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Genetic, Immune and Environmental Risk Factors in Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is a progressive, neurodegenerative disease and the most common form of dementia. It is characterized by a decline of memory, language, problem-solving and other cognitive skills that affect individual ability to perform everyday activities.

The two main neuropathological hallmarks of AD are extracellular deposits of amyloid-*beta* (Aβ) in diffuse plaques and plaques containing elements of degenerating neurons (neuritic plaques), and neurofibrillary tangles (NTFs) consisting of hyperphosphorylated tau protein accumulation inside neurons. Activation of microglia, astrocytes, loss of neurons and synapses are also widespread in AD brains.

Several genes, environmental factors and interactions among genes and these components may be involved in AD occurrence and progression.

AD is a complex multi-factorial, heterogeneous disease and among environmental factors likely associated with it, persistent virus infections, the progressive decline of immune competence with advancing age and chronic psychological stress exposures might play a pivotal role.

Infective agents, both bacteria and viruses, have been suggested to play a role in the clinical progression of AD. Impaired immune responses are associated with ageing and decreased defensive immunity against peripheral persistent bacterial infections and/or latent and reactivating viruses might expose the brain to an increased risk of microorganism invasion and chronic infections. These alterations may contribute to impair brain inflammatory responses leading to neurodegenerative processes and dementia. However, the role of brain innate immunity in the pathogenesis and clinical history of AD remains to be clarified.

The present study focused on genes involved in antimicrobial defences, especially against virus infections, such as interferon regulatory factor (IRF) 7, mediator complex

(MED) 23, interferon (IFN)-λ3, or IL28B (interleukin 28B), and IFN-α genes. We attempted to evaluate the potential association of their expression in hippocampus and in temporal cortex AD brain samples. Since our recent findings showed that diverse genetic backgrounds in genes regulating antiviral responses were associated with an increased risk of AD, the focus of this thesis was set on single nucleotide polymorphisms (SNPs) located upstream of the IRF7, MED23 and IL28B genes and on SNPs of APOE gene, and their potential effect on the brain gene expression profiles.

Most AD patients showed an impaired brain expression of these major antiviral response genes. Carriers of the APOE ε4 allele showed a significant decrease of MED23, IL28B and IFN-α gene expression in hippocampus area. Moreover, the presence of GA and AA genotype in the upstream IRF7 variant (*rs*6598008) was associated with further decreased hippocampal expression of IRF7, MED23, IL28B and IFN-α, which are all involved in the innate immune control of HSV-1 infection.

The present findings suggest that brains from AD patients have defective antimicrobial immune defences and individual genetic makeup, such as positivity for the APOE ε4 and IRF7 A alleles, further decreases brain immune efficiency. A decreased brain immune efficiency may increase the susceptibility to chronic infections during ageing, which in turn may activate a vicious neuroinflammatory circle leading to neuronal death, neurodegeneration and clinical dementia. Maintenance of efficient immune responses in the elderly might slowdown neurodegenerative mechanisms associated with the age-related cognitive decline and influence both prevalence and incidence of AD.

While most cases of AD occur at late onset and older ages, increasing evidences support the notion that the neurodegenerative alterations precede AD clinical manifestation by many decades. In addition to genetics and environmental factor, several epidemiological reports have indicated that chronic stress and stress-related disorders can influence the progression of AD-related symptoms and pathologies.

Different studies have shown that chronic stress has a strong impact on different cognitive processes ranging from attention to memory and social cognition. Moreover, chronic stress at different stages of life, including intrauterine life, has a negative impact on AD pathology. The early-life environment is one of the most important factors affecting life-long health and prenatal stress (PNS) is an important programming factor in brain development and function.

The second part of this thesis shows that in mouse model, environmental factors, such as PNS exposure, can be controlled and AD-related behaviour can be monitored throughout the disease progression. It has been investigated the long-term cognitive consequences of PNS in AD mice and the PNS-early neurobiological effects in wild type (WT) animals. As these, mice are a useful model to suggest that PNS affects the onset of cognitive deficit in AD mice in a sex-dependent manner. Furthermore, our findings highlight that the impairment of fetal neurodevelopment might influence adult mental health and brain ageing. Based on these results, AD is probably best considered in a life-course framework, with important influences beginning also at early-life moments.

Introduction

An Introductory Overview of Alzheimer's Disease

Epidemiology

According to the World Alzheimer Report 2015 it has been estimated that the number of people living with dementia will almost double every 20 years, reaching 74.7 million in 2030 and 131.5 million in 2050 (https://www.alz.co.uk/research/world-report-2015).
The regional estimates of dementia prevalence in people aged 60 years and over range from 4.6% in Central Europe to 8.7% in North Africa and the Middle East, though all other regional estimates fall between 5.6% and 7.6%. The regional distribution of new dementia cases is 4.9 million (49% of the total) in Asia, 2.5 million (25%) in Europe, 1.7 million (18%) in the Americas and 0.8 million (8%) in Africa. The incidence of dementia increases exponentially with increasing age, from 3.9 per 1000 person-years at age 60-64, to 104.8 per 1000 person-years at age 90 and over.
Today 47 million people live with dementia worldwide. There is a clear and urgent need to improve the coverage of healthcare around the world, for people living with dementia now and those who will be in the future. Through cost modelling, the World Alzheimer Report 2016 shows that these improvements are affordable and achievable, but governments and societies need to effect transformative change to deliver them (https://www.alz.co.uk/research/world-report-2016).

Alzheimer's disease (AD)

AD was first identified more than 100 years ago, but 70 years passed before it was recognized as the most common cause of dementia[1]. Researchers believe that early detection will be key of preventing, slowing and stopping Alzheimer's disease. Nowadays, there are no definitive diagnostic tests or validated biological markers for this disease[2]. Revised criteria and guidelines for diagnosing Alzheimer's were

proposed and published in 2011[3-6]. Because scientific evaluation of the proposed criteria is ongoing, we refers to AD as defined by the earlier criteria[7].

AD symptoms

The AD common symptoms are:

memory loss that disrupts daily life; challenges in planning or solving problems; difficulty completing familiar tasks at home, at work or at leisure; confusion with time or place; trouble understanding visual images and spatial relationships; new problems with words in speaking or writing; misplacing things and losing the ability to retrace steps; decreased or poor judgment; withdrawal from work or social activities; changes in mood and personality, including apathy and depression.

Brain changes AD-related

The two primary neuropathological hallmarks of AD are amyloid-*beta* (Aβ) plaques and neurofibrillary tangles (NTFs): the first ones consist of extracellular deposits of Aβ, which is derived from the β-amyloid precursor protein (APP), in diffuse plaques and plaques containing elements of degenerating neurons (neuritic plaques)[2], while the second ones are primary composed of hyperphosphorylated tau protein accumulation inside neuronal cells[8]. Activation of microglia, astrocytes and loss of neurons and synapses is also widespread[9]. The fundamental pathogenic mechanisms underlying these changes are still under debate, but it is clear that environmental and genetic components play a pivotal role[2].

In AD, information transfer at synapses begins to fail, the number of synapses declines, and neurons eventually die. The accumulation of Aβ is believed to interfere with the neuron-to-neuron communication at synapses and to contribute to cell death. NTFs block the transport of nutrients and other essential molecules inside neurons and are also believed to contribute to cell death. The brains of people with advanced AD show dramatic shrinkage from cell loss and widespread debris generated by dead and dying neurons[10].

AD and genetic mutations

A small percentage of AD cases (an estimated 3% or less)[11] develops as a result of mutations to any of three specific genes. These mutations involve the amyloid precursor protein (APP) gene, presenilin 1 (PSEN1) and presenilin 2 (PSEN2) genes. People inheriting a mutation to the APP or PSEN1 genes are guaranteed to develop Alzheimer's. Those inheriting a mutation in the PSEN2 gene have a 95% chance of developing the disease[12]. Individuals with mutations in any of these three genes tend to develop AD symptoms before age 65, sometimes as early as age 30. On the other hand, the majority of individuals with sporadic AD shows a late-onset disease, occurring at age 65 or later.

Known AD risk factors

With the exception of the rare cases of Alzheimer's caused by genetic mutations, experts believe that AD develops as a result of multiple factors rather than a single cause like other common chronic degenerative diseases. Researchers have outlined several factors that affect risk of developing dementia.

- Age

Age is the major risk factor for AD. Most patients develop AD after 65 years or at older ages. AD is not a normal part of ageing and age alone is not sufficient to cause the disease[10].

- ApolipoproteinE (APOE) ε4 allele

ApolipoproteinE (ApoE) protein is the major cholesterol carrier in the brain. ApoE is involved in neuronal maintenance and repair. This protein binds several receptors on the cell surface, which are involved in lipid delivery and transport, glucose metabolism, neuronal signaling and mitochondrial function. Normally, ApoE also binds Aβ peptide and plays a role in its clearance[13].

There are three main polymorphisms of APOE gene, called APOE ε2, ε3 and ε4 alleles.

The ε3 is the most common; the ε4 is carried by an estimated 20-30% of individuals, while the ε2 form is carried by an estimated 10-20% of the population[12,14]. Allele ε4 positivity increases AD risk, while having the ε2 allele may decrease the disease's risk. Those who inherit one copy of the ε4 allele have a 3-fold higher risk of developing AD than those without it, while individuals who inherit two copies of the ε4 form have an 8- to 12-fold increased disease's risk[15,16]. In addition, subjects with the ε4 allele are more likely to develop AD at a younger age than those with the ε2 or ε3 alleles[17]. Unlike inheriting a genetic mutation that causes Alzheimer's, inheriting the ε4 form of the APOE gene does not guarantee that an individual will develop AD.

- Family History

Subjects in which multiple members of the family are affected by AD are at increased risk for dementia, but the distribution of secondary cases is not consistent with Mendelian inheritance. AD is more frequent among monozygotic than dizygotic twins and first-degree relatives of patients with AD have approximately twice the expected lifetime risk of developing the disease[18-21]. Individuals who have a parent, brother or sister with AD are more likely to develop the disease than those who do not have a first-degree relative with Alzheimer's[16,22]. However, when a disease runs in families, heredity along with shared environmental and lifestyle factors, play a role[10].

- Cardiovascular Disease Risk Factors

Growing evidence suggests that the brain health is linked to the heart and blood vessel health. Many risk factors of cardiovascular disease are also associated with an increased risk of dementia. These factors include smoking[23-25], obesity in midlife[26,27] and diabetes[24,28-31]. Growing evidence also implicates midlife hypertension[27,32] and midlife high cholesterol[33,34] as risk factors. Conversely, factors that protect the heart (e.g. physical activity, a diet low in saturated fats and rich in vegetables and fruits[29,35-37]) are also protective for the brain and reduce the risk of developing AD and other dementias.

- Social and Cognitive Engagement

Additional studies suggest that social and mental activity throughout life may reduce the risk of AD and other dementias[38-44]. Remaining socially and mentally active may help build cognitive reserve; however, underlying mechanisms are still unknown[10].

- Traumatic Brain Injury (TBI)

TBI is a mechanical insult to the brain caused by external forces and associated with inflammation and oxidative stress[45]. Moderate TBI increases twice the risk of developing AD and other dementias, and severe TBI increases 4.5 times the AD risk[46,47]. Individuals who have experienced repeated head injuries, such as boxers, football players and combat veterans, are at increased risk of developing dementia, cognitive impairment and neurodegenerative diseases[48-54]. Some of these neurodegenerative diseases, such as chronic traumatic encephalopathy, can only be distinguished from AD upon post-mortem brain autopsy.

AD and Genetics

Early Onset Alzheimer's Disease (EOAD)

AD is a complex and heterogeneous neurodegenerative disease. Several genes, environmental factors and interactions among genes and these components may be involved in AD occurrence and progression[55]. Studies have been performed on mono- and di- zygotic twins to estimate the role of genetics in AD, the environmental influences and the disease heritability. Variation in the age of onset, neuropathological patterns and the disease duration may be ascribed to genetic–environmental interactions[56-58].

AD can be categorized into early (age at onset <65 years) and late onset; among early onset families, patterns of AD inheritance have been consistent with Mendelian autosomal dominant inheritance. Studies from late onset familial cases suggested a multifactorial inheritance, involving both genetic and non-genetic factors[59-61].

Three mutations are considered main risk factors for EOAD: these mutations are on amyloid precursor protein (APP)[62], presenilin 1 (PSEN1) and 2 (PSEN2)[63-65] genes.

Mutations in APP gene appear to cause shifts in its proteolytic cleavage toward amyloidogenic pathways, leading to accumulation of the Aβ42 isoform which is less soluble and more neurotoxic[66] than the common Aβ40[67] isoform.

PSEN1 and PSEN2 share 67% homology; both genes contain twelve exons with ten coding exons (exons 3–12), for a ~450 amino acids protein. PSEN1 and PSEN2 are transmembrane (TM) proteins with, at least, seven TM domains[68]. Most AD mutations have been detected in PSEN1 gene (approximately 30%–70% of familial EOAD), located on chromosome 14[69]. Patients with PSEN1 mutations might develop AD symptoms in their 40s or early 50s, with a few cases occurring in individuals in their late 30s and early 60s[70]. PSEN2, on chromosome 1, is another EOAD gene, among a

very small European population. The most well-known AD group with the PSEN2 mutations is from families with the Volga German ancestry. AD patients with PSEN2 mutations may show disease onset between 40 and 75 years[71,72].

PSEN mutations contribute to a partial loss of function in the γ-secretase complex, which affects APP processing and increases the brain vulnerability to Aβ toxicity[73]. However, the roles of presenilin mutations continue to be investigated[74].

Late Onset Alzheimer's Disease (LOAD)

LOAD comprises the majority (90–95% or more) of AD cases and there is considerable evidence to support a genetic component in its etiology[59]. Identifying genetic contributors to LOAD has posed great challenges; while EOAD is characterized by highly penetrant mutations in a few risk genes, LOAD is likely caused by multiple low penetrance genetic variants[74].

The first confirmed LOAD susceptibility gene was the APOE gene, located on chromosome 19 and coding for ApoE protein, which is involved in the transport, storage and metabolism of lipids[74].

To identify other genomic contributors to AD risk, a variety of approaches have been used: regional and genome-wide linkage studies (GWLS) in multiplex pedigrees, candidate gene association studies, meta-analyses of linkage and association studies, genome-wide association (GWA) studies and, most recently, whole genome sequencing (WGS) and whole exome sequencing (WES) studies[74].

The first two sets of large-scale GWA studies identified CLU (clusterin), PICALM (phosphatidylinositol-binding clathrin assembly protein), CR1 (complement receptor 1), BIN1 (bridging integrator 1), MS4A4A (membrane spanning 4-domains A4A), ABCA7 (ATP-binding cassette sub-family A member 7), CD2AP (CD2 associated protein), CD33 and EPHA1 (Ephrin type-A receptor 1 precursor) as AD susceptibility loci[75-78].

The largest GWA study to date was performed by the International Genomics of Alzheimer's Project (IGAP)[2]. This consisted of a large, two-stage meta-analysis of the major GWA studies (GWAS) of individuals of European ancestry that included a total of 74,046 subjects. In the first stage, 7,055,881 single nucleotide polymorphisms (SNPs), genotyped or imputed in at least 40% of AD cases and 40% of control (ctrl) samples, were used to perform a fixed-effects inverse variance-weighted meta-analysis on four previously published GWAS data sets, consisting a total of 17,008 AD cases and 37,154 ctrl. In total, 11,632 SNPs associated with AD risk, exhibiting a p-value of $<1 \times 10^{-3}$, were genotyped and tested for association in the second stage, in an independent set of 8,572 AD cases and 11,312 ctrl[2]. Additionally to the APOE locus, nineteen loci reached genome-wide significance, defined as $p < 5 \times 10^{-8}$, in the combined stage 1 and 2 analysis [CR1, BIN1, CD2AP, EPHA1, CLU, MS4A6A (membrane spanning 4-domains A6A), PICALM, ABCA7, HLA-DRB5/HLA-DRB1(major histocompatibility complex, Class II, DR beta 5/1), PTK2B (protein tyrosine kinase 2 beta), SORL1 (sortilin related receptor 1), SLC24A4/RIN3 (Solute Carrier Family 24 - Sodium/Potassium/Calcium Exchanger - Member 4/Ras and Rab Interactor 3), INPP5D (inositol polyphosphate-5-phosphatase D), MEF2C (myocyte enhancer factor 2C), NME8 (NME/NM23 family member 8), ZCWPW1 (zinc finger CW-type and PWWP domain containing 1), CELF1 (CUGBP Elav-like family member 1), FERMT2 (fermitin family member 2) and CASS4 (Cas scaffolding protein family member 4)]. Out of these, eleven (HLA-DRB5/HLA-DRB1, PTK2B, SORL1, SLC24A4/RIN3, INPP5D, MEF2C, NME8, ZCWPW1, CELF1, FERMT2 and CASS4) were new association risk factors.

The odds ratio (OR) ranged from 1.08 to 1.29; examining the genetic effect attributable to all the associated loci, the most strongly associated SNPs at each locus, other than APOE, had population-attributable fractions or preventive fractions between 1.0 and 8.0% in the stage 2 sample. The cumulative population attributable fraction was 89.4%[2]. In summary, this meta-analysis of major AD GWAS identified eleven new susceptibility loci. These new loci underline the significance of specific pathways

already shown to be enriched for association signal in AD GWAS, such as immune response and inflammation (HLA-DRB5/DRB1, INPP5D, MEF2C), cell migration (PTK2B), lipid transport and endocytosis (SORL1), and reinforced the relevance of some additional previously suggested pathways including APP (SORL1 and CASS4) and tau (CASS4 and FERMT2) pathology, hippocampal synaptic function (MEF2C and PTK2B), cytoskeletal function and axonal transport (CELF1, NME8, CASS4), regulation of gene expression and post-translational modification of proteins, microglial and myeloid cell function (INPPD5)[2].

Exome and genome sequencing approaches have recently yielded novel insights into the genetic contributors to AD. Rare loss-of-function mutations R47H and R62H in the chromosome 6p21.1 gene TREM2 (triggering receptor expressed on myeloid cells 2) have recently been found to lead to an increased AD risk of as much as 400%[79-82]. TREM2 implicates innate immunity pathways[83], the regulation of phagocytic pathways and it has also been found to inhibit microglia cytokine production and secretion reducing inflammatory response[84]. The identification of this gene has strengthened the growing consensus that microglia induced neuroinflammation is a critical component of AD pathogenesis[74]. A whole-genome sequencing study examining genotypes of 1,795 Icelanders found a coding mutation in the APP gene (A673T) that protects against AD and cognitive decline in the elderly without Alzheimer's[85]. SORL1, which was first an AD candidate gene[86] and then observed in the IGAP GWAS, was recently found through exome sequencing to carry mutations causing a form of EOAD with autosomal dominant inheritance[87]. Furthermore, exome sequencing of a Turkish family with AD identified a mutation (R1231C) in NOTCH3 (notch homolog 3 (Drosophila)) gene [81], which has previously been implicated in a subtype of vascular dementia, the CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy).

The next generation of AD rare variant studies is currently in development. The upcoming Alzheimer's Disease Sequencing Project (ADSP; https://www.niagads.org/adsp/content/home) is a joint project by the National Institute on Aging and National Human Genome Research Institute to identify novel risk and protective variants for AD in nearly 600 whole genomes from 110 multiplex pedigrees heavily burdened with AD, and approximately 10,500 whole exomes on unrelated AD cases and non-demented ctrl. This kind of studies promise to characterize the yet unknown heritable component in Alzheimer's susceptibility, that has been defined as the "dark matter" of the AD genetics[74].

In previous works of Licastro's team[88,89], genetic data from four AD GWAS[75-77,90] were discussed and it was suggested that the above genes[75-77,90] might be linked to herpes viral infections. In particular, it has been argued that the association of these genes with AD is suggestive of a pivotal role of environmental factors in the pathogenesis of the disease and, according to a non conventional interpretation of AD etiology, virus infection[88,89] is one AD etiologic component. In other words, the genetic signature revealed by GWA studies discloses a network of genes that might influence the ability of the central nervous system (CNS) to cope with and fight against the invasion by virus of the herpes family[88,89].

In spite of the great number of AD patients and ctrl subjects from GWA studies, each SNP alone showed a low OR for the disease. Potential interactions between several SNPs in different genes could be more illuminating to understand the etiopathogenesis of this multi-factorial neurodegenerative disease, since the aforementioned genes are involved in different aspects of AD pathogenesis and clinical history. On the other hand, environmental AD risk factors, as microbial infections, might trigger several of these genes, depending on the presence and/or the absence of the above interacting SNPs, and influence individual immune responses. Therefore, it has been suggested

that virus belonging to the herpes family might be one of the missing link between these genetic variants and the individual AD clinical history.

AD and Inflammation

The modulation of the neurodegenerative disease course by specific immune molecules in preclinical experimental approaches and the up-regulation of inflammatory genes in arrays on tissues derived from patients with neurodegenerative diseases are indicative of a relationship between inflammation and these diseases and implicate early immune actions in their pathogenesis[91-95].

Classically AD has been viewed as a neurodegenerative disease of the elderly, characterized by the extracellular deposition of misfolded Aβ peptide and by intraneuronal accumulation of NTFs. Thereafter, neuroinflammation has emerged as an important component of AD pathology[8,93]. Experimental, genetic and epidemiological data indicate a crucial role for activation of the innate immune system as a disease-promoting factor[93]. The inflammatory reaction observed in AD is primarily driven by CNS-resident immune cells, included microglia, perivascular myeloid cells and other elements such as astrocytes, and reflects the tissue reaction to pathological events that occur during the disease[8].

Microglia cells are the CNS macrophages that continuously scrutinize their environment for damage. Microglia colonize the cephalic mesenchyme during embryogenesis and actively shape the developing neuronal network by immune-mediated mechanisms. During CNS maturation, microglia drastically change phenotype and function. Adult microglia contribute to brain homeostasis, but also the establishment and resolution of inflammatory conditions[96]. Under pathological conditions, CNS environment changes and microglia respond by activation and potential redirection of their phagocytic activity from synaptic pruning to clearing dangerous factors[96-98]. Long-lasting chronic inflammation has been proposed to drive microglial physiological functions off balance[96]. Microglia are equipped to sense the so-called *danger signals* (Damage Associated Molecular Pattern, DAMP) and changes in

neuronal health by adopting a set of different morphological and functional attributes[99]. Microglia cells are the most intimately associated with tissue changes that are observed in AD[8]. In both *in vitro* and *in vivo* experiments, microglia exhibit receptor-dependent interactions[100-103] with various forms of Aβ (monomers, oligomers, protofibrils, fibrils and plaques) as well as non-receptor mediated interactions, particularly with Aβ oligomers[104]. Aβ species can stimulate changes in microglial functions through signalling receptors[105], that induce production of inflammatory mediators by other cells, such as astrocytes[106]. Aβ peptides also affect post-phagocytic processes within microglia, including lysosomal injury, which acidifies the cytosol and contributes to activating the NLRP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome[107]. Receptor-mediated interactions between microglia and Aβ monomers induce a “primed state”, characterized by heightened responses to subsequent DAMP or cytokine stimuli[8]. Some microglia function, such as motility and phagocytic activity, are impaired in APP/PS1 AD mouse model[108], by oligomeric Aβ[109]. This functionally compromised state affects the microglial cells during neurodegenerative processes and microglia become “dystrophic”; such phenotype consists of microglial *burn out*[8]. It is more appropriate to view the transformed microglia as being indicative of a loss of tissue homeostasis. Because microglia carry out critical physiological tasks in the healthy brain[110,111], the phenotypically transformed microglia may contribute to CNS tissue pathology[8].

According to this view, rare structural variants of genes encoding the immune receptors TREM2[80,82,112,113], CD33[77,114] and CR1[115], all of which are expressed on microglia, have been found associated with AD risk. TREM2 is involved in regulating microglial phagocytosis[116,117] and has also a relevant impact *in vivo* in different AD mouse models, by promoting the survival of activated microglia and their peripherally derived myeloid counterparts[118,119] and by recruiting these cells around Aβ plaques[118-120] and dying neuronal cells[118]. CD33 variants have also been associated with an increased

risk of developing AD[77,114]. All these findings support the concept of impaired microglial function in AD.

During ageing, number and density of microglia increase significantly and the regularity of the microglia distribution also appears to deteriorate[121]. Aged microglia undergo changes in their ramified morphology and decreased dynamic motility[121]. Multiple lines of evidence have indicated that microglia in the aged CNS show increased basal states of activation and increased expression levels of inflammatory cytochine[121]. It has also been suggested that the ageing of microglia might be partially driven by a cumulative history of environmental influences such as systemic infections over a life-time[121]. Therefore, microglial cells in AD brains might be primed by infectious agents challenging the CNS and/or by temporary permeabilization of selected districts of blood brain barrier (BBB) induced by the persistence of peripheral subclinical inflammatory responses[122].

AD and Environmental Risk Factors

Health and disease in the brain are influenced by multiple factors. These factors are genetic or environmental. In sporadic AD, different genetic and environmental factors might interact.

Over the past decade, epidemiological surveys have identified several risk factors for AD, including ageing, systemic infections, inflammation, midlife obesity, brain trauma, chronic periodontitis, toxic chemical exposures, reduced physical activity and chronic psychological stress exposures[93,123-129]. Interestingly, most of these risk factors involve the activation of innate immunity. Experimental evidence suggests that systemic inflammation exerts detrimental effects on brain functions[93]. Post-mortem analysis of brains from patients who had suffered septicemia has shown a distinct increase in microglial activation[130]. Those data are complemented by findings showing persistent cognitive alterations, hippocampal atrophy and electroencephalograph changes in patients who survive sepsis[131]. Brain trauma is also characterized by a strong neuroinflammatory reaction[45,93]. Moreover, trauma-induced activation of microglia has been shown to increase the Aβ deposition in an AD mouse model[132] and underlying mechanisms might include cytokine release and impaired microglial clearance[93].

Infections

An infection/immune component might play a role in AD etiopathogenesis. There are several studies, mainly on humans, implicating specific microbes in the AD etiology, notably herpes simplex virus type 1 (HSV-1), *Chlamydia pneumonia*, *Helicobacter pylori* and several types of spirochetes[133-137]. The presence of fungal infection in the brain of AD patients has also been observed[138,139]. On the other hand, plasma concentrations of TNF and antibodies to periodontal bacteria were found to be higher in

AD patients compared to non-demented ctrl subjects and were independently associated with AD[140,141].

Virus of Herpes Family and Dementia

Herpes simplex virus type 1 (HSV-1) and AD

The first observations of HSV-1 in AD brain were reported in 1991[142]. The increasing number of these studies warrants re-evaluation of the infection and AD concept [143]. As previously described[88,89], Licastro's group discussed genetic data from four GWAS on AD[75-77,90]. From these investigations a set of SNPs associated with AD emerged and it has been hypothesised that the concomitant presence of these SNPs might result in a genetic signature predisposing to AD, via complex and diverse mechanisms each contributing to an increased individual susceptibility to herpes virus infection[88,89].

An AD viral etiology, especially involving herpes virus, has been already proposed and most works have shown an association between HSV-1 and AD[144-148].

HSV-1 is a ubiquitous virus that infects more than 80% of over 65-year-old individuals worldwide[149]. HSV-1 is a neurotropic double-stranded DNA virus that primarily infects epithelial cells of oral and nasal mucosa. Here, the virus undergoes lytic replication. The newly produced viral particles may enter sensory neurons and, by axonal transport, reach the trigeminal ganglion where usually establish a latent infection. The virus undergoes periodic reactivation cycles, in which the newly formed viral particles are transported back to the site of primary infection through the sensory neurons, causing the typical clinical lesions, commonly referred to as cold sores. However, the bipolar trigeminal ganglion neurons also project to the trigeminal nuclei located in the brainstem. From here, neurons project to the thalamus to finally reach the sensory cortex. This represents the path through which the reactivated virus may reach the CNS, where it may cause acute neurological disorders like encephalitis (Herpes Simplex Encephalitis, HSE), mild and clinically asymptomatic infection or establish life-

long latent infection[150,151]. Recent findings showed a significant association of HSV-1 infection with AD risk[150]. A reactivation of HSV-1 infection, assessed by increased serum levels of specific anti-HSV-1 antibodies, was found associated with an increased AD risk in a longitudinal study on 3,432 Swedish elderly[150]. Another study from Italy reported that elevated serum HSV-1 antibody titers correlated with reduced cortical grey matter volume as assessed by MRI[152].

Itzhaky and colleagues in their recent editorial[143] discussed relevant evidences in favour of an HSV-1 infectious component in AD etiopathogenesis. HSE causes damage in localized CNS regions related to the limbic system[153], the same regions as those affected in AD[143]. In brain of AD patients, the presence of HSV-1 DNA specifically colocalize with AD pathology[148]. In mice and in cell cultures, Aβ and NTFs were observed after HSV-1 infection[143] and a direct interaction between APP and HSV-1 has been reported[154]. Antiviral drugs, including acyclovir, *in vitro* block HSV-1-induced Aβ and tau pathology[155]. Olfactory dysfunction is an early symptom of AD and the olfactory nerve, the initial site from where characteristic AD pathology subsequently spreads through the brain, is another potential portal of entry of HSV[143]. Further, brainstem areas that harbour latent HSV directly project the brain regions involved in AD pathology: brainstem virus reactivation would thus disrupt same tissues as those affected in AD[143].

Moreover, others herpes viruses share the ability to become latent in the infected host and eventually latently infect neurons[149], but investigations focused on herpes viruses such as human cytomegalovirus (CMV), Epstein-Barr virus (EBV) or human herpes virus 6 (HHV-6) in AD are scarce.

Cytomegalovirus (CMV) and AD

CMV is ubiquitously distributed in human population and the most frequent cause of brain infection in immune compromised patients or in infants with congenital virus transmission[156,157]. Postnatal acute peripheral CMV infection is usually asymptomatic, however, the virus, once established, remains latent in blood monocytes[158,159]. Several studies described CMV as a potential risk factor for AD[122]. For instance, an increased rate of cognitive decline over a four year period in subjects with elevated CMV antibody levels has been reported[160]. However, results regarding this topic have been conflicting[122]. Lin and colleagues, analysing brain frontal and temporal cortex samples, found that both AD patients and elderly healthy subjects were positive for CMV, with no statistically significant difference between the two groups[161]. On the other hand, brain positivity for CMV was found in a greater proportion of patients with vascular dementia than normal elderly and these findings suggested a virus role in the disease[162]. CMV was also present in cerebrospinal fluid (CSF) of subjects with encephalitis or meningitis or other neurological condition[163]. A recent investigation reported increased CMV antibody levels in the elderly who developed clinical AD during a five-year follow-up[164]. Furthermore, findings from a longitudinal follow-up of 849 participants in the USA showed that CMV infection doubled the risk of developing AD[165], even if some criticisms to this type of data have been presented[166].

Epstein-Barr virus (EBV) and AD

EBV infects more than 95% of human beings within the first years of life. The virus is the etiological agent of acute infectious mononucleosis in a minority of immune competent subjects, while the majority develops a life-long asymptomatic infection. EBV remains latent in B lymphocytes[149,167,168]. Data describing an association between EBV and AD are very limited; only two papers from 1992 described a possible correlation between EBV and AD, however, with discordant results[169,170]. Recently, our

findings showed a positive association of peripheral blood positivity for EBV genome and AD, and elevated levels of EBV specific antibodies positively associated with an increased risk of developing AD[164].

Human herpes virus (HHV)-6 and AD

HHV-6 is a neurotropic virus, present in two different variants[171], with a very high sero-prevalence, involving almost 100% of population by the age 3[172]. HHV-6 establishes latency in the brain and may reactivate under conditions of immunosuppression[171]. It has been associated with multiple neurological diseases including seizures, encephalitis, mesial temporal lobe epilepsy and multiple sclerosis[173]. HHV-6 has been found in a higher proportion of AD than age-matched ctrl brains[161]. Recently, Agostini and colleagues showed no difference in serum HHV-6 IgG antibody titers and the avidity index between AD patients, MCI and ctrl individuals[174]. Another study showed an higher value of HHV-6 levels in ctrl brains[175]. On the other hand, our findings showed an elevated positivity for HHV-6 genome in the brains and in peripheral blood cells of AD patients and an increased sero-positivity associated with increased risk of developing AD[164].

In summary, research in epidemiology, neuropathology, molecular biology and genetics, regarding the hypothesis that pathogens interact with susceptibility genes and contribute to sporadic AD, is conspicuous but underestimate[135]. Sporadic AD is a complex multi-factorial neurodegenerative disease with evidence indicating coexisting multi-pathogen and inflammatory etiologies[135]. Pathogens like those aforementioned are able to evade destruction by the host immune system, leading to persistent infection. Viral DNA, but also viral RNA and/or bacterial ligands, increase the expression of proinflammatory molecules and activate the innate and adaptive immune systems. Chronic brain infections like those caused by HSV-1, *Chlamydophila pneumonia* and spirochetes result in complex processes inducing uncontrolled

neuroinflammation and neurodegeneration. Infections such as those caused by CMV, *Helicobacter pylory* and periodontal pathogens induce production of systemic proinflammatory cytokines that may cross the BBB to promote neurodegeneration. Moreover, pathogen-induced inflammation and CNS accumulation of Aβ damage the BBB, which in turn contributes to the pathophysiology of AD[135].

Antiviral Defence and The New Interferon Lambda (IFN-λ) Family

Interferon lambda (IFN-λ), also called type III IFN or IFNL, represents the most recently described family of IFNs and plays pivotal roles in host-pathogen interactions[176].
The IFN-λ family comprises four homologous members: IFNL1 (Interleukin IL29), IFNL2 (IL28A), IFNL3 (IL28B) and the more recently described IFNL4[176]. They belong to a cytokine family that shares functional similarities with the family of type I IFNs (IFN-α/β)[177]. Genes encoding type I IFNs characteristically lack introns (with the exception of IFNK) and are located in a single locus on human chromosome 9 and mouse chromosome 4. Genes encoding IFN-λ are located on human chromosome 19 and mouse chromosome 7 and share the 5-exon gene structure characteristic of IL-10 (Interleukin 10) cytokine family members[178].

IFN-λ biology partially differs from IFN- α/β biology. First, the effects of IFN-λ are most evident on epithelial cells, suggesting that it contributes to the specialized immune mechanisms that protect epithelial surfaces, which are constantly exposed to commensal and pathogenic microbes[179-181]. Second, because of the more focused nature of its signalling effects, IFN-λ might share the therapeutic benefits of IFN-α/β without side effects that have limited the clinical use of IFN-α/β[180,182-184]. Third, GWA studies have reported several IFN-λ SNPs that are linked to clearance of hepatitis C virus (HCV) infection and possibly improved outcomes with other viral infections, including hepatitis B virus (HBV), CMV and HSV-1[176,185-187]. Although the mechanisms by which these SNPs affect IFN-λ production and activity remain unclear, these

associations reinforce the notion that IFN-λ contributes to the control of chronic viral infections in humans[177].

Stimuli inducing expression of IFN-λ encoding genes are similar to those inducing expression of IFN-α/β genes. As would be expected from an antiviral cytokine, IFN-λ can be induced by a wide range of viruses in different cell types[179,188,189]. IFN-λ is expressed in a variety of primary human cell types of the hematopoietic lineage, that also produce type I IFNs in abundance[190]. Among non hematopoietic cells, epithelial cells are potent producers of IFN-λ[190]. The induction of IFNs is mediated by pattern-recognition receptors (PRRs) that recognize the invading virus[177] and the set of PRRs and transcription factors expressed by a cell contributes to its specific capabilities and magnitude of the IFN release following infection[191]. Early studies have shown that IFN-λ genes have binding sites for the transcription factors NF-κB, IRF3, IRF7 and AP-1 in their promoter regions[192-194] and can therefore be coexpressed with type I IFNs. Additionally, it was suggested that IFN-λ2 and –λ3 are more dependent on IRF7 and seem to have delayed expression kinetics[190]. Human hepatocytes infected with HCV or treated with polyinosinic-polycytidylic acid (polyI:C), released IFN-λ2 and –λ3 by induction of IRF3 and IRF7, whereas the induction of IFN-λ1 was also dependent on NF-κB[195]. Finally, another group has identified Med23, a subunit of the mediator complex, as a direct interaction partner for IRF7, showing how Med23 and IRF7 synergistically increase IFN-λ transcription and inhibit HSV-1 replication[196].

The proximal signaling events and downstream transcriptional responses are similar between IFN-α/β and IFN-λ, even though these cytokines and their receptors are structurally and genetically distinct. The IFN-λ structure resembles that of members of IL-10 family, although the primary amino acid sequence of IFN-λ is more similar to that of IFN-α/β[177]. Whereas all type I IFNs signal through a shared heterodimeric receptor, IFNAR (IFNAR1 and IFNAR2), IFN-λs bind to IFNLR, a unique heterodimeric receptor. IFNLR is formed by one subunit shared with other IL-10 family cytokines (IL10Rb) and

one subunit specific for IFN-λ (IFNLR1, also called IL28Ra). Although IFNAR and IL10Rb are expressed broadly on many cell types and tissues, IFNLR1 is expressed mainly on epithelial cells[181,197]. Consistent with this pattern, the antiviral effects of IFN-λ are more evident against pathogens targeting epithelial tissues[177].

Although epithelial cells produce IFN-λ, myeloid-lineage cells are major sources of the cytokine in response to double-stranded RNA (polyI:C) or viral infections[198]. In the small intestine, epithelial and immune cells both respond to polyI:C stimulation, suggesting that multiple cell types produce IFN-λ cooperatively[181]. Whereas plasmacytoid dendritic cells (DCs) produce nearly all IFNs, including IFN-λ, monocytes and myeloid DCs more selectively express IFN-β, IFN-λ1 and IFN-λ2[191].

A recent study on West Nile virus (WNV) infection demonstrated an antiviral effect of IFN-λ in correspondence to the BBB[199]. Ifnlr1$^{-/-}$ mice exhibited increased BBB permeability after WNV infection and sustained higher viral titers in CNS tissues. Although endothelial cells, including those composing the BBB, did not express high levels of IFNLR, administration of exogenous IFN-λ tightened the BBB, restricted viral neuroinvasion, reduced viral titers in the CNS and protected mice from lethal viral infection. These observations are consistent with those of a study demonstrating that IFN-α/β can also exert a tightening effect on the BBB[200]. Ultimately, our recent findings showed that SNPs in genes regulating antiviral responses such as IFN-λ, IRF7 and MED23 are differently distributed in AD and influence a differential positivity to EBV and HHV-6 DNA in the elderly [201]. Moreover, risk alleles were increased in elderly progressing to AD [201]. These findings support the hypothesis that individual genetic background may play a role in the progression of cognitive impairment by influencing the efficiency of immune responses to persistent viruses.

Prenatal Stress (PNS)

Stress is ubiquitous in our daily lives and has a strong impact on different cognitive processes ranging from attention to memory and social cognition[202-204].

Epidemiological data have indicated that environmental factors, such as chronic stress exposures and stress-related disorders, can impact on the progression of AD-related symptoms and pathologies[205-207]. Recently, a meta-analysis of 20 studies found that a history of depression approximately doubled the risk for the later development of AD[206]. Similarly, recent prospective cohort studies found that the tendency to experience psychological distress was associated with a 10-fold increased risk of episodic memory decline[128] and a 2.7-fold increased risk of developing AD[127].

One key mechanism linking stress with increased AD risk and cognitive decline is the hypothalamic pituitary adrenal (HPA) axis, also known as stress-response axis. Stress triggers the activation of the HPA axis, culminating in the production of glucocorticoids (GCs) by the adrenal gland. Receptors for these steroids are expressed throughout the brain and they can act as transcription factors regulating gene expression[208]. Thus, glucocorticoids potentially have long-lasting effects on the functioning of the brain regions that regulate their release[208] such as the limbic system, hypothalamus and cortex[209].

Every day, parents observe the growing behavioural repertoires of their infants and young children, and the corresponding changes in cognitive and emotional functions. These changes are thought to relate to normal brain development, particularly the development of the hippocampus, the amygdala, the frontal lobes and of the complex circuitry that connects these brain regions. At the other end of the age spectrum, we observe changes in cognition in our grandparents and parents during ageing, which are related to both normal and pathological brain processes ageing-associated. Studies in

animals and humans have shown that during both perinatal life and old age the brain is particularly sensitive to stress, probably because CNS undergoes important changes during these life periods[208]. Furthermore, research now relates exposure to early-life stress with increased reactivity to stress and cognitive deficits in adulthood and elderly, indicating that the effects of stress at different periods of life interact[208]. Many studies have shown that stressful events during the intrauterine life exert long-lasting effects on the brain by affecting the nervous, neuroendocrine and immune systems[209-211]. Moreover, studies in humans and rodents, revealed a clear impact of early-life stress on the development of neuropsychiatric disorders such as autism, mood disorders and schizophrenia[208].

Investigating early-life stress exposure-related effects on AD progression is challenging, particularly for the epidemiological setting, because of the large and extremely variable time span between early stress exposures and clinical manifestation of AD. In addition, the number, type, and frequency of the exposures cannot be controlled, making it difficult understanding the contribution of each individual exposure to the disease. Indeed, by using animal models, environmental exposure can be strictly controlled and AD-related behaviour and neuropathology can be monitored throughout the disease progression[212]. However, the effects of prenatal stress (PNS) on the development of AD have been poorly investigated in animal models, even though it is well documented that prenatal maternal stress exposure can strongly contribute to an individual's predisposition for developing adult psychopathology[208,213,214].

Interestingly, two animal studies showed a causal link between disturbances of embryonic development by maternal stress and the risk of AD-like neuropathology[214,215]. In the first one, Krstic and colleagues provided the first evidence that a maternal immune challenge during the late-gestational time window predisposes wild type (WT) mouse offspring to develop AD-like neuropathology during ageing[215]. Animals prenatally stressed by maternal immune challenge displayed chronic elevation

of inflammatory cytokines, an increase in the levels of hippocampal APP and its proteolytic fragments, altered tau phosphorylation, mis-sorting to somatodendritic compartments and significant impairments in working memory in old age. If the prenatal maternal infection is followed by a second immune challenge in adulthood, the phenotype is further exacerbated and mimics AD-like neuropathologic changes[215]. Furthermore, Sierksma and colleagues explored whether the combination of genetic variations and exposure to stress during prenatal life could affect behavioural and neuropathological phenotype of APPswe/PS1dE9 (AD mouse model) offspring[214]. They studied the effects of PNS in both male and female APPswe/PS1dE offspring in terms of cognition, affect and AD-related neuropathology. They demonstrated that PNS exerts a sex-dependent effect: PNS induced spatial memory deficits and a blunted HPA axis response in male offspring. PNS improved spatial memory performance, increased depressive-like behaviour and decreased hippocampal plaque load in females[214].

PNS exposure is likely to alter AD development by modifying the effects of an individual's genetic predisposition[216,217] likely through epigenetic regulation of gene expression[218,219]. Interestingly, the prenatal period is characterized by high levels of epigenetic programming and imprinting, enabling perturbations to exert long-lasting effects. In this light, it was suggested that the origin of most diseases that are expressed later in life, such as AD, may find their origin in altered epigenetic regulation in response to environmental cues arising during critical periods of development, such as the pre- and neonatal period[220-222].

Materials and Methods

Antiviral innate immune response in the brain of Alzheimer’s disease (AD) patients

Human Brain Sampling

Post-mortem brain tissues of AD patients and age-matched non-demented controls (ctrl) were obtained from the Netherlands Brain Bank (NBB; Amsterdam, The Netherlands), which abides all rules and regulations laid down in the ethical Code of Conduct of BrainNet Europe. Patients or their next of kin gave written informed consent to NBB for brain autopsy and use of tissue and clinical information for research purposes. This study was approved by NBB scientific committee and all conditions for the transfer and the use of the material are laid down in the Material Transfer Agreement (MTA). Staging of AD pathology was evaluated according to Braak and Braak criteria for neurofibrillary tangles (NFTs)[223,224] and Thal criteria for amyloid deposition[223,224], and based on neuropathological evaluation of formalin-fixed, paraffin-embedded tissues from eighteen brain areas.

AD cases were selected based on clinical and neuropathological reports, and availability of tissues stored in liquid nitrogen. 20 μm thick frozen tissue slices of the hippocampus and temporal cortex were cut at -20°C and stored at -80°C for the following molecular analyses. The final selection was based on the availability of DNA and RNA samples of good quantity and quality.

Twenty-nine AD hippocampus brain samples, nineteen AD temporal cortex brain samples, and six hippocampus, four temporal cortex samples from ctrl cases were included in this study.

Table 1. Post-mortem AD brain samples and general data summary

ID	Age (years)	Gender	Clinical diagnosis	DD	PMI (h)	BW (gr)	Cause of death	Brain Area
05-082	78	F	MID	7	4.35	1043	Pneumonia, cachexia, decubitus	Hip; Temp
04-087	78	F	AD	10	4.50	1105	Gastrointestinal bleeding	Hip; Temp
06-037	81	M	AD	5	4.50	1253	Probable CVA and sepsis with unknown underlaying disease	Hip
03-020	95	F	MID likely mixed with AD	7	3.40	1032	Aspiration pneumonia	Hip
05-002	86	M	MID	6	3.40	1206	Metabolic disturbances, dehydration, metastasized prostate carcinoma	Hip; Temp
05-008	84	F	MID	NA	3.40	1109	Dehydration, cachexia	Hip; Temp
06-214	92	F	VD	3	3.40	1115	Bleeding gastric ulcer	Hip
07-094	73	F	AD	12	3.40	1082	Sepsis, cachexia	Hip
04-062	67	F	presenile AD	15	3.40.	945	Cachexia	Hip; Temp
05-039	93	M	AD	1	3.40	1210	NA	Hip
05-095	93	M	MID	9	3.40	1220	Cardiac decompensation caused by pneumonia	Hip
02-307	69	M	NA	NA	3.40	1241	Dehydration, cachexia	Hip
04-111	89	F	AD	11	3.40	1211	NA	Hip
05-090	84	F	AD	NA	3.40	1098	Dehydration	Hip
06-248	91	F	AD	5	3.40	1101	Dehydration, cachexia and probable CVA	Hip; Temp
05-040	93	F	AD	4	2.30	1045	Cachexia	Hip; Temp
04-185	86	F	AD	10	5.05	998	Uncontrolled anti-coagulation in combination with general deterioration after hip prosthesis	Hip
04-166	94	F	VD after CVA	6	5.05	1170	Cachexia and dehydration, decubitus	Hip
03-051	75	F	AD	9	6.00	1129	Dehydration	Hip
05-050	89	F	VD	19	4.30	1185	Pneumonia	Hip; Temp
09-198	74	M	AD	2	5.35	1380	Anaemia, hepatic dysfunction, extensive lymphoadenopathy by M. Kimura	Hip
06-132	75	F	AD	6	15.0	1230	Cardiac arrest	Hip
09-307	94	F	AD	6	8.05	1053	Cachexia	Hip
09-285	90	F	AD	6	5.40	1100	Cerebro vascular accident, advanced AD	Hip
09-019	88	F	NA	1	6.45	1170	Natural death	Hip
09-271	66	F	AD	6	6.30	1190	Acute heart failure by advanced dementia syndrome	Hip
07-036	82	F	VD	6	5.55	1225	Cardiac arrest with dehydration after MI	Hip
07-053	99	F	AD	5	3.30	1150	Airway infection	Hip
07-116	60	M	AD likely of Lewy Body type	3	6.15	1241	Cachexia and dehydration by dementia syndrome	Hip; Temp
09-010	81	F	AD	2	6.10	1295	Cachexia and dehydration by dementia syndrome	Temp
08-289	57	M	presenile AD	6	3.50	1055	Aspiration pneumonia	Temp
10-050	69	M	VD, CI	3	7.10	1173	Aspiration pneumonia	Temp
10-036	86	M	AD	6	6.15	1331	Possible cardiac arrest after gastroenteritis by AD	Temp
10-002	92	F	AD	5	3.25	1105	Probable CVA	Temp
01-032	88	F	AD	12	12.15	935	Cachexia and decubitus	Temp
01-145	85	F	MID	5	4.45	1310	Shock due to acute abdomen	Temp

Table 1. Post-mortem AD brain samples and general data summary

ID	Age (years)	Gender	Clinical diagnosis	DD	PMI (h)	BW (gr)	Cause of death	Brain Area
02-314	94	F	NA	NA	5.00	1410	Double sided pneumonia	Temp
10-030	80	M	NA	NA	4.00	1328	Unknown	Temp
01-111	85	M	AD likely combined with MID	5	4.25	1458	Sudden death (suspected heart-failure	Temp

Abbreviations: DD, Disease Duration; PMI, Post-mortem Interval; BW, Brain Weight; F, female; M, male; MID, Multi-Infarct Dementia; AD, Alzheimer's Disease; VD, Vascular Dementia; CI, Cognitive Impairment; CVA, Cerebrovascular Accident; MI, Myocardial Infarction; Hip, hippocampus; Temp, temporal cortex; NA, Not Applicable.

Table 2. Post-mortem AD brain samples and neuropathological details

ID	Pathological Diagnosis	Grade (Braak, NTFs)	Grade (Thal, Aβ)
05-082	AD	5	C
04-087	AD	5	C
06-037	AD	4	C
03-020	AD; meningioma	4	C
05-002	AD; cerebral contusion	3	A/B
05-008	AD	5	C
06-214	AD; infarction	4	C
07-094	AD	5	C
04-062	AD	6	C
05-039	AD	5	C
05-095	AD; caa; arg	4	C
02-307	AD	6	C
04-111	AD	5	C
05-090	AD; infarction	5	C
06-248	AD	4	C
05-040	AD	4	C
04-185	AD	4	C
04-166	AD; infarction	4	C
03-051	AD; caa	5	C
05-050	AD	6	C
09-198	AD	6	C
06-132	AD	5	C
09-307	AD	4	C
09-285	AD; infarction	6	C
09-019	AD	5	C
09-271	AD	5	C
07-036	AD	4	C
07-053	AD	4	C
07-116	AD	6	C
09-010	AD	5	C
08-289	AD	6	C
10-050	AD; infarction	5	B
10-036	AD	5	C
10-002	AD; ischaemia	5	C
01-032	AD	4	C
01-145	AD	5	C
02-314	AD	4	C
10-030	AD	4	C
01-111	AD	5	B/C

Abbreviations: caa, congophilic angiopathy; arg, argyriphilic grain disease.

Genomic DNA Isolation

20 μm thick frozen tissue slices of hippocampus and temporal cortex were cut at −20 °C and collected in RNAse free eppendorf vials. Genomic DNA was obtained from frozen samples and purified according to the Phenol:Chloroform:Isoamyl Alcohol (25:24:1) extraction's protocol (Sigma-Aldrich, St. Louis, MO) after overnight incubation with proteinase K 10 mg/ml (Roche, Basel, SW) and ATL buffer (Qiagen, Hilde, DE). Absorbance measurements were made on NanoDrop 1000 (Thermo Scientific, Wilmington, DE) and the ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA samples, further stored at -80°C.

RNA Isolation

20 μm thick frozen tissue slices of hippocampus and temporal cortex were cut at −20 °C, and collected in RNAse free eppendorf vials, whereafter RNA-Bee (AMSBIO, Cambridge, MA) was added.

Totally RNA extraction from frozen hemi-brain hippocampus or temporal cortex samples was performed using RNA-Bee kit (AMSBIO, Cambridge, MA) according to manufacturer's instructions. Total RNA was purified according to phenol-clorophorm standard extraction after overnight incubation with proteinase K. Absorbance measurements were made on NanoDrop 1000 (Thermo Scientific, Wilmington, DE) and the ratio of absorbance at 260 nm and 280 nm was used to assess the purity of RNA samples, further stored at -80°C.

Quantitative Reverse - Transcription Polymerase Chain Reaction

Total RNA (50 ng) was retro-transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions. PrimerPCR™ assay for real-time PCR gene expression analysis was performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Bio-Rad pre-validated primer pairs for target genes IRF7 (*qHsaCED0007783*), MED23 (*qHsaCID0007348*), IL28B (*qHsaCED0038284*), IFN-α (*qHsaCED0037471*) and for two reference genes were used. CYC1 (*qHsaCED0047348*) and EIF4A2 (*qHsaCED0023870*) were selected as reference genes for normalization as suggested by Penna and colleagues[225].

Quantitative Real-Time pcr assay (qRT-pcr) for gene expression analysis was realized in a Bio-Rad CFX96™ instrument and all reactions were run in triplicate in 96-well optical plates.

Data from qRT-pcr experiments were analyzed by relative quantification with Bio-Rad CFX Manager software. Using the $2^{-\Delta\Delta Ct}$ method[226], the gene expression data are presented as the fold change in gene expression normalized to the two reference genes (CYC1 and EIF4A2) and relative to the non-demented control (ctrl) samples.

SNPs detection

TaqMan® SNP Genotyping Assay (Applied Biosystems, Foster City, CA) was used to genotype AD patients according to the manufacturer's instructions. It included an unlabelled PCR primer pair to detect specific target SNP and two different Taqman probes that distinguished two alleles of the SNP: one probe labelled with VIC® dye and the other one labelled with 6-FAM® dye. Allelic discrimination was based on the

generated signal from each probe at the end of the real-time PCR (RT-pcr) using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA).

APOE genotyping for the ε allele (*rs*429358 and *rs*7412) from AD hippocampus and temporal cortex DNA samples was assessed by RT-pcr using Taqman® probes according to the manufacturer's instructions (table 3). The upstream variant of IRF7 (*rs*6598008 A/G), MED23 (*rs*3756784 T/G), IL28B (*rs*12979860 C/T) genes were also analyzed by RT-pcr using Taqman® probes according to the same manufacturer's instructions. Thereafter, qRT-pcr data showing relative expression ($2^{-\Delta Ct}$ values[227]) to the reference genes (CYC1 and EIF4A2) of AD patients were grouped in APOE ε4 alelle carrier/non carrier and according to SNP genotypes of IRF7 (*rs*6598008), MED23 (*rs*3756784) and IL28B (*rs*12979860).

Table 3. The APOE genetic variants

APOE genotyping for ε allele		
APOE genotype	***rs*429358**	***rs*7412**
ε2/ε2	T/T	T/T
ε2/ε3	T/T	C/T
ε2/ε4	C/T	C/T
ε3/ε3	T/T	C/C
ε3/ε4	C/T	C/C
ε4/ε4	C/C	C/C

Statistical analysis

Statistical analysis was performed using the *Statistical Package for the Social Sciences* (version 22.0; SPSS Inc, Chicago, IL) and statistical significance level was set at 0.05. After a careful exploration of the quality of the normalized data, a generalized linear model analysis (ANOVA) followed by Bonferroni *post* test or unpaired *t*-test were used to analyzed differences in gene expression data between groups.

Effects of prenatal stress (PNS) on adult cognitive health

Investigation of PNS effect on cognitive ability of wild type and APPswe/PSEN1dE9 offspring

Animals

The experiments were performed with APPswe/PSEN1dE9 (Tg) and wild type (WT) offspring mice at 6 and 9 months of age.

The generation of the mouse line expressing the human mutated forms APPswe and PSEN1dE9 has already been described[228]. Animals were maintained under standard animal housing conditions in a twelve-hour dark-light cycle with an average temperature of 22 °C, relative humidity of 42% and with food and water provided *ad libitum*. All studies were performed according to protocol approved by the Animal Ethical Committee of École polytechnique fédérale de Lausanne (Switzerland) and by cantonal authorities (licence number: VD-2875).

To obtain offspring, virgin C57BL/6 female mice were mated with APPswe/PSEN1dE9 male mice at 8–12 weeks of age. Presence of a copulation plug was considered embryonic day 0.5 (E0.5). Thereafter, females were housed individually and assigned to a treatment group (prenatal stress or control).

Prenatal stress paradigm

Half of pregnant dams underwent prenatal stress (PNS) by chronic restraint stress paradigm[214] during the last week of pregnancy (embryonic days E12.5 – E18.5). PNS consisted of daily restraint stress of the mothers, by placing the pregnant mouse in a well-ventilated plastic tube for 45'/day at unpredictable times of the day during the last week of pregnancy, a paradigm that has previously been shown to have long-lasting effects on adult cognitive health (J.Gräff, unpublished). Simultaneously, control time-pregnant females were handled in the same way, but not exposed to chronic restraint

stress paradigm. After each restraint stress session, the animals were placed back into their home cage, while control dams were left undisturbed in their home cage.

At birth, PNS-exposed pups were cross-fostered with a non-exposed stress dam and non-PNS (ctrl) litters were cross-fostered with another non-exposed stress dam, so that they spent the entire postnatal period until weaning with a control non-stressed mother. This allowed excluding any postnatal influence of dams that have been stressed during pregnancy.

The offspring of the dams exposed to stress during pregnancy will be referred to as prenatally stressed (PNS), offspring of the control dams will be referred to as controls (ctrl).

Behavioural testing

Offspring were tested at 6 and 9 months of age. To assess overall locomotor function and anxiety-like behaviour, an open-field test (OFT) was performed[229-231]; to test for long-term recognition memory[232,233], the novel object recognition test (NOR) was used (see scheme 1 for a global time line).

The following groups of animals were included in these experiments: wild type (WT) and APPswe/PSEN1dE9 (Tg) male and female offspring, of both prenatal stress (PNS) and control (ctrl) conditions.

Novel object recognition task (NOR)

The NOR test was carried out in three sessions divided by an interval of twenty-four hours. Mice were placed in the testing room, thirty minutes before each session to ensure their acclimation to the room.

During the first day (habituation session), the mouse was placed in the empty testing apparatus and it was allowed to explore the open-field for 10 minutes. The testing apparatus was a circular arena, 43 cm in diameter, with black plexiglas walls. During

the second day (familiarization session), the animal was free to explore two identical objects and during the last session (retention), one of the objects was replaced by a novel, unfamiliar one. This test is a one-trial task, as it does not involve learning of rules. In addition, it does not require reinforces and is purely based on the innate preference of the rodent to explore the novel object rather than the familiar one. Thus, a rodent that remembers the familiar object will spend more time exploring the novel object[232,233].

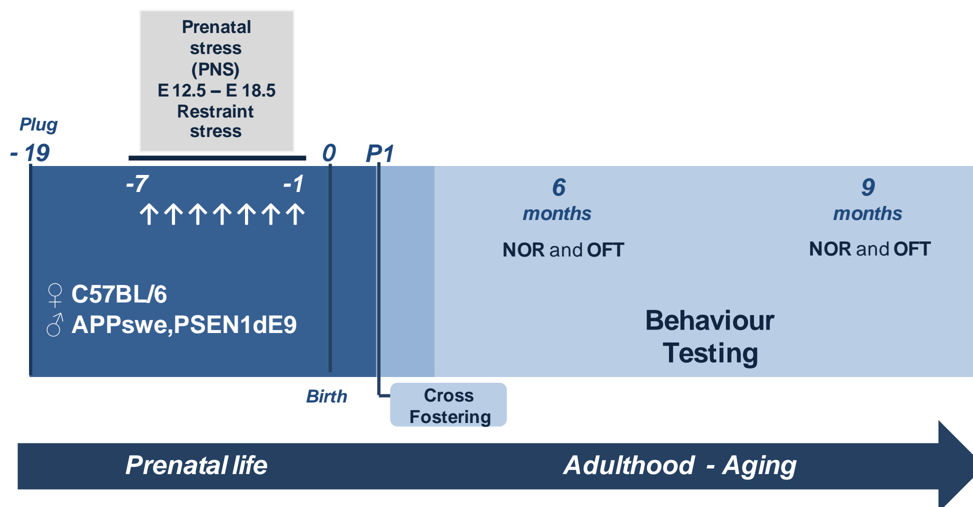
In particular, on the 2nd day of testing (familiarization session), two identical objects were placed in the circular arena equidistant from each other and the arena walls. The animal was then placed in the arena and allowed to explore the objects for 10 minutes. To prevent coercion to explore the objects, mice were released against the center of the opposite wall with its back to the objects.

On the last day of testing (retention session), the animal was introduced in the testing apparatus containing one familiar object (identical to those used in the familiarization) and one novel object for 10 minutes. Between uses, the experimental apparatus and all objects were thoroughly cleansed with 5% ethanol to minimize olfactory cues. The pair of objects used during familiarization was randomized between each mouse and each group tested as well as the position of the novel object during retention (left or right).

Locomotor activity and object investigation were video-recorded using a camera mounted above the testing apparatus and analyzed using EthoVision pro video tracking system and software (Noldus Inc., Leesburg, VA). Object exploration measures for the retention session were scored manually (nose-point detection) by the experimenter who was blinded to the treatment group.

Open-field task (OFT)

During the first day of the NOR test the general locomotor activity and the exploratory behaviour of animals in the open-field, represented by the empty circular arena, was assessed[229]. In particular, mouse was placed in the empty open-field, facing the wall that is nearest to the experimenter, and allowed to explore the open-field for 10 minutes. Between uses, the experimental apparatus was thoroughly cleansed with 5% ethanol to minimize olfactory cues. Distance moved and time spent in the center and in the periphery of the open-field were recorded using the EthoVision pro video tracking system and software (Noldus Inc., Leesburg, VA).



Scheme 1. Investigation of PNS effects on cognitive ability of wild type and APPswe/PSEN1dE9 offspring. Pregnant dams underwent daily restraint stress for 45'/day at embryonic days 12.5 to 18.5. At birth, PNS-exposed and ctrl pups were cross-fostered with a non-stressed dam to exclude any postnatal influence of dams that have been stressed during pregnancy. At 6 and 9 months of age the same prenatally stressed (PNS) animals have been compared to non-stressed (ctrl) animals for their cognitive health. The following behavioural tests were employed: to test for anxiety-like behaviour and animal overall locomotor function, an open-field test (OFT) was used; to test for memory capacities, novel object recognition test (NOR) was performed.

Investigation of acute PNS effects in wild type E18.5 and P1 offspring

Animals

The experiments were carried out on wild type (WT) litter at embryonic day 18.5 (E18.5) and at postnatal day 1 (P1). To obtain offspring, virgin C57BL/6 female mice were mated with C57BL/6 male at 8–12 weeks of age. Presence of a copulation plug was considered embryonic day 0.5 (E0.5). Thereafter, females were housed individually and assigned to a treatment group (prenatal stress or control). Animals were maintained under standard animal housing conditions in a twelve-hour dark-light cycle with an average temperature of 22 °C, relative humidity of 42% and with food and water provided *ad libitum*. All studies were performed according to protocol approved by the Animal Ethical Committee of École polytechnique fédérale de Lausanne (Switzerland) and by cantonal authorities (licence number: VD-2875).

Prenatal stress paradigm

Half of pregnant dams underwent prenatal stress (PNS) by the same chronic restraint stress paradigm[214], already mentioned, during the last week of pregnancy (embryonic days E12.5 – E18.5) (see scheme 2 for a global time line).

The offspring groups included in the following experiments were: wild type E18.5 (embryonic day 18.5) embryos and P1 (postnatal day 1) pups, males and females, of both prenatal stress (PNS) and control (ctrl) conditions.

Tissue collection

Both dams and litters were sacrificed immediately after the last session of restraint stress (E18.5 time point) and one day after birth/delivery (P1 time point) in order to investigate the acute PNS effects on HPA axis function and on brain gene expression.

Blood sampling

Blood samples were collected from dams, E18.5 embryos and P1 pups to determine corticosterone concentrations by ELISA assay. Blood samples were drawn from trunk at sacrifice using heparinized blood collection tubes (Microvette CB300, Sarstedt, DE), kept on ice and subsequently centrifuged at 3000 rpm for 10 minutes at 4°C. Subsequently, plasma was isolated, frozen down to -80°C and stored until further processing.

Brains

E18.5 and P1 brains were removed with the skull, immediately frozen on powdered dry ice and stored at -80°C.

E18.5 whole brains were dissected from the decapitated embryos and the cerebellum was discarded. Subsequently, E18.5 brains were divided into three regions: a frontal portion (dissected by a coronal cut at the level of the first most anterior third of the brain), the remaining caudal portion was further cut in the middle along the dorsal-ventral axis. The dissection was performed using a 16-gauge stainless steel needle based on the coordinates for developing mouse brain atlas[234]. Brains were maintained at -20°C during the whole duration of the dissection.

The prefrontal cortex (PFC), dorsal hippocampus (HPC) and amygdala (AMY) from P1 brains were isolated from 20 µm thick frozen coronal brain sections using brain punches 0.50 – 1 mm (Stoelting CO, Wood Dale, IL) basing on the coordinates for developing mouse brain atlas[234].

RNA Isolation

Total RNA from P1 brain tissue punches and E18.5 brain frontal region was carried out using RNeasy Tissue Mini Kit (Qiagen, Hilden, DE) according to manufacturer's instructions. Absorbance measurements were made on NanoDrop 1000 (Thermo Scientific, Wilmington, DE) and the ratio of absorbance at 260 nm and 280 nm was used to assess the purity of RNA samples, further stored at -80°C.

Quantitative Reverse - Transcription Polymerase Chain Reaction

Total RNA was retrotranscribed using the qScript™ cDNA Synthesis Kit (Quantabio, Beverly, MA) following the protocol provided by the supplier. Quantitative Real-Time pcr assay (qRT-pcr) for gene expression analysis was realized using primer pairs listed in table 4 and SYBR Green Master Mix (Thermo Scientific, Wilmington, DE), according to the manufacturer's instructions. All qRT-pcr reactions were run in triplicate in 384-well optical plates and performed in a 7900HT Fast Real-Time PCR System instrument (Applied Biosystems, Foster City, CA). TBP (forward: 5'CTGGAATTGTACCGCAGCTT3'; reverse: 5'CAGTTGTCCGTGGCTCTCTT3') and EEF1a (forward: 5'TCCACTTGGTCGCTTTGCT3'; reverse: 5'CTTCTTGTCCACAGCTTTGATGA3') were used as reference genes and fold change was determined by qBase relative quantification software[235].

Corticosterone ELISA assay

For the quantitative determination of plasma corticosterone levels the Corticosterone ELISA kit (Enzo Life Science, Farmingdale, NY) was used. The assay was performed according to the manufacturer's instructions.

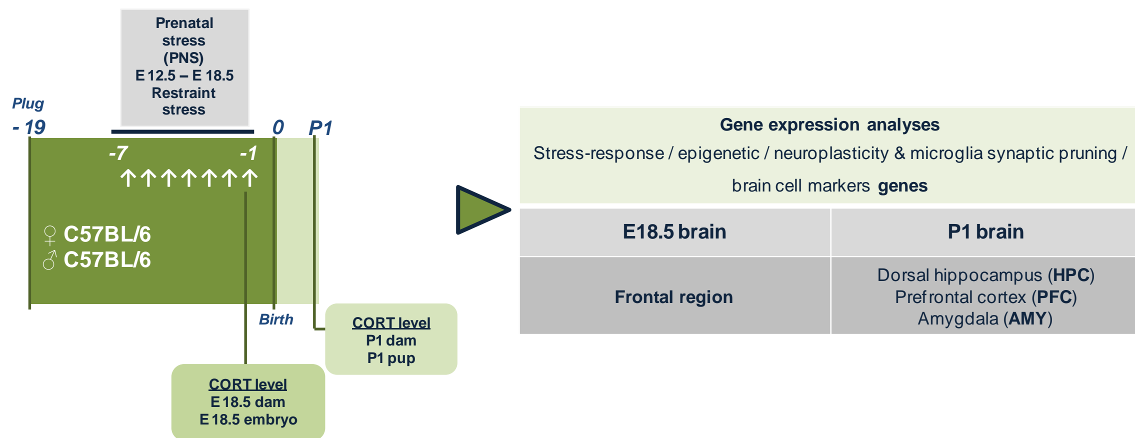
Statistical analysis

Statistical analysis was performed using the *Statistical Package for the Social Sciences* (version 22.0; SPSS Inc, Chicago, IL) and statistical significance level was set at 0.05. Behavioural data were analyzed by *two*-way analysis of variance (ANOVA) with genotype and PNS as factors, followed by Bonferroni *post* test, or by unpaired *t*-test when required. Unpaired *t*-test was used to analyze differences in gene expression data between groups.

Table 4. Primer pairs for quantitative real-time pcr (qRT-pcr) assay

Target	Product size (bp)	Forward primer (5'-3')	Reverse primer (5'-3')
HDAC2	92	GGGACAGGCTTGGTTGTTTC	GAGCATCAGCAATGGCAAGT
HDAC1	416	CAAAGGACACGCCAAGTGTG	CACAGGCAATGCGTTTGTCA
DNMT1	91	CAGAGGAGAGAGACCAGGATAA	CGTGTTACCTCTTCCAGTTTCT
GR	90	GGCTTCTGGGTGTCACTATGG	CACAGATAGTTGTGTTGTCCTTCCA
MR	170	AGGCCGCTCAGTGTTTTCTA	TACAGCTTCCACACGTCAGC
BDNF	118	GCGCCCATGAAAGAAGTAAA	TCGTCAGACCTCTCGAACCT
BDNF IV	276	CTCCGCCATGCAATTTCC	GCCTTCATGCAACCGAAG
CX3CR1	65	CAGCATCGACCGGTACCTT	GCTGCACTGTCCGGTTGTT
CX3CL1	71	CCGCGTTCTTCCATTTGTGT	GCACATGATTTCGCATTTCG
TREM2	64	TGGGACCTCTCCACCAGTT	GTGGTGTTGAGGGCTTGG
NeuN (Rbfox3)	160	GGCAAATGTTCGGGCAATTCG	TCAATTTTCCGTCCCTCTACGAT
GFAP	57	GGAGATGCGGGATGGTGAG	ACCACGTCCTTGTGCTCCTG
CNP	147	GAAGAATACGCCCAGCAGGA	CAGATCACTGGGCCACAACT
GAD67	174	CTTCTTCAGGCTCTCCCGTG	CAGGAACAGGCTCGGTTCAG

HDAC2 (histone deacetylase 2); HDAC1 (histone deacetylase 1); DNMT1 (DNA methyltransferase 1); GR (glucocorticoid receptor); MR (mineralcorticoid receptor); BDNF (brain derived neurotrophic factor); BDNF IV (brain derived neurotrophic factor exon IV); CX3CR1 (C-X3-C motif chemokine receptor 1); CX3CL1 (C-X3-C motif chemokine ligand 1); TREM2 (triggering receptor expressed on myeloid cells 2); NeuN or Rbfox3 (RNA binding protein, fox-1 homolog 3); GFAP (glial fibrillary acidic protein); CNP (2',3'-cyclic nucleotide 3' phosphodiesterase), GAD67 (glutamate decarboxylase 67).



Scheme 2. Investigation of acute PNS effects in wild type E18.5 and P1 offspring. A second cohort of animals underwent daily restraint stress for 45'/day at embryonic days 12.5 to 18.5. Then dams and litters were scarified immediately after the last session of restraint stress (embryonic day E18.5) and one day after birth/delivery (postnatal day P1) in order to investigate the acute PNS effects on HPA axis function and on brain gene expression.

Results

Antiviral innate immune response in the brain of Alzheimer’s disease (AD) patients

Impaired antiviral gene expression in AD brains

To reinforce the notion of an individual defective immune response against microorganisms in AD, the expression of genes involved in antimicrobial defences in the hippocampus and temporal cortex of patients with clinical and neurological defined diagnosis of AD and non-demented controls was investigated.

We focused on genes involved in antiviral responses: IRF7, MED23, IFN-λ3, also known as IL28B, and IFN-α. A differential genetic background in these genes, regulating antiviral responses, was previously associated with an increased risk of cognitive decline and AD[201].

In particular, mRNA levels of IRF7, MED23, IL28B and IFN-α were analyzed in 29 AD hippocampus and 19 AD temporal brain samples.

Most AD patients showed a down-regulation of these major antiviral immune response genes both in the hippocampal (figure 1) and temporal (figure 2) brain areas (table 5). Interestingly, mRNA levels of the transcription factor involved in innate immunity IRF7, MED23, a key regulator of interferon-expression, and the antiviral cytokines IL28B and IFN-α were hypo-expressed, at the same time, in the hippocampus of 55,2% (16/29) and in the temporal cortex of 26,3% (5/19) of AD patients.

AD patients were stratified according to their down- (fold change < 1) or up- (fold change > 1) regulation of the analyzed genes compared to the ctrl group. Most of the patients showed a significant down-regulation of IRF7 ($p=0.04$), MED23 ($p=0.0001$), IL28B ($p=0.0001$) and IFN-α ($p=0.0005$) in the hippocampus (fig. 1), but also in the temporal cortex (fig. 2: IRF7, $p=0.0006$; MED23, $p=0.005$; IL28B, $p=0.002$; IFN-α, $p=0.0009$). A small group of AD patients showed up-regulation of MED23 ($p=0.0009$), IL28B ($p=0.004$) and IFN-α ($p=0.002$) genes only in the hippocampus (fig. 1).

Hippocampus

	Gene	N	Fold change
Down-regulation in the mRNA expression	IRF7	19	0.62 ± 0.06
	MED23	20	0.28 ± 0.04
	IL28B	21	0.22 ± 0.05
	IFN-α	19	0.22 ± 0.06
Up-regulation in the mRNA expression	IRF7	10	1.52 ± 0.14
	MED23	9	1.85 ± 0.15
	IL28B	7	2.07 ± 0.22
	IFN-α	9	2.39 ± 0.26

Temporal cortex

	Gene	N	Fold change
Down-regulation in the mRNA expression	IRF7	16	0.40 ± 0.07
	MED23	11	0.37 ± 0.09
	IL28B	12	0.44 ± 0.10
	IFN-α	9	0.40 ± 0.07
Up-regulation in the mRNA expression	IRF7	3	1.94 ± 0.50
	MED23	8	1.90 ± 0.26
	IL28B	4	1.45 ± 0.19
	IFN-α	5	1.90 ± 0.35

Table 5. Antiviral gene expression profiles in hippocampus and temporal cortex of AD patients. Values are given as fold change ± SEM

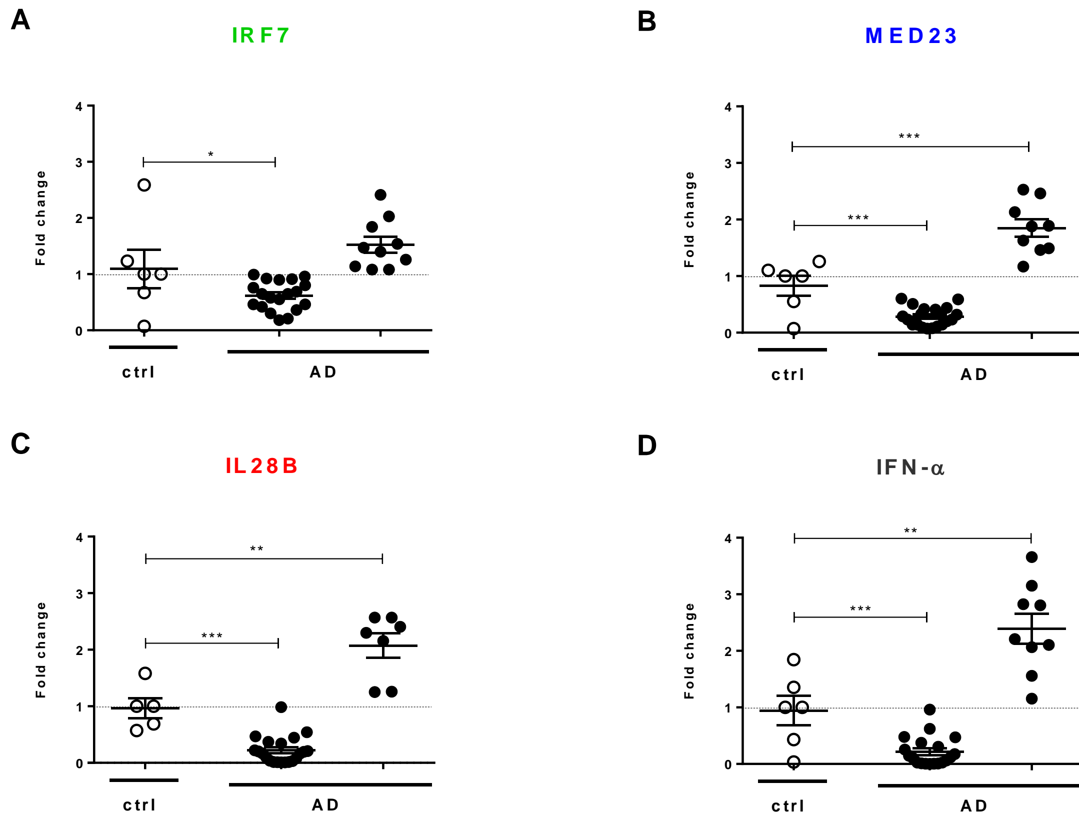


Figure 1. Hippocampal differential expression of antiviral response genes in AD patients and healthy individuals. Fold change (qRT-pcr $2^{-\Delta\Delta Ct}$ method) of IRF7 (**A**), MED23 (**B**), IL28B (**C**), IFN-α (**D**), normalized to two reference genes (CYC1 and EIF4A2) and relative to non-demented healthy individuals (ctrl), in hippocampus of patients with clinical and neurological defined diagnosis of AD. Values are given as fold change ± SEM. *p<0.05; **p<0.01; ***p<0.001 (unpaired *t*-test).

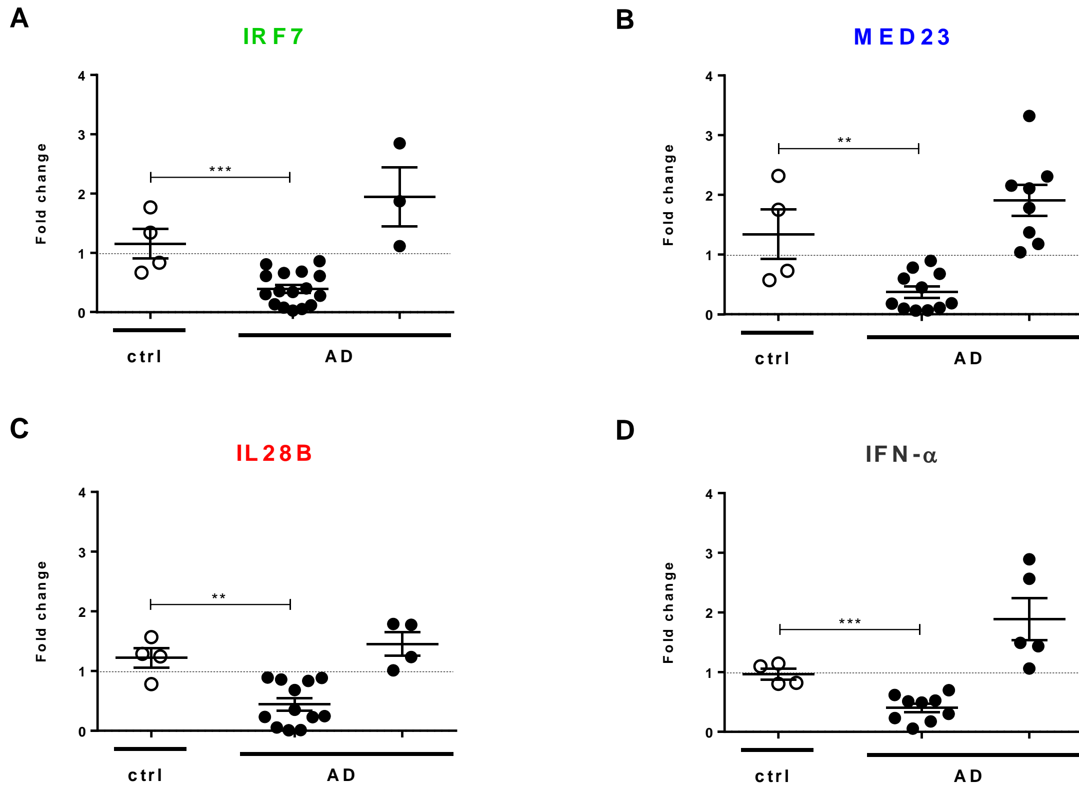


Figure 2. Temporal differential expression of antiviral response genes in AD patients and healthy individuals. Fold change (qRT-pcr $2^{-\Delta\Delta Ct}$ method) of IRF7 (**A**), MED23 (**B**), IL28B (**C**), IFN-α (**D**), normalized to two reference genes (CYC1 and EIF4A2) and relative to non-demented healthy individuals (ctrl), in temporal cortex of patients with clinical and neurological defined diagnosis of AD. Values are given as fold change ± SEM. **p<0.01; ***p<0.001 (unpaired *t*-test).

Single nucleotide polymorphisms (SNPs) of antiviral genes and their effects on gene expression profiles in AD brains

DNA from brain tissues (hippocampus and temporal cortex) was extracted and AD patients (table 1 and 2) were genotyped for APOE ε allele (*rs*429358 and *rs*7412; table 3) and for SNPs of IRF7 (*rs*6598008), MED23 (*rs*3756784), IL28B (*rs*12979860) genes (tables 6 and 7) that have been previously associated with increased risk of cognitive decline and AD[201]. We analyzed these genetic variants in order to investigate a potential effect of these SNPs on the brain antiviral gene expression profiles.

The presence of APOE ε4 allele influenced the hippocampus mRNA levels of MED23, IL28B and IFN-α. The relative expression of MED23, the antiviral component of the Mediator complex, IL28B and IFN-α, belonging to the group of type III and I IFNs respectively, were significantly lower in ε4 allele carrier AD patients when compared to ε4 allele non carriers (MED23, p=0.010; IL28B, p=0.010; IFN-α, p=0.0076; figure 3B, C, D).

APOE ε4 carrier AD patients showed also significantly lower temporal mRNA levels of MED23 gene than that of APOE ε4 non carriers (p=0.04; figure 4B). However, temporal mRNA expression levels of IL28B (p=0.88) and IFN-α (p=0.06) of APOE ε4 carriers did not significantly differ from that of non carrier AD patients (figure 4C and D).

mRNA levels of antiviral factors from hippocampus and temporal cortex samples from AD patients were also stratified according to IRF7 (*rs*6598008) (figures 5 and 6), MED23 (*rs*3756784) (figures 7 and 8), IL28B (*rs*12979860) (figures 9 and 10) genotypes (tables 6 and 7).

AD A carriers of IRF7 SNP (*rs*6598008) showed significantly lower mRNA relative levels of IRF7 (p=0.027), MED23 (p=0.005), IL28B (p=0.02) and IFN-α (p=0.006) in hippocampus than those observed from GG carriers (figure 5). However, these differences were not observed in temporal cortex from AD samples (figure 6).

The effects of MED23 (*rs*3756784) and IL28B (*rs*12979860) SNPs on AD brain antiviral gene expression were also investigated. However, no statistically significant differences in mRNA profiles were found between G carriers and G non carriers of MED23 gene variant, and between T carrier and T non carrier patients of IL28B SNP, as shown from hippocampus (figure 7 and 9) and temporal cortex samples (figures 8 and 10).

Hippocampus

APOE genotype

	ε2/ε3		ε3/ε3		ε3/ε4		ε4/ε4		ε4 non carrier		ε4 carrier	
	N	%	N	%	N	%	N	%	N	%	N	%
	2	6.9	7	24.1	15	51.7	5	17.2	9	31	20	69

IRF7 *rs*6598008 genotype

	AA		GA		GG		A carrier		G carrier	
	N	%	N	%	N	%	N	%	N	%
	5	17.2	16	55.2	8	26.6	21	72.4	24	82.8

MED23 *rs3756784* genotype

	GG		GT		TT		G carrier		T carrier	
	N	%	N	%	N	%	N	%	N	%
	1	3.4	11	37.9	17	58.6	12	41.4	28	96.6

IL28B *rs12979860* genotype

	TT		CT		CC		T carrier		C carrier	
	N	%	N	%	N	%	N	%	N	%
	3	10.3	14	48.3	12	41.4	17	58.6	26	89.7

Table 6. Genotype and allele distribution of SNPs of APOE, IRF7, MED23 and IL28B genes from AD patients whose hippocampus samples have been analyzed.

Temporal cortex

APOE genotype

	ε2/ε3		ε3/ε3		ε3/ε4		ε4/ε4		ε4 non carrier		ε4 carrier	
	N	%	N	%	N	%	N	%	N	%	N	%
	1	5.3	10	52.6	7	36.8	1	5.3	11	57.9	8	42.1

IRF7 *rs*6598008 genotype

	AA		GA		GG		A carrier		G carrier	
	N	%	N	%	N	%	N	%	N	%
	1	5.3	11	57.9	7	36.8	12	63.2	18	94.7

MED23 *rs3756784* genotype

	GG		GT		TT		G carrier		T carrier	
	N	%	N	%	N	%	N	%	N	%
	2	10.5	5	26.3	12	63.2	7	36.8	17	89.5

IL28B *rs12979860* genotype

	TT		CT		CC		T carrier		C carrier	
	N	%	N	%	N	%	N	%	N	%
	2	10.5	9	47.4	8	42.1	11	57.9	17	89.5

Table 7. Genotype and allele distribution of SNPs of APOE, IRF7, MED23 and IL28B genes from AD patients whose temporal cortex samples have been analyzed.

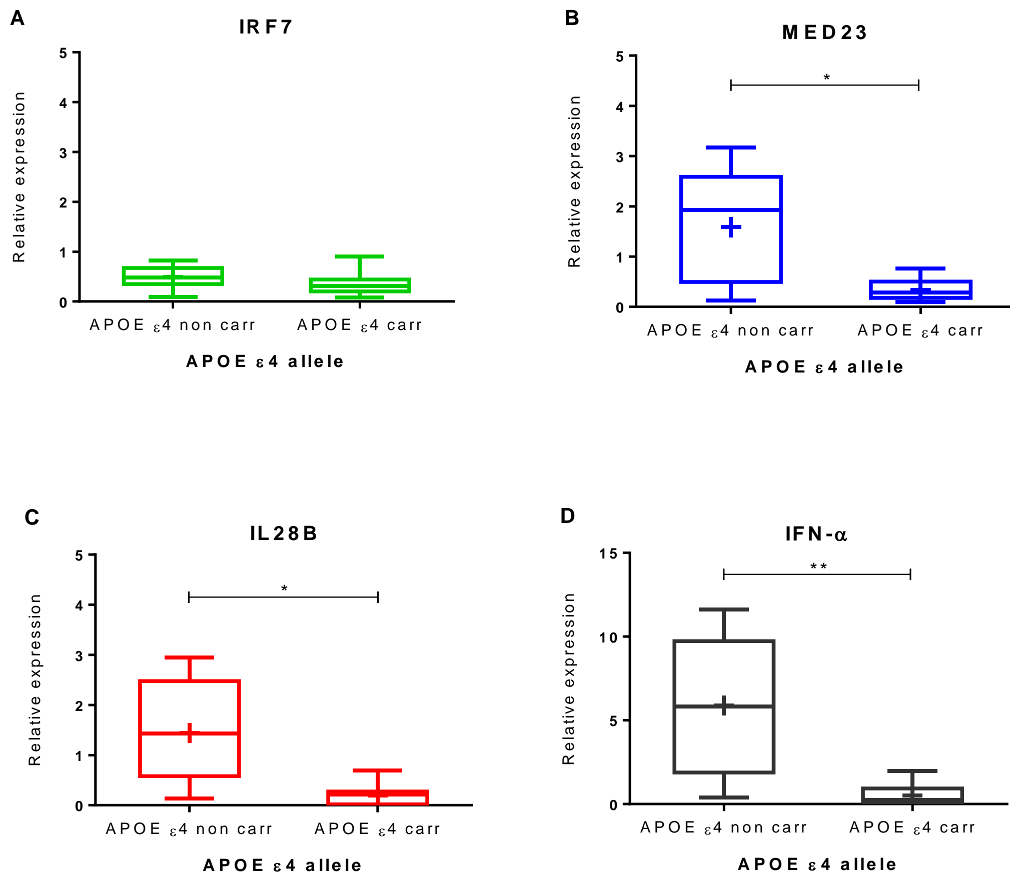


Figure 3. Effect of APOE ε4 allele on antiviral immune gene expression from AD hippocampus samples. qRT-pcr data showing relative expression ($2^{-\Delta Ct}$ values using CYC1 and EIF4A2 as reference genes) of IRF7 (**A**), MED23 (**B**), IL28B (**C**), IFN-α (**D**) from hippocampus of AD patients grouped in APOE ε4 non carrier/or APOE ε4 carrier. Data from each group are shown as a box and whiskers plot with the ends of the whiskers represent the minimum and maximum data values, the horizontal line represents the median and the "+" represents the mean relative expression values. *p<0.05; **p<0.01 (unpaired *t*-test).

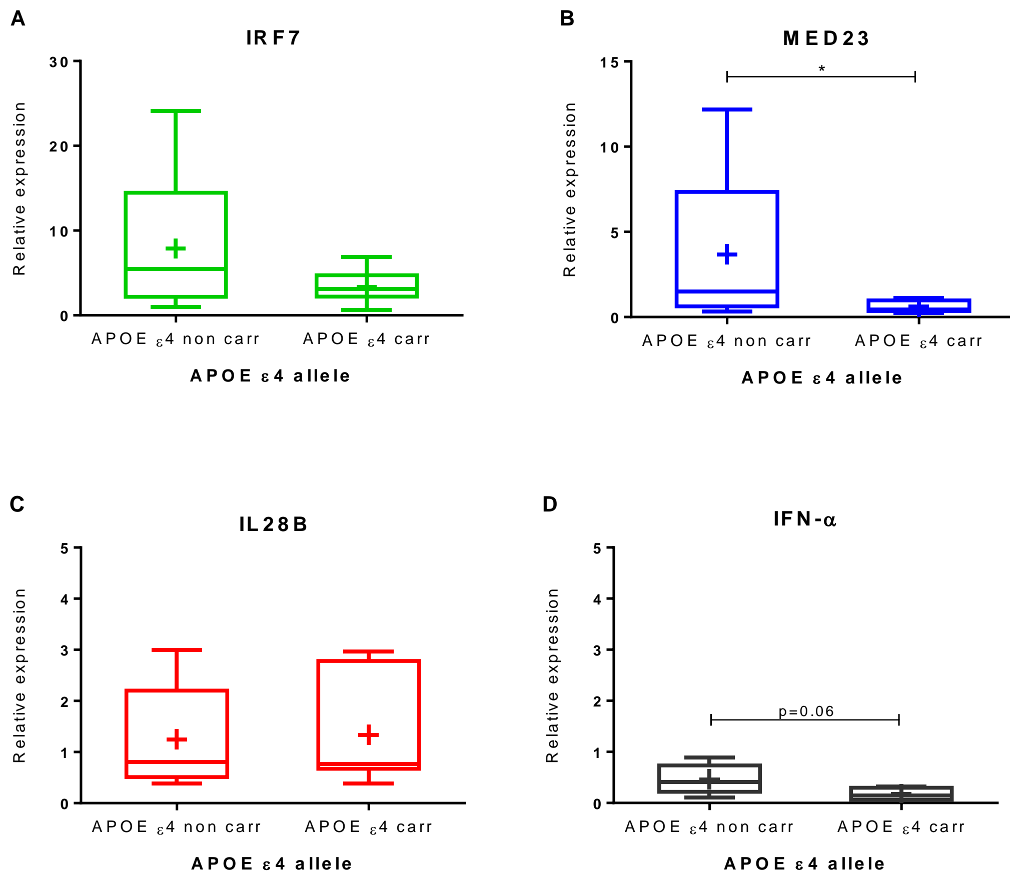


Figure 4. Effect of APOE ε4 allele on antiviral immune gene expression from AD temporal cortex samples. qRT-pcr data showing relative expression ($2^{-\Delta Ct}$ values using CYC1 and EIF4A2 as reference genes) of IRF7 (**A**), MED23 (**B**), IL28B (**C**), IFN-α (**D**) from temporal cortex of AD patients grouped in APOE ε4 non carrier/or APOE ε4 carrier. Data from each group are shown as a box and whiskers plot with the ends of the whiskers represent the minimum and maximum data values, the horizontal line represents the median and the "+" represents the mean relative expression values. *p<0.05 (unpaired *t*-test).

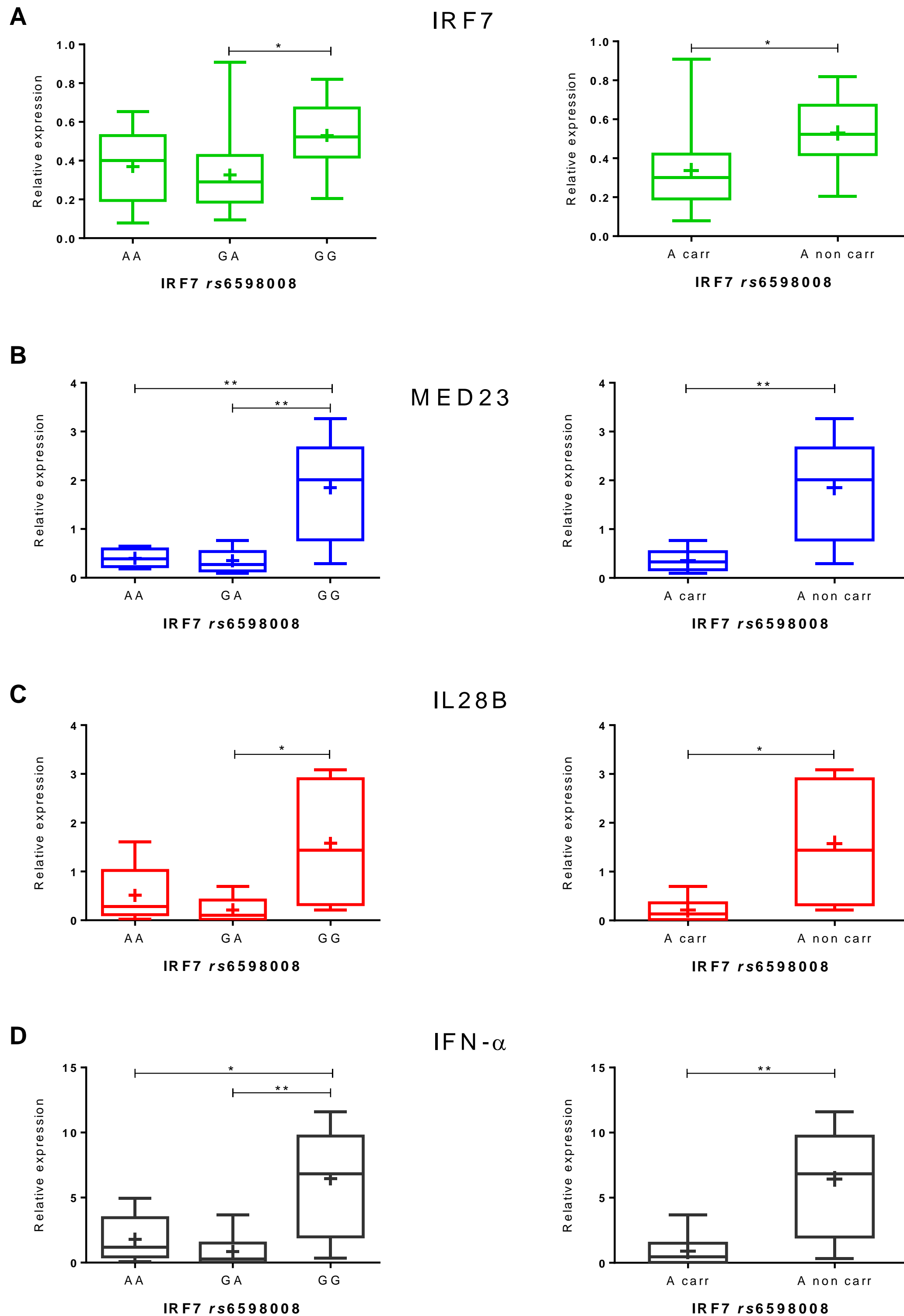


Figure 5. Effect of IRF7 SNP (*rs*6598008) on antiviral immune gene expression from AD hippocampus samples. qRT-pcr data showing relative expression ($2^{-\Delta Ct}$ values using CYC1 and EIF4A2 as reference genes) of IRF7 (**A**), MED23 (**B**), IL28B (**C**), IFN-α (**D**) from hippocampus of AD patients grouped in AA, GA, GG genotypes on the left panels and in A carrier/or A non carrier on the right panels. Data from each group are shown as a box and

whiskers plot with the ends of the whiskers represent the minimum and maximum data values, the horizontal line represents the median and the “+” represents the mean relative expression values. *p<0.05; **p<0.01 (unpaired *t*-test; *one-way* ANOVA followed by Bonferroni *post* test).

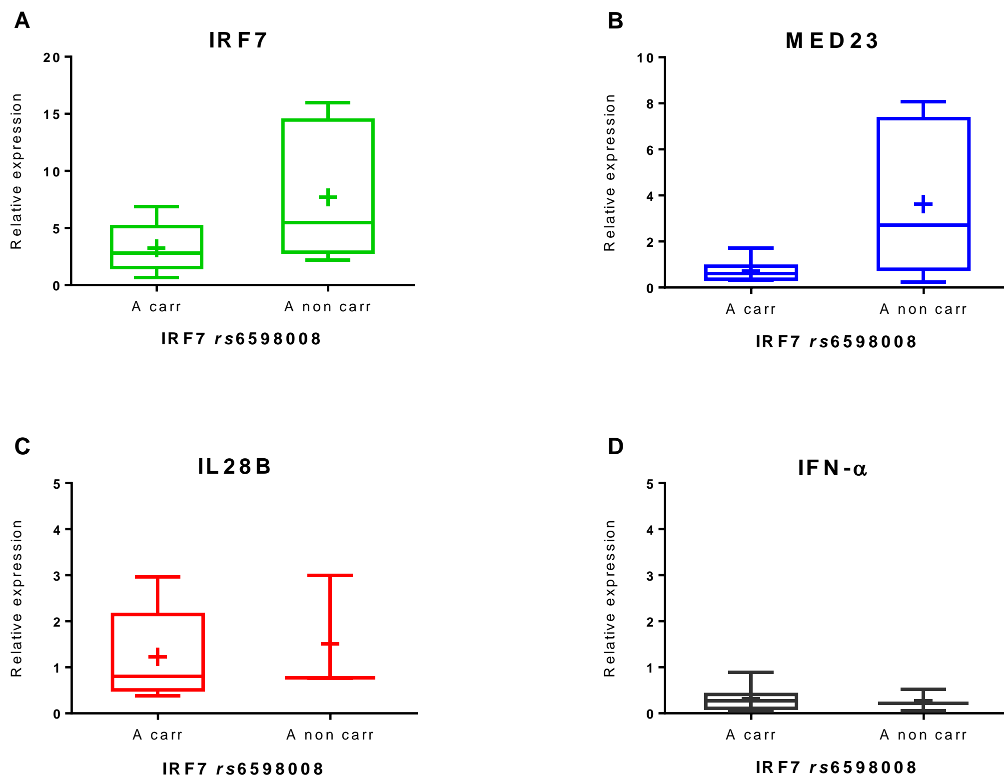


Figure 6. Effect of IRF7 SNP (*rs*6598008) on antiviral immune gene expression from AD temporal cortex samples. qRT-pcr data showing relative expression ($2^{-\Delta Ct}$ values using CYC1 and EIF4A2 as reference genes) of IRF7 (**A**), MED23 (**B**), IL28B (**C**), IFN-α (**D**) from temporal cortex of AD patients grouped in A carrier/or A non carrier. Data from each group are shown as a box and whiskers plot with the ends of the whiskers represent the minimum and maximum data values, the horizontal line represents the median and the “+” represents the mean relative expression values. Unpaired *t*-test.

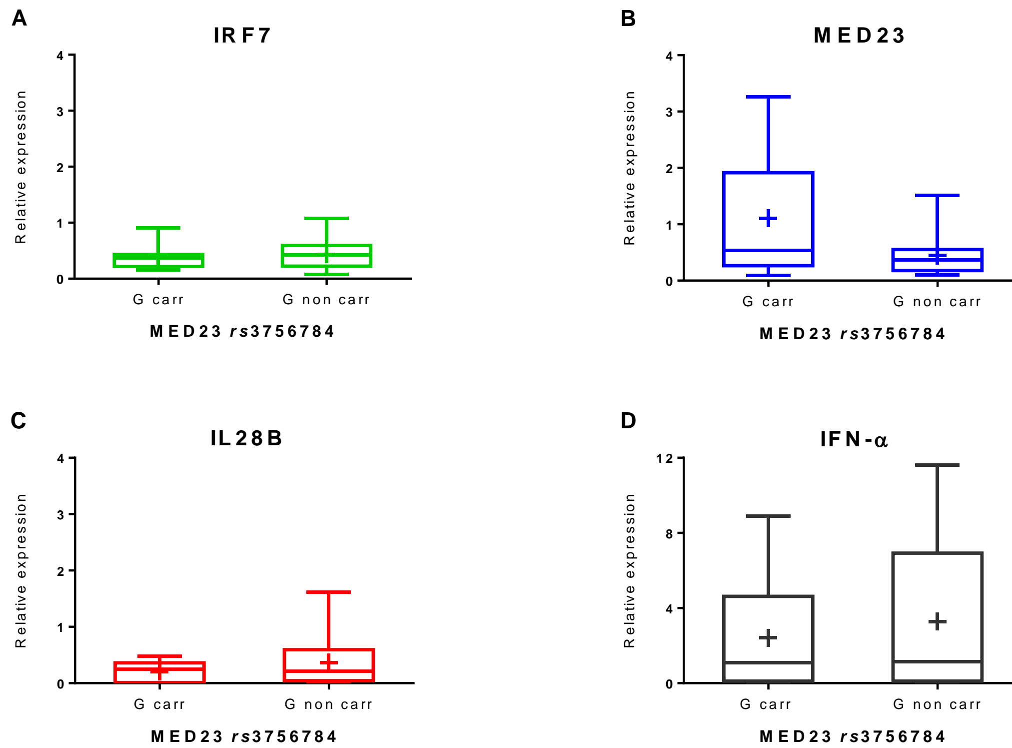


Figure 7. Effect of MED23 SNP (*rs*3756784) on antiviral immune gene expression from AD hippocampus samples. qRT-pcr data showing relative expression ($2^{-\Delta Ct}$ values using CYC1 and EIF4A2 as reference genes) of IRF7 (**A**), MED23 (**B**), IL28B (**C**), IFN-α (**D**) from hippocampus of AD patients grouped in G carrier/or G non carrier. Data from each group are shown as a box and whiskers plot with the ends of the whiskers represent the minimum and maximum data values, the horizontal line represents the median and the "+" represents the mean relative expression values. Unpaired *t*-test.

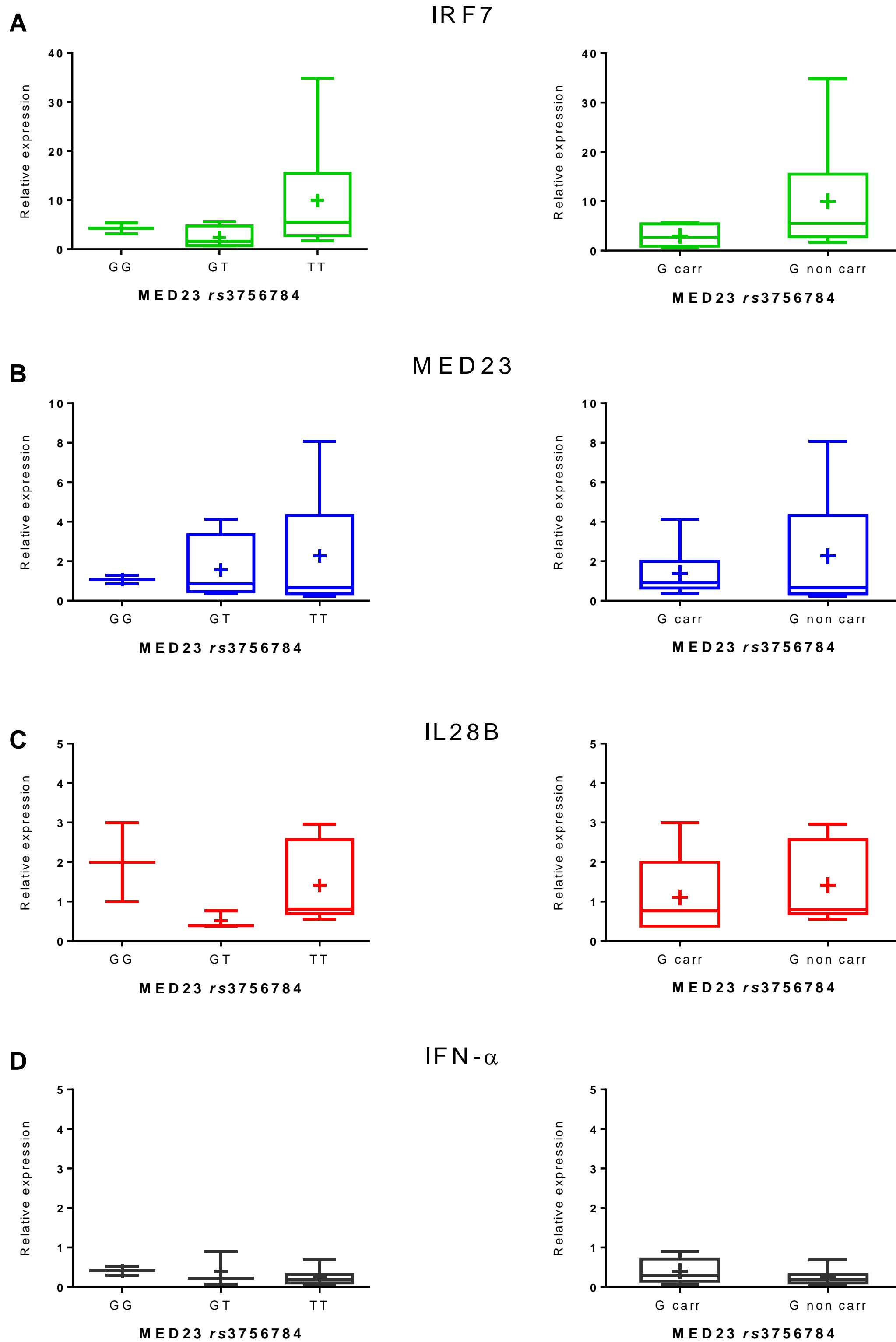


Figure 8. Effect of MED23 SNP (*rs*3756784) on antiviral immune gene expression from AD temporal cortex samples. qRT-pcr data showing relative expression ($2^{-\Delta Ct}$ values using CYC1 and EIF4A2 as reference genes) of IRF7 (**A**), MED23 (**B**), IL28B (**C**), IFN-α (**D**) from temporal cortex of AD patients grouped in GG, GT, TT genotypes on the left panels and in

G carrier/or G non carrier on the right panels. Data from each group are shown as a box and whiskers plot with the ends of the whiskers represent the minimum and maximum data values, the horizontal line represents the median and the "+" represents the mean relative expression values. Unpaired *t*-test; *one-way* ANOVA followed by Bonferroni *post* test.

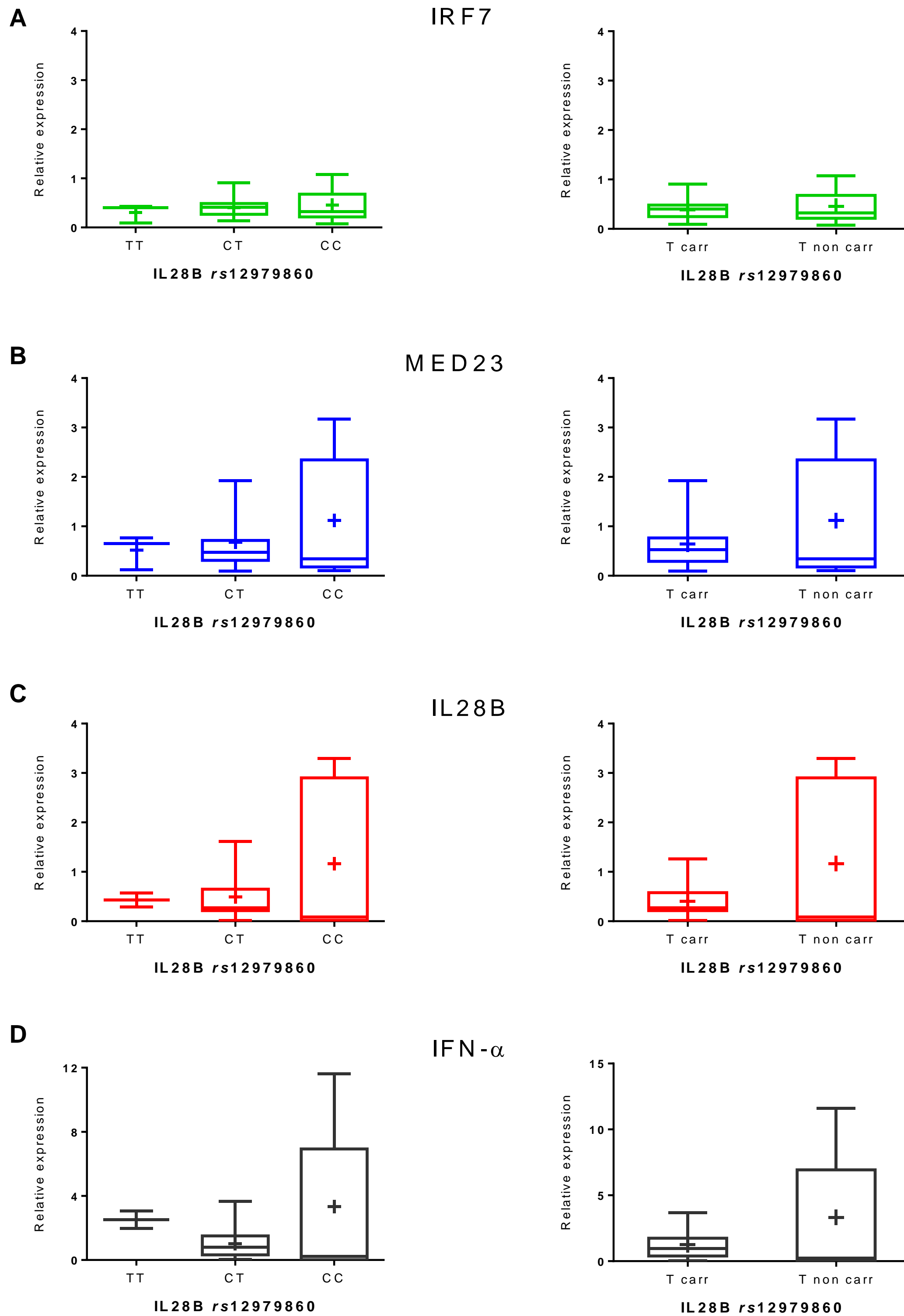


Figure 9. Effect of IL28B SNP (*rs*12979860) on antiviral immune gene expression from AD hippocampus samples. qRT-pcr data showing relative expression ($2^{-\Delta Ct}$ values using CYC1 and EIF4A2 as reference genes) of IRF7 (**A**), MED23 (**B**), IL28B (**C**), IFN-α (**D**) from hippocampus of AD patients grouped in TT, CT, CC genotypes on the left panels and in T carrier/or T non carrier on the right panels. Data from each group are shown as a box and

whiskers plot with the ends of the whiskers represent the minimum and maximum data values, the horizontal line represents the median and the "+" represents the mean relative expression values. Unpaired *t*-test; *one-way* ANOVA followed by Bonferroni *post* test.

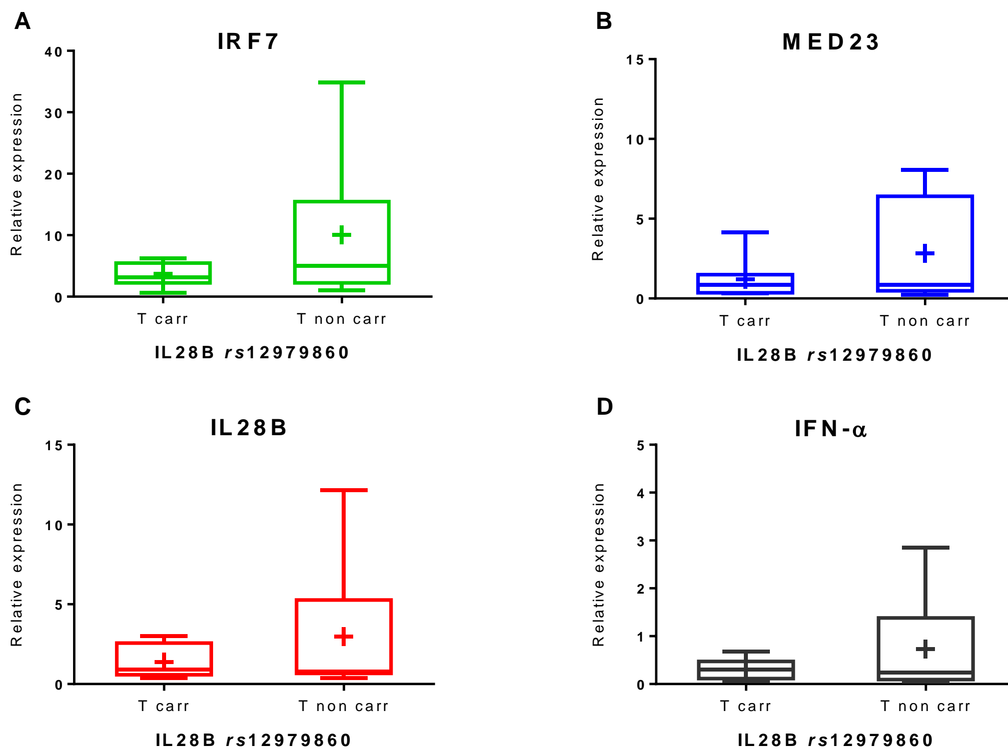


Figure 10. Effect of IL28B SNP (*rs*12979860) on antiviral immune gene expression from AD temporal cortex samples. qRT-pcr data showing relative expression ($2^{-\Delta Ct}$ values using CYC1 and EIF4A2 as reference genes) of IRF7 (**A**), MED23 (**B**), IL28B (**C**), IFN-α (**D**) from temporal cortex of AD patients grouped in T carrier/or T non carrier. Data from each group are shown as a box and whiskers plot with the ends of the whiskers represent the minimum and maximum data values, the horizontal line represents the median and the "+" represents the mean relative expression values. Unpaired *t*-test.

Effects of prenatal stress (PNS) on adult cognitive health

Long-term effects of prenatal stress (PNS) on cognitive ability of WT and APPswe/PSEN1dE9 offspring

Basal locomotion is not altered by prenatal stress (PNS) in wild type and APPswe/PSEN1dE9

The open-field test (OFT) is a common measure of exploratory behaviour and general locomotor activity in rodents. The OFT is used to assess locomotor activity level as a "control" experiment for behavioural tests that involve activity[231,236,237] but it also provides an initial screen for mouse anxiety-related behaviour[230]. Rodents typically spend a significantly greater amount of time exploring the periphery of an open-field (empty arena), usually in contact with the walls (thigmotaxis), than the unprotected center area. The proportion of time spent avoiding the center of the arena is therefore taken as a measure of anxiety-like behaviour[229].

At adulthood, at 6 months and 9 months of age, PNS-exposed wild type (WT) and APPswe/PSEN1dE9 (Tg) offspring were compared to non-stressed ones (ctrl) for their locomotor activity and exploratory behaviour in the open-field context, and total distance moved and time spent in the center and the periphery of the open-field arena were measured.

At 6 months, animal locomotor activity was not affected (figure 11, A and B) and time spent exploring central and peripheral zone of the open-field arena didn't reveal significant differences between prenatally stressed and control offspring both in WT and Tg groups (fig. 11, C and D). Examination of the open-field center time exploration as a preliminary screen for anxiety-like behaviour revealed no significant PNS differences both in male and female offspring.

Likewise, at 9 months of age, the anxiety-like behavioural measures didn't highlight any differences due to PNS. Animal general locomotor activity was not affected (figure 12, A and B) and central and/or peripheral zone activity within the open-field arena didn't reveal significant differences between prenatally stressed and control offspring both in WT and Tg mice (figure 12, C and D).

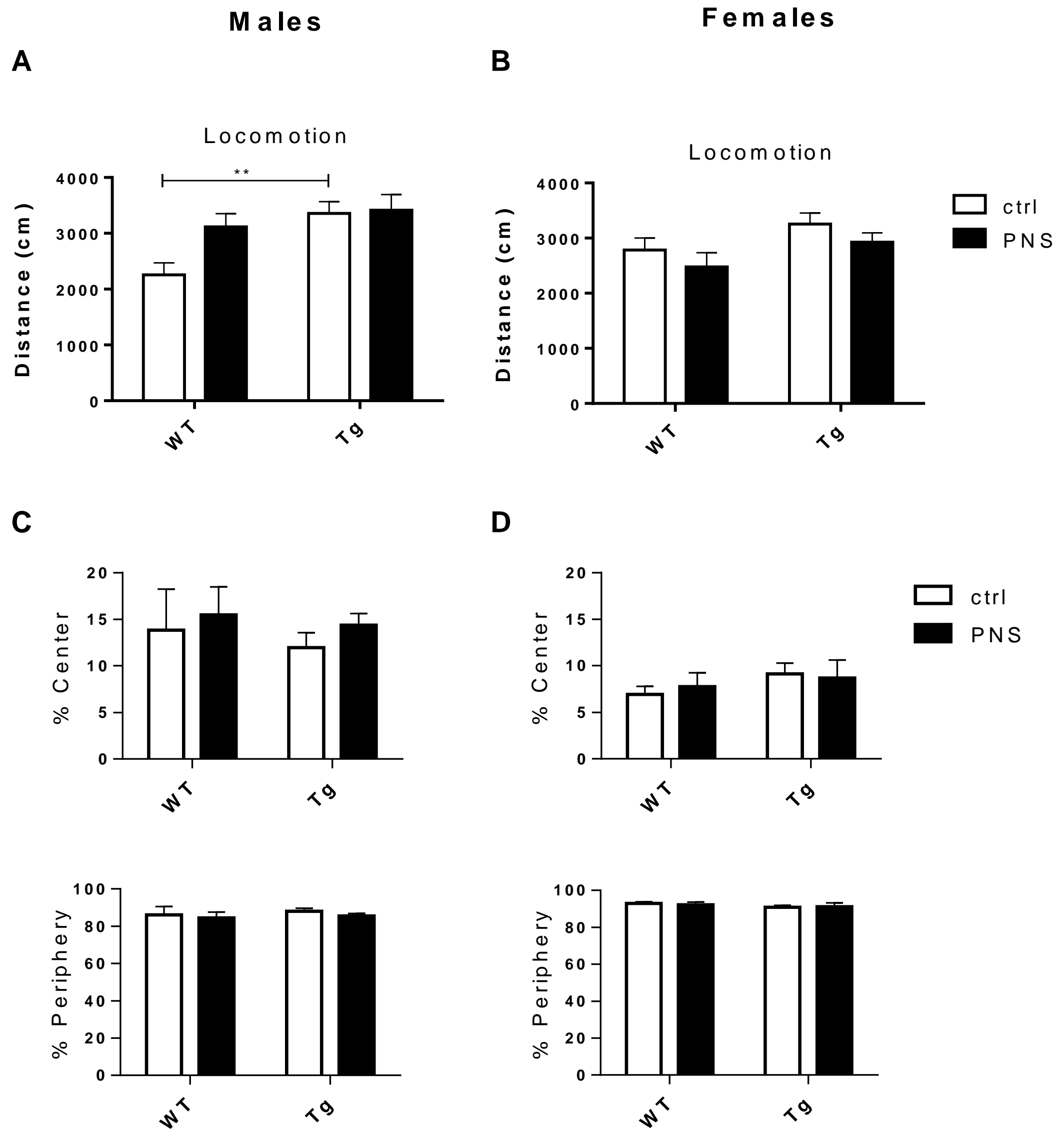


Figure 11. No effect of PNS on open-field exploration offspring at 6 months of age. Data show locomotor activity during OFT of prenatally stress and control male (**A**) and female (**B**) offspring at 6 months of age. Panels **C** and **D** show time spent exploring the center and the periphery of the open-field by prenatally stress and control male (**C**) and female (**D**) offspring at 6 months of age. No significant PNS differences on central and peripheral zone activity were found.

Male offspring: WT ctrl n=8; WT PNS n=8; Tg ctrl n=16; Tg PNS n=11. Female offspring: WT ctrl n=11; WT PNS n=13; Tg ctrl n=14; Tg PNS n=8. Data are shown as mean ± SEM. ** $p<0.01$ (*two-way* ANOVA followed by Bonferroni *post* test).

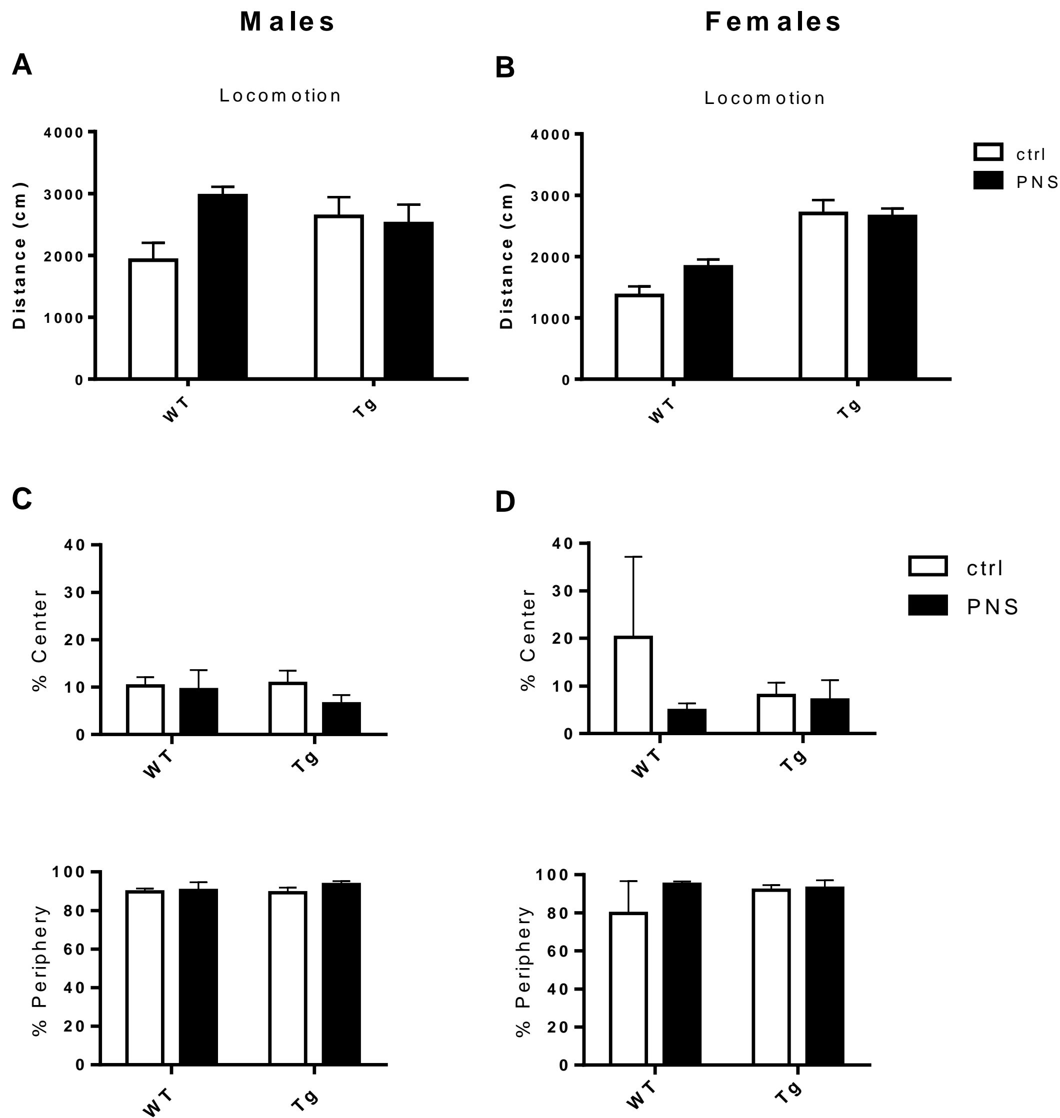


Figure 12. No effect of PNS on open-field exploration offspring at 9 months of age. Data show locomotor activity during OFT of prenatally stress and control male (**A**) and female (**B**) offspring at 9 months of age. Panels **C** and **D** show time spent exploring the center and the periphery of the open-field by prenatally stress and control male (**C**) and female (**D**) offspring at 6 months of age. No significant genotype / PNS differences on central and peripheral zone activity were found.

Male offspring: WT ctrl n=4; WT PNS n=2; Tg ctrl n=6; Tg PNS n=4. Female offspring: WT ctrl n=2; WT PNS n=9; Tg ctrl n=6; Tg PNS n=2. Data are shown as mean ± SEM. *Two-way* ANOVA followed by Bonferroni *post* test.

PNS accelerates long-term memory deficit in APPswe/PSEN1dE9

The novel object recognition (NOR) test was used to assess the potential effect of PNS on long-term recognition memory of wild type (WT) and APPswe/PSEN1dE9 (Tg) offspring at 6 and 9 months of age. We performed this behavioural test to characterize the potential cognitive impairment caused by PNS in APPswe/PSEN1dE9 mice at early stages of amyloid-β (Aβ) deposition[238], where recognition memory is normally comparable between Tg and WT animals[239]. NOR test is a typical behavioural test used to assess memory function in AD mouse model[240]. This test is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one. The choice to explore the novel object reflects the use of learning and recognition memory[232,233].

Familiar object exploration time (Time Familiar, TF) and novel object exploration time (Time Novel, TN) was scored manually with the EthoVision software (Noldus Inc., Leesburg, VA) by the experimenter blinded to the treatment group. Exploration of an object was defined as directing the nose toward the object, while climbing onto the object (unless active object sniffing is observed) or chewing the object didn’t qualify as exploration. Mice that didn’t satisfy the criterion of 20 s exploration time of both items within familiarization and/or retention test sessions were excluded from the following analyses.

We used the Discrimination Index (DI) and Recognition Index (RI) as two estimations of recognition process and to measure long-term recognition memory of each animal group. The DI [DI = (TN-TF)/(TN+TF)] is a measure of discrimination between the novel and familiar objects and it calculates the difference between exploration time for novel and familiar objects, dividing it by the total amount of exploration time during retention session. The RI [RI = TN/(TN +TF)] is the time spent investigating the novel object relative to the total exploration time and it is considered the main index of retention[232].

In most cases, mice were able to distinguish the novel from the familiar object both at 6 (figure 13, A and B) and 9 (figure 14, A and B) months, indicating a functionally intact memory as shown by DI higher than 0.

When comparing discrimination performance between groups, at 6 months we observed a significant decreased novel object recognition of prenatally stressed Tg males, and not of prenatally stressed WT males, when compared to non-stressed controls (fig.13 A, $F(1,39)=5.845$, $p<0.05$), while the decrease of DI was not statistically significant in prenatally stressed female offspring (fig.13 B). Prenatally stressed Tg male offspring demonstrated also a significant memory deficit for the familiar object (fig.13 C, $F(1,39)=5.884$, $p<0.05$; fig.13 E, $F(1,39)=8.070$, $p<0.05$). On the other hand, female offspring, both WT and Tg, showed intact object recognition memory (fig. 13, D and F) at 6 months. Object recognition memory was not significantly influenced by genotype, as indicated by the absence of significant differences between WT and Tg groups, after *two-way* ANOVA analysis. It seems that PNS influenced memory performance of Tg male offspring by accelerating the onset of the cognitive deficts.

At 9 months a smaller number of animals per group were tested because some mice died before reaching 9 months of age and a fraction of the animal cohort had not reached the 9 month age yet. No significant difference of recognition performance between prenatally stressed Tg male offspring and control was found. We observed a decreased discrimination ability of PNS WT males compared to ctrl WT and of ctrl Tg males when compared to the ctrl WT ones (figure 14 A). On the other hand, PNS-exposed Tg female offspring showed an increasing DI and RI trend (fig. 14, B and D) even if significant differences were not found. The improved discrimination ability of PNS Tg female offspring compared to the corresponding ctrl, as indicated by their significant decreased exploration of familiar object (fig. 14 F, $F(1,15)=5.042$, $p<0.05$), points to a possible PNS positive impact on Tg female recognition performance.

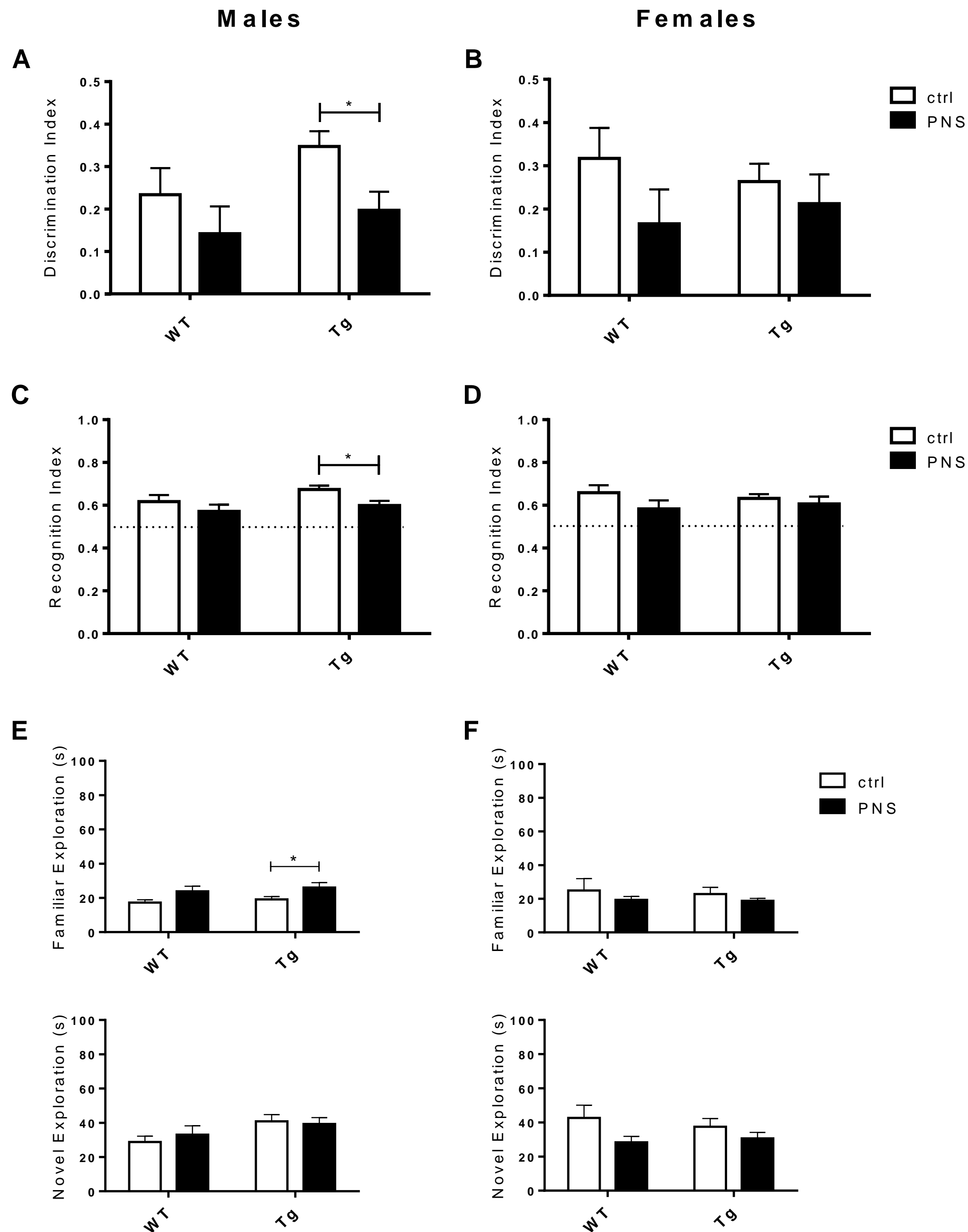


Figure 13. Effect of PNS on long-term recognition memory at 6 months of age. Discrimination Index (**A**, **B**), Recognition Index (**C**, **D**), exploration time of familiar and novel objects (**E**, **F**) during the retention session of NOR test at 6 months in prenatally stressed and control animals. Discrimination Index: (time novel – time familiar)/(time novel + time familiar); Recognition Index (time novel)/(time novel + time familiar). Male offspring: WT ctrl n=8; WT PNS n=8; Tg ctrl n=16; Tg PNS n=11. Female offspring: WT ctrl n=11; WT PNS n=13; Tg ctrl n=14; Tg PNS n=8. Data are shown as mean ± SEM. *$p<0.05$ (*two*-way ANOVA followed by Bonferroni *post* test).

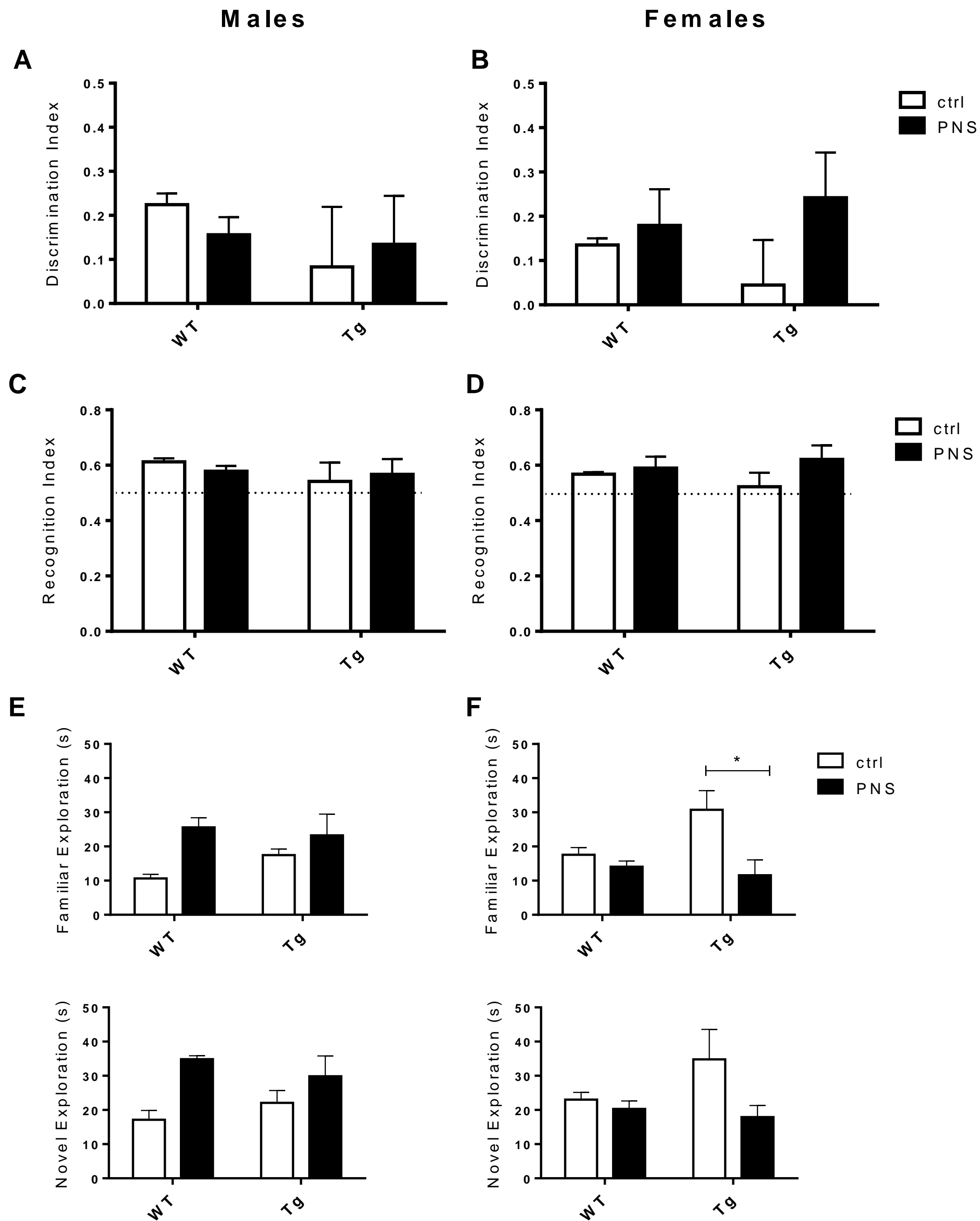


Figure 14. Effect of PNS on long-term recognition memory at 9 months of age. Discrimination Index (**A**, **B**), Recognition Index (**C**, **D**), exploration time of familiar and novel objects (**E**, **F**) during the retention session of NOR test at 9 months in prenatally stressed and control animals. Discrimination Index: (time novel – time familiar)/(time novel + time familiar); Recognition Index (time novel)/(time novel + time familiar). Male offspring: WT ctrl n=4; WT PNS n=2; Tg ctrl n=6; Tg PNS n=4. Female offspring: WT ctrl n=2; WT PNS n=9; Tg ctrl n=6; Tg PNS n=2. Data are shown as mean ± SEM. *p<0.05 (*two-way* ANOVA followed by Bonferroni *post* test).

Early neurobiological effects of prenatal stress (PNS) in embryos at E18.5 and pups at P1

Corticosterone secretion displays sex-dependent alterations in response to PNS-exposure

Pregnant dams underwent prenatal stress (PNS) by the same chronic restraint stress paradigm[214] mentioned in *Materials and Methods* chapter during the last week of pregnancy (embryonic days E12.5 – E18.5) (see scheme 2). The offspring groups included in the following experiments were: wild type E18.5 (embryonic day 18.5) embryos and P1 (postnatal day 1) pups, males and females, of both PNS and control conditions. Blood samples of these animals were collected immediately after the last session of restraint stress (embryonic day 18.5) and one day after delivery (postnatal day - P1) in order to measure corticosterone (CORT) plasma levels and to investigate the potential acute effects of PNS on HPA axis function.

First of all, we planned to determine CORT levels in plasma of stressed (PNS) and ctrl dams immediately after the last day of restraint stress paradigm (E18.5 time point) and one day after delivery (P1 time point) to assess whether the PNS paradigm had properly worked. As indicated by the plasma CORT surge of PNS dams at E18.5, 45 minutes after the beginning of the last stress session, we verified the validity of the PNS paradigm (figure 15 A). Moreover, the obtained data showed an increase of CORT plasma levels in female embryos measured at this time point (fig. 15 A). Embryonic CORT plasma levels upon-PNS (E18.5 time point) were significantly influenced by PNS exposure (fig. 15 A, *two*-way ANOVA: PNS factor, $F(1,19)=9.23$, $p<0.05$).

Furthermore, a sex effect (F(1,18)=26.60, p<0.0001), a PNS-exposure effect (F(1,18)=11.10, p<0.01) and also a sex X PNS-exposure effect (F(1,18)=18.06, p<0.01) were observed at rest in P1 offspring. In particular, PNS significantly increased basal CORT plasma levels only in male P1 pups (fig. 15 B). .

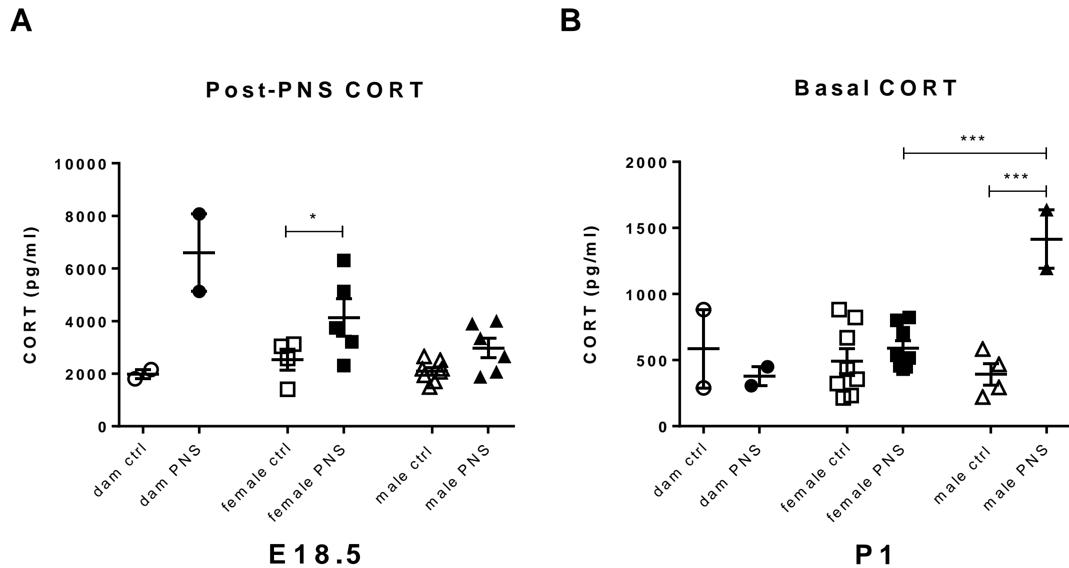


Figure 15. Serum corticosterone concentration after PNS and at rest. A) Corticosterone (CORT) plasma levels in stressed and non-stressed (ctrl) mothers and E18.5 embryos. Blood samples were collected immediately after restraint stress on day E18.5. **B**) Corticosterone (CORT) plasma levels in stressed and ctrl mothers and P1 offspring. Blood samples were collected one day after delivery (postnatal day 1). Data are shown as mean ± SEM. *p<0.05; ***p<0.001(*two*-way ANOVA followed by Bonferroni *post* test).

PNS affects hippocampal gene expression profiles in P1 females

To identify early effects of PNS on brain gene expression and subsequently clarify trigger mechanisms of the potential cognitive impairment caused by PNS during ageing, we analysed expression levels of a number of genes in PNS E18.5 embryos and P1 pups compared to non-PNS controls.

During the prenatal period, the HPA axis is particularly susceptible to programming by glucocorticoids (GCs), that are important for normal maturation in most fetal organs including the developing brain[208]. Moreover, early-life stressful events produce long-term effects on the developing brain, in part mediated by epigenetic modification[241-244], increasing subsequent risk of neuropsychiatric disorders throughout life[245] which are all virtually associated with changes in neuroplasticity and neuroinflammatory processes[246,247]. Based on these notions, we focused on genes implicated in epigenetic regulation, stress-response, neuroplasticity and microglia synaptic pruning pathways. In addition, we determined relative mRNA expression of genes that are specifically expressed in different brain cell subtypes.

E18.5 brain area of interest was the frontal part, while P1 brain areas analyzed were dorsal hippocampus (HPC), prefrontal cortex (PFC) for cognitive functions, and amygdala (AMY) for anxiety-like and impulsive behaviours.

Acute PNS consequences on brain gene expression were different depending on the timing and brain region. Moreover, the PNS impact on developing brain gene expression was different between male and female offspring, since it significantly affected brain gene expression only of P1 females.

In particular, PNS mostly affected gene expression in the hippocampus (figure 16), where down-regulation of genes encoding for the mineralcorticoid receptor (MR) ($p=0.018$), the chemokine C-X3-C motif receptor 1 (CX3CR1) ($p=0.048$) and the DNA methyltransferase 1 (DNMT1) ($p=0.012$) was observed. On the other hand, histone

deacetylase-1 (HDAC1) (p=0.007) gene was up-regulated in hippocampus of P1 female offspring prenatally exposed to stress (fig. 16 A). The tendency for the up-regulation of HDAC1 gene (p=0.055) was already present in the frontal brain region of PNS female embryos even if, in this case, the relative expression difference between PNS and ctrl groups was not statistically significant (figure 17 A).

P1 PNS female offspring showed a significant decreased mRNA level of glucocorticoid receptor (GR) (p=0.004) and BDNF exon IV (BDNF IV) (p=0.02) genes, in the prefrontal cortex (figure 18 B) and amygdala (figure 19 B) respectively. An increased expression of the glial fibrillary acidic protein (GFAP) gene (p=0.037) (fig. 18 D), an astrocytic marker, was also present in the prefrontal cortex of P1 PNS females.

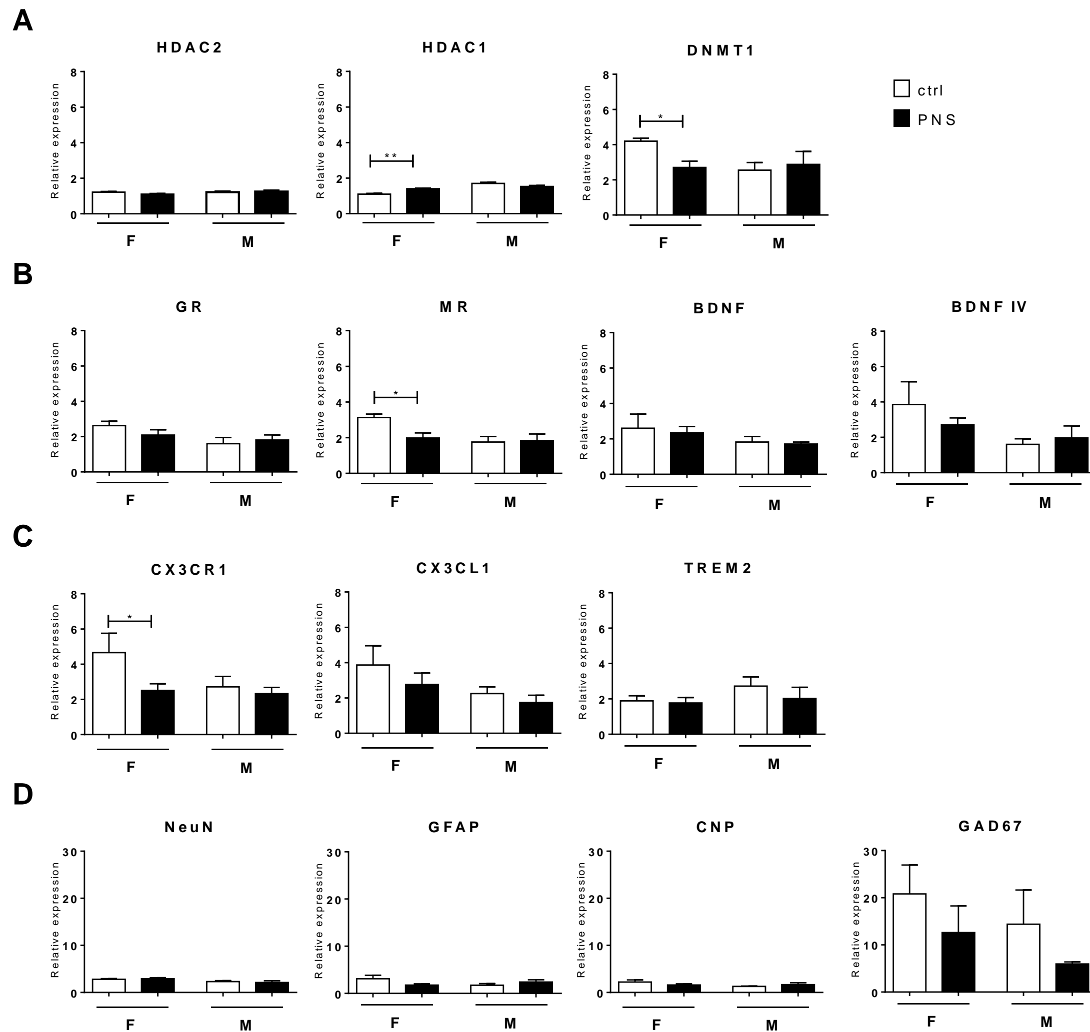


Figure 16. Gene expression analyses in dorsal hippocampus (HPC) of prenatally stressed P1 wild type offspring. qRT-pcr data show relative expression ($2^{-\Delta\Delta Ct}$ method), using EEF1 and TBP as reference genes, of (**A**) epigenetic regulation, (**B**) stress-response, (**C**) neuroplasticity and microglia synaptic pruning genes and (**D**) brain cell subtype markers.

F, female offspring: WT ctrl n=3/4; WT PNS n=5/6. **M**, male offspring: WT ctrl n=4; WT PNS n=4. Data are shown as mean ± SEM. *p<0.05; ** p<0.01 (unpaired *t*-test).

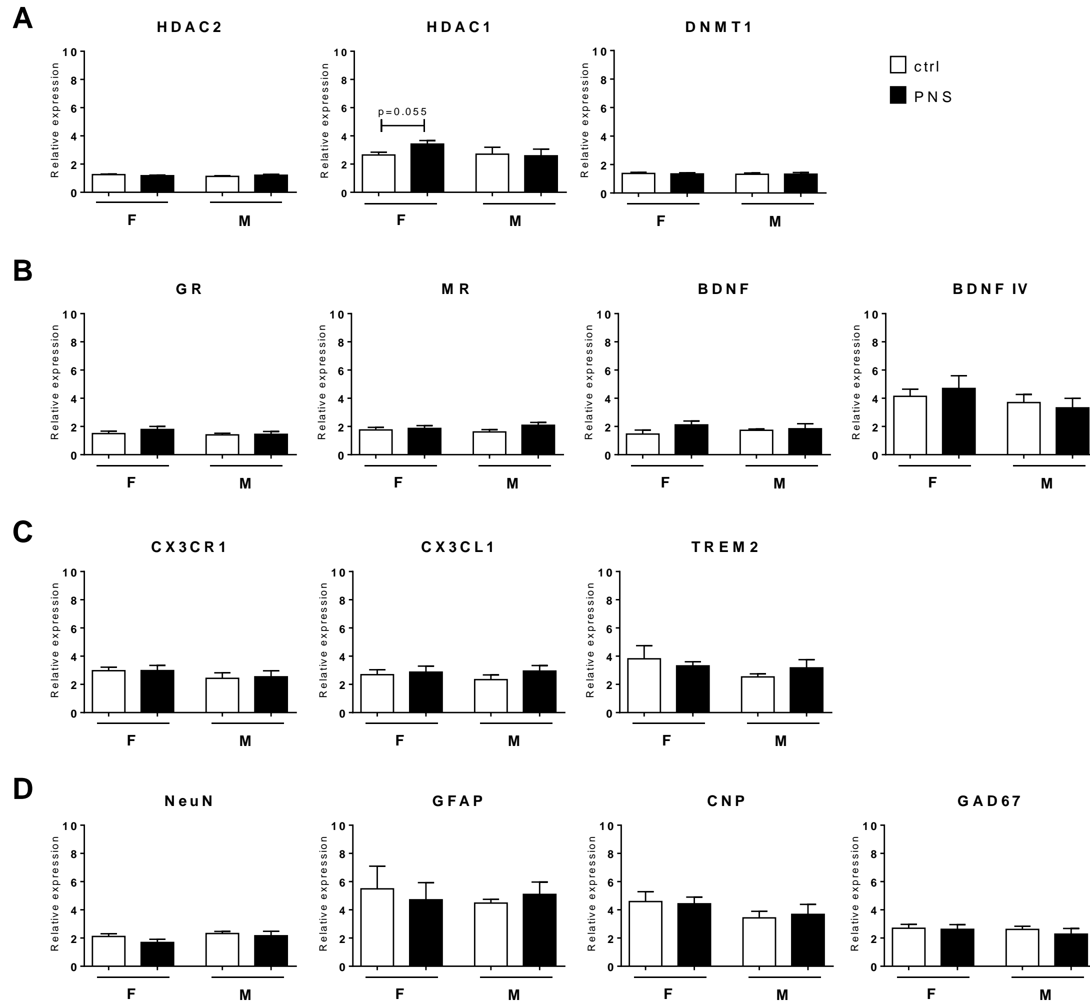


Figure 17. Gene expression analyses in frontal region of prenatally stressed E18.5 wild type embryos. qRT-pcr data show relative expression ($2^{-\Delta\Delta Ct}$ method), using EEF1 and TBP as reference genes, of (**A**) epigenetic regulation, (**B**) stress-response, (**C**) neuroplasticity and microglia synaptic pruning genes and (**D**) brain cell subtype markers.

F, female embryos: WT ctrl n=4; WT PNS n=6. **M**, male embryos: WT ctrl n=4/7; WT PNS n=3/6. Data are shown as mean ± SEM. Unpaired *t*-test.

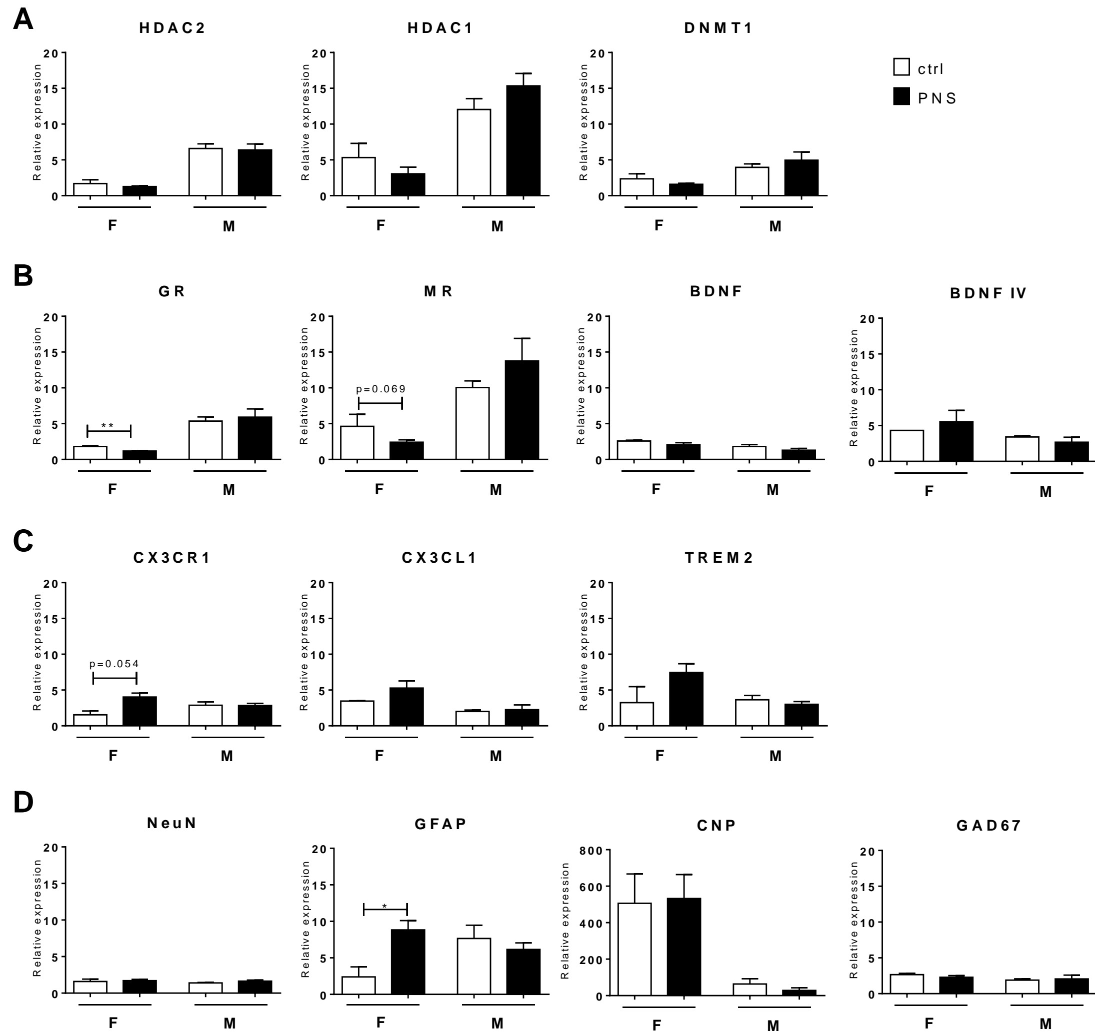


Figure 18. Gene expression analyses in prefrontal cortex (PFC) of prenatally stressed P1 wild type offspring. qRT-pcr data show relative expression ($2^{-\Delta\Delta Ct}$ method), using EEF1 and TBP as reference genes, of (**A**) epigenetic regulation, (**B**) stress-response, (**C**) neuroplasticity and microglia synaptic pruning genes and (**D**) brain cell subtype markers.

F, female offspring: WT ctrl n=2; WT PNS n=3/6. **M**, male offspring: WT ctrl n=4; WT PNS n=4. Data are shown as mean ± SEM. *p<0.05; ** p<0.01 (unpaired *t*-test).

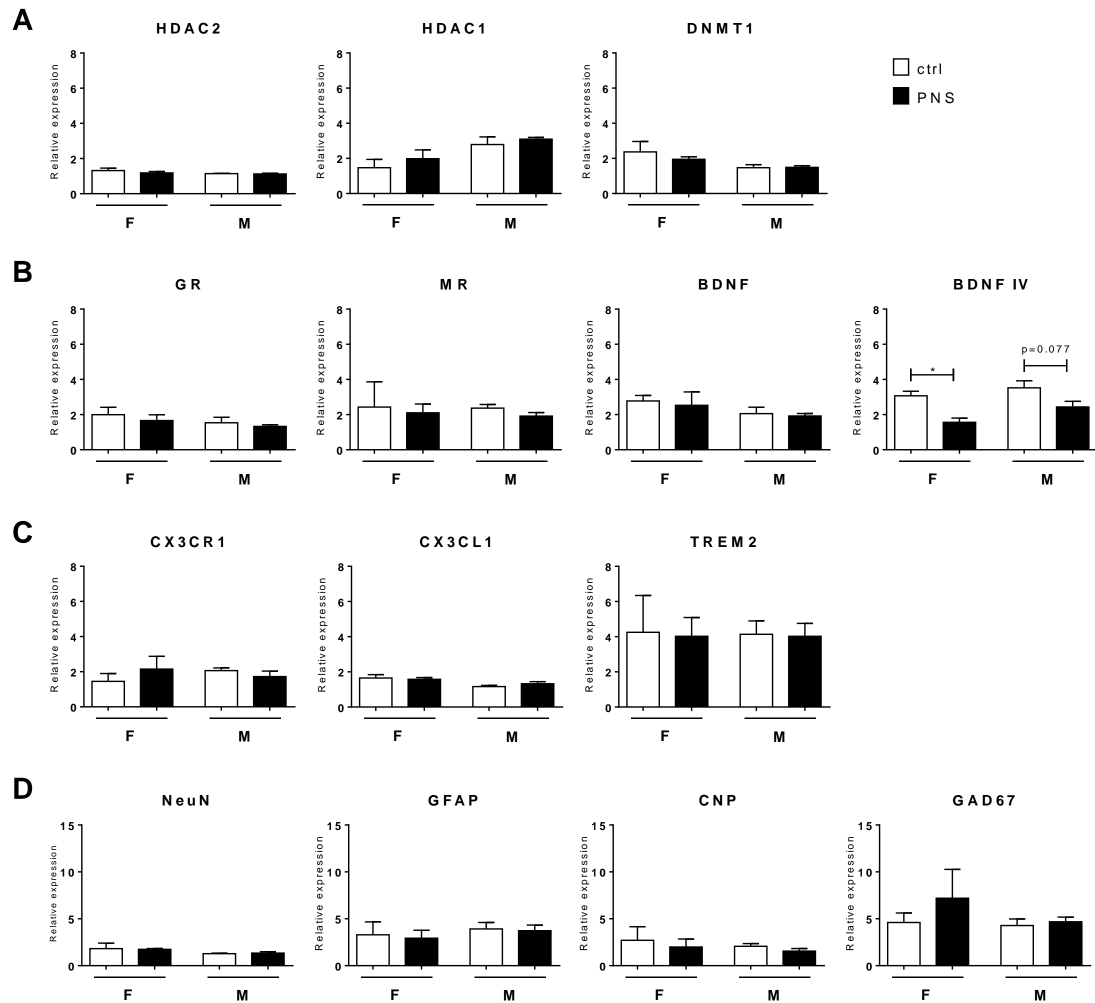


Figure 19. Gene expression analyses in amygdala (AMY) of prenatally stressed P1 wild type offspring. qRT-pcr data show relative expression ($2^{-\Delta\Delta Ct}$ method), using EEF1 and TBP as reference genes, of (**A**) epigenetic regulation, (**B**) stress-response, (**C**) neuroplasticity and microglia synaptic pruning genes and (**D**) brain cell subtype markers.

F, female offspring: WT ctrl n=2; WT PNS n=4. **M**, male offspring: WT ctrl n=4; WT PNS n=4. Data are shown as mean ± SEM. *p<0.05 (unpaired *t*-test).

Discussion

Antiviral innate immune response in the brain of Alzheimer's disease (AD) patients

Alzheimer's disease (AD) is a progressive, degenerative disorder of the CNS and represents the most common form of dementia. Neurodegenerative diseases primarily occur in the later stages of life, positioning ageing as an essential co-factor in their pathogenesis[248]. Ageing affects all physiological systems, of which one of the most important is the immune system[249]. No all immune responses show the same rate of ageing or senescence. Innate immunity is partially affected by human ageing, while adaptive immune response progressively decline with age[149].

Over the past years, GWA studies of sporadic AD cases have shown that several genes with immune regulatory functions were associated with differential risk of AD[75-77,80,82,90]. In this context, it should be mentioned that the well known genetic risk factor for AD, the APOE ε4 allele, may be involved in the dysregulation of phagocytosis and inflammatory responses in the brain[93]. However, the role of innate immunity in the pathogenesis and in clinical history of AD remains to be clarified.

AD is a multi-factorial and heterogeneous disease and among environmental factors likely associated with it, persistent virus infections and the progressive decline of immune competence with advancing age may play a pivotal role[122]. The ageing of the immune system is a dynamic process which partially reflects adaptation of the immune response to an evolving pathogen milieu[249,250]. Several studies suggest that ageing is associated with increased memory T cell compartment in part due to continuous consumption of existing immunological resources by persistent infections[249,251]. Persistent virus infections continue mainly because the viral source is not completely removed by the immune system and usually reside inside certain cell types as immune, neuronal and epithelial cells[249]. Viruses of the herpes family, largely and commonly present in old individuals, undergo frequent cycles of reactivation and latency over the life-time and lead to the accumulation of memory T cells. However, the immune system is not able to completely eradicate these viruses and repeated antigen stimulation induced by them activates a peripheral chronic inflammatory response that

progressively impairs defensive immune ability and aggravates the senescence of the immune system[156,249].

Chronic subclinical infections represent relevant environmental factors for the clinical progression of AD[122]. A declining immune system resulting in a chronic brain inflammation might therefore contribute to neurodegeneration[122,149].

The peripheral immune dysfunction induced by persistent subclinical infections may impair brain functioning by priming microglia and/or by increasing the leaking of the BBB[122]. An altered resolution of inflammatory state has been recently found in the brain of AD patients and such impairment correlated with cognitive performances[252]. Moreover, elevated levels of CNS and CSF inflammatory markers have been reported in preclinical stages of AD[253]. Recent findings reinforced the notion that brain inflammation, as assessed by CSF markers, increases in normal ageing and is associated with markers of neurodegeneration in the preclinical stages of AD[254]. Neuroinflammation in Alzheimer's disease may be partially caused by already primed microglia that may easily switch to a damaging M1 phenotype[255,256]. Animal studies from aged mice showed an exaggerated brain inflammatory and oxidative stress responses to peripheral stimuli[257], increased concentrations of interleukin 1β (IL1β) in the CNS and neuronal apoptosis in the ME7 prion mouse, after peripheral challenge with lipopolysaccharide (LPS) or polyinosinic-polycytidylic acid (polyI:C)[258-260].

AD microglia might also be primed by infectious agents challenging the CNS as viruses belonging to the herpes family. There are several studies implicating HSV-1 in the etiology of AD[133,134,136,261]. The first observations of HSV-1 in AD brains were reported almost thirty years ago[142]. HSV-1 is a ubiquitous virus that affects more than 80% of people over 65 worldwide. It is a neurotropic double-stranded DNA virus that primarily infects epithelial cells of oral and nasal mucosa. Here, HSV-1 undergoes lytic replication; the newly produced viral particles thereafter enter sensory neurons and, by

axonal transport, reach the trigeminal ganglion where usually establish a latent infection. HSV-1 undergoes periodic reactivation cycles in which the newly formed viral particles are transported back to the site of primary infection through the sensory neurons, causing typical epithelial lesions. However, the bipolar trigeminal ganglion neurons also project to the trigeminal nuclei located in the brainstem. From here, neurons project to the thalamus to finally reach the sensory cortex. This is the path through which the reactivated virus may reach the CNS, where it may cause acute neurological disorders like encephalitis or a mild, clinically asymptomatic infection, or establish a life-long latent infection[149,262]. A reactivation of HSV-1 infection, assessed by increased serum levels of specific anti-HSV-1 antibodies, was found associated with an increased AD risk in a longitudinal study on 3432 elderly[151].

Other pathogens have been implicated in the pathogenesis of AD[135,164] and chronic infections, in a context of senescent immune system, are emerging risk factors for this disease[143]. A recent review by Harris et al. confirmed that infection agents such as CMV, HSV-1, HHV-6, *Helicobacter pylori*, *Chlamydophila pneumonia* and several periodontal pathogens are able to induce the production of peripheral proinflammatory cytokines that, by crossing the BBB, might promote neurodegeneration[135]. In addition, spirochetes have also been proposed to be associated with AD[137]. Moreover, oral infections have been recently suggested as potential causes of BBB disruption and brain inflammation. These pathogens may also infect the brain via trigeminal and/or olfactory nerves[135,263].

On the other hand, the Aβ peptide has been shown to function as a defensive immune factor in the brain[264]. The physiological function of the APP and the biological role of its proteolytic derivatives are still uncertain[265]. In mice and in cell culture, Aβ deposition and tau abnormalities, typical of AD, are observed after infection with HSV-1[266-275] or bacteria[276-279] and a direct interaction between APP and HSV-1 has been reported[154]. Recent investigations confirmed that Aβ is an antimicrobial peptide (AMP) with potent

activity against bacteria and yeast[264] but also against viruses. Experimental evidences showed that Aβ peptides inhibit influenza virus[280] and HSV-1[281,282] replication. Carrano and colleagues suggested that APP and/or its cleaved products are necessary to mount a complete and effective innate immune response to inflammatory injury in the brain, being involved in microglia activation[283]. These data reinforced the notion that the Aβ peptide might be a component of the innate immunity against brain pathogens, using a classic AMP mechanism characterized by reduce microbial adhesion to host cells, and agglutination and entrapment of microbes by Aβ fibrils[284,285].

The above findings are compatible with the hypothesis that neurodegenerative mechanisms associated with clinical AD might be in part induced or affected by chronic infections of the ageing brain. In previous publications, Licastro et al. discussed genetic data from four AD GWA studies[75-77,90]. From these investigations a set of SNPs associated with AD emerged and they suggested that the concomitant presence of these SNPs might result in a genetic signature predisposing to AD, via complex and diverse mechanisms, each contributing to an increased individual susceptibility to herpes virus infection[88,89]. Therefore, neurotropic herpes viruses may directly infect and damage selected brain areas in genetically susceptible elderly and induce neurodegenerative mechanisms.

However, it is also relevant how the host responds to these microorganisms. In fact, the individual genetic background plays a pivotal role in the maintenance of the chronic inflammation both in the brain and in the peripheral tissues[149]. In this context, as already mentioned, GWA studies in AD showed that several immune factors were associated with increased risk of the disease, but each single immune gene showed a low OR (odds ratio < 1.7) of association with AD. The only exception was the APOE ε4 allele, which is also a well known susceptibility factor for several virus infections[286-288]. The weak association of immune genes with AD can be simply explained as no immune factor is the cause of the disease. Nevertheless, the concomitant presence of

several genetic factors in the same individual might show a stronger association and individual infection susceptibility may be affected by the concomitant presence of alleles resulting in decreased immune efficiency[88,89]. As infections appear to be involved in AD pathogenesis, the link between a given pathogen and the host susceptibility to its infectivity might be one missing link in the clinical progression from cognitive decline to AD[149].

At the present, little is known about the efficiency of immune factors involved in antimicrobial defences in the human brain.

In the current study, we have attempted to evaluate the role of interferon regulatory factor (IRF) 7, mediator complex (MED) 23, interferon (IFN)-λ3, also known as IL28B, and IFN-α genes in the brain of AD patients as well as the potential association of their expression in hippocampus and temporal cortex. Since our recent findings showed that diverse genetic backgrounds in genes regulating antiviral responses were associated with an increased risk of AD[88,89,164,201], the focus of this thesis was set on SNPs of APOE gene and SNPs located upstream of the IRF7, MED23 and IL28B genes, and their potential effect on the brain gene expression profiles.

IFN-α and IL28B are interferons belonging to the type I and III IFN family respectively and they induce a strong antiviral state in responsive cells. Whereas almost all nucleated cells respond to type I IFNs, responses to type III IFNs are restricted to tissues with an increased propensity to viral exposure and infection, such as those at mucosal surfaces[190]. There is mounting evidence for a role of the type III IFNs in the regulation of virus infection[188], particularly in the case of HCV infection[289,290]. Moreover, individuals with recurrent HSV-1 reactivation have been shown to be deficient in IFN-λ expression[291] and it has also been found an association between a SNP in the promoter of IL28B and ethnically Italian patients suffering recurrent and severe reactivations of HSV-1-related oral herpes outbreaks[196]. Type I IFNs, mainly produced

by HSV-infected keratinocytes[292] and pDCs (plasmacytoid dendritic cells)[293], inhibit the spread from neurons to epithelial cells and between epithelial cells[294]. Type III IFNs are also able to directly inhibit HSV-1 infection in primary neurons, astrocytes, macrophages and dendritic cells[295,296] and are mainly expressed by myeloid dendritic cells (mDC) and monocyte-derived macrophage[191]. Since primary HSV-1 infection and reactivation affect skin and mucosa in the majority of cases, IFN-λ may play a role in the control of HSV-1 infection and reactivation.

Using a genome-wide approach to identify host factors that functionally influence HSV-1 infection *in vitro*, Griffiths and collegues identified a subunit of the Mediator multi-protein complex, Med23, as a key regulator of IFN-λ induction and of control of HSV-1 infection both *in vitro* and *in vivo*[196]. This study identified Med23 as a novel antiviral factor which acts as a key regulator of IFN-λ expression by interacting with and enhancing the activity of IRF7. Investigations into the mechanism of action revealed that Med23 inhibits HSV-1 replication by preferentially inducing a IFN-λ response at the mRNA and protein level. This IFN-λ induction was mediated via a direct interaction with IRF7, which resulted in a synergistic increase in IFN-λ expression. Interestingly, the inhibitory effect of Med23 was specific to HSV-1[196]. The failure to induce IFN-λ and thereby control HSV-1 in the brain may be a potential cofactor for the development of dementia of Alzheimer's type[89].

The study presented here showed that the expression of genes involved in antimicrobial defences, especially against virus infections, such as IRF7, MED23, IL28B and IFN-α was defective in the majority of AD brains. There was a tendency for the down-regulation of these antiviral innate response genes in the brain of AD patients, particularly accentuated in the hippocampus area. The defective brain gene expression found in most AD patients might compromise the efficiency of immune responses and retard or impair the eradication of brain invading microbes. It is intriguing reminder that type I IFN has been shown to increase autophagy, an emerging

antiviral defense mechanism of neurons[297]. Moreover, autophagy-lysosome defects have been reported to occur early in the AD pathogenesis[298]. Therefore, decreased signaling by IFN molecules in the AD brain might reverberate upon other antimicrobial mechanisms and increase neuronal susceptibility to infections and neuroinflammation.

Possible regulatory mechanisms of the innate immune genes upon Aβ peptide expression in normal or AD brains have been poorly explored. However, it cannot be excluded that Aβ peptide might be an emergency defensive mechanism compensating the declining expression of other specialized defensive immune genes in the ageing brain.

Among the genetic risk factors for AD, the presence of the APOE ε4 allele plays a major role and the APOE status might influence different mechanisms involved in neurodegeneration. Here, we showed that brain hippocampal expression of MED23, IL28B and IFN-α mRNAs in APOE ε4 carrier AD patients was significantly decreased.

It is known that APOE affects immunity, since increased systemic proinflammatory states and altered immune responses have been found in APOE deficient mice[299,300]. Therefore, we speculate that, at least, part of the increasing AD risk effect of the APOE ε4 allele might be mediated by a negative influence on the brain immune efficiency in selected CNS areas, such as the hippocampus, where replicating neurons[301] might be more susceptible to virus infections or microbial products/toxins.

Some studies have suggested that in people carrying the APOE ε4 allele and, therefore, predisposed to develop AD, occurrence of HSV-1 infection was increased[302-304]. However, this correlation was not always confirmed[305]. APOE seems to affect the outcome of different infections[303,306] and, interestingly, APOE ε4 is also a risk factor for cold sores[307]. Animal studies demonstrated that APOE4/4 mice had an impaired microglia immune functionality[308] and ApoE4 presence also influenced the viral load in the brain[309]. In a subsequent study, Burgos et al. showed that during acute infection

with HSV-1, ApoE4 was more efficient than ApoE3 in promoting viral colonization of the brain[144].

To explore the influence of individual genetic background on the observed impaired expression of antiviral response genes in AD brains, we have stratified their gene expression data with SNPs genotypes of IRF7 (*rs*6598008), MED23 (*rs*3756784) and IL28B (*rs*12979860).

The presence of GA and AA genotype in the upstream IRF7 variant (*rs*6598008) was associated with decreased expression, in the hippocampus, of IRF7, MED23, IL28B and IFN-α, which are all involved in the innate immune control of HSV-1 infection[196]. Therefore, we suggest that AD patients with A allele for IRF7 *rs*6598008 may be more prone to deficient innate immune response and predisposed to brain infections with severe course leading to neurodegeneration.

Individual genetic variations as APOE ε4 and IRF7 SNP (*rs*9568008) might be relevant in affecting antiviral gene responsiveness in the presence of specific stimuli as microbial infections.

Susceptibility to complex heterogeneous diseases, like AD, depends on both genetic predisposition and exposure to environmental factors, with interactions between the two components that likely contribute substantially to the degree of disease risk[310,311]. However, the extent and mechanisms by which common human genetic variants affect the host response to environment factors remain inadequately explored and have been difficult to detect in clinical studies[310,312].

A minority of hippocampal AD samples included in this study showed up-regulation of MED23, IL28B and IFN-α genes. Such increased levels of mRNAs were not correlated with the presence of APOE ε4 or IRF7 A alleles (*rs*6598008). Therefore, AD shows heterogeneous alteration of IFN gene expression. Increased or decreased expression might be related with different clinical stages of the disease and parallel cycles of

reactivation and latency of neurotropic viruses. In this context, we hypothesize the existence of other candidate genetic variants potential associated *in trans* with type I and III interferon induction, in response to stimulus such as the persistence of virus and/or bacterial infections, and potentially linked to exacerbated brain immune actions. Alternatively, oral infections, that were reported as potential causes of BBB distruction[135], might induce an exacerbated IFN-λ response of chroid plexus epithelial cells[313] in the presence of neuro-invasive viruses, as those belonging to herpes family.

In animal models, differences in treatment with various stimuli have revealed the existence of reQTLs[314-317] (response expression Quantitative Trait Loci), defined as QTLs associated with the change in expression after stimulation. The reQTLs identified by Lee and colleagues provide genetic explanations for inter-individual variation in innate immune responses[318]. In particular, their study revealed the effects of a *trans*-reQTL in the IRF7 locus on target antiviral genes in the context of a particular cell type, such as DCs, and in response to specific ligand, such as influenza virus. The changes in this immune response were, in turn, likely to affect organism's phenotypes that are driven by the IFN module, including susceptibility to viral infections[318].

This work indirectly supports the notion that a defective brain response to microorganisms is a risk factor for developing clinical AD. Microbial infections may play a role in AD clinical progression and impaired innate immune responses against viruses, bacteria or their products may increase neurodegenerative mechanisms in the elderly. However, these preliminary results should be interpreted with caution and they should be replicated in a larger population of patients.

Effects of prenatal stress (PNS) on adult cognitive health

AD is probably best considered in a life-course framework, like other chronic diseases, with important influences beginning at conception and early-life moments. While most cases of AD occur at late onset and older ages, increasing evidences support the notion that the neurodegenerative alterations precede AD clinical manifestation by many decades[319]. Both genetic and environmental factors are involved in the onset of sporadic AD. Several epidemiological studies have indicated that environmental factors, such as chronic stress and stress-related disorders, can influence the progression of AD-related symptoms and pathologies[205-207].

AD mouse models at different life stages have demonstrated that stress exposure can alter the neuropathological process causing reduction of neurons in memory regions, along with increased deposits of Aβ peptide and hyperphosphorylated microtubule associated tau protein[320-322]. Contrary to infancy and adulthood mouse models, only few studies investigated the role of stress prenatally experienced on the development of AD[319].

There is growing evidence that proper organism development is strongly influenced by early-life environment[323]. It has been observed that infants with low birth weight and small head circumference are at higher risk of suffering coronary heart disease, hypertension, stroke, insulin resistance, diabetes and other diseases in adulthood[324]. According to this experimental evidences, Barker proposed the *fetal origins* hypothesis in which the intrauterine environment has significant impact on the development of chronic diseases[325]. Lahiri and colleagues suggested that many neurobiological disorders, including AD, share a similar mechanistic etiology: the *Latent Early-life Associated Regulation* or LEARn model[126,221]. Under LEARn, early-life stressors modify potential expression levels of disorder-associated genes in a latent manner. Latent changes in these genes are maintained by epigenetic mechanisms. This model makes the greatest allowance for sporadic appearance of a disorder, especially if it is actually

”many” or “n hit”, where “n” can be any number of individual risk factors, each with the potential to instil or activate a latent alteration[326].

Many studies have shown that stressful events during the intrauterine life can exert long-lasting effects on the brain by affecting the nervous, neuroendocrine and immune systems[209-211]. Moreover, studies in humans and rodents, revealed a clear impact of early-life stress on the development of neuropsychiatric disorders such as autism, mood disorders and schizophrenia[208,327,328]. Interestingly, few studies have also linked prenatal stress (PNS) to adult cognitive impairment[214,215,329].

Based on these grounds, we planned to get a deeper insight in the molecular mechanisms at the basis of potential cognitive impairment caused by prenatal stress in mice.

In this study we investigated the effects of prenatal stress (PNS) on the onset and progression of AD-related behavioural deficits, focusing also on potential sex-dependent differences. To address this problem, we assessed the consequences of PNS on adult behaviour in wild type (WT) and APP/PSEN1dE9[330,331] (Tg) offspring, testing them for anxiety-related behaviour and for learning and recognition memory at six and nine months of age.

PNS during late gestation (embryonic days 12.5 – 18.5) did not elicit anxiety-related behaviour during late adulthood. Indeed, the open-field test and the following analyses of locomotor, central and/or peripheral activities of WT and Tg offspring (male and female) did not reveal any significant differences between PNS and ctrl groups neither at six nor at nine months. Similarly, Sierksma et al. showed that anxious behaviour remained unchanged in both prenatally stressed male and female Tg offspring. On the other hand, other studies showed that PNS induces anxiety-traits in young adulthood in WT offspring and the discrepancy with our results might depend on the kind of PNS, its timing and on the age of the tested offspring[332-335].

Furthermore, at six months we found that PNS exposure impaired significantly long-term recognition memory performance only of Tg male offspring, affecting the onset of AD-related recognition memory deficit. The evidences of memory stress-damage in six month old mice, for which the AD has not entailed recognition memory deficit yet, was not observed in nine month old mice. The nine month mouse memory has been already damaged by the AD neurodegeneration, minimising therefore the gap between the stress-exposed and non-exposed stress animals. In summary, these data showed a decreasing tendency in novel object recognition in Tg males that have been prenatally stressed, while prenatal stress seems to exert a protective effect on recognition memory of APPswe/PSEN1dE9 females.

These results are in line with Sierksma and colleagues who found that repetitive restraint stress during the first week of gestation exerted a sex-dependent effect on behaviour and AD-related neuropathology in APPswe/PS1dE9 mice. Prenatally stressed male offspring showed spatial memory deficits and a blunted HPA axis response, while female offspring showed increased depressive-like behaviour and improved spatial memory performance with a decrease in hippocampal plaque load[214]. According to our results, it has been shown that AD mouse models exposed to stress during various stages of life alter AD-related symptoms and pathology[320-322,336-338]. Restraint stress during adulthood resulted also in elevated hippocampal concentrations of Aβ40 and Aβ42, higher plaque deposition, and increased tau phosphorylation. In addition, these mice displayed augmented neuronal degeneration with concomitant cognitive deficits[338-342]. Furthermore, several animal studies showed that prenatal maternal stress correlates with a series of neurological impairments, such as cholinergic neuronal damage, cognitive decline, hippocampal neuronal loss, increased tau protein phosphorylation and A*β* peptide deposits[343-345].

As a part of this ongoing project, the next step will be neuropathological phenotyping, by determing hippocampal plaque load and intracellular Aβ, of the WT and Tg offspring

to assess whether impact of PNS on the onset of AD-related cognitive decline correlates with amyloid burden.

Prenatal life is a critical period characterized by increased vulnerability to stressors[213,346]. The process by which perinatal life events can have long-term effects on physiological system has been described as perinatal programming. During the perinatal period, the HPA axis is particularly susceptible to programming by glucocorticoids (GCs) that are important for normal maturation of most fetal organs including the developing brain. Thus, GCs are prime candidates for perinatal programming[347] and exposure of the developing fetus or neonate to these glucocorticoid signals in excess or at an incorrect stage of maturation might lead to substantial alterations in normal developmental trajectories, resulting in altered physiological function throughout life and pathology in some circumstances[348,349]. In the brain, excess exposure to GCs leads to cognitive decline and has been associated with hippocampal neuron endangerment, dendritic atrophy and synaptic loss[350,351].

To find out PNS early target genes in the brain and to shed light on neurodevelopment trigger mechanisms of the cognitive impairment caused by PNS in APP/PS1 offspring, PNS neurobiological effects on WT E18.5 and P1 offspring were investigated. In particular, we focused on acute PNS effects on HPA axis function and brain gene expression.

We found a different PNS influence on HPA axis between male and female WT offspring. PNS led to enhanced embryonic female HPA axis stress-response and to increased P1 male basal HPA activity, and this could result in an impaired programming of HPA function. It is important to consider that several confounding factors are present in the literature on this field. Within a given species, the stress-timing, -dose and –source often differ among studies. The stage of development at which the outcome is measured and the sex of the offspring are other critical factors[211].

All of these aspects will lead to differences in the measured outcomes and the presence of such confounding factors clearly require further repetition of the same experiment in order to improve the validity of our results.

PNS consequences on brain gene expression of WT E18.5 and P1 offspring were different depending on the brain region, timing and sex. PNS significantly affected gene expression profiles of hippocampus only in P1 female offspring. The hippocampus is crucial for learning and memory processes. The susceptibility of the developing hippocampus to increased levels of GCs is linked to the high expression levels of their receptors[209,211,319]. We observed significant down-regulation of mineralcorticoid receptor (MR), DNA methyltransferase 1 (DNMT1) and of chemokine C-X3-C motif receptor 1 (CX3CR1) genes and up-regulation of histone deacetylase-1 (HDAC1) gene in P1 female offspring prenatally exposed to stress.

The hippocampal decrease of MR mRNA expression of PNS P1 female WT offspring might be involved in feedback control of HPA axis during stress mediating alterations in glucocorticoid feedback sensitivity. Historically, because of different affinities for GCs, MR was considered important in regulating basal HPA activity, with GRs being occupied under conditions of elevated GCs (e.g. after stress); however, more recent evidences indicate that both receptor types are involved in HPA axis feedback control during stress[204,352,353]. The mechanisms reducing hippocampal MR mRNA expression in PNS mice are not defined; it is likely that increased levels of in utero corticosterone might affect MR gene promoter by influencing its expression. Whether PNS leads to MR gene methylation, as proposed for early-life programming of the GR gene[244], is still far to be understood[354].

Different studies have linked PNS to impairments in adult cognitive heath, but little is known about the effects of PNS on epigenetic regulation despite prenatal life being a critical period for the programming of the epigenome[355]. DNA methylation players, including DNMT1, have a fundamental role in pre- and post-natal neurodevelopment[356-

[360]. In dividing cells, DNMT1 ensures that the parental DNA methylation pattern is maintained in the daughter cells, while in adult CNS neurons it is required for synaptic plasticity, learning and memory[357]. An histone modifying enzyme shown to interact with DNMT1 is HDAC1[361]. Fuks and colleagues identified a transcriptional repression domain in DNMT1 that functions, at least partly, by recruiting HDAC1 and they suggested that the process of DNA methylation mediated by DNMT1 may depend on or generate an altered chromatin state via histone deacetylase activity[361]. Akhtar et al. proposed that HDAC1, which is predominantly expressed in neural progenitor cells and glia[362], together with HDAC2 forms a developmental switch that controls synapse maturation and function acting in a manner dependent on the maturational states of neuronal networks[363]. Our data have shown a PNS-induced altered expression of DNMT1 and HDAC1 genes in hippocampus of P1 female WT offspring and we hypothesize that PNS could affect or modulate synapse development during perinatal life modifying their expression. Evidence is accumulating regarding the opposite effects of PNS in males and females[208,209,211,364], but the precise molecular mechanism underlying this sex-dependent difference is still unknown. The difficulty to unravel this mechanism is then increases by the fact that in the few studies found in the literature, researchers do not differentiate PNS effects according to the sex of the offspring[215,335,357,363].

Microglia were found to be crucial for the elimination of redundant dendritic spines by phagocytosis during brain development[365]. This so-called “synaptic pruning” is necessary for the proper formation and maturation of neural circuits and it is thought to involve CX3CL1, expressed by neurons during synapse maturation, and its receptor CX3CR1 on microglia[365]. However, the mechanisms that underlie microglial–synapse or microglial–axonal interactions remain unsolved[96]. CX3CR1 gene was found to be up-regulated in the hippocampus of PNS P1 female WT offspring. Matcovitch-Natan et al. have identified a stepwise developmental program of microglia in synchrony with the developing brain and suggested that genetic or environmental perturbations of these

pathways can disrupt stage-specific functions of microglia and lead to loss of brain homeostasis, which may be associated with neurodevelopmental disorders[366].

The trend resulting from different animals studies suggest that exposure to prenatal stress can lead to significant increases in microglial activity[327,367,368]. Of these studies, the two by Diz-Chaves et al. found potentiated responses in prenatally stressed animals that were subsequently given an immune challenge in adulthood[327,367]. The effects of maternal/early-life stress on microglial activity closely resembled that caused by pathogenic immune challenge in rodents[369,370]. Such studies support the idea that stress leads to microglial priming. The initial prenatal stimulation primes microglia inducing an exaggerated microglia response to a second inflammatory stimulus[371]. This underlies the “n-hit” hypothesis of the LEARn model[326] according to which early-life stress sensitises microglial cells so inducing an exaggerated microglial responsiveness to stress in late adolescence or adulthood. This abnormal microglial activity may lead to brain changes that underlie the development of CNS chronic diseases[372]. As demonstrated by the preclinical studies, many forms of psychosocial stress are able to stimulate microglial activity throughout the brain, and in several cases, prenatal stress exposure is sufficient to cause lasting changes in microglial response[372].

Based on collected data, we suggested that the molecular players of PNS consequences in the brain have to seek within microglial synaptic plasticity pathways during neurodevelopment. Furthermore, in the attempt to discover the “hot spots” of PNS neurodevelopment impairment, it would be appropriate to focus on microglia cells and their role in synaptic plasticity both in female and male offspring. Future research will focus on the role of the same genes in synaptic remodelling and loss in APP/PSEN1dE9 offspring prenatally exposed to stress in order to test their roles in PNS-induced cognitive impairment.

Conclusion

AD is a complex multi-factorial disease in which several pathogenetic, clinical, environmental and stochastic factors are involved.

It is on record that persistent virus infections and the immune competence decline with ageing might play a pivotal role in AD[122]. In particular, defective immune defences against viruses may play a role in triggering chronic inflammatory responses and directly or indirectly activate neuroinflammation[261]. Here, we show that in patients with clinical and neurological defined diagnosis of AD, antiviral immune response appears to be defective in the majority of AD brain samples. Moreover, gene variants of APOE and IRF7 genes strongly affect mRNA levels of IRF7, MED23, IL28B and IFN-α in hippocampus area. This brain area shows an extensive and early neurodegeneration during AD clinical progression[373]. Therefore, these findings indirectly support the notion that microbial infections may play a role in AD clinical progression and deficient innate immune responses against viruses, bacteria or their products may increase neurodegenerative mechanisms in the elderly.

The second part of this thesis shows that experimental animal research has the advantage of enabling strict control of environmental factors, such as prenatal stress (PNS) exposure, that might have a role in AD-related behaviour and neuropathology[127,128,205]. In particular, we investigated the long-term cognitive consequences of PNS in AD mice and the PNS-early neurobiological effects in wild type animals. As these, mice are a useful model to suggest that PNS affects the onset of cognitive deficit in AD mice in a sex-dependent manner. Our findings highlight that preventing the impairment of fetal neurodevelopment might be important for ensuring not only the future adult mental health[374], but also for ageing well. Based on these results, AD is probably best considered in a life-course framework, with important influences beginning also at conception and early development.

In conclusion, the presented study gives new perspectives for prevention and treatment of the ageing-associated cognitive decline and AD. Protecting women from chronic stress during pregnancy, on the one side, and maintenance of efficient immune responses during ageing, on the other one, might slowdown neurodegenerative mechanisms associated with senile dementia and positively influence both prevalence and incidence of AD.

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