AD progression, our molecule of interest stand as an important subject of study to further understand the mechanisms. However, every question answered creates a new inquiry. One of the most exciting observations from our work is the expression of galectin-3 in microglial cells in the vicinity or in contact with Aβ plaques in AD, either in human or mouse samples. This observation caught our attention, as not that many markers of inflammation are so clearly related with Aβ plaques. Some of our future questions would be what is the set of used by microglial cells in the vicinity of amyloid plaques and what is the role of these microglial cells in the dynamics of plaque formation? Are galectin-3 positive microglia involved in the formation or the degradation of the amyloid plaques? This is not a minor question cause it would be very useful to elucidate if microglial cells are involved in the plaque formation, in order to predict where the plaques will emerge and follow the disease progression.

To achieve our goals, the application of new tools and techniques would be necessary. Isolating microglial cells expressing galectin-3 from AD brains to analyze single cells novel technologies would be one of the first steps to clarify the role of galectin-3 in the plaque formation. Moreover, by knowing the exact profile we can move further and describe the potential proinflammatory factors interacting with neurons leading to their dysfunction. Taken together, this will

bring additional comprehension to the research field of neuroninflammation as well as new therapeutic possibilities.

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