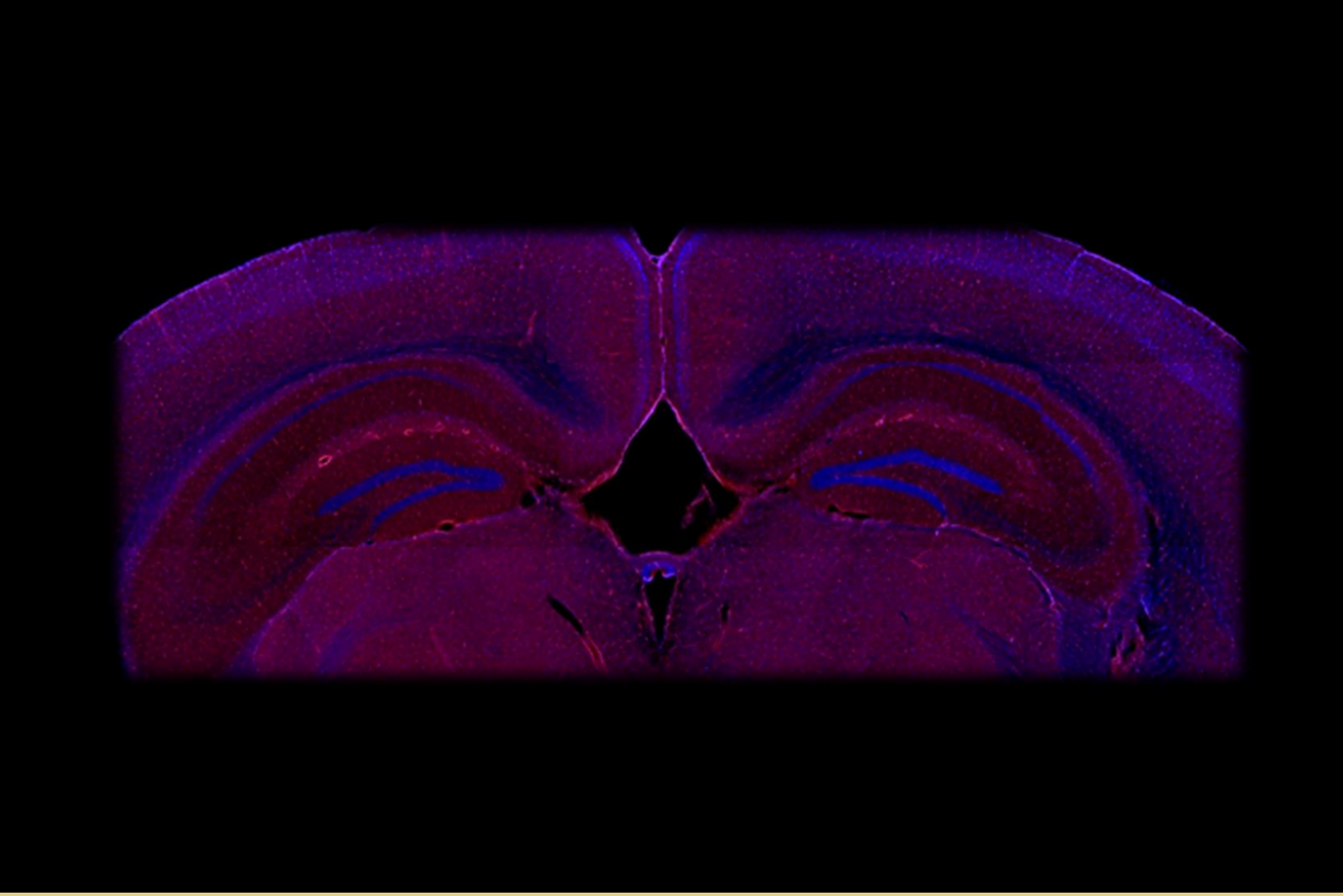

Tiago André Antunes David

Role of Auto-Antibodies to the Nicotinic Receptor in Neurological and Psychiatric Diseases

Thesis presented to Medicine Faculty of Coimbra in order to acquire the Master's Degree in Experimental Pathology under Academic orientation of Prof. Doctor António Silvério Cabrita and Scientific Orientation of Doctor Uwe Maskos.

Master Thesis in Experimental Pathology

May of 2017

· U C ·

UNIVERSIDADE DE COIMBRA

Tiago André Antunes David

Master Thesis
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Purpose: Thesis submitted to Coimbra University, Medicine Faculty to acquire the Master's Degree in Experimental Pathology.

English Title: Role of Auto-Antibodies to the Nicotinic Receptor in Neurological and Psychiatric Diseases

Portuguese Title: Papel dos Autoanticorpos no Receptor Nicotínico em Doenças Neurológicas e Psiquiátricas.

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Place of work: Institut Pasteur, Paris, France

May 2017

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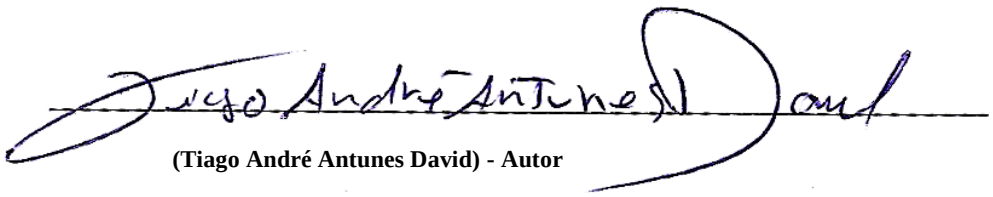
Declaration of Authenticity

I, Tiago André Antunes David, confirm that the work presented to the University of Coimbra - Medicine Faculty, is my own.

I also confirm, there is information derived from other sources which are indicated in the thesis.



(Tiago André Antunes David) - Autor

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… *my beloved* ***Graça Barão***.

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Table of Contents

Abbreviations and Acronyms

AA	AutoAntibody
ACh	AcetylCholine
ACNA7	α7 nAChR KO mice line
AD	Alzheimer's Disease
ANS	Autonomic Nervous System
BBB	Blood Brain Barrier
BSA	Bovine Serum Albumin
CBA	Cell-Based Assays
CFU	Colony Forming Units
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
CSF	CerebroSpinal Fluid
DAPI	4',6-diamidino-2-phenylindole
DPI	Days Post Infection
ECD	ExtraCellular Domain
ELISA	Enzyme-Linked Immunosorbent Assay
GFAP	Glial Fibrillary Acidic Protein
GWAS	Genome-Wide Association Study
HEK293	Human Embryonic Kidney 293
HLA	Human Leukocyte Antigen
HRP	HorseRadish Peroxidase
IAV	*Influenza A Virus*
IBA1	Ionized calcium-Binding Adapter molecule 1
IgG	Immunoglobulin G
IgM	Immunoglobulin M

IkB	Inhibitor of kappa B
IL-6	InterLeukin 6
KO	Knock Out
LPS	LipoPolySaccharide
MHC	Major HistoCompatibility complex
MS	Multiple Sclerosis
nAChR	nicotinic ACh Receptor
NF-kB	Nuclear Factor kappaB
NGS	Normal Goat Serum
NISC	Group of Integrative Neurobiology of Cholinergic Systems
Nm	*Neisseria meningitidis*
NMDAR	N-Methyl-D-Aspartate Receptor
NMDAR1-AA	NMDA Receptor 1 AA
NR1	NMDAR subunit 1
NR2	NMDAR subunit 2
OD	Optical Density
OPD	o-Phenylenediamine Dihydrochloride
PBS	Phosphate Buffered Saline
PFA	ParaFormAldehyde
PFU	Plaque Forming Unit
PNS	Peripheral Nervous System
RT	Room Temperature
SP	*Streptococcus pneumoniae*
TNF-α	Tumor Necrosis Factor alpha

Resumo

Nas ultimas décadas o receptor nicotínico pertencente no sistema colinérgico tem sido alvo de grande interesse em investigação nas áreas de neurociências. Esta família de canais iónicos, os receptores nicotínicos, tem como função mediar os efeitos de transmissão do neurotransmissor de acetilcolina.

O aparecimento do autoanticorpo (AA), um tipo de proteína produzido pelo sistema imunitário que actua contra as suas próprias células, tem tido um grande impacto em neurociências. Com apenas diferenças nas suas propriedades imunológicas, os AA estão relacionados com doenças auto-imunes.

O facto disso foi o estabelecimento de uma nova patologia nomeada de encefalia do receptor NMDA. Esta descoberta ajudou a elucidar o papel dos AA para com as proteínas expressas no cérebro. Por outro lado, outros estudos realizados sobre estes AA, têm ligado muitas outras grandes patologias do foro neurológico e psiquiátrico, tais como, Alzheimer, Esquizofrenia e até mesmo o ciclo do vicio de drogas.

O laboratório NISC tem se dedicado nos últimos tempos ao estudo das origens e das consequências funcionais dos AA. Em vez de NMDAR, NISC elaborou a hipótese de que os receptores nicotínicos de acetilcolina (nAChRs) tem um igual papel de importância em algumas patologias.

O presente estudo começou por perseguir a hipótese de que os AA são gerados contra nAChRs expressos no pulmão durante uma infecção ou em doenças crónicas como o caso da doença pulmonar obstrutiva crónica (COPD).

O ensaio experimental tem como modelo a determinação dos AA e a sua penetração através da barreira hematoencefálica em condições fisiológicas e patológicas. Detém ainda a sua acção sobre os nAChRs no cérebro de humanos e de ratinhos, uma vez que estes são expressos em tecidos neuronais e não neuronais.

Compreender a origem dos AA e o mecanismo de penetração no cérebro, poderá permitir o uso de tratamentos com probióticos abordando as origens infecciosas e o aumento da permeabilidade do BBB.

Os resultados provindos das amostras de humanos sugerem que os pacientes diagnosticados com encefalites do receptor NMDA podem de facto produzir AA contra o subtipo homomérico α7 dos nAChR.

Os resultados procedentes dos ratinhos sugerem que existe uma resposta cruzada contra outros receptores cuja a expressão e/ou exposição são modificados nos ratinhos KO.

Palavras-chave: Colinérgico; Alpha 7 nAChR subunidade; Autoanticorpo; Neuro-inflamação; Alzheimer; Esquizofrenia; CSF; ELISA; Cérebro; Murganhos; Células Monoclonais.

Abstract

The nicotinic acetylcholine receptors (nAChRs) are a key component of the cholinergic system. Over the last decades, they have become an important target for neuroscience research. The function of ligand-gated ion channels family is to mediate the neurotransmission of acetylcholine.

The autoantibodies (AAs) discovery, a type of protein produced by the immune system against its own cells, has also started to have a big impact in neuroscience. AAs have been related to autoimmune pathologies.

This discovery led to a better understanding of a new pathology called "NMDA receptor encephalitis" and helped to elucidate the role of AAs as proteins able to enter the brain. Other studies focusing on these AAs related them to a number of neurological and psychiatric pathologies such as Alzheimer's disease, schizophrenia or drug addiction.

Recently, the NISC team has focused on the origin and functional consequences of AAs, and their relationship with nAChRs instead of NMDA receptors. The Human working hypothesis is that nAChRs can have an equal importance in some pathologies.

Other present study started to demonstrate that the AAs are elicited against nAChRs appearing during an infection through the lungs, as frequently observed in chronic pathologies such as Chronic Obstructive Pulmonary Disease (COPD) for example.

The experiment was designed to determine the AAs and their ability to cross the blood-brain barrier (BBB) in both physiological and pathological conditions.

This Thesis approaches the AAs action on nAChRs in human and mouse brains, expressed in neuronal and non-neuronal cells. Understanding the origin of AAs and the mechanisms used to reach the brain will allow possible therapies, like

the use of probiotic against the infections, and also the increased permeability of the BBB.

The results obtained from wild-type mice may support this hypothesis. However, the data from infected α7 knockout mice suggests there are a crossed-reactivity against other nicotinic receptor types, and whose expression and/or exposition is modified in these genetically modified animals.

A parallel study was focused on the relationship between autoimmune pathologies and the α7 nAChR in humans. The analysis of human samples showed that patients diagnosed with NMDA receptor encephalitis can produce AAs not only against NMDA receptor but also against α7 nAChR.

With the access to patient serum obtained from schizophrenic patients, this should now allow further understanding of the contribution of nicotinic receptor AAs in psychiatric disease.

Keywords: Cholinergic; Alpha 7 Nicotinic Acetylcholine Receptor Subunit; Autoantibody; Neuroinflammation; Alzheimer's; Schizophrenia; CSF; ELISA; Brain; Mice; Monoclonal cells.

Aim of The Thesis

This thesis is divided into two main parts:

I) The analysis of CSF monoclonal human samples from NMDAR encephalitis patients;

II) The characterization of AAs against α7 nAChR using a α7 knock-out mice line (ACNA7).

The objectives can be summarised as:

I)

a) Determination of a positive reaction between α7 extracellular domain (ECD) with AAs from 27 pooled samples, using ELISA;
b) Further characterization of the 141 individual samples;
c) Identification of high-affinity AAs using serial dilutions;

II)

a) The role of AAs in WT vs. ACNA7 mice;
b) The presence of AAs in the CSF;
c) Immunohistochemistry setup for neuroinflammation studies;

The following chapter describes the current knowledge regarding the nAChR AAs and their involvement in neurological and psychiatric disease, before presenting the results.

Chapter 1

Introduction

Chapter 1. Introduction

The following chapter is intended to provide a general overview of the relationships between autoantibodies (AAs) and brain receptors such as N-methyl-D-aspartate (NMDA) receptor, glutamate receptors, sodium and calcium channels, and nicotinic acetylcholine receptors (nAChRs).

This project was focus on the possible interaction between AAs and α7 nAChRs, and its role in neurological and psychiatric diseases.

1.1. Cholinergic System – The nAChRs

Acetylcholine (ACh) is a chemical mediator of synapses in the Central Nervous System (CNS), Peripheral Nervous System (PNS), at the skeletal neuromuscular junction, and on the Autonomic Nervous System (ANS). ACh, the ACh receptor and the enzyme responsible for its synthesis and degradation constitute the cholinergic neurotransmission system.

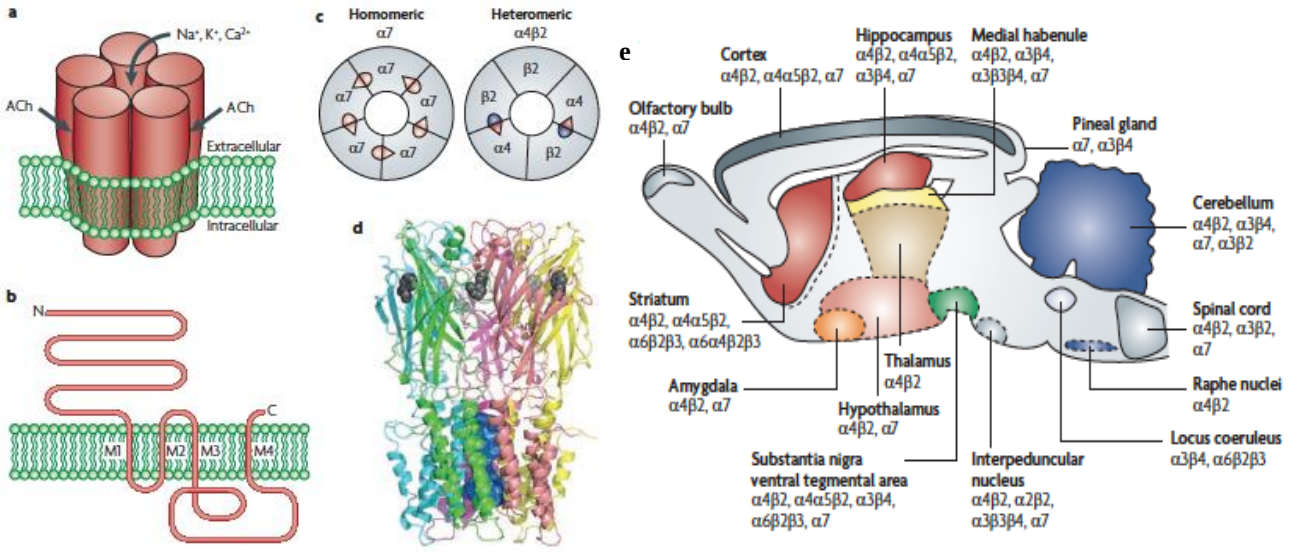
Nicotinic ACh receptors (nAChRs) in muscle and in autonomic neurones have been characterised in detail. CNS receptors have become the focus of intense research efforts since the 80's. The nAChR family in the brain and spinal cord is progressively documented to have therapeutic applications, while some physiological functions of these nAChRs remain enigmatic. Their subunit composition, assembly, trafficking and regulation, and the significance of their heterogeneity need to be dissected further. [1,2]

The nAChRs are ligand-gated ion channels, containing integral allosteric membrane proteins with a molecular weight of ~290 kDa, with five homologous subunits symmetrically arranged around a central ionic channel. In mammals, several types of nAChRs exist which differ in their subunit compositions according to their expression in different organs (Figure 1a).

As illustrated in Figure 1e, the nAChRs are extensively expressed in the mouse brain, and the receptor subtypes are dependent on the specific brain region. The 17 genes encoding the nAChR subunits have been identified as 8 **α** subunits (**α2 – α7, α9 – α10**), the so-called principal components, and 3 **β** subunits (**β2 – β4**), the complementary components, forming heteropentamers or homopentamers depending on their association with α and β subunits (Figure 1c).

Each subunit is highly conserved through evolution and contains a large amino-terminal extracellular domain (ECD), a transmembrane domain made up of four segments (M1–M4), and a variable cytoplasmic domain (Figure 1b). Amino acids of the ACh-binding site are grouped into sequence stretches

referred to as loops, forming the principal component (loop A, B and C) and the complementary component (loop D, E and F). These loops form a compact pocket, which is located in the centre of the interface of the ECD and capped by loop C (Figure 1d). [1–4]



(JP Changeux, 2010) *(Antoine Taly et al., 2009)*

Figure 1 – **nAChRs Structure**. **a)** Nicotinic acetylcholine receptors are transmembrane oligomers consisting of five subunits assembled around a central pore that is permeable to ions Na^+, K^+ and Ca^{2+}; **b)** Each subunit consists of an amino-terminal extracellular domain, a transmembrane domain and a variable cytoplasmic domain. The extracellular domain carries the ACh-nicotine binding sites at the borderline between subunits, there are 4 transmembrane segments (M1-M4); **c)** The two main types of brain nAChRs are the α7 homopentamers, and the α4β2 heteropentamers; **d)** Side view of an α7 nAChR pentamer, showing five nicotine molecules (dark grey); **e)** Variability of nicotinic binding sites and receptor subunits in mouse brain. (Adapted from *JP Changeux, 2010* and *Antoine Taly et al., 2009).*

1.2. Nicotine Addiction and Nicotinic Receptors

The World Health Organization (WHO) reported that tobacco epidemic is one of the biggest public health threats the world has ever faced, killing more than 7 million people a year. An obstacle to the prevention of these deaths is that tobacco contains nicotine, a compound responsible for driving the strong addiction to smoke, one of the major additions in worldwide. [5]

The nAChRs (homopentamers and heteropentamers) interact with nicotine throughout the PNS and CNS. These allosteric membrane proteins respond to ACh and nicotine agonist by the fast opening (μs to

ms) of a cationic channel that is permeable to Na^+, K^+ and Ca^{2+} ions. nAChRs mediate the rapid local phasic effects, short-lasting with high ACh levels in PNS. More data is required to understand the fast transmission in the brain. Brain nAChRs are also the target of released ACh, in lower modular concentrations (an important key for the understanding of nicotine addiction). Chronic exposure to ACh, nicotinic drugs and others nicotine addiction are known to affect ionic response (gradual decrease of the rate 100ms to minutes), leading to a high-affinity, desensitised, closed state of the receptor and to additional long-term changes in receptor properties. It also causes an upregulation of the number of high-affinity receptors in the brain. [6]

Chronic Obstructive Pulmonary Disease (COPD) is a term used to describe progressive lung diseases including emphysema, chronic bronchitis, asthma, and some forms of bronchiectasis. This disease is characterised by increasing breathlessness and producing large amounts of a slimy substance called mucus. COPD can be caused by inhaling pollutants, that includes smoking (cigarettes, pipes, cigars, etc.), and second-hand smoke. COPD most often occurs in people 40 years of age and older who have a history of smoking. Most of the individuals who have COPD (about 90%) have smoked, although not everybody who smokes gets COPD. People who have COPD are more susceptible to influenza, pneumonia, lung cancer and cystic fibrosis, among others. [7,8]

Human lung epithelial cells express α7 nicotinic receptor and regulate airway epithelium differentiation by controlling basal cell proliferation. Moreover, the α7 subtype has been identified to provoke dysfunction of the airway epithelium under nicotinic expose. [9–11]

1.3. α7 nAChRs and the Regulation of Inflammation

The α7 nAChR belongs to the superfamily of well-characterized neurotransmitter-gated ion channels, and is localised in neurones pre- and post-synaptically: pre-terminal axon regions, axon terminals, in dendrites and in the soma. [1]

The human gene encoding α7 nAChR has been mapped and described by Gault in 1998, as a α7 gene expressed in human brain, but also in leukocytes. However, the assembly of the protein to functional nAChR, from the expression of the α7 subunit gene, has revealed complex. The α7 nicotinic subtype is highly expressed in mammalian brain regions like the amygdala, olfactory bulb, cerebral cortex, hypothalamus and hippocampus. [12–14]

α7 nAChRs are expressed on non-neuronal cells such as astrocytes in the hippocampus which could play a role in neuroprotection and inflammation. [15,16]

Since 1999, the hippocampus has been described as the most prevalent area of α7 and α4β2 subtypes as well. nAChRs with α7 or α4β2 can be involved in Alzheimer's Pathology. Studies have shown that AAs can be produced against α7 and/or α4 subunits. [17–19,1,20]

Until recently, neuronal α7 proteins were considered to be functional exclusively as a homopentamer, but in 2002 the co-expression and assembly of α7 nAChR subunits with β2-subunits has been discovered, giving rise to functional heteropentamers (if for example overexpressed in HEK293 cells). When comparing these two types of homo- and heteropentamers, the desensitisation properties and ion permeability appear to be different. Co-expression of the β2 subunit with the α7 subunit slowed the rate of nAChR desensitisation significantly and altered its pharmacological properties. [13,21,22]

The link between α7 nAChRs and the immune system starts with the bone marrow being innervated by the parasympathetic cholinergic nerve fibres and several other types, such as myeloid and lymphoid cells which also express the α7 receptor. [12,23]

α7 subunits of non-neuronal cells (especially cells of the immune system) have a rapid desensitisation and a high permeability to Ca^{2+} to Na^{+} (permeability ratio exceeding NMDARs). The second messenger-mediated calcium from intracellular stores or from voltage-gated Ca^{2+} channels is inducing the opening of the α7 nAChR channels, increasing significantly the intracellular concentration of Ca^{2+}. These types of non-neuronal cells capable of ion channel function suggest that the intracellular domain mediates signal transduction directly. This signalling modality may also arise in neurones. [12,13]

One of the main questions on this topic is "how does acetylcholine modulate the activation of immune cells". The answer must come with the complete understanding of the intracellular signalling pathways, involving the anti-inflammatory potential of ACh, even though some mechanisms remain to be established.

Nicotine is an agonist of nAChRs. Nicotine has been studied as a possible anti-inflammatory mediator. As described before, α7 nAChR activation is mediated via its ion channel, although it can also activate neuronal and non-neuronal cells. A nicotinic activation of the α7 receptor elicits an increase of intracellular Ca^{2+} in microglia and macrophages.

The largest class of phagocytes within the CNS are the myeloid cells. Microglia activation can have an outcome of neuroprotection or an outcome of neurotoxicity. Normally, microglia recognises healthy neurones and astrocytes in the brain microenvironment, regulating the magnitude of innate immune responses via α7 nAChRs, among other neurotransmitters. In healthy brains, microglia shows a resting phenotype in comparison to other immune cells. However, the response against acute and chronic insults can be rapid. In CNS pathologies, activated microglia can be important via the release of free radicals along with cytokines and toxic effects. Alternatively, microglia can also exert neuroprotection properties, by

secreting anti-inflammatory mediators or secreting growth factors, helping to resolve inflammation and restore homoeostasis. Cytokines in CNS regulate synaptic activity while in peripheral organs act as proinflammatory. [12,24]

Some studies have described in monocytes, macrophages and endothelial cells, that the anti-inflammatory effects of the α7-nAChR are mediated by the inhibition of the transcription factor NF-kB. In brief, the activation of the NF-kB requires the ubiquitination of the co-factor IkB. This process will allow the nuclear translocation of the p65 and/or p50 subunits in order to modulate the transcription of NF-kB responsive genes such as Interleukin 6 (IL-6). Indeed, it has been shown that the anti-inflammatory action of ACh is associated with the inhibition of the LPS-induced activation of the NF-kB pathway. α7 nAChR activation may prevent IkB breakdown and p65 nuclear translocation and this mechanism explains the anti-inflammatory potential of ACh and also nicotinic agonists in monocytes, macrophages and endothelial cells. [12,25–29]

Studies in mice have shown that inflammation decreases the α7 nAChR levels in the brain and makes them more susceptible to apoptosis induction. [30]

It is known that dendritic cells and activated T cells have the ability to synthesise ACh using choline acetyltransferase. The role of the non-neuronal cholinergic system expressed in immune cells has been implicit in the regulation of immune cell function. The knowledge about α7 nAChRs to negatively regulate synthesis and release of tumour necrosis factor (TNF)-α in macrophages (acute phase reaction) has also been described.

The IgG and IgM are antibodies that the body produces when it comes in contact with some kind of invading microorganism. The difference between them is that IgM is produced in the acute phase of infection, while IgG, which also arises in the acute phase, is more specific and serves to protect the person from future infections and remains throughout life. [31–33]

1.4. The role of AAs in Neuroinflammation

With the implication of α7 nAChR in mediating the CNS inflammation, its role in significant diseases such as Alzheimer, schizophrenia, COPD and multiple sclerosis (MS), gain clinical importance.

Clinical research and animal models of diseases have been contributing to understanding the brain-reactive antibodies in human pathobiology. Currently, 5–7% of the world's population is affected by autoimmune diseases; in most diseases, there are AAs circulating. Brain-reactive antibodies are present in

a fair percentage of the general population but do not usually contribute to brain pathology. Circulating AAs have been documented to be directed against brain epitopes. [34,35]

The most common approach involving antibodies in brain disease is to find them in cerebrospinal fluid (CSF), yet the presence of autoantibodies (AAs) in CSF is not sufficient to consider them as pathogenic agents. [34]

It is described that the induction of brain-reactive antibodies arises in 3 situations:

a) in individuals with autoimmune diseases often linked to a specific HLA composition;

b) exposure to exogenous antigens, microbial antigen and perhaps also food antigen, may trigger expression of antibodies that cross-react with brain antigens (genetic susceptibility to these diseases has not yet been fully characterized); and lastly

c) cryptic tumours may exhibit paraneoplastic syndromes, where the tumour expresses an inciting antigen resulting in cross-reacting antibodies.

B cells have two main functions. One is to assist in the removal of cellular debris, and the other to neutralise and destroy invading pathogens and their toxins. To protect against pathogenic microorganisms, an enormous range of antibodies is required. After B cells encounter an antigen, antigen-specific T cells help them to differentiate further to a germinal centre response in which immunoglobulin genes undergo class-switch recombination and somatic hypermutation. The final process generates auto-specificities that need to be eliminated through negative selection mechanisms. These antibodies can penetrate brain tissue only in early development or under pathologic conditions, as discussed before. At last, these considerations apply only to antibodies directed against CNS antigens, antibodies to peripheral nerve antigens do not need to penetrate the BBB to mediate pathogenic effects. [34]

The circulation and immune cells passage to the brain are very restricted. Unlike other myeloid cells, microglia does not derive from bone marrow precursors, instead, descend from primitive macrophages that invade the neural tube in early development (and afterwards are replenished by self-replication in the adults). In non-neuronal cells, nAChRs are expressed in spleen regulating B lymphocyte propagation and activation. [34,36]

In human astrocytes, IL-6 is stimulated by α7 nAChR specific antibody thru the p38-dependent pathway. α7 nAChRs control the inflammatory cytokine production in macrophages and astrocytes. Maryna Skok's group showed that the agonist choline attenuates the IL-6 production, stimulated by bacterial lipopolysaccharide (LPS) in monocytes and astrocytes (cell lines U937 and U373, respectively). In contrast, in the absence of LPS from bacteria, the α7(179–190)-specific antibody stimulated the IL-6 production in a

p38 kinase-dependent manner. They suggest that α7-specific antibody can provoke neuroinflammation within the brain by inducing IL-6 production in astrocytes. [37]

The work of Lars Eriksson and colleagues from the University of California, San Francisco, also helps to understand the mechanism underlying neuroinflammation and the disruption of the BBB. In Eriksson's work, we can see the demonstration that peripheral surgery can indeed disrupt the BBB facilitating the migration of macrophages into the parenchyma (activation of TNFα signalling pathway). Surgery and other forms of trauma can initiate a systemic inflammatory response leading to neuroinflammation by activation of the α7 subtype of nAChRs, an endogenous inflammation-resolving pathway, as described previously. [38]

1.5. Neurological and Psychiatric Diseases

AAs can be associated with neurological disorders by acting on ligand-gated ion channels (receptors) or on voltage-gated ion channels. These disorders can, as discussed above, become the antibody markers for an inflammatory process (targeting neurones) and those which are directly pathogenic. This distinction is important since immunotherapies are limited.

AAs act by increasing the turnover of the antigens and subsequently depleting the neural tissues of their function. The opening of the channel can be affected if the AAs bind to the agonist site or to the ion channel pore. [39]

The defects in crucial elements such as ion channels in neuronal signalling and synaptic transmission are known to underlie rare genetic disorders (epilepsy can be an example). Emerging evidence for AA-mediated mechanisms with central nervous system disorders, involving defects in cognition or sleep is often associated with epilepsy. [40,41]

Neurological AA prevalence in autoimmune epilepsy has an unknown incidence. However, among adult patient samples, a significant amount of neurological AAs in the serum was detected, suggesting an autoimmune aetiology. [42]

The second class of channelopathies, characterised by AAs against ligand- and voltage-gated ion channels cause a variety of defects in peripheral neuromuscular and ganglionic transmission. Studies established a long time ago that AAs against muscle-type nAChRs are the main reason of myasthenia gravis.

NMDA receptors, glutamate receptors, sodium and calcium channels and nAChRs are crucial elements in neuronal signalling and synaptic transmission, and defects in their function are known to be related to neurological disorders. [40,43,44]

Marina Skok published that α7 nAChR AAs can also have a relevance in Alzheimer's disease (AD) pathology. She concluded that α7 (1-208) nAChR-specific antibodies can be present and originated in human blood plasma after a common infection (accompanied with respiratory epithelium destruction). AD has been characterised by the loss of α4β2 and α7 nAChRs in the brain and severe memory impairments. In the brains of mice, antibodies produced against ECD of α7 nAChR subunit (1-208 amino acids) decreased the number of α7 nAChRs and accumulation of β-amyloid peptide. [17,19,45]

The AAs also have a potential role in psychiatric diseases such as Bipolar Affective Disorder, Autoimmune Encephalopathies, Psychosis, Schizophrenia, multiple sclerosis, etc. A new treatment strategy can emerge if the understanding of the interaction antibody-antigen within the CNS is clarified.

To understand the neurobiological basis of associated psychiatric symptoms, as endogenous, bioactive, highly specific, receptor-targeting molecules, provides a valuable opportunity to the psychopharmacological perspective. The effects of the AAs on their target antigens are described as resulting in hypofunction, because of the effects on neuronal receptor function at synapses, for example through internalisation.

To support relevant genetic and pharmacological evidence, the psychiatric effects of the antibodies need to be related to known functions of the receptor target or its complexed proteins. [46]

In the specific case of α7 nAChRs, the key role of the agonist nicotine in cognitive, attentional deficits, and in the deficient processing of repetitive sensory information has been an object of study. The preference of schizophrenic patients for tobacco (extremely heavy cigarette smokers) may be a way to compensate the reduction of α7 nAChRs in the hippocampus by delivering exogenous ligand to the remaining receptors. Rodent models have confirmed the role of α7 subtype in these processes. Therapeutic agents selected for α7 nicotinic activity may have utility in treating certain symptoms of schizophrenia. [47,48]

Genome-wide Association Study (GWAS) published data from 108 schizophrenia-associated genetic loci. The evidence established the association of major locus of the human immune system, the MHC complex, with schizophrenia. [49]

Multiple sclerosis (MS) is an unpredictable, often disabling disease of the central nervous system that disrupts the flow of information within the brain, and between the brain and body. Most people with MS are diagnosed between the ages of 20 and 50. The cause of MS is still unknown. The disease is triggered by as-yet-unidentified environmental factors in a person who is genetically predisposed to respond. MS is an autoimmune disease characterised by recurrent episodes of demyelination and axonal lesion. $CD4^+$ T cells with a proinflammatory T helper, macrophages, and soluble inflammatory mediators have been considered key players in the pathogenesis. Only recently, it has become evident that B cells have a major role of contribution, yet the target antigens of B cells need to be identified. [50]

Microglial cells are the first response to neuronal injury. The CNS is constituted by about 10% of these cells. Antibodies are frequently observed in acute lesions of MS patients. The CSF of MS patients is also characterised by the presence and accumulation of Ig molecules. The production of AAs, particularly those that react with myelin, is relevant since they can contribute to the process of demyelination. [51,52]

The AAs (IgG and IgM) localised against demyelinated axons, oligodendrocytes and antibody-antigen immune complexes were detected in macrophages in active lesion areas. [52–54]

Potential therapies such as monoclonal antibodies in MS treatment have been tried to prevent or delay the disease, although there are still problems to be solved such as immunogenicity, infusion reaction, and of course CNS penetration. [55]

1.6. Working Hypothesis 1

"*A patient with encephalitis associated with NMDA receptor antibodies*", a Dalmau paper from 2007, described for the first time a new category of autoantigens to a treatment-responsive paraneoplastic encephalitis. [56,57]

This new finding in neuroscience led to new case studies and analysis of the effects of antibodies being pursued by Josep Dalmau, Angela Vincent, Hannelore Ehrenreich, Harald Prüss, and others. It still remains unclear how AAs appear in the CSF, crossing the BBB. But then again, anti-NMDA-receptor encephalitis became a new disease associated with antibodies against NMDA receptor (NR1-NR2 heteromers). Circulating AAs were documented against brain epitopes, connecting them with classical autoimmune diseases or paraneoplastic syndromes. Diseases groups from AD, schizophrenia and major depression, to diabetes, hypertension and stroke, as well as in healthy individuals, share a seroprevalence with NMDAR1-AA but also with immunoglobulin class (IgM, IgA, IgG and titer range). [58–61]

Harald Prüss went a bit further, and in order to understand better the AAs elicited against NMDAR, he decided to use cell cloning and sequencing assays. The cell cloning involved the full-length immunoglobulin heavy and light genes and generated a panel of recombinant monoclonal NR1 antibodies from cerebrospinal fluid memory B cells, and antibodies secreting cells from NMDAR encephalitis patients. The immunohistochemical functional data using the CSF patient against brain section from mice was sufficient to prove the antibody pathogenicity. However, a vast majority of antibody-secreting cells and memory B cells produced antibodies (from patients diagnosed with NMDAR encephalitis) did not bind to NR1 (non-NR1 antibodies), yet react against others brain-express epitopes as hippocampus and cerebellum neuronal surfaces. This finding suggested that these cells are specified in the CNS in which the antigen is present. Future work is needed to understand the target protein specificity of these non-NR1 antibodies. [59,62]

Taking into consideration the work described above, including also the work of Marina Skok, our working hypothesis is to test these non-NR1 antibody samples from the human cerebrospinal fluid monoclonal NMDAR AAs against nAChRs, more specifically the α7 subtype. [63,64]

1.7. Working Hypothesis 2

Previous work in the lab proposed to model the generation of AAs in mouse models of infectious and pulmonary disease (COPD), and then comprehensively characterised the AAs against nicotinic receptors, especially the α7 subtype. [13]

A novel mechanism of how microbes can influence brain activity and pathology was addressed. Instead of directly penetrating the brain, microbes can cause infectious disease with concurrent B-cell activation.

Infections imply the generation of AAs against neural proteins (neuronal nicotinic receptors) and can be expressed in peripheral tissues, as the lung. B cells and AAs are then able to penetrate the brain, and neuroinflammation can ensue because of an activation of microglia or other non-neuronal cells. [62]

In Figure 2 the experimental data of these experimental infections in inducing AAs in mice upon respiratory infections are shown. Mice of two strains, BALB/c and C57BL/6J, were exposed to repeated respiratory infections using *Influenza A virus* (IAV), *Neisseria meningitidis* (Nm), and *Streptococcus pneumoniae* (SP) agents. Mice were sampled after the infectious episodes. The kinetics of the appearance and the levels of AAs in blood and in the CSF was evaluated by ELISA. **unpublished data**

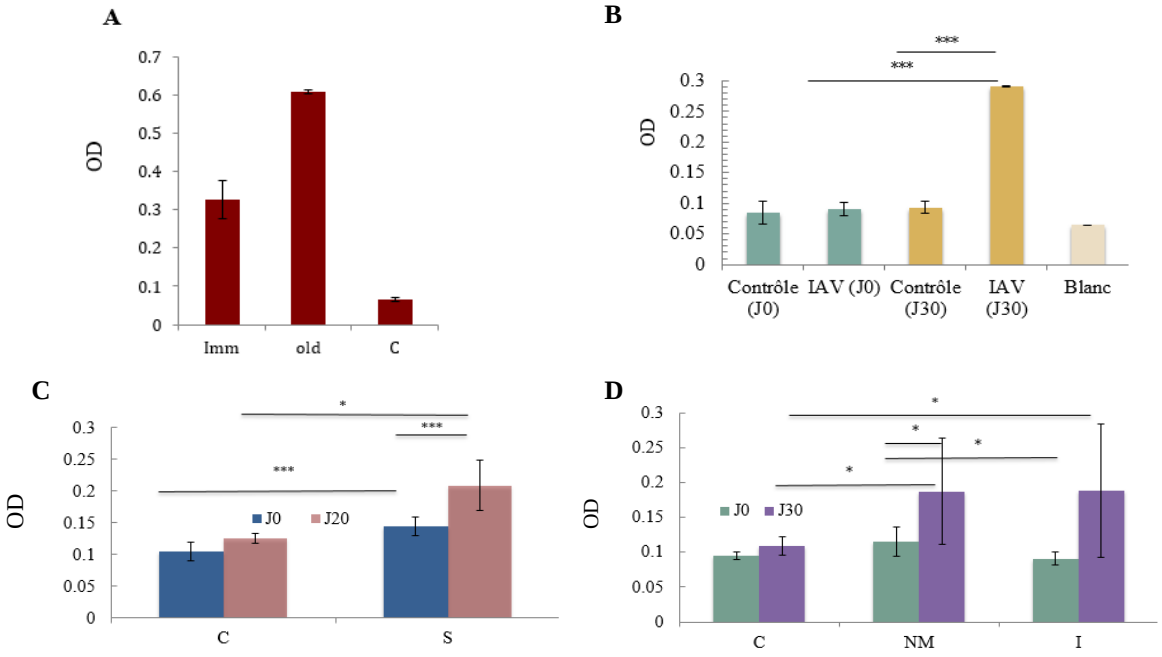


Figure 2 - Experimental data of infections inducing AAs in mice upon respiratory infections and immunization with α7 subtype ECD. **A)** average of the α7 antibody levels, in 3 different group of mice; mice immunize with α7 ECD (Imm); mice with 2 years old (old) and control group injected with PBS (C); **B)** level of circulating IAV antibodies in the control group mice (contrôle) and IAV infected group mice (IAV), on day 0 (J0) and day 30 (J30) dpi; **C)** Levels of α7 antibody circulating in control and IAV + SP infected groups mice, on day 0 and 30 after intra-nasal administration of PBS (C) or IAV (S); **D)** α7 antibody levels in three groups (control, Nm and IAV) 0 and 30 days after intra-nasal administration of PBS (C) or IAV + Nm (I).
The differences between the different conditions were tested by Student's T-test (* P <0.05; ** P <0.01; *** P <0.001)

Carrying on the previous work, a new strain of mice was added to test if virus and bacteria are able to elicit AAs against α7 nAChRs. α7 KO mice were infected with *Influenza A virus* to provoke the generation of AAs (bronchial infection), and they were subsequently superinfected with the bacteria *Neisseria meningitides*, as before, to elicit additional AAs generation.

Mice homozygous for the α7 nAChR null allele (α7 KO mice) are a good animal model to study the exact implications and influences of the immune system. This animal model has also been used in research of cognitive impairment, therapies for schizophrenia, AD, nicotine addiction and other studies related to α7 nAChR. [65–68]

With the α7 KO mice, it was possible as well to understand the effects of serum antigen-specific IgG and proinflammatory cytokine production depending on the α7 subunit gene. Deducting that appears to regulate cytokine production, modulating TNF-α, IFN-γ and IL-6 productions. This leads to an antibody modification production. [69]

Chapter 2

Materials and Methods

Chapter 2. Materials and Methods

The current chapter is presenting the methods and techniques in 2 parts: in **2.1)** analysis of human cerebrospinal fluid monoclonal samples using ELISA method to qualify the presence of α7 antibodies against nAChRs and **2.2)** analysis of CSF and blood serum samples from infected α7 KO mice (ACNA7), using also ELISA method to detect the presence of α7 antibodies against nAChR as well and mouse brain slices to search for neuroinflammation.

The ACNA7 is translated to a total inactivation of α7 subtypes in neural and non-neuronal cells.

For the *in vivo* trials, the present experiment described using KO and WT mice (**2.2**) was conduct in accordance with the guidelines on the ethical use of animals from the *European Community Council Directive* of 24 November 1986 (86/609/EEC) and in accordance with institutional animal welfare guideline that were approved by the *Animalerie Centrale and Médecine du Travail* at the *Institut Pasteur,* France. [70]

2.1. Human Cerebrospinal Fluid Monoclonal Samples

The 141 human CSF monoclonal samples were kindly provided by our collaborator Harald Prüss lab. from the *German Center for Neurodegenerative Diseases* (DZNE) Berlin, Germany, which has a partnership with the *NISC* lab. at *Institut Pasteur*.

As above mentioned, Harald Prüss lab. has identified samples from CSF human monoclonal that did not cross-react with NMDARs. The *NISC* lab had the opportunity to test these samples and assess if some of these NMDAR encephalitis patient samples cross-react against α7 nAChRs.

The ELISA assay was the technique used to explore the hypothesis.

2.1.1. Determination by ELISA assay

The ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. In ELISA assay, an antigen must be immobilised to a solid surface and then complexed with an antibody that is linked to an enzyme. The detection is accomplished by assessing the conjugated enzyme activity via incubation with a

substrate to produce a measurable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction.

The present protocol describes an indirect ELISA to detect the link between α7 antibody with human α7 (1-208) ECD (antigen), provided by the *Hellenic Institut Pasteur,* Athens, Greece. [17,19,63,64,60]

In more detail, the working hypothesis is to test if human CSF monoclonal samples from NMDAR encephalitis patients, that did not cross-react with NMDARs, have α7 antibody present. To test this hypothesis ELISA protocol was chosen since it is the screening method for evaluating antibody/antigen association.

2.1.2. ELISA protocol 1

Due to the enormous number of samples, 27 pools were created and evaluated. Each pool contained on average 5 human monoclonal antibodies ([20 – 600] μg / ml) isolated from human CSF resident B-lymphocytes (detailed protocol at *Kreye et al., 2016*).

ELISA Nunc MaxiSorp™ 96-well flat bottom was used with standard serology, plates surface treatment and high affinity to molecules with mixed hydrophilic/hydrophobic domains (600 to 650 ng d'IgG per cm^2 of affinity), *Dominique Dutsher* provider.

The plates were coated with α7 (1-208) ECD 5 μg / ml or BSA (3 %), in 50 μl of PBS per well by evaporation overnight at 37 °C and then blocked with 3 % of BSA (1h30min., 37 °C).

The samples were applied in 1:5 dilution in 0,05 % Tween 2-containing PBS for 2 h at 37 °C.

The bound antibodies were detected with HRP peroxidase-conjugated antibodies against Human IgG (*SIGMA*), and OPD solution (*SIGMA*) with 10 minutes' reaction was used to reveal it.

Finally, to stop the reaction, 25 μl of 1M H_2SO_4 solution (*FLUKA)* were added.

The optical density (OD) was read at 490 nm.

(according to *Koval et al., 2011*)

As a positive control, ELISA plate was coated with a human sample (serum) from an 8.8 years old boy, confirming the link of the 1st antibody with the same Anti-Human IgG (*SIGMA*).

As a negative control, besides BSA (3%), the ELISA plate was also coated with α7 (1-208) ECD and IgG anti-Human (*SIGMA*), with no human CSF monoclonal sample (1^{st} antibody).

2.2. Blood serum and CSF samples from mice

The previous work described in 1.7 was carried in order to improve knowledge and information about the generation and the characteristics of AAs in infected mice models against α7 nAChRs.

A new approach was settled for extending preceding data. The analysis of blood serum samples and CSF from infected α7 KO mice (ACNA7) and WT (C57BL/6J) were used to compare the WT AAs against α7 nAChRs. The ACNA7 mice line was selected since the genetic modification was already obtained and available for mouse models experiments.

2.2.1. The mouse as a model organism

WT and ACNA7 mice were kept in the animal facility of *Institut Pasteur*, France. They were housed in a quiet, temperature-controlled room (23°C) and were provided with water and dry food pellets *ad libitum*. ACNA7 mice were bred at *Charles River Laboratories* (L'Arbresle, France) and WT mice at *Institut Pasteur*. Before experimentation, mice were kept in definitive cages for 1 week without manipulation.

First experiment, mice were organized in 5 groups:

I)	WT and ACNA7 control group	(n=3) (n=2);
II)	WT and ACNA7 superinfected group by IAV + Nm	(n=5) (n=4);
III)	ACNA7 group infected with IAV	(n=4);

Male mice had 8 weeks of age at the beginning of the experiments. In total 8 WT mice and 10 ACNA7. Control groups were inoculated with PBS 1x.

During the experiment, at the second infection with Nm, WT mice died. Since they were too young, the superinfection was excessively violent to the organism. Although, α7 KO mice with the same age and weight survived in good physical shape.

Second Experiment, mice were organized in 6 groups:

I)	WT and ACNA7 control group	(n=2) (n=2);
II)	WT and ACNA7 infected group by IAV	(n=5) (n=4);

III) WT and ACNA7 superinfected group by IAV + Nm (n=5) (n=4);

Male mice had 12 weeks of age at the beginning of the experiments. In total 12 WT mice and 10 ACNA7. Control groups were inoculated with PBS 1x.

2.2.2. Infections and collection of samples

Both experiments were performed with the same infection protocol.

Respiratory infections were caused by intranasal administration of 50 µl of viral (IAV – 130 pfu, per mouse) or bacterial inoculum (Nm – $2.5x10^7$ CFU, per mouse) to the respective groups.

Mice were lightly anaesthetized with a mixture of Xylazine 2 % and Ketamine 100 mg/ml (*Sanofi*, France) in PBS in every single infection.

Figure 3 represents a chronogram of respiratory infections in the groups of mice.

On day 1, mice from all groups were weighed and blood was collected. Next, groups II) and III) were infected with IAV.

Blood samples taken by intra-orbital sampling on day 1 were centrifuged. The plasma was recovered and frozen at -20 °C.

Weight was controlled on days 5, 7, 20 and 30.

On day 7, mice of group III were superinfected with Nm and 30 dpi and all the mice were sacrificed. CSF was collected followed by blood collection from the heart and finally the brains. The blood samples were processed as before. Brains were fixed with 4 % PFA (*SIGMA*) for 5 days and then saved in PBS 1x at 4 °C.

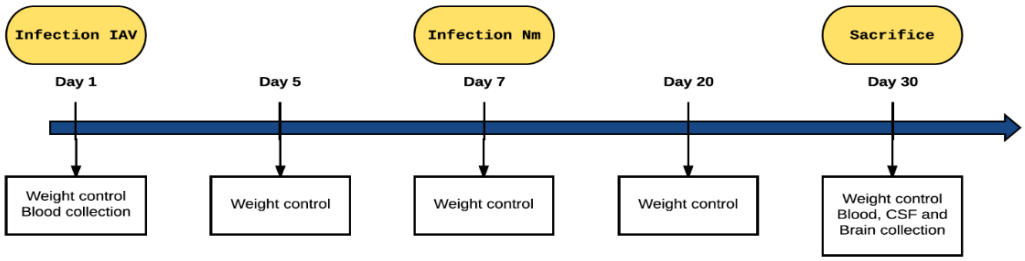


Figure 3 - Chronogram representing the respiratory infections of mice groups (ACNA7 and WT). On day 1 mice were: weighed, blood was collected, and then infected. On day 7 group III) of ACNA7 mice was infected with Nm. 30 days after the 1st infection all mice were sacrificed. PBS1x was used for control groups.
White boxes designate the procedures, yellow boxes the days of infections experiment and the blue arrow labels the time points.

2.2.3. ELISA protocol 2

Blood and CSF samples were analysed using the ELISA indirect method:

ELISA Nunc MaxiSorp™ 96-well flat bottom was used with standard serology, plates surface treatment, high affinity to molecules with mixed hydrophilic/hydrophobic domains (600 to 650 ng d'IgG per cm^2 of affinity), Dominique Dutsher provider.

The plates were coated with α7 (1-208) ECD 5 µg / ml or BSA (3 %), in 50 µl of PBS per well by evaporation overnight at 37 °C and then blocked with 3 % of BSA (1 h 30 min., 37 °C).

The samples, collected before and after infections, were applied in 1:5 dilution in 0,05 % Tween 2-containing PBS for 2 h at 37 °C.

The bound antibodies were detected with HRP peroxidase-conjugated antibodies against Mouse IgG (SIGMA) and OPD solution (SIGMA) with 10 minutes' reaction. Finally, to stop the reaction, 25 µl of H_2SO_4 1M solution (FLUKA) were added.

The optical density (OD) was read at 490 nm.

(according to Koval et al., 2011)

As a positive control, ELISA plate was coated with a positive mouse sample from the previous results shown in Figure 2.

As a negative control, besides BSA (3%), the ELISA plate was also coated with α7 (1-208) ECD and IgG anti-mouse (*SIGMA*), with no mouse sample (1^{st} antibody).

2.2.4. Immunohistochemistry set-up

To search for neuroinflammation, immunohistochemistry technique was setup for mouse brain sections. After PFA 4 % fixation, the brain sections were cut on a vibratome (*LEICA VT1000 S*) with 55 µm step size and 1 mm/s speed. Slices were permeabilizated with 10% NGS (*ThermoFisher*), saturated with 0,2 % Triton, 1h at RT. Followed by 2 washes with PBS 1x 2% NGS + 0,2% Triton also a RT.

Staining included anti-GFAP, anti-Mouse (1:400, *SIGMA*) to astrocytes and anti-IBA1, anti-Rabbit to microglia (1:1000, *WAKO*) incubated overnight at 4 °C under agitation. Followed by 3 washes with PBST 1x, 5 min at RT.

Reveal with *Alexa 488* (anti-Mouse, green-fluorescent dye) and *Alexa 594* (anti-Rabbit, red--fluorescent dye) (work solution 1:500, *ThermoFisher*). Followed by 3 washes with PBST 1x, 5 min at RT.

Lastly, with prolong with Dapi slices were mounted onto microscope slides.

2.3. Data analysis

The results were statistically evaluated using the student's *t*-test. The data are presented as average values and standard errors (M ± SE) and are considered significant at $p < 0.05$.

Chapter 3

Results

Chapter 3. Results

The results obtained from the different experiments are shown below. The working hypothesis 1 about the human CSF monoclonal samples and the working hypothesis 2 about blood serum and CSF of mice is indicated in 3.1 and 3.2, respectively.

3.1. Human Cerebrospinal Fluid Monoclonal Results

Samples from Harald Prüss lab were tested by indirect ELISA as shown in Figure 4. Samples of non-NR1 antibody from the patients diagnosed with NMDAR encephalitis were tested against nAChRs of

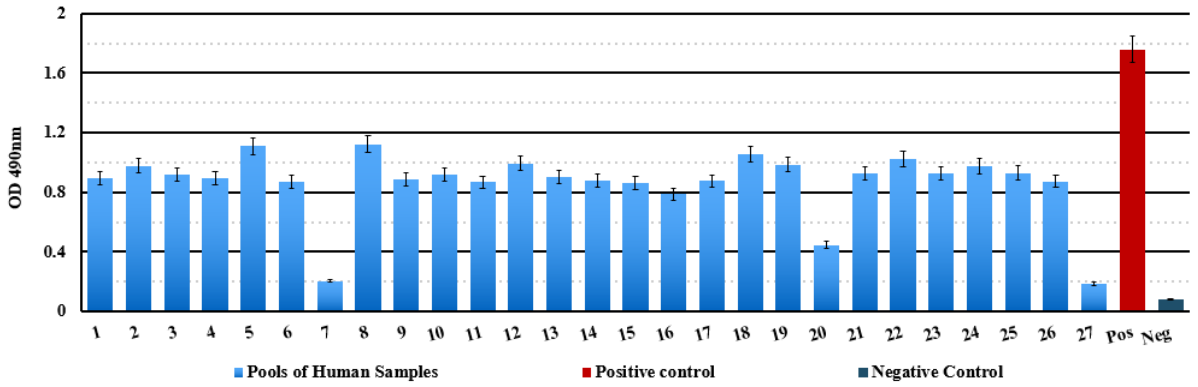


Figure 4 - Pools of 27 samples; Each sample contains on average 5 human monoclonal non-NR1antibodies. **BLUE** is representing the 27 Human pools; **RED** and **GREEN** represent positive and negative controls respectively.

the α7 subtype.

In order to understand the activity of the human pools or a possible cross-reaction, Figure 5 shows the individual samples from pool no.7 (considered negative) and pool no.8 (considered positive). The individual human samples were tested in the same conditions as previously.

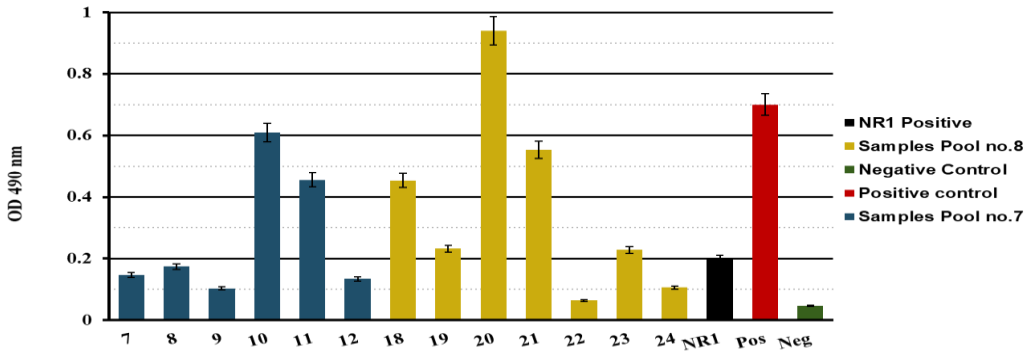


Figure 5 – Individual human samples from pools no. 7 and no. 8. Columns in **BLUE** represents individual samples from pool no. 7; in **YELLOW** the samples from pool no.8; **BLACK** the NR1 antibodies positive to NMDA receptors; **RED** the Positive control and **GREEN** the Negative control.

The test of the 141-individual samples was conducted after the evaluation of the 27 pools and the individual human samples (from pools no. 7 and no. 8).

One more assay was carried out to understand the activity of the human pools. Figure 6 shows the effect of dilutions in the human samples (pool no.8 and no.14) in indirect ELISA.

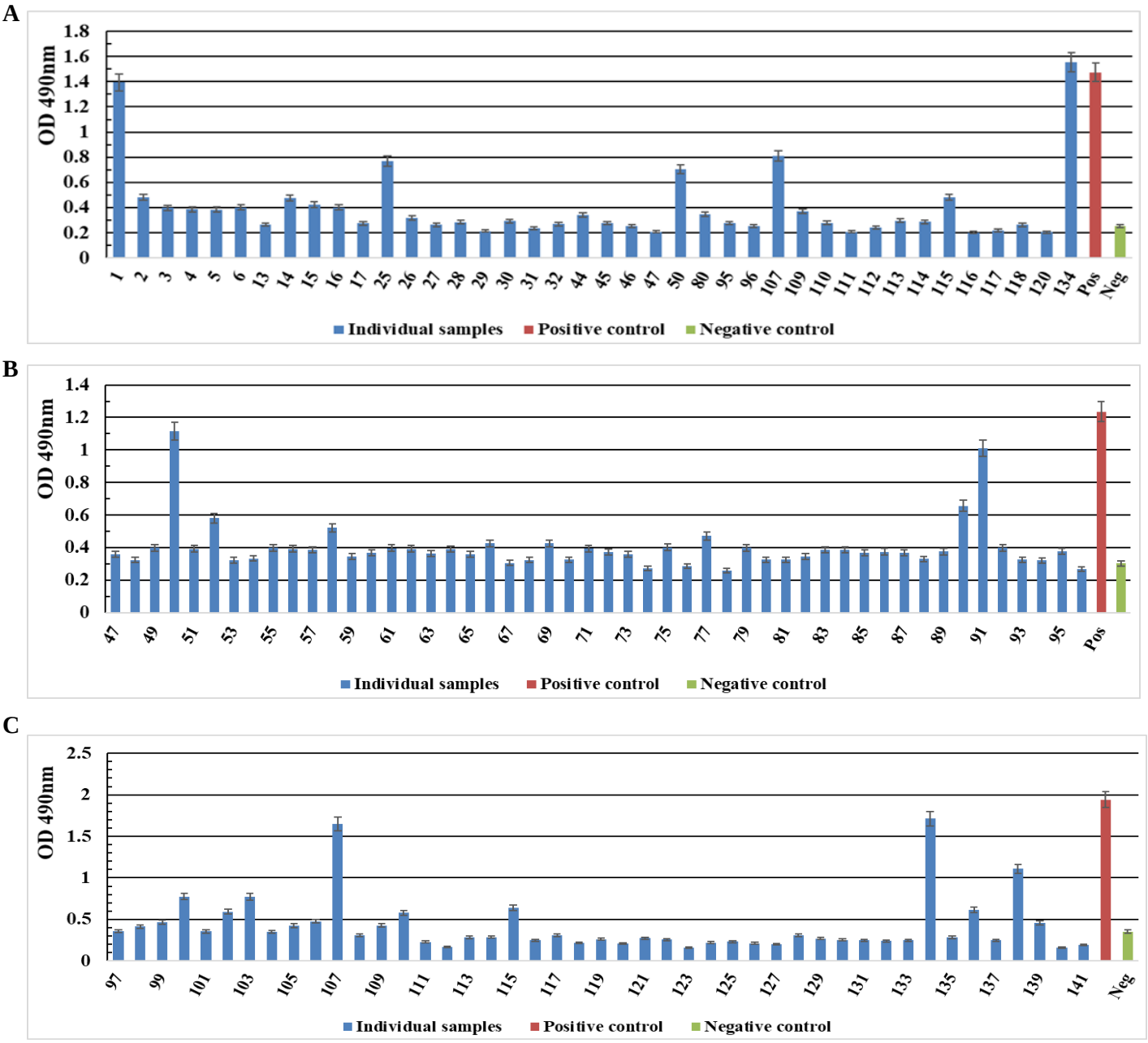


Figure 6 – The 141-individual human CSF monoclonal samples were tested. Columns in **BLUE** represents the individual samples; **RED** the Positive control and GREEN the Negative control. **A)** sample no. 1 and no. 134 have evidence of being positive; no. 25, 50 and 107 are indeterminate, the rest appears to be negative. **B)** sample no. 50 and no. 91 have evidence of being positive; no. 52 and 90 are indeterminate, the rest seems to be negative also. **C)** sample no. 107 and no. 100 and 134 have evidence of being positive; no. 138 is indeterminate; the rest looks negative.

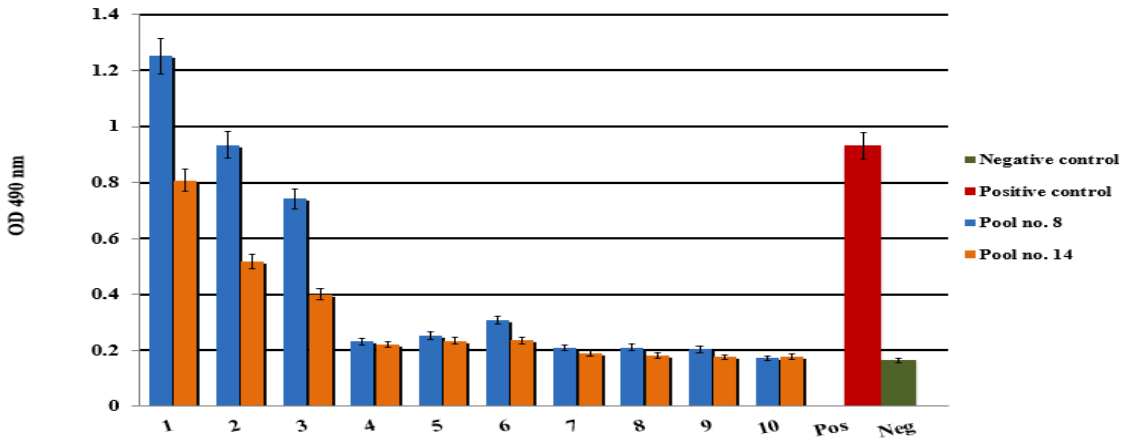


Figure 7 - – Pools no. 8 and no. 14 from human monoclonal samples. Columns in **BLUE** correspond to sample no.8 and Columns in **ORANGE** correspond to sample no. 14; **RED** the Positive control and **GREEN** the Negative control.
Dilutions groups: **1** – 1:1, **2** - 1:2, **3** - 1:5, **4** – 1:10, **5** – 1:50, **6** – 1:100, **7** – 1:500, **8** – 1:1000, **9** – 1:5000, **10** – 1:10000.

3.2. Blood serum and CSF results in mice

The data are organised into 3 parts: the first experiment, followed by the second experiment. In the end of this chapter, the immunohistochemistry set-up can as well be found.

3.2.1. First Experiment

Mice infected and not infected were weighted at the time points shown in Figure 3.

Weight from 8 WT mice and 10 ACNA7 is shown in Figure 8.

Control groups were inoculated with PBS 1x. Peripheral blood was collected at the beginning of the experiment. Heart blood and CSF were collected at the end of the experimentation.

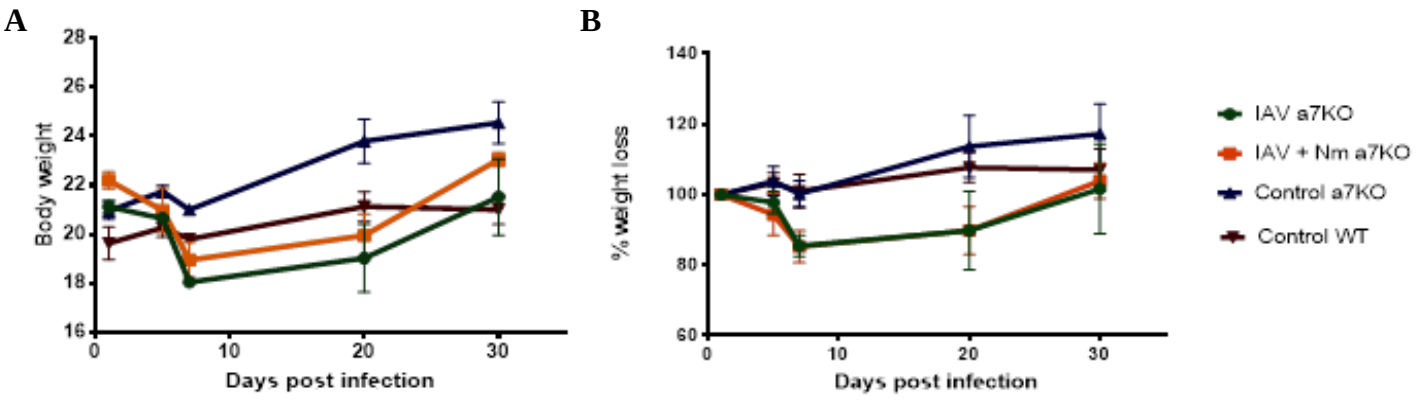


Figure 8 – Body weight of 13 infected and 5 not infected mice. **A)** Body weight during the 30 days of post infections; **B)** Body weight loss percentage also during the 30 days of experiment.

A correlation between the weight variation and the ELISA titers is shown in Figure 9. The ratio Day30/Day1 per mouse was used and then normalised by the same ratio of the control mice.

Figure 10 shows the CSF and the blood serum samples tested by indirect ELISA.

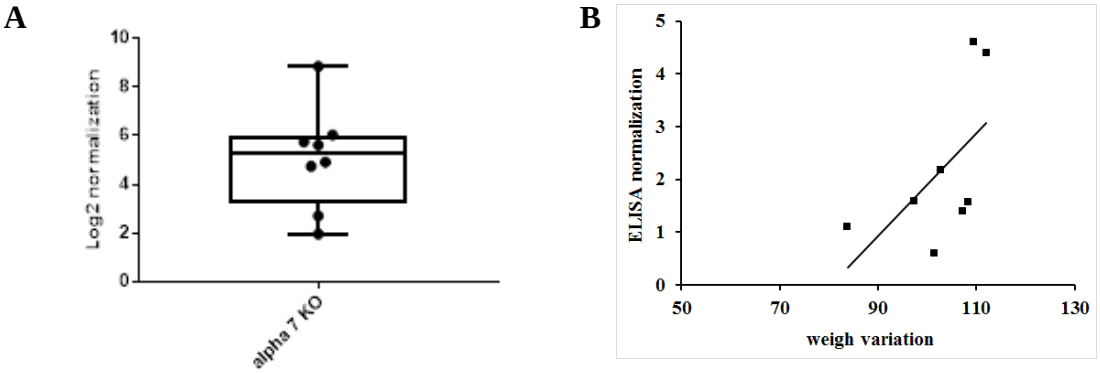


Figure 9 – Normalization of the α7 KO mouse titers and its correlation with the weight variation. **A)** Log2 normalization graph of ratio Day30/Day1 **B)** Correlation weight variation/ELISA titers.

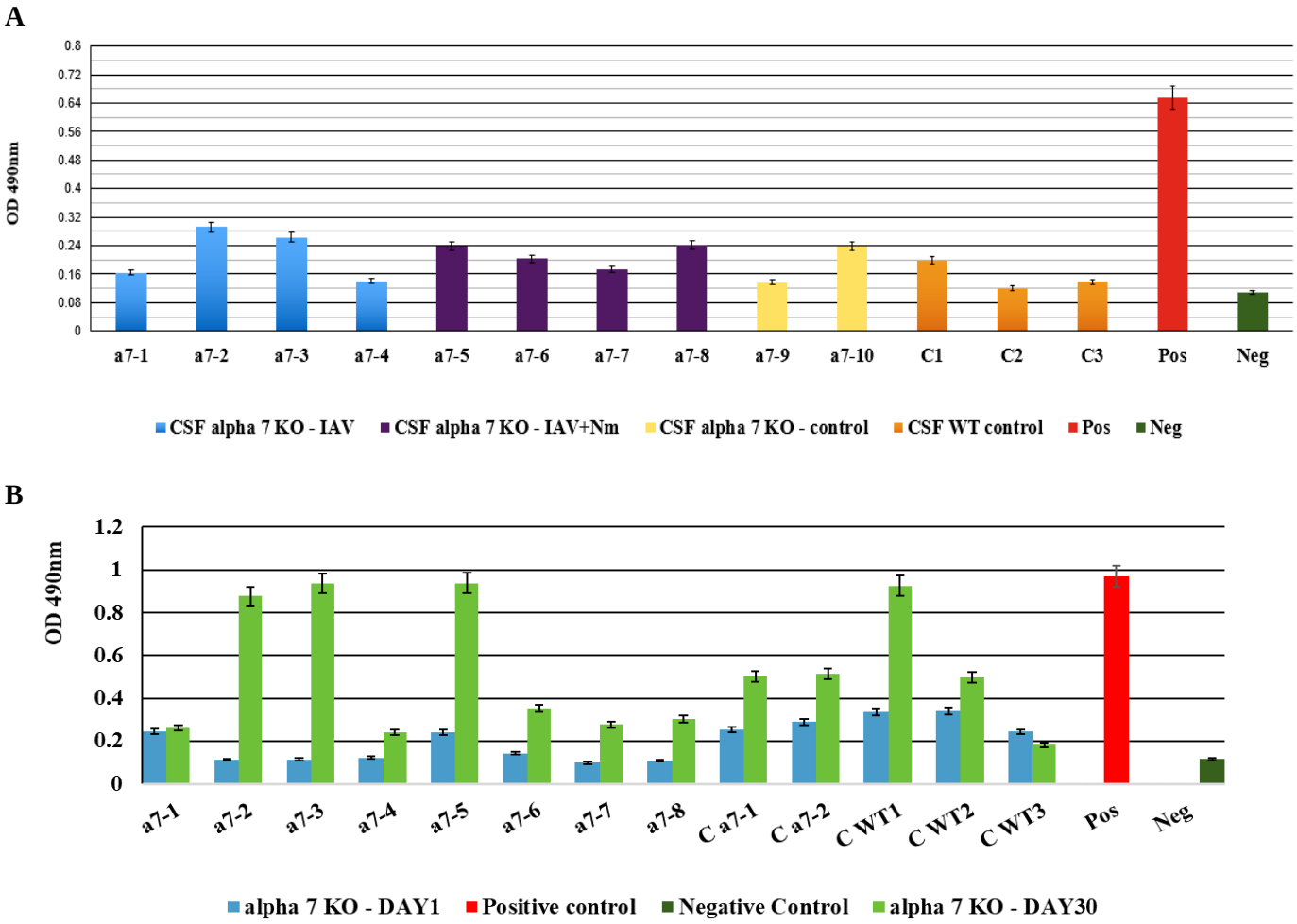


Figure 10 – Indirect ELISA titers of CSF and blood serum samples. **A)** CSF samples displayed by 4 groups; **BLUE** – mice infected only with IAV at day 1; **PURPLE** – mice infected at day with IAV and at day 7 with Nm; **YELLOW** – Control group (PBS 1x) and ORANGE – WT control group. **B)** Blood serum presented as before: a7-1 to a7-4 IAV; a7-5 to a7-8 IAV + Nm; C a7-1 and C a7-2 groups control for α7 KO mice. WT control: C WT1 to C WT3; **LIGHT GREEN**. **RED** and **DARK GREEN** is representing positive and negative controls respectively.

3.2.2. Second Experiment

Experiment accomplished as before presented, however, CSF was not collected because trials are still ongoing.

Weights from 12 WT mice and 10 ACNA7 are shown in Figure 11.

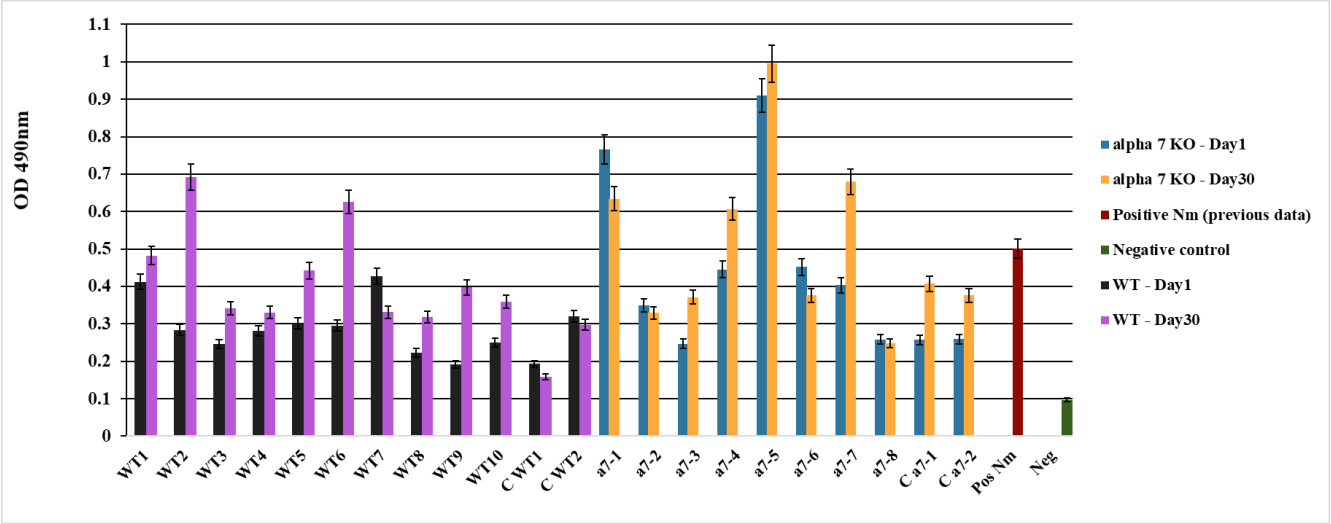


Figure 11 – Second experiment indirect ELISA titers of blood serum samples. Blood serum presented as before: a7-1 to a7-4 infected with IAV; a7-5 to a7-8 - IAV + Nm; C_7-1 and C_a7-2 groups control for α7 KO mice. WT control: C_WT1 to C_WT3; IAV infection = WT1 to WT5; IAV + Nm = WT6 to WT10;
BLUE - α7 KO before infection; **YELLOW** - α7 KO day 30; **BLACK** - WT before infection; **PURPLE** - WT day 30; **RED** and **GREEN** are representing positive and negative controls respectively.

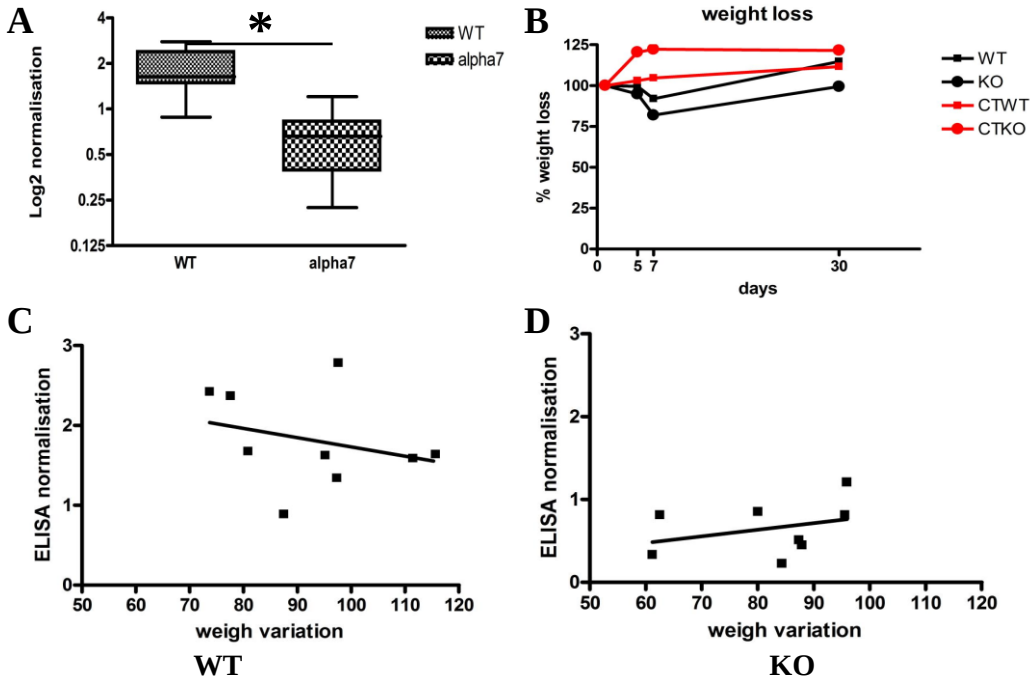


Figure 12 – statistical data analysis. **A)** Samples normalizations – ratio Day30/Day1; **B)** weight loss percentage; **C)** correlation weight variation/ELISA titers from WT samples; **D)** correlation weight variation/ELISA titers from α7 KO samples
* *(p=0,0002)*

3.2.3. Immunohistochemistry

The following subchapter presents the images of immunohistochemistry set-up for neuroinflammation. Astrocytes in green, microglia in red and nuclei in blue were stained.

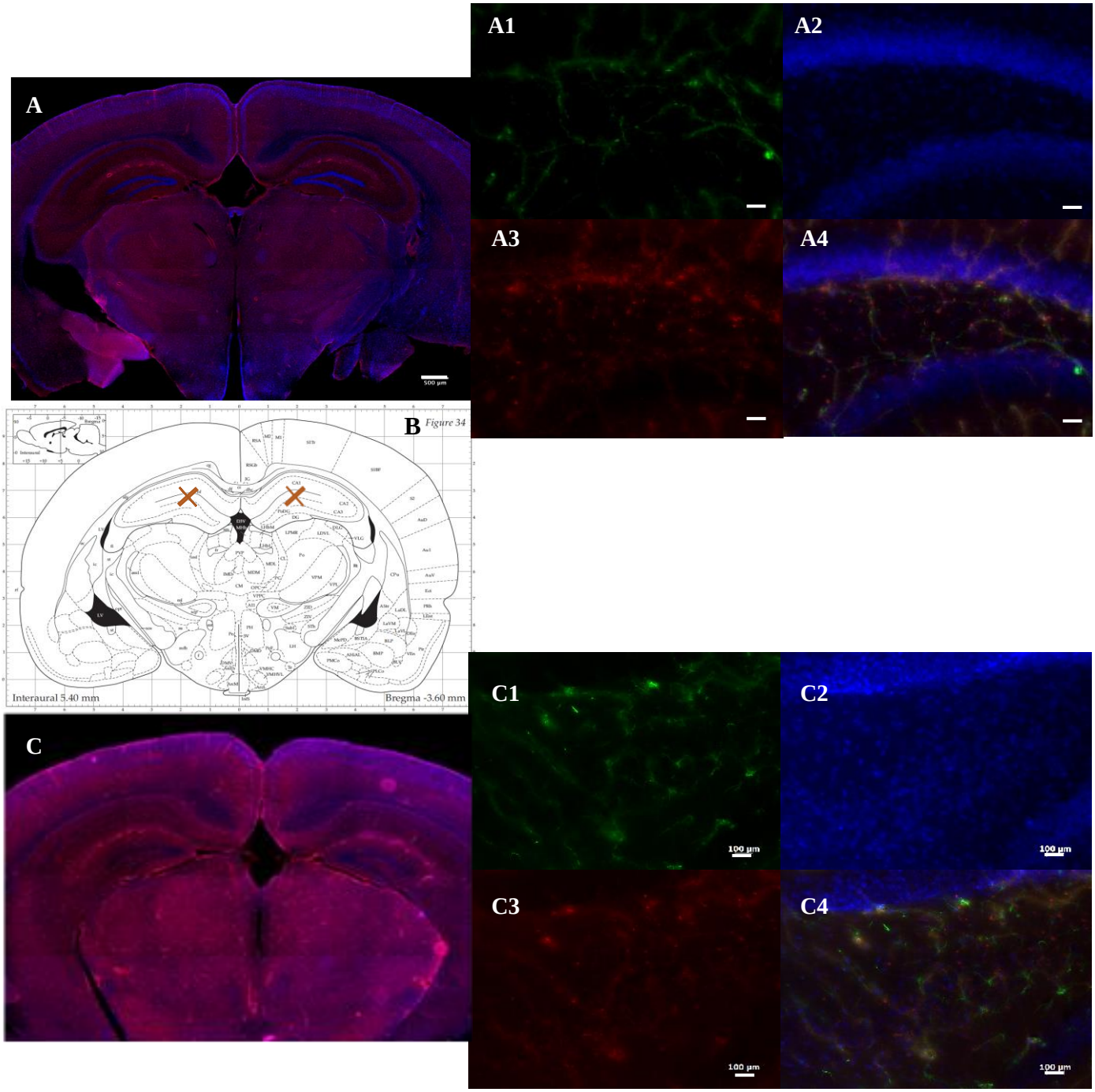


Figure 13 **–** Immunofluorescence on fixed hippocampus sections using *Alexa 488* (green) anti-Mouse anti-GFAP to stain astrocytes and *Alexa 594* (red) anti-Rabbit, anti-IBA1 to microglia. Nuclei were marked by Prolong with DAPI (blue). **A)** 5x magnification of Control brain slice; A1 – astrocytes, 20x; A2 – DAPI, 20x; A3 – microglia, 20x; A4 – merge; **B)** Mice ATLAS (*Bregma,* -3.60mm); C) 5x magnification of IAV + Nm brain slice, scale bar = 500 µm; C1 – astrocytes, 20x; C2 – DAPI, 20x; C3 – microglia, 20x; C4 – merge;
scale bar = A-B = 500 µm; A1-A4, C1-C4= 100 µm.

Chapter 4

Discussion

Chapter 4. Discussion

The next chapter aims to discuss the results obtained and shown in the previous chapter. The same guiding principle is used, human CSF monoclonal analysis is discussed first, and then the experiments of blood serum and CSF of mice.

4.1. Human Cerebrospinal Fluid Monoclonal analysis

The present results of ELISA are showing that almost all the human pools of the non-NR1 antibodies react against α7 ECD. This can provide evidence that patients who were diagnosed with NMDA encephalitis are also affected not only by NMDAR but also, at least, by nAChR antibodies.

In the process of exploring more these pooled samples, and before testing all individual samples, pools with the lowest, considered negative, and pools with the maximum OD value, considered positive, were tested under the same as for the previous pools. More precisely, the pools no. 7, no. 20 and no. 27 presented the minimum OD values while the rest of the pools 24 of 27 presented similar higher values. Pools no. 5 and no. 8 had the maximum OD values.

As expected, results from individual samples (Figure 5) were consistent with the first ELISA results. The Pool no. 7 gave lower values compared to pool no. 8. To complement these results, the NR1 positive sample was tested as well, showing a negative response to nAChR as anticipated.

To be clear, the rule defined to classify the samples as positive or negative was the comparison of the samples presenting proximal values to the positive control. From the 141-individual samples, it was possible to identify some positive reaction against α7 ECD (individual human samples no. 1, 50, 91, 107 and 134). It is clear that some samples from NMDAR encephalitis patients had a very good response to the α7 nAChR.

To check the range of work dilution two similar higher pools were tested (Figure 7). Pool no. 8 and pool no. 14 were chosen confirming that the best working dilution is 1:5.

Further dilution gave rise to indistinguishable values.

More experiments and data are required to have solid conclusions and facts about these patients producing AAs against α7 nAChRs.

4.2. Mouse Blood serum and CSF Results

The first experiment on α7 KO mice was quite inconclusive. The facts were: the ACNA7 mouse line seemed to be more resistant to infection related sickness than WT mice based on physical signs and weight loss; ELISA CSF samples from ACNA7 line showed that OD of controls and infected mice were very similar, the presence of AA are questionable; the detection of AAs in blood were also unexpected in the ELISA experiments, as α7 KO mice displayed some reactivity with α7 ECD.

Therefore, a new set of experiments was carried out. The age of WT mice was adjusted in order to better compare results with α7 KO mice. The ACNA7 line seemed to be resistant to infections again and all WT mice survived to the superinfection since they were older and subsequently stronger. ELISA results of this experiment were not clear-cut again. A mathematical approach was used in order to try better and clearly understand these results. The statistical analysis showed that the correlation of weight variation with ELISA titers from α7 KO samples and WT were significantly different. WT responded with an increased titre when compared with KO mice (Figure 12). The hypothesis proposed to explain this fact was a possible cross-reactivity against other receptors whose expression and/or exposure are modified in the ACNA7 mouse line, as proposed before by Maryna Skok. [36]

This also ties in with the enhanced production of antibodies as described in the α7 KO line. [69]

The set-up of immunohistochemistry worked. The hippocampus was chosen since it is the area of the brain with the highest expression of α7 nAChR. Further technical and statistical analyses and staining are now necessary to correlate more accurately neuroinflammation with AAs, and infections.

Chapter 5

Concluding Statement and Future Directions

Chapter 5. Concluding Statement and Future Directions

The working hypothesis 1 consisted in testing non-NR1 antibody samples from human cerebrospinal fluid monoclonal NMDAR encephalitis patients. The results showing the possibility of AAs being produced against α7 nAChRs is supportive. There is, in fact, a possibility for the AAs to be produced and elicited against α7 nAChRs in this context.

Nevertheless, future experiments are required. Upcoming experiments will consist of CBA and two-photon imaging.

The ELISA technique was a good method to test the link between proteins. However, CBA will be a better system to test the efficiency and affinity of the aforementioned. Through this method, the HEK293 cells can mimic the entire α7 nAChR reproducing an experimental environment similar to the native configuration.

The functional two-photon imaging experiment in the awake mice will continue the analysis of AA actions. At first stage, mouse models (WT and ACNA7) will receive an intracerebroventricular or intravenous infusion of the individual samples previously marked as positive (by indirect ELISA and CBA). It is expected that AAs against the α7 subtype will lead to a (partial) reduction of α7 subtype activity. This may be translated as neuronal hyperactivity, as shown already for the ACNA7 mice. Further work will test these samples on also α4 and β2 KO mice.

The working hypothesis 2 is not, so far, clearly certain. The ACNA7 results did not match exactly with the theory. More experiments using additional ECD from other receptors, like α4, can be pursued to understand exactly what is happening in the α7 KO mouse line. By means of the Maryna Skok, a cross-reaction is one of the explanation for now. [36,69]

Chapter 6

References

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