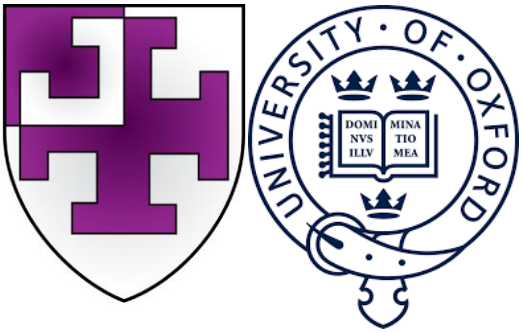
CENTRAL NERVOUS SYSTEM AUTOIMMUNITY IN NEUROPSYCHIATRIC DISORDERS

Maria Ester Freitas Barbosa Pereira Coutinho

St. Cross College



A thesis submitted for the degree of
DOCTOR OF PHILOSOPHY IN CLINICAL NEUROLOGY

Nuffield Department of Clinical Neurosciences
Medical Sciences Division
University of Oxford

Trinity term, 2016

Central nervous system autoimmunity in neuropsychiatric disorders

A thesis submitted for the degree of Doctor of Philosophy

Trinity term, 2016

Abstract

The recent history of autoimmune neurology is marked by the discovery of many central nervous system (CNS) antibody-mediated diseases. These disorders are caused by antibodies that target important proteins expressed in the neuronal surface, which are believed to be directly pathogenic. These antibodies are immunoglobulin G (IgG) isotype and, as such, have the potential to cross the placenta during gestation. Foetal exposure to CNS-targeting antibodies could alter developing neuronal circuits, leading to disease. However, the consequences of exposure to these antibodies during neurodevelopment has hardly been considered. To study the relationship between maternal antibodies towards neuronal surface proteins and neurodevelopmental disorders in the foetus a dual approach was undertaken. First, pregnancy serum samples from mothers of children later diagnosed with a neurodevelopmental disorder and from mothers of children with typical development were screened for the presence of neuronal surface antibodies. Next, the effects of pathogenic neuronal surface antibodies in the offspring were assessed in a maternal-to-foetal transfer mouse model. Antibodies to neuronal surface proteins in the gestational serum, particularly CASPR2 antibodies, were found to associate with an increased risk of mental retardation and disorders of psychological development in the progeny. The animal model showed that mice exposed *in utero* to CASPR2 antibodies have long term behavioural sequelae and histological findings suggestive of abnormalities in brain development.

These findings support a model in which maternal antibodies towards foetal neuronal proteins cause long-term behavioural deficits and permanent abnormalities at the cellular and synaptic level in a subset of children with neurodevelopmental disorders.

Acknowledgments

This thesis was made possible through the help and encouragement I received from others, to whom I will forever be indebted to.

First, I would like to thank the Gulbenkian Programme for Advanced Medical Education, which not only provided 8 months of graduate courses that improved my scientific background, but also funded me for 3 years of my DPhil. It is a very special programme and I'm humbled to have been a part of it.

Next, I would like to thank my supervisors, Prof Angela Vincent and Prof Paul Harrison. It would have not been possible to conclude this thesis without Angela's support and encouragement throughout the years. She was a magnificent supervisor who gave me the freedom to pursue my own research interests, even when results were disappointing at first. It is truly inspirational to observe your continuous enthusiasm as a scientist and the caring way you help fellow students and other members of your lab. Likewise, Paul was also extremely supportive in very crucial moments. It is not easy for a neurologist to venture into "Psychiatry-land" but I could always count on him for good guidance.

I also want to extend my acknowledgments to Prof Bethan Lang who not only allowed me to work on the animal model under her project license, but also took on the role of supervisor at a later stage. I also thank Dr Isabel Leite who first introduced me to research in 2010, during my EFNS fellowship, and later encouraged me to pursue it.

I was also very fortunate to have met and worked with Drs Teresa Moloney and David Menassa. Without any formal obligation towards me, Teresa patiently taught me most of what I've learnt in the lab. Most importantly, she also had the extraordinary capacity of making anything bad sound a thousand times worse; so much worse that we invariably ended up laughing about it. Thank you, I don't think I would have survived without your humour. David came at a later stage in my DPhil and was truly a godsend in a period of

much need. He shared with me all his histology / imaging expertise and I can say that this work would literally look “paler” if not for him. If this wasn’t enough, he also charitably proof-read this manuscript. I’m very grateful for all your work, enthusiasm and friendship.

I am extremely grateful for the help I received from all the other members of the neuroimmunology lab. I would like to acknowledge in particular Dr Leslie Jacobson who has always been extremely helpful, both in the study of human cohorts and in the animal model initial experiments. I can almost forgive him for his numerous attempts to give me a heart attack by sneaking upon me and giving me a fright. I also want to thank Dr Joana Domingos who kept me going during the insane month of neonatal behavioural experiments. Not only was she by my side conducting these experiments every single evening for over a month, she did it with contagious joy and enthusiasm.

My acknowledgments go beyond the limits of my lab. During the years of my DPhil I was truly blessed by many researchers in the University who generously spent their time discussing my work, giving me advice or practical help. I am extremely grateful to Prof David Bannerman and his group for all the help I received during the behavioural studies. After the first negative results, I would probably have not persevered if not for David’s encouragement. I also would like to thank Prof David Bennet and particularly Dr Steven West for the help with synaptic profiles quantification.

I am also grateful to our collaborators in Denmark, Marianne Pedersen, Michael Benros, Profs Mortensen and Norgaard-Pedersen for allowing me access to the fantastic resources of the Danish Biobank and Registries.

And finally, my gratitude goes to those that in the backstage made it all possible: my family. To my mother and my brother, whose continuous love and encouragement forms the trampoline that sends me flying and cushions my falls. And to Quan, who suffered all my disappointments and exulted with my most modest triumphs; you were my most significant discovery.

Statement

The vast majority of the experiments and analyses conducted for this thesis were my own work, under the invaluable advice of my supervisors and many colleagues.

The work described on chapter 3 was made possible through a collaboration with Dr Marianne Pedersen, Dr Michael Benros, Prof Mortensen and Prof Norgaard-Pedersen at the Danish Statens Serum Institute and the Danish National Centre for Register-based Research.

Dr Leslie Jacobson was the second reader for the cell-based assays described in chapter 3 and he administered the ip injections with coded IgG for experiments 2 and 3 of the animal model.

Dr John Dawes and Professor David Bennett kindly provided tissue from the *Cntnap2* knockout mouse for the staining described in chapter 4.

I received help and instruction from Dr David Menassa for the experiments described in chapter 6. Dr Menassa did all the confocal imaging, the staining for cortical layer markers, calbindin and PSD-95, and the cell counts.

Dr Steven West and Professor David Bennett shared their expertise in the staining of synaptic proteins and have performed the quantification of PSD-95 profiles.

No part of this thesis is being submitted, or has already been accepted, for any other degree at this, or any other, university.

Oxford, January 2017

Ester Coutinho

Publications

The experimental work contained in this thesis generated the following publications:

- Coutinho E, Menassa D, Jacobson L, West SJ, Domingos J, Moloney T, Lang B, Harrison PJ, Bennett DLH, Bannerman D, Vincent A. Maternal-to-foetal transfer of CASPR2-antibodies is associated with neurodevelopmental abnormalities in mouse offspring. In preparation
- Coutinho E, Jacobson L, Pedersen MG, Benros ME, Nørgaard-Pedersen B, Mortensen PB, Harrison PJ, Vincent A. CASPR2 autoantibodies are raised during pregnancy in mothers of children with mental retardation and disorders of psychological development but not autism. Under revision at JNNP.

The bibliographical revision done for this thesis generated the following reviews / book chapters:

- Pollak T, Coutinho E, Palmer-Cooper E, Vincent A. Inflammatory and autoimmune disorders in neuropsychiatry. Oxford Textbook of Neuropsychiatry. In preparation.
- Coutinho E, Vincent A. Autoimmunity in neuropsychiatric disorders. Handb Clin Neurol. 2016;133:269-82.
- Coutinho E, Harrison PJ, Vincent A. Do neuronal autoantibodies cause psychosis? A neuroimmunological perspective. Biol Psychiatry. 2014 Feb 15;75(4):269-75.
- Coutinho E, Vincent A. Central Nervous System Neuronal Surface Antibodies (2014). In Autoantibodies (3rd edition, pp 595-603). MA, USA: Elsevier.

Table of contents

List of tables

List of figures

Abbreviations

AChR	Acetylcholine receptor
ADHD	Attention deficit and hyperactivity disorder
AMC	Arthrogryposis multiplex congenita
AMPAR	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of variance
ASD	Autism spectrum disorder
BBB	Blood-brain barrier
CASPR2	Contactin-associated protein-like 2 (protein)
CBA	Cell-based assay
CDFE	Cortical dysplasia-focal epilepsy
CNS	Central nervous system
CNTNAP2	Contactin-associated protein-like 2 (gene)
CSF	Cerebrospinal fluid
CUX1	Cut Like Homeobox 1
D2R	Dopamine 2 receptor
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DIV	Days *in vitro*
DPD	Disorders of psychological development
DPPX	Dipeptidyl-peptidase-like protein-6
DSM	Diagnostic and Statistical Manual of Mental Disorders
EEG	Electroencephalography
EMG	Electromyography
EPM	Elevated Plus-maze
FOXP2	Forkhead box protein P2
GABAAR	Gamma-aminobutyric acid A receptor
GABABR	Gamma-aminobutyric acid B receptor
GFAP	Glial fibrillary acidic protein
HC	Healthy control
HEK	Human embryonic kidney
HLA	Human leukocyte antigen
ICC	Immunocytochemistry
ICD-10	International Classification of Diseases and Related Health Problems (10th edition)
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IvIg	Intravenous immunoglobulins
LD	Light-dark
LE	Limbic encephalitis
LGI1	Leucine-rich glioma-inactivated 1
MAC	Mothers of autistic children
MG	Myasthenia gravis
MGLUR1	Metabotropic glutamate receptor 1
MGLUR5	Metabotropic glutamate receptor 5
MHC	Major histocompatibility complex

MoS	Morvan’s syndrome
MR	Mental retardation
MRI	Magnetic resonance imaging
MuSK	Muscle specific kinase
n.s.	non-significant
NeuN	Neuronal Nuclei
nFcR	Neonatal Fc Receptor
NMDAR	N-methyl-D-Aspartate receptor
OCB	Oligoclonal band
PBS	Phosphate buffered saline
PSD-95	Postsynaptic density protein 95
RIA	Radioimmunoassay
SCLC	Small cell lung carcinoma
SEM	Standard error of mean
TD	Typical development
USVs	Ultrasonic vocalisations
VGKC	Voltage-gated potassium channel
VWF	von Willebrand factor
WB	Western blot

Chapter 1: Introduction

Throughout the first half of the twentieth century, autoimmune disease was not believed to occur naturally in human beings, a dogma termed by Paul Erlich “the horror autotoxicus” (reviewed in (Mackay 2010)). Autoimmunity was only to receive scientific acceptance in the 50s, largely due to the documentation of anti-thyroglobulin antibodies in thyroiditis (Roitt, Doniach et al. 1956). In neurology, the recognition and acceptance of autoimmunity progressed from the peripheral to the central nervous system, from the description of antibody-mediated disorders of the neuromuscular junction to the autoimmune synaptopathies and associated brain disorders (reviewed in (Vincent, Dalton et al. 2003)).

In this introductory chapter, an overview of the expanding field of antibody-mediated neurological disorders is given, followed by an introduction to the immunology of pregnancy and to the relevance of maternal immunity in the physiopathology of neurodevelopmental disorders.

1.1 On the definition of autoimmune disease

A disease is termed autoimmune if an abnormal adaptive immune response to a self-antigen, either by auto-reactive T cells or autoantibodies, causes or is associated with the observed pathology. Knowing that natural autoantibodies and self-reacting T cells exist in normal individuals (reviewed in (Cohen and Young 1991, Coutinho, Kazatchkine et al. 1995)), many questions arise on the fine border that separates physiological auto-reactivity and autoimmune disease. In the case of antibody-mediated diseases, it is possible that disease-relevant autoantibodies had escaped normal primary repertoire selection or that natural autoantibodies had undergone affinity maturation, and by a process of multiple somatic mutations had become highly reactive to autoantigens. A

practical approach to establish the diagnosis of autoimmune disease, invoked many times, is based on the Koch/Witebsky postulates (reviewed in (Rose and Bona 1993)). These postulates were later adapted to antibody-mediated diseases (Drachman 1990) and are based on the following principles:

1) The autoantibody is present during the clinical manifestations and detectable in circulation and/or affected tissue;

2) The autoantibody interacts with a receptor, ion channel, or other important protein expressed on the cell membrane;

3) Antibody transfer replicates the disease in an animal model or gestational maternal to foetal transfer induces disease in the neonate;

4) Active immunization produces a model of the disease;

5) Prevention of disease progression or clinical improvement occurs with immunosuppression or other therapies aiming to eliminate or suppress the autoimmune response (for instance plasma exchange).

This set of criteria in neurology can be applied to several antibody-mediated disorders, such as myasthenia gravis and the autoimmune encephalitides.

1.2 Importance of extracellular antigenic targets and relevance for methods of detection

The subcellular location of the antigenic target is of great importance to support or refute the potential pathogenicity of an antibody (reviewed in (Lancaster and Dalmau 2012)). Pathogenic autoantibodies can only target extracellular epitopes. They impact on the function of the targeted cells through one or a combination of pathogenic molecular mechanisms: direct modulation of function of the targeted protein, cross-linking and internalisation or complement activation (Figure 1-1). By contrast, intracellular antigens

imply a different primary phenomenon, with the autoantibodies likely to be secondary to cell destruction (reviewed in (Roberts and Darnell 2004)). To detect reliably pathogenic autoantibodies, the method of detection must take into account this difference. An ideal method is the one that preserves the native structure of the protein, allowing the antibodies to recognize the extracellular portion of the protein and its conformational epitopes. Western blots or peptide Enzyme-Linked Immunosorbent Assays (ELISAS), by using denatured linearized proteins, fail to conform to these requirements and are usually valueless for detection of pathogenic autoantibodies. Cell-based assays (CBAs) or flow cytometry, using live cells that preserve the structure of membrane proteins, have been shown to be superior methods for the detection of these (Waters, McKeon et al. 2012). In this thesis, live CBAs have been used for antibody detection. These assays use live cells transiently transfected with the protein of interest and then incubated with patient's serum, and binding is detected visually with an immunofluorescent anti-human antibody (Figure 1-2). These assays are labour intensive and the majority of diagnostic laboratories uses a commercial alternative with fixed cells. However, it is important to note that, in those assays, the cells are fixed and permeabilised, and there might be changes in the protein expression that can lead to reduced sensitivity and specificity.

An overview of the neurological disorders caused by pathogenic autoantibodies is given in the following sections, with particular focus on the central nervous system (CNS) disorders.

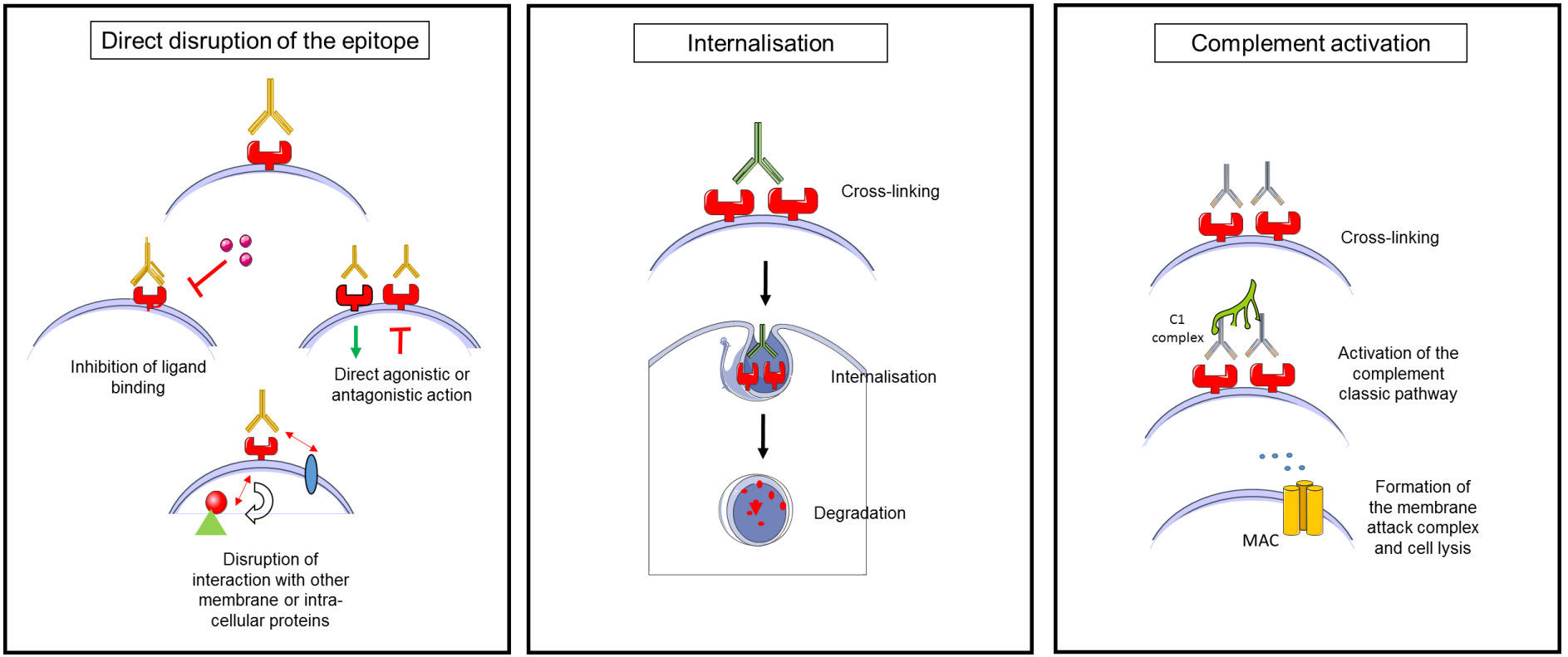


Figure 1-1: Pathogenic mechanisms in antibody-mediated disorders

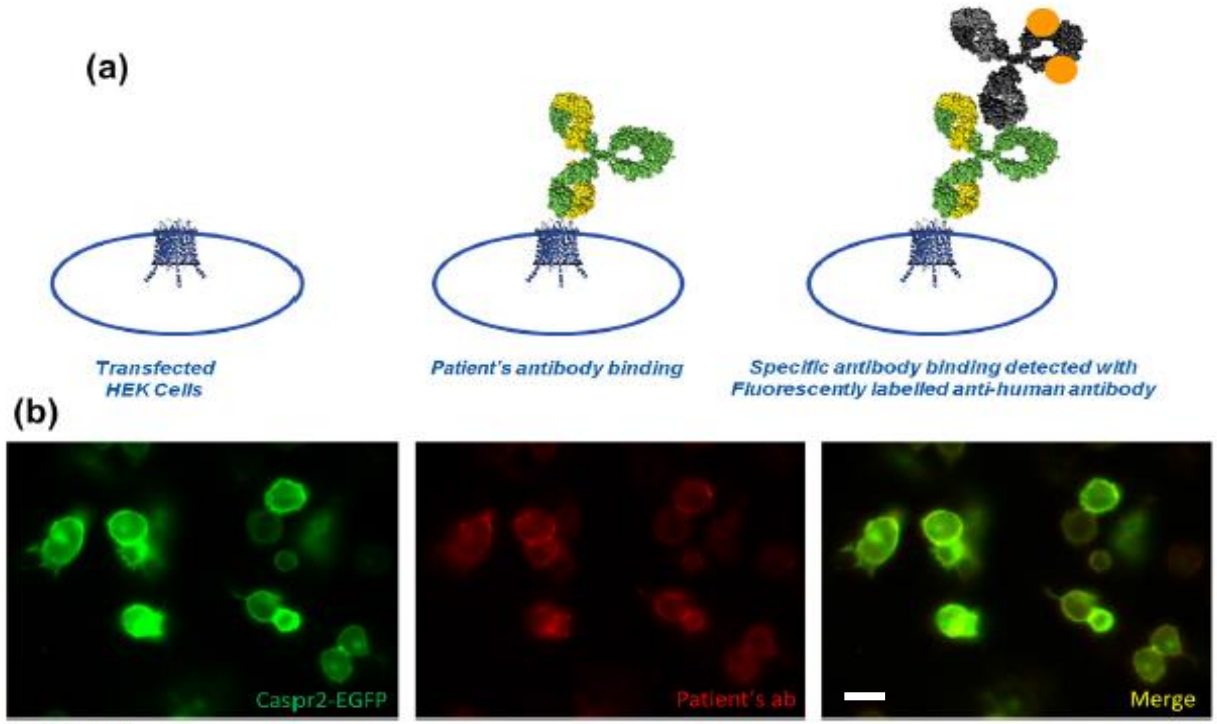


Figure 1-2: Routine cell-based assay (CBA) for detection of antibodies.

(A) Schematic representation of a CBA. Live human embryonic kidney (HEK) cells are transiently transfected with the protein of interest, which is expressed in the cell membrane. The transfected HEK cells are then incubated with patient's serum or CSF. If there are antibodies binding to the membrane protein, they are detected by visual fluorescence using a secondary antibody. (B) Photomicrographs of a CBA detecting antibodies binding to CASPR2-transfected cells.Scale bar: 50 μm

1.3 Autoantibody-mediated disorders: from the periphery to the CNS

1.3.1 Myasthenia Gravis and other peripheral antibody-mediated disorders

Myasthenia gravis (MG) remains the paradigm of antibody-mediated disorder. Patients present clinically with fatigable muscle weakness that can affect the ocular, palpebral, bulbar or limb muscles and usually respond to immunosuppressive therapy. This muscular weakness results from a defect in neuromuscular transmission and is characterized electrophysiologically by a gradual decrease of muscle action potential amplitude in response to repetitive nerve stimulation (reviewed in (Gilhus and Verschuuren 2015)). Clinical and experimental evidence of its association with antibodies targeting the acetylcholine receptor (AChR) was discovered in the 1970s (Patrick and Lindstrom 1973, Bender, Ringel et al. 1975), thus fulfilling the first two criteria for antibody pathogenicity. Transfer of antibodies causing neonatal MG is well documented both in patients and in animal models (reviewed below). Additionally, several animal models of active and passive immunization have already been established (reviewed in (Christadoss, Poussin et al. 2000)). Antibodies to another synaptic protein, the muscle-specific kinase (MuSK), are present in an AChR antibody-negative subgroup of patients and are also believed to be directly pathogenic (Hoch, McConville et al. 2001).

Other peripheral neurological disorders are also antibody-mediated. Lambert-Eaton myasthenic syndrome is another antibody-mediated disorder of the neuromuscular junction, characterized by proximal muscle weakness, areflexia and autonomic dysfunction (Titulaer, Lang et al. 2011). A proportion of patients, 60% of cases, have an associated tumour, most commonly a small cell lung carcinoma (SCLC). Antibodies to the P/Q-type voltage-gated calcium channel (VGCC), in the pre-synaptic membrane, are

present in 85-90% of patients (Motomura, Johnston et al. 1995). Neuromyotonia, also known as Isaacs-Merton syndrome, is a peripheral nerve hyperexcitability syndrome that associates with voltage-gated potassium channel (VGKC) antibodies (Shillito, Molenaar et al. 1995). Patients present with muscle involuntary contractions, cramps, stiffness, peripheral pain and dysautonomia; they respond to plasma exchange and have neuromyotonic discharges on electromyography (Maddison 2006). Although a clinical phenotype was not detected in passive immunization experiments in mice, purified IgG or sera from VGKC-positive neuromyotonia patients were shown to affect potassium currents *in vitro* and to induce spontaneous repetitive firing of action potentials in dorsal root ganglion cells (Sinha, Newsom-Davis et al. 1991, Shillito, Molenaar et al. 1995, Sonoda, Arimura et al. 1996, Tomimitsu, Arimura et al. 2004), indicating a disruption in the channel function. Both VGCC and VGKC antibodies can also be associated with CNS disorders, namely paraneoplastic cerebellar degeneration and limbic encephalitis, respectively, as discussed below.

1.3.2 Central nervous system antibody-mediated disorders

Despite the recognition of antibody-mediated peripheral neurological disorders for several decades, the brain was largely considered immunologically privileged until fairly recently. The first CNS disorders associated with autoantibodies were described in the 1980s (Greenlee and Brashear 1983, Grunwald, Klein et al. 1985, Jaeckle, Graus et al. 1985). These neurological syndromes are usually the remote manifestation of a tumour, and they are often the first manifestation of the associated cancer. They are termed paraneoplastic syndromes. Many different syndromes have been described, affecting either or both peripheral and central nervous systems (reviewed in (Graus, Saiz et al. 2010)). Classical CNS syndromes are encephalomyelitis, limbic encephalitis (LE), subacute cerebellar degeneration and opsoclonus-myoclonus, but many others have been described. The antibodies found in those patients, despite being useful diagnostic

markers, are not considered to be directly pathogenic. Those antibodies target proteins inside the cell, cytoplasmic or nuclear in location, making it unlikely that the antibody is the primary pathogenic mechanism in the disease. In fact, evidence is towards a cytotoxic T cell mechanism in these disorders (reviewed in (Dalmau and Rosenfeld 2008)). Likewise, passive transfer of those antibodies to rodents have failed to reproduce the disease (Graus, Illa et al. 1991, Tanaka, Tanaka et al. 1995) and the patients usually do not respond to immunotherapies (Vedeler, Antoine et al. 2006).

In the last 15 years, another type of antibody-mediated disorders have been described (reviewed in (Crisp, Kullmann et al. 2016)). In these, the antibodies bind to the extracellular epitopes and are believed to be directly pathogenic, they are associated with cancer in some patients, but are mostly non-paraneoplastic, and patients usually respond to therapies such as plasma exchange and immunosuppression, particularly if started early in the course of the disease. The antibodies in these disorders usually target important synaptic proteins expressed on the neuronal surface, such as neurotransmitter receptors or ion channel complexes, and are believed to cause the disease due to disturbances in synaptic transmission or neuronal excitability. In this thesis, I will focus on those disorders associated with neuronal-surface antibodies and a predominantly encephalitic-type phenotype. An overview of these neuronal-surface antibody-mediated disorders is provided in Table 1-1.

Table 1-1: Neuronal surface antibody-mediated autoimmune encephalitides

Antigen	Demographics	Most common clinical Phenotypes	Paraclinical findings	Tumour association	Proposed pathogenic mechanisms	Animal models
N-methyl-D-Aspartate receptor (NMDAR)	80% females Age range: <12 months to 85 years (median 21 years)	Whole brain encephalopathy (psychiatric symptoms, seizures, memory and language deficits, followed by movement disorders, autonomic instability, and decreased level of consciousness)	CSF: lymphocytosis (~70%) in the early stages and OCBs later (~50%) EEG: generalized slowing, epileptiform activity in early stages MRI: normal or mild signs of inflammation (cortical or subcortical)	Teratoma (~40-50%)	Cross-linking and internalization. Direct disruption of epitope.	Yes
VGKC-complex associated protein Leucine-rich glioma-inactivated 1 (LGI1)	65% males Age range: 30 to 80 years old (median 60 years)	Limbic encephalitis. Faciobrachial dystonic seizures.	Hyponatremia CSF: usually normal, occasional OCB MRI: medial temporal lobe increase of FLAIR signal (80%)	Rare	Direct disruption of epitope. Complement activation and internalization.	No (but natural animal model)
VGKC-complex associated protein Contactin-associated protein-like 2 (CASPR2)	85% males Age range: 46 to 77 years (median 60 years)	Neuromyotonia. Morvan's syndrome. Limbic encephalitis. Idiopathic ataxia.	MRI: medial temporal lobe increase of FLAIR signal (40%) in limbic encephalitis EMG: spontaneous muscular hyperactivity in neuromyotonia	Thymomas and SCLC (uncommon)	Complement activation and internalization.	No
Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)	75% females Age range: 23 to 87 years (median 60 years)	Limbic encephalitis (prominent psychiatric manifestations)	CSF: lymphocytosis, raised protein and OCBs occasionally. MRI: medial temporal lobe increase of FLAIR signal (90%)	Thymomas / Thymic carcinomas, breast cancer and SCLC (~50%)	Cross-linking and internalization	No
Gamma-aminobutyric acid B receptor (GABABR)	50% females Age range: 24 to 75 years (median 62 years)	Limbic encephalitis (prominent seizures)	CSF: lymphocytosis, raised protein and OCBs occasionally MRI: medial temporal lobe increase of FLAIR signal (~66%)	SCLC	Unknown	No

Table 1.1 (cont.)

Gamma-aminobutyric acid A receptor (GABAAR)	66% males Age range:2-74 years	Undefined clinical picture (seizures, memory deficits, psychiatric abnormalities, stiff-person syndrome)	EEG: ictal activity (in epileptic cases) MRI: non-specific inflammatory changes or normal GAD-65 antibodies and other serological markers of autoimmunity	No	Reduced protein surface expression	No
Dopamine 2 receptor (D2R)	75% males Age range: 0.4-17 years	Basal ganglia encephalitis Sydenham chorea Tourette Syndrome	Abnormal MRI in 1/3 of cases	No	Unknown	No
Dipeptidyl-peptidase-like protein-6 (DPPX)	66% males Age range: 13-75 years (median: 53)	PERM, cerebellar ataxia, encephalitis / romboencephalitis. Prodromal Diarrhoea and weight loss.	EEG: focal or diffuse slowing CSF: pleocytosis	B-cell neoplasms (in 2 patients)	Cross-linking and internalization	No
Metabotropic glutamate receptor 1 (MGLUR1)	3 females, 2 males Age range: 19-69 years (median: 50)	Cerebellar ataxia	MRI: cerebellar atrophy CSF: mononuclear pleocytosis and OCB	Hodgkin's lymphoma T-cell lymphoma	Unknown	Yes
Metabotropic glutamate receptor 5 (MGLUR5)	2 females and 2 males Age range: 15-46 years	Limbic encephalitis (Ophelia's syndrome)	MRI: inflammatory changes or normal CSF: OCB	Hodgkin's lymphoma	Unknown	No

Abbreviations: CSF: cerebrospinal fluid; EEG: electroencephalography; MRI: magnetic resonance imaging; EMG: electromyography; FLAIR: fluid-attenuated inversion recovery; OCB: oligoclonal band; SCLC: small cell lung carcinoma

1.3.2.1 Voltage-gated potassium channel (VGKC) complex encephalitis and associated disorders

1.3.2.1.1 VGKC and associated proteins

VGKCs are transmembrane channels responsible for the regulation of neuronal excitability. Kv1 channels, also known as the Shaker family, are of particular importance since they form the complex targeted by antibodies in autoimmune encephalitis (Irani, Alexander et al. 2010). Kv1 channel clusters are predominantly found at the juxtaparanodes of myelinated axons and in the basket cell terminals of the cerebellum, but are also expressed in the axonal initial segment (Rasband 2010). In each of this locations, they form a complex structure (called the VGKC-complex) with scaffolding proteins in the pre and post-synaptic terminals, amongst which are the proteins contactin-associated protein 2 (CASPR2) and leucine-rich glioma inactivated 1 (LGI) (Figure 1-3).

CASPR2 is a highly conserved protein among mammals, with approximately 94% homology between human and mouse (reviewed in (Rodenas-Cuadrado, Ho et al. 2014)). It is a cell adhesion transmembrane protein, member of the neurexin family, which clusters VGKCs (mainly Kv1.1 and Kv1.2) at the juxtaparanodes of myelinated neurons (Poliak, Salomon et al. 2003). The extracellular domain of CASPR2 interacts with contactin2 /TAG1, which is expressed both in neurons and glia. On the cytoplasmic site, CASPR2 binds to cytoskeleton protein 4.1 and to the VGKC via a PDZ domain-containing protein (Horresh, Poliak et al. 2008). Together, those proteins form a scaffold necessary to maintain the VGKC at the juxtaparanode. The stabilization of the VGKCs allows them to modulate action potential conduction at the nodes of Ranvier, helping to maintain the internodal resting membrane potential and avoiding repetitive firing from damaged or developing myelinated neurons (Rasband 2010).

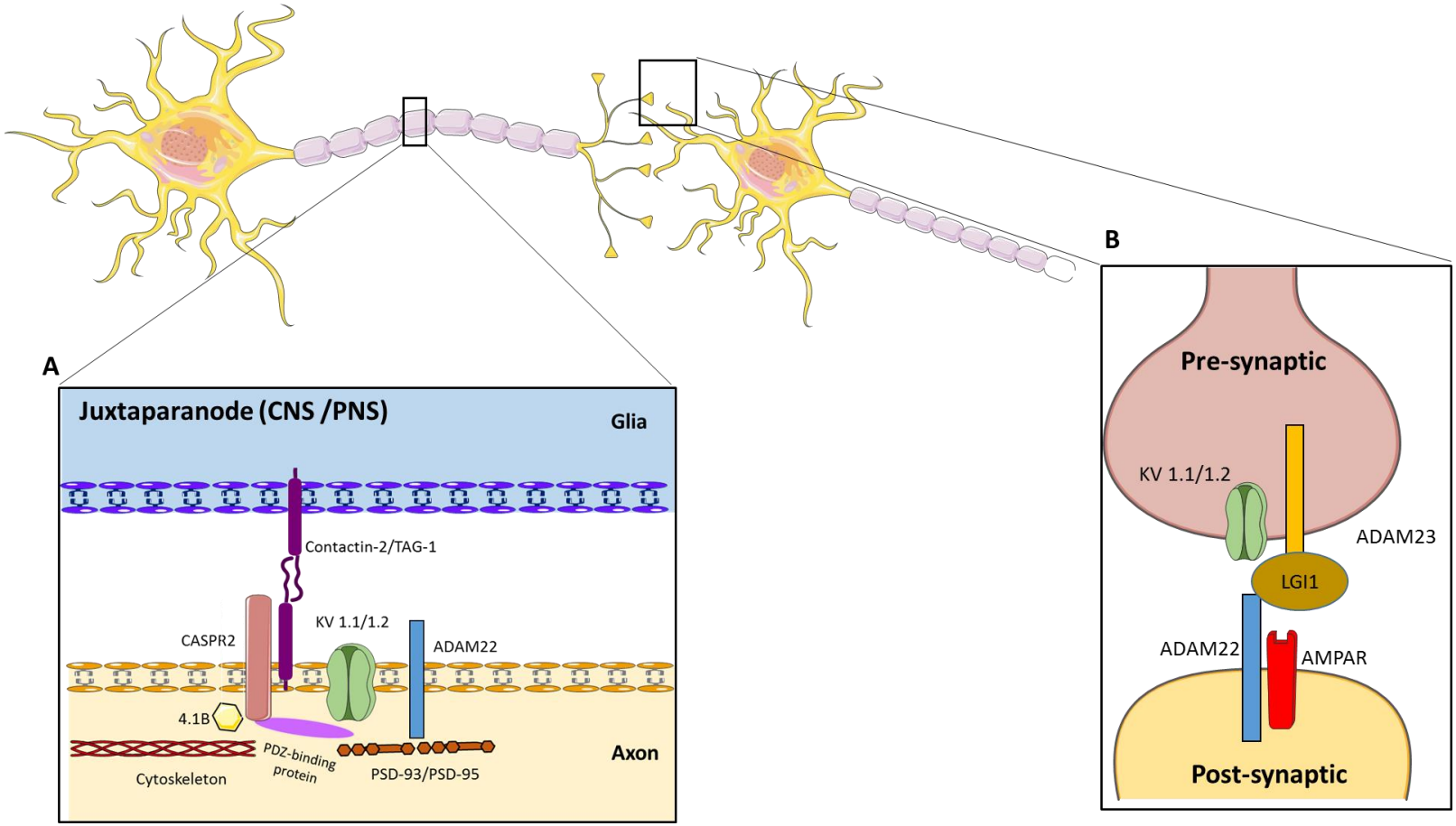


Figure 1-3: Voltage-gated potassium channel and associated proteins.

(A) CASPR2 interactions in the juxtaparanode. CASPR2 binds to contactin-2/TAG-1 via its extracellular domain and links to PDZ-binding proteins, and to the cytoskeleton via protein 4.1B. (B) LGI1 interactions in the synaptic cleft. LGI1 is a secreted protein that interacts pre-synaptically with ADAM23 and post-synaptically with ADAM22. This complex interacts with the pre-synaptic VGKC and the post-synaptic AMPAR.

LGI1 is a secreted protein that in the adult brain is highly expressed in the cortex and hippocampus (Senechal, Thaller et al. 2005). It interacts pre-synaptically with a disintegrin and metalloprotease domain 23 (ADAM23) and post-synaptically with ADAM22, forming a trans-synaptic complex that interacts with the pre-synaptic VGKC and the post-synaptic alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) (Fukata, Lovero et al. 2010). An autosomal dominant mutation of LGI1 has been found in families with a focal epileptic syndrome, with onset in infancy/adolescence and characterized by auditory auras and ictal aphasia, called autosomal dominant lateral temporal epilepsy (ADLTE) or autosomal dominant partial epilepsy with auditory features (ADPEAF) (Nobile, Michelucci et al. 2009). LGI1-null mice have spontaneous myoclonic seizures and die shortly after 2 weeks, while the heterozygous mice do not have spontaneous seizures but do have auditory-induced seizures (Fukata, Lovero et al. 2010).

1.3.2.1.2 Clinical characteristics

The first patient with a CNS disorder associated with a putative pathogenic neural-surface complex antibody had Morvan's syndrome (MoS) and VGKC antibodies (Liguori, Vincent et al. 2001). MoS, a very rare disorder, encompasses both peripheral and central manifestations, such as neuromyotonia, peripheral pain, sleep disturbance, neuropsychiatric symptoms and dysautonomia. Soon afterwards, two other patients with VGKC antibodies and immunotherapy-responsive LE, presenting with memory loss, psychiatric disturbances and seizures of subacute onset, and no obvious peripheral disease, were described (Buckley, Oger et al. 2001). At the time of these descriptions, antibodies targeting the VGKC were detected using a radioimmunoprecipitation assay using brain tissue lysates containing the VGKC-complex labelled with radio-iodinated α-dendrotoxin (Hart, Waters et al. 1997). Later, it was shown that the targets of those antibodies are in fact proteins that associate with the channel – LGI1, CASPR2 and

Contactin-2 – and not the channel subunits themselves (Irani, Alexander et al. 2010, Lai, Huijbers et al. 2010, Lancaster, Huijbers et al. 2011). It is this pleiotropy of antigenic targets that partially justify the diverse clinical phenotypes associated with what was previously described as simply VGKC antibodies, and that include LE, MoS, neuromyotonia, faciobrachial dystonic seizures (Irani, Michell et al. 2011) and cerebellar ataxia (Becker, Zuliani et al. 2012). Despite some overlap, patients with typical LE are more likely to have LGI1 antibodies, consistent with the predominant cortical and hippocampal localisation of this protein. On the other hand, patients with antibodies targeting CASPR2, a protein expressed in the central and peripheral nervous system, are more likely to have NMT and MoS, although they may also have LE (Irani, Alexander et al. 2010). Other clinical features might also point to one or other antigenic target. LGI1 antibodies are preferentially associated with a form of epilepsy, manifested by anti-epileptic treatment resistant, adult-onset, high-frequency, faciobrachial dystonic seizures, that often allows intervention before development of other limbic dysfunction symptoms(Irani, Stagg et al. 2013). Although uncommon in any form of VGKC-complex associated encephalitis, if the patient has cancer, particularly a thymoma, CASPR2 antibodies are more likely to be present, and this is also true if the patient has cerebellar signs. Early recognition and treatment are usually associated with good prognosis, although less so in the relatively rare paraneoplastic cases (Irani, Alexander et al. 2010).

1.3.2.1.3 Pathogenic mechanisms of LGI1 and CASPR2 antibodies

Most studies looking at the pathogenic mechanisms of the VGKC-complex antibodies were performed before the discovery of specific antigenic targets LGI1 / CASPR2 and were done in the context of neuromyotonia (Nagado, Arimura et al. 1999, Tomimitsu, Arimura et al. 2004). However, both LGI1 and CASPR2 antibodies decrease the surface expression of the targeted protein in cultured neurons after exposure to IgG for 24h (Pettingill, P. 2013.Unpublished DPhil thesis. University of Oxford). Another study, using

whole-cell patch-clamping and extra-cellular recordings in rat hippocampal acute slices, showed an increase in cell excitability in the CA3 area with IgG from one patient with LE and high VGKC / LGI1 titres, but not IgG from a control; the effects mimicked the actions of pharmacological VGKC antagonists (Lalic, Pettingill et al. 2011). It was shown by another *in vitro* study that LGI1 antibodies from a number of patients disrupt the interaction of LGI1 with ADAM22 and ADAM23 and that this leads to a reduction in synaptic AMPARs (Ohkawa, Fukata et al. 2013).

No animal model of LGI1 or CASPR2 antibody-mediated disease has been reported to date. However, cats who naturally develop a form of encephalitis have VGKC-complex/LGI1 antibodies (Pakozdy, Halasz et al. 2013, Klang, Schmidt et al. 2014, Pakozdy, Glantschnigg et al. 2014). These cats suffer from treatment-resistant, complex partial seizures and abnormal behaviour, and serum antibody titres seem to mirror the clinical course. Interestingly, a neuropathological study of the cats' brains showed hippocampal lesions with IgG infiltration and complement deposition on hippocampal neurons but minimal T-cell infiltrates, associated with neuronal loss (Klang, Schmidt et al. 2014), which is very similar to that described in human patients with VGKC-complex antibodies (Bien, Vincent et al. 2012).

1.3.2.2 N-methyl-D-Aspartate receptor (NMDAR) antibody encephalitis

1.3.2.2.1 NMDAR

Glutamate receptors mediate excitatory synaptic transmission in the CNS. They comprise 2 types: the metabotropic glutamate receptors and the ionotropic receptors. The NMDARs, together with the AMPAR and the kainate receptors, are ionotropic receptors. The different NMDAR subunits - NR1, NR2 and NR3 (now re-named as GluN1, GluN2 and GluN3) – co-assemble to form ligand-gated ion channels containing an agonist recognition site, a transmembrane ion pathway, and gating elements

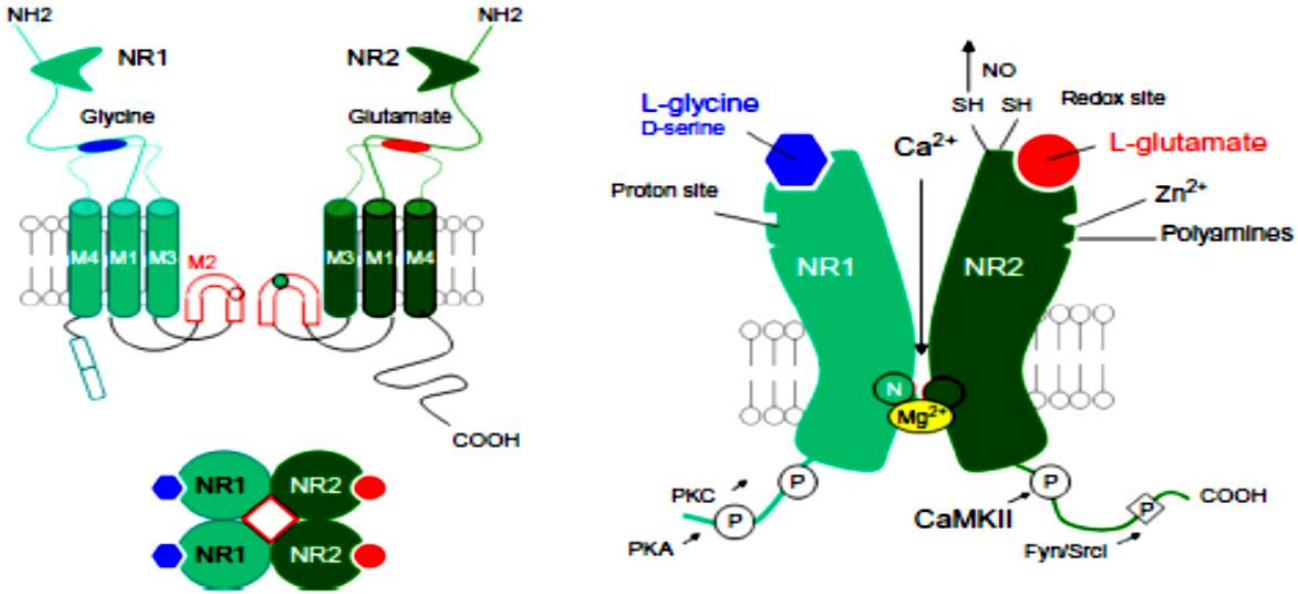


Figure 1-4: Structure and modulation of the NMDAR.

Different NMDAR subunits – (NR1 / NR2 depicted in this figure) co-assemble to form ligand-gated ion channels containing an agonist recognition site, a transmembrane ion pathway, and gating elements. Activation requires binding from 2 agonists, L-glutamate and L-glycine. Figure adapted from chapter 2, page 28, Handbook of Clinical Neurology (J. Pittock and Vincent 2016)

(Traynelis, Wollmuth et al. 2010). Activation of the NMDAR requires binding from 2 agonists, NMDA and glycine (or D-serine) that bind to their respective extracellular domains of NR1 and NR2, respectively. The transmembrane domain is responsible for the receptor's high-calcium permeability and voltage-dependent magnesium block (Figure 1-4). The NR1 subunit is obligatory and it assembles with NR2 subunits (NR2A-D), coded by 4 genes, or NR3 (NR3A-B) subunits, coded by two separate genes (Schuler, Mesic et al. 2008). The existence of several subunits, added to the alternative splicing of the NR1 subunit, results in great heterogeneity of the NMDAR. The subunit composition and expression in the brain changes during development as a consequence of neuronal activity or sensory experiences and this determines the receptor properties (reviewed in (Paoletti, Bellone et al. 2013)). For instance, NR2B is well known to be preferentially expressed in the developing cortex, hippocampus and cerebellum, while NR2A is more abundant in the adult brain (Jantzie, Talos et al. 2015).

1.3.2.2.2 Clinical characteristics

The first clinical description of NMDAR encephalitis was reported in 2005 (Vitaliani, Mason et al. 2005). The authors described four female patients with acute psychiatric symptoms, seizures, hypoventilation and decreased consciousness in the context of ovarian teratoma. Two years later, this syndrome was shown to be associated with antibodies targeting the NMDAR (Dalmau, Tuzun et al. 2007). Although initially only recognized as a neurological syndrome in the context of ovarian teratoma, it is now known that the majority of patients have no neoplasia (around 60-70%) and that the disease can affect men and women of all ages (Irani, Bera et al. 2010, Titulaer, McCracken et al. 2013). Neurological features usually evolve from acute or subacute psychiatric symptoms, such as psychosis, seizures and memory disturbance, to movement disorders, dysautonomia and in the later stages decreased levels of consciousness. Paraclinical findings are often unremarkable. MRI is frequently normal,

although 40% of patients have some evidence of inflammation. Electroencephalograms can show ictal discharges, most commonly at disease onset, or generalized slowing, consistent with encephalopathy and decreased conscious level. Patients usually respond to immunotherapy, with tumour removal in the paraneoplastic cases, particularly if initiated early in the course of disease. Despite this, many patients have relapsing or residual symptoms, most often cognitive and psychiatric, and in about 5% the outcome is fatal (Titulaer, McCracken et al. 2013).

1.3.2.2.3 Pathogenic mechanisms of NMDAR antibodies

Several studies investigated the NMDAR antibodies' pathogenic effects *in vitro* and *in vivo*. The first *in vitro* study showed a reduction in the expression of surface NMDAR in hippocampal neurons exposed to CSF or IgG from patients with NMDAR encephalitis for 3 or 7 days, but not controls; this decrease was reversible upon removal of the antibodies (Dalmau, Gleichman et al. 2008). This was confirmed by a subsequent study (Hughes, Peng et al. 2010) that showed that NMDAR internalization was mediated by cross-linking of the IgG molecules *in vitro*, and reduced protein expression *in vivo* in the hippocampus of rats infused with patient's CSF for 2 weeks. A selective decrease of NMDAR currents *in vitro* was also documented in this study. Another group, however, revealed a somewhat different mechanism of action, showing that the NMDAR antibodies displaced the receptor to extrasynaptic sites and consequently blocked synaptic plasticity by prevention of long-term potentiation (LTP) (Mikasova, De Rossi et al. 2012). The disruption of the interaction between NMDAR and ephrin-B2 receptors, a post-synaptic transmembrane protein that is believed to stabilise the NMDAR, was shown to cause the displacement. The suppressive effects on LTP was confirmed in another publication (Zhang, Tanaka et al. 2012). A hyperglutamatergic state, characterized by increased concentrations of glutamate in the extracellular space, was also documented by direct infusion of NMDAR CSF in the CA1 area and premotor cortex in rats (Manto, Dalmau et

al. 2010). The NMDAR encephalitis clinical phenotype was partially reproduced in a passive immunisation model that showed memory deficits and anhedonic behaviours in mice infused intraventricularly with patient's CSF but not control's CSF (Planaguma, Leypoldt et al. 2015). A similar study, using patient's and control's plasma purified IgG given in a single injection intraventricularly, assessed the epileptogenic potential of the antibodies, and showed an increased susceptibility to seizures in the NMDAR antibody-exposed animals (Wright, Hashemi et al. 2015). Interestingly, it seems that NMDAR encephalitis can also occur naturally in animals, since a description of a similar clinical syndrome with NMDAR antibodies was reported recently in a polar bear (Pruss, Leubner et al. 2015).

1.3.2.3 Other antibody-mediated encephalitides

Since the description of NMDAR encephalitis, many other neuronal surface antibodies causing CNS disease have been found. AMPAR antibodies were described in patients with psychiatric and cognitive changes, mostly anterograde memory deficits and confusion, although a few had more atypical symptoms and signs like cerebellar dysfunction, nystagmus or a speech disturbance. This condition is associated with cancer in about 50% of cases (mostly a SCLC) and is more common in female patients in their sixth decade (Joubert, Kerschen et al. 2015). Interestingly, thymus abnormalities (thymus hyperplasia, thymoma and thymic carcinoma) were present in 15% of the patients reported so far. Patients with gamma-aminobutyric acid receptor B (GABARB) antibodies usually present with LE, although some cases of cerebellar ataxia, opsoclonus-myoclonus, rapidly progressive encephalomyelitis and status epilepticus have also been documented (Hoftberger, Titulaer et al. 2013, Jeffery, Lennon et al. 2013). Around half of the patients with GABARB encephalitis have an associated tumour, usually a SCLC. Other antibodies (N-type VGCC, GAD65, thyroperoxidase, SOX1) are commonly found in the paraneoplastic cases. GABA receptor A (GABARA) antibodies

have also been described, although the clinical phenotype is broader, including encephalitis with seizures, stiff-person syndrome, opsoclonus-myoclonus and other uncharacterized psychological disorders (Ohkawa, Satake et al. 2014, Petit-Pedrol, Armangue et al. 2014, Pettingill, Kramer et al. 2015). Antibodies to the Glycine receptor (GlycR), which are mainly associated with progressive encephalomyelitis with rigidity and myoclonus, have also been identified in a few patients with limbic encephalitis or epileptic encephalopathy (Carvajal-Gonzalez, Leite et al. 2014).

Other neuronal surface antibodies that apparently are less frequent, and are of uncertain clinical utility include: metabotropic glutamate receptor 5 (mGLUR5) and 1 (mGLUR1) antibodies, dipeptidyl-peptidase-like protein-6 (DPPX) antibodies and dopamine 2 receptor (D2R) antibodies. Antibodies to mGLUR5 s were detected in a few patients with LE and Hodgkin's lymphoma, an association also called Ophelia Syndrome (Lancaster, Martinez-Hernandez et al. 2011, Mat, Adler et al. 2013, Pruss, Rothkirch et al. 2014), while mGLUR1 antibodies were associated with cerebellar ataxia(Sillevis Smitt, Kinoshita et al. 2000). DPPX, a protein that associates and regulates Kv4.2 channels, was also found to be an antibody target in patients with a form of LE (Boronat, Gelfand et al. 2013, Piepgras, Holtje et al. 2015). Severe diarrhoea and weight loss preceding the neurological symptoms seems to be common in this condition, and are attributed to the high-level of expression of this protein in the myenteric plexus. Finally, D2R antibodies were reported in a cohort of children with basal ganglia encephalitis, characterized by predominant movement and psychiatric disease, Sydenham chorea, and Tourette syndrome (Dale, Merheb et al. 2012).

1.4 Neuropsychiatric features of autoimmune encephalitides and expanding phenotype

1.4.1 Neuropsychiatric phenotype of patients with autoimmune encephalitis

All antibody-mediated encephalitis have profound psychiatric and cognitive manifestations, although generally in the context of encephalopathy and other focal neurological symptoms (reviewed in (Kayser and Dalmau 2011)). These neuropsychiatric manifestations are more prominent in patients with NMDAR encephalitis and was highlighted by the fact that, when the syndrome was first described, the majority of patients had been admitted to psychiatric units in the first instance (Dalmau, Tuzun et al. 2007). In a large observational cohort study (Titulaer, McCracken et al. 2013), 65% of all adult patients and 50% of the children had behavioural symptoms at disease onset. The percentage of patients with behavioural or cognitive symptoms at any stage of the disease was over 95% in both adults and children. Importantly, in a small number of cases, patients had isolated psychiatric symptoms at disease onset (0.9%) or during relapse (3.2%) (Kayser, Titulaer et al. 2013). Psychiatric problems ranged from psychotic illness, with visual and auditory hallucinations, delusions, personality changes or catatonia, to mood disturbances.

VGKC complex encephalitis can also present with psychiatric symptoms. In a recent cohort study (Somers, Lennon et al. 2011), 44% of all patients had neuropsychiatric problems that the authors classified into two groups: the first had florid psychiatric symptomatology at onset in the context of a clinical picture of LE, while the second group had more subtle neuropsychiatry, such as depression and anxiety, and a lower percentage of neurological focal symptoms or signs. Several case reports have also described patients with a more marked psychiatric phenotype, from obsessive-

compulsive disorders (Spinazzi, Argentiero et al. 2008) to mood disorders (Kitten, Gupta et al. 2011), to anxiety (Parthasarathi, Harrower et al. 2006).

The psychiatric phenotype of other antibody-mediated encephalitides has not been so extensively described. One AMPAR encephalitis publication reported two patients with rapidly-progressive, psychotic-like behaviour and aggressiveness (Graus, Boronat et al. 2010). In a more recent report of 22 patients (Hoftberger, van Sonderen et al. 2015), one had psychosis with bipolar features. Those patients with GABARA and GABARB antibodies can also present with behavioural changes in the context of LE, and one patient with GABAAR encephalitis had paranoid schizophrenia (Pettingill, Kramer et al. 2015). Paediatric patients with basal ganglia encephalitis and other related disorders and D2R antibodies were also described to have prominent psychiatric symptoms, such as attention deficit, emotional lability, and psychosis (Dale, Merheb et al. 2012).

Although the majority of patients with antibody-mediated encephalitis have pronounced psychiatric manifestations together with multifocal CNS disease and encephalopathy, it seems clear that a smaller proportion can have isolated psychiatric manifestations at presentation or in posterior relapses. However, whether patients with primarily psychiatric disorders can have an antibody-mediated illness is still under debate.

1.4.2 Autoantibodies in psychiatric patient groups

Of all patients with psychiatric illnesses, patients with schizophrenia and first-episode psychosis are the ones that have been most extensively studied for the presence of neuronal surface antibodies, particularly NMDAR antibodies (Table 1-2). This likely results from the known implication of NMDAR dysfunction in the pathophysiology of schizophrenia (reviewed in (Javitt, Zukin et al. 2012)). The first study (Zandi, Irani et al. 2011) assessed the presence of these antibodies in patients with first episode psychosis who later received a final diagnosis of schizophrenia, and found two positives among 46

screened. Since then, several publications have reported contradictory results, which likely results from different patient inclusion criteria and different methods for antibody detection, since many have used the commercial fixed assay. A second study (Tsutsui, Kanbayashi et al. 2012) reported four NMDAR-antibody positive patients among 51 patients with schizophrenia or schizophreniform disorders, but those patients also had seizures and teratomas. In another cohort of 121 patients diagnosed with schizophrenia, 4 had NMDAR antibodies, although in hindsight 2 patients had a broader clinical picture compatible with NMDAR encephalitis (Steiner, Walter et al. 2013). Another study (Hammer, Stepniak et al. 2013) found 7 NMDAR antibody positive-patients among 1081 patients with schizophrenia but also found a similar percentage of positivity (0.4%) among healthy and disease controls. In this last study, factors associated with blood-brain barrier permeability were postulated to be responsible for the difference in the relevance of antibodies among patients and controls. Other cohort studies (Rhoads, Guirgis et al. 2011, Haussleiter, Emons et al. 2012, Masdeu, Gonzalez-Pinto et al. 2012, de Witte, Hoffmann et al. 2015, Kruse, Lapid et al. 2015, Masopust, Andrys et al. 2015), however, did not detect NMDAR antibodies in schizophrenic patients or controls.

Other autoantibodies have not been so extensively studied in psychiatric cohorts. One cohort study (Kruse, Lapid et al. 2015) identified 12 VGKC-positive patients among a total of 36 patients with several psychiatric manifestations. Of these, five had other evidence suggestive of autoimmunity and a specific antigenic target was determined in two, in both of them the target was LGI1. In the first study of autoantibodies in psychosis (Zandi, Irani et al. 2011), 1 patient was VGKC-positive among the 46 studied. AMPA receptor antibodies were screened in a large cohort of 459 patients with several psychiatric diagnoses, but none was found to be positive (Steiner, Walter et al. 2013). D2R antibodies were reported in 3 out of 43 children with first-episode psychosis (Pathmanandavel, Starling et al. 2015), but none in another cohort of adult schizophrenic

Table 1-2: Publications on neuronal surface autoantibodies in psychiatric patients.

Publication	Population	Assay / Criteria for positivity	Antibodies screened	Findings	Comments
Zandi et al., 2011	First episode psychosis (n=46)	CBA (Oxford)	NMDAR (IgG) and VGKC	2 patients with low NMDAR antibody scores and one patient with VGKC antibodies	Lack of control population. Patients tested early in disease onset and positive patients retrospectively interviewed and investigated. No atypical features found.
Rhoads et al., 2011	DSM-IV schizophrenia (n=7) and healthy controls (n=3)	Euroimmun AG slides (Luebeck)	NMDAR (IgG)	All patients negative	Small case series. Patients with long disease course.
Masdeu et al., 2012	First episode psychosis that later developed schizophrenia (n= 80) and controls (n=40)	CBA, Immunohisto-chemistry and neuronal cultures (Barcelona)	NMDAR (IgG)	All patients and controls negative	Strict laboratorial criteria for positivity.
Tsutsui et al., 2012	Patients with typical anti-NMDAR encephalitis (n=5); with narcolepsy with severe psychosis (n=5) and with schizophrenia or schizo-affective disorders, with seizures, atypical presentation, or treatment resistance (n=51)	Some patients tested by CBA, Immunohisto-chemistry and neuronal cultures (Barcelona), others unclear.	NMDAR (IgG)	7 positive patients in the psychiatric groups: 3 patients with narcolepsy with severe psychosis and 4 patients with schizophrenia or schizo-affective disorder.	All positive psychiatric patients had atypical features. Lack of control population.
Haussleiter et al., 2012	DSM-IV schizophrenia (n= 45), schizo-affective disorder (n= 4), delusional disorder (n= 1)	Euroimmun AG slides (Luebeck)	NMDAR (NR1A/NR2A and NR1A/NR2B), AMPAR (GluR1/GluR2) , GABA receptor (B1), LGI1 and CASPR2	All patients negative for all antibodies tested.	Lack of control population. Patients with long disease course.

Table 1.2 (cont.)

Steiner et al., 2013	DSM-IV schizophrenia (n=121), MD (n=70), or BLPD (n=38) and healthy controls (n=230)	Euroimmun AG slides (Luebeck)	NMDAR (NR1 or NR1A/NR2B – IgG, IgA and IgM) and AMPAR (GluR1/2)	Four patients with paranoid schizophrenia had IgG antibodies (2 retrospectively reclassified as NMDAR encephalitis). Seven patients with schizophrenia, 2 patients with MD and 1 control had IgM or IgA NMDAR antibodies. All patients and controls negative for AMPAR antibodies.	Antibodies towards NR1 epitope alone were not seen among psychiatric patients or controls.
Hammer et al., 2013	Patients with schizophrenia and schizo-affective disorder (n=1081), affective disorders (n=148), healthy Controls (n=1325) and Parkinson Disease (n=263)	Euroimmun AG slides (Luebeck)	NMDAR (NR1 or NR1/NR2B (IgG, IgA, IgM)	No difference in seropositivity between patients and controls (7 patients with Schizophrenia with IgG ab; 5 patients with affective disorder, 5 controls, 1 patient with Parkinson disease). IgA and IgM seropositivity was frequent among patients and controls.	Phenotype attributed to factors influencing BBB integrity
Muller et al., 2014	Schizophrenia patients (n=184); MD (n=99), BLPD (n=42), and matched controls (n=357)	Euroimmun AG slides (Luebeck)	Dopamine receptors (DRD1, DRD2, DRD3, DRD4, and DRD5)	D1 receptor autoantibodies were detected in the sera of two healthy controls. Antibodies against DRD2-5 absent in all subjects.	
Masopust et al., 2015	First episode psychosis (n=50) and healthy controls (n=50)	Euroimmun AG slides (Luebeck)	NMDAR (IgG) antibodies	All patients and controls negative	Lack of (positive) control population
de Witte et al. 2015	3 cohorts (combined n=475): schizophrenia / schizophreniform disorder/ schizo-affective disorder / first episode pychosis	In-house CBA (cohort 1 and 2); Euroimmun AG slides (cohort 3)	NMDAR (IgG) antibodies	All patients negative using in-house CBA; 2 positive patients using Euroimmune slides (but negative with cross-validation studies)	Lack of (positive) control population.
Pathmanandavel et al., 2015	Children with first episode psychosis (n=43) and pediatric healthy and disease controls (n=43)	Flow Cytometry Live CBA and neuronal cultures	Dopamine receptor (D2R) and NMDAR (NR1) – IgG, IgM and IgA antibodies	D2R antibodies in 3 psychosis patients (3 IgG, 1 IgM) and NMDAR antibodies in 6 patients (5 IgG, 1 IgM, 1 IgA)	

Abbreviations – CBA: Cell-based assay; NMDAR: N-Methyl-D-Aspartate receptor; VGKC – Voltage-gated potassium channel; MD – Major depression; BLPD – Borderline Personality Disorder; AMPAR - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; LGI1 - leucine-rich glioma inactivated-1; CASPR2 - contactin-associated protein-like 2; GABA - gamma-aminobutyric acid; BBB – Blood-brain barrier

patients (Muller, Teegen et al. 2014). Other neuronal surface targeting antibodies have not been studied in cohorts of patients with a strictly psychiatric diagnosis.

1.4.3 Autoantibodies in postpartum psychosis

Postpartum or puerperal psychosis is a severe mental illness manifested by episodes of mania, psychotic depression or mixed affective symptoms that affects women 4 to 6 weeks after childbirth (Jones, Chandra et al. 2014). It has unique characteristics from a neuroimmunological perspective. It occurs in a period of immune system hyperactivity and presents with very “organic” symptoms such as rapid deterioration, catatonia and fluctuating confusion resembling an encephalopathy. Additionally, most patients have good short-term outcome with complete symptom remission (Boyce and Barriball 2010). The aetiology is not yet completely understood, but immune system dysfunction has been implicated (Bergink, Kushner et al. 2011, Bergink, Burgerhout et al. 2013). An association with other pregnancy complications of possible immune aetiology, such as pre-eclampsia, has also been reported (Bergink, Laursen et al. 2015). Interestingly, three cases of NMDAR encephalitis presenting as postpartum psychosis have been reported (Yu and Moore 2011, Shaaban, Choo et al. 2012, Koksal, Baybas et al. 2015), and a cohort study of 96 patients with postpartum psychosis identified 4 patients with neuronal surface-binding antibodies, two of which were targeting the NMDA receptor (Bergink, Armangue et al. 2015). Only one case (Gotkine, Ben-Hur et al. 2011) of VGKC encephalitis manifesting as postpartum psychosis was reported and these antibodies have not been tested in large patient cohorts. There are no published studies on the long-term outcome of the children of women with NMDAR antibodies who suffered from postpartum psychosis, even though IgG antibodies are known to be transferred from mother to foetus during pregnancy.

1.5 The immunology of pregnancy

It is a basic immunological principle that a normal organism will mount a strong immune response against foreign tissue. On the other hand, the maternal immune system needs to tolerate the paternal semi-allogeneic tissues of the foetus. The mechanisms for this apparent immunological contradiction have intrigued many immunologists since the first theories were raised by Peter Medawar in the 1950s (reviewed in (Moffett and Loke 2004)). Many factors have been shown to play a role in this maternal 'immunosuppression', including local, uterine, evasion mechanisms (such as the absence of classical MHC alloantigens in the trophoblast, suppression of T cells by local depletion of tryptophan, apoptosis of activated lymphocytes and mechanisms of complement suppression), the presence of uterine NK cells during placentation (important regulators of decidua implantation and vascularization) and the expansion of regulatory T cells (reviewed in (Trowsdale and Betz 2006)). However, this maternal 'immunosuppression' is exclusive to responses against the foetus, since both mother and child continue to need protection against pathogenic microbial agents. In fact, the maternal protection of the child is prolonged after birth, since the newborn relies heavily on the maternal antibodies acquired *in utero* and via milk for defence against pathogens.

1.5.1 Mother to foetus transfer of immunity

Another eminent immunologist, Paul Erlich, showed in the later part of the 19th century that immunity is transferred from the mother to her foetus (reviewed in (Silverstein 1996)). Using simple, but very elegantly designed rodent immunization experiments, he succeeded in demonstrating that immunity is given from the mother and not the father, that it is not inherited in a genetic sense and that is transient in the newborn life. Next, by a series of cross-fostering experiments, he also managed to establish that it is through the maternal milk that the majority of immunity is transferred in rodents. Others have since explored the routes of immunity transfer in different animal species. In some

animals, like rats, mice and dogs, the transfer occurs primarily after birth through the maternal milk although some antibodies are transferred, to a lesser extent, through the inverted yolk sac (Brambell and Halliday 1956). In humans, rabbits and guinea pigs, however, this transport occurs antenatally, via the placenta, and less so through the maternal milk. Despite these differences in the timing of antibody transfer, the immunoglobulin G (IgG) is the only antibody isotype transferred (Brambell and Hemmings 1949, Brambell and Halliday 1956, Morphis and Gitlin 1970) and the molecule responsible for the transport of IgG is the neonatal Fc receptor (nFcR).

1.5.2 The neonatal Fc receptor

The nFcR molecule has a MHC class I-like structure. In fact, it is a heterodimer of a MHC class-I like heavy chain and the β2 microglobulin light chain (Simister and Mostov 1989). As expected by its function as transporter of maternal IgG and the routes of transmission in rodents and humans, the nFcR is highly expressed in the epithelial-cell layer of the proximal small intestine in the former, and in the placental syncytiotrophoblasts in the latter (reviewed in (Roopenian and Akilesh 2007)). The nFcR binds to the Fc portion of IgG only in an acidic milieu (pH <6.5). In rodents, the optimal pH for binding is provided by the gastric acid contents in the duodenum, and in humans by the endosomal vesicles where it is expressed in the synciotrophoblasts. Once the IgG transcytosis occurs, the physiologic pH of the foetal circulation will promote IgG release from the nFcR (Figure 1-5). Interestingly, the nFcR also protects IgG from catabolism, prolonging the half-life of this immunoglobulin (7-22 days) well beyond the half-life of the other isotypes (2-6 days). This is accomplished by the nFcR expressed in the endothelial cells that, by binding to IgG, prevents degradation and allows recirculation of this molecule (Lencer and Blumberg 2005).

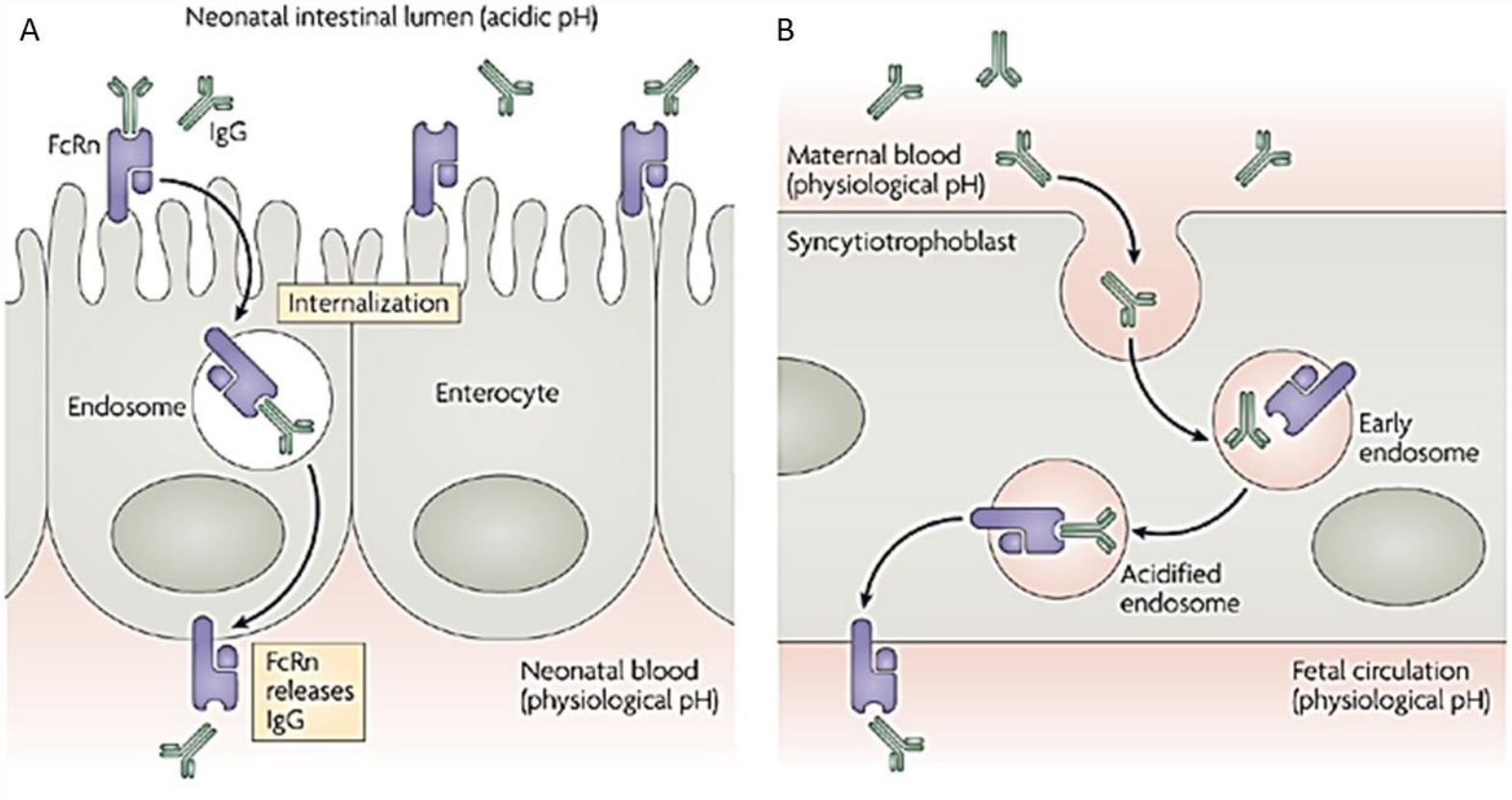


Figure 1-5: Neonatal Fc receptor-mediated transcytosis of IgG.

(A) Maternal IgG transcytosis at the neonatal intestinal lumen in rodents. (B) Maternal IgG transcytosis at the syncytiotrophoblast in humans. IgG binding to the neonatal Fc receptor is dependent on an acidic pH. Figure adapted from (Roopenian and Akilesh 2007)

1.5.3 Transfer of IgG throughout pregnancy in humans and rodents

In humans, maternal IgG transfer is almost negligible until the 13th week of gestation and remains at a low level until the third trimester, when a sharp increase occurs until birth. At this stage, the foetal total IgG concentration typically reaches or even exceeds the maternal concentration. The IgG1 subclass is most efficiently transported, and then IgG4, IgG3, and lastly IgG2, that has a much slower rise (Garty, Ludomirsky et al. 1994, Palmeira, Quinello et al. 2012). The gestational materno-foetal transfer of mouse IgG has not been so extensively investigated, but it is known that in rats there is also an increase in foetal IgG concentration up until birth, albeit less pronounced (Halliday 1955). However, the heterologous transfer of human IgG in the mouse occurs from day 12 of gestation and increases continuously until birth (Jacobson, Polizzi et al. 1999). It is unknown whether there is subclass selectivity in the mouse, although this is quite possible given how minute molecular changes can affect the nFcR affinity (a fact often explored by the pharmaceutical industry for the creation of new monoclonal antibodies).

1.6 Transfer of pathogenic antibodies during pregnancy

Considering the physiology of antibody transfer during pregnancy, it is perhaps a logical conclusion that pathogenic antibodies are transferred from mother to foetus in the same manner as protective antibodies, as long as they are of the IgG isotype, and if these pathogenic antibodies target foetal antigens, they will cause disease in the foetus or the newborn. In fact, there are many such examples of neonatal antibody-mediated disorders, some of them supported by maternal to foetal transfer animal models.

1.6.1 Neonatal autoimmune diseases

The haemolytic disease of the newborn is perhaps the classic example of induction of disease in the foetus / newborn by maternal antibodies. During the gestational period, a

small amount of the foetal blood can enter the maternal circulation. In these disorders, the mother suffers alloimmunization to paternally-derived red blood cell antigens and produces IgG antibodies that will cause mild to severe haemolytic disease in the foetus / newborn (reviewed in (Murray and Roberts 2007)). Similarly, neonatal thrombocytopenia can also occur in a mother who produces antibodies targeting paternally derived platelet antigens (a condition called foeto-maternal alloimmune thrombocytopenia) and if the mother herself suffers from immune thrombocytopenia, there is also a risk for thrombocytopenia in the neonate (reviewed in (Curtis 2015)).

Mothers who suffer from other antibody-mediated disorders also have an increased risk of having a baby with a neonatal autoimmune disorder. A well-documented case is neonatal lupus (reviewed in (Johnson 2014)). The neonatal lupus syndrome is a consequence of transplacental passage of anti-SSA/Ro and anti-SSB/La antibodies, or indeed other yet unknown cell-surface antibodies present in the sera of these mothers (Buyon, Winchester et al. 1993). It can occur in the offspring of women who suffer from systemic lupus erythematosus, Sjogren's syndrome, undifferentiated connective tissue disease or even in asymptomatic mothers who have those antibodies in circulation. The most feared complication is congenital heart block, which can be persistent through life and even fatal in a few cases, while other symptoms, such as cutaneous, hepatobiliary or haematological manifestations, are usually transient. The risk of a mother with anti-SSA/Ro and anti-SSB/La antibodies to have a child with congenital heart block is low (around 1-2% of cases) but this rises dramatically in further pregnancies of a mother with a previously affected neonate (to approximately 25%), suggesting some other inherent risk factors (reviewed in (Brucato, Cimaz et al. 2011)). Interestingly, an association between maternal lupus and neurocognitive problems in the offspring has been reported (McAllister, Kaplan et al. 1997, Neri, Chimini et al. 2004, Urowitz, Gladman et al. 2008), with the offspring showing deficits in memory and learning, and behaviour. Following the report of anti-dsDNA antibodies cross-reacting with the NR2A/NR2B subunits of the

NMDAR in lupus patients (DeGiorgio, Konstantinov et al. 2001, Kowal, Degiorgio et al. 2006), a maternal lupus mouse model was reported where dams that harboured these antibodies had offspring with cognitive impairment (Lee, Huerta et al. 2009). However, the association of these antibodies with neurocognitive deficits was studied in different cohorts of lupus patients with conflicting results (reviewed in (Lauvsnes and Omdal 2012)). Likewise, they haven't been undoubtedly shown to bind extracellular epitopes, nor have they been identified in mothers of children with neurodevelopmental problems.

The antiphospholipid syndrome is also a known cause of pregnancy complications, such as miscarriage, late foetal losses and pre-eclampsia but, even though 30% of babies born to mothers with antiphospholipid syndrome show evidence of passive transfer of antiphospholypid antibodies, neonatal thrombosis is an extremely rare event (reviewed in (Levy, Dos Santos et al. 2015)). Other disorders believed to be associated with transfer of maternal antibodies are the neonatal thyroid diseases, neonatal Bechet's disease, neonatal dermatomyositis or neonatal scleroderma, although their occurrence is much less frequent and doubts remain on the causal role of the antibodies (Chang 2012).

Neonatal myasthenia gravis is the best studied neurological disease caused by maternal transfer of antibodies. This condition is detected in 10-20% of neonates born to AChR antibody-positive mothers (Vernet-der Garabedian, Lacokova et al. 1994) and was also described in MuSK antibody-positive mothers (Niks, Verrips et al. 2008, O'Carroll, Bertorini et al. 2009, Murray, Kedar et al. 2010). It is usually transient, lasting the time necessary for clearance of the antibodies from the neonatal circulation, and the clinical symptoms range from mild to severe. Poor swallowing and suckling, hypotonia and mild respiratory distress are common in the mild forms, and can lead to need for oral gavage and assisted mechanical ventilation in severe cases. A much more severe, related condition occurs if the mother has antibodies that block the function of the foetal form of the AChR, which can cause decreased foetal movements *in utero*. This condition, known as arthrogryposis multiplex congenita (AMC), can lead to foetal or neonatal death or

cause permanent deformities due to multiple joint contractures (Riemersma, Vincent et al. 1996). Likewise, other features such as hearing impairment and even cognitive impairment can persist (Hacohen, Jacobson et al. 2015), clearly showing how a time-limited antibody-mediated attack during neurodevelopment can lead to permanent disability. Interestingly, this disorder can affect children born to asymptomatic or only mildly symptomatic mothers (Dalton, Clover et al. 2006).

A rodent model of mother to foetus transfer of foetal AChR antibodies (Jacobson, Polizzi et al. 1998, Jacobson, Polizzi et al. 1999) has confirmed the association between AMC and these antibodies. In this model, a significant number of foetuses from dams injected with plasma from AChR antibody-positive women, whose offspring had AMC, were stillborn and showed fixed joints and other deformities, mimicking the disease phenotype.

1.6.2 Foetal outcome in pregnant patients with antibody-mediated CNS diseases

The discovery of CNS disorders associated with neuronal-surface antibodies is still recent and, consequently, there is a lack of information on the pregnancy outcomes of these patients in the literature. The pregnancy outcome in patients with neuromyelitis optica spectrum disorders, a CNS demyelinating disorder caused by antibodies towards aquaporin 4 (a water channel expressed in astrocytes), is the best documented so far. Not only was the effect of pregnancy on the disease studied (Kim, Kim et al. 2012), showing an increase in relapse rate and an increase in disease onset in the first and second trimester after delivery, but also adverse effects on pregnancy were documented (Nour, Nakashima et al. 2016). A retrospective cohort showed an increased risk for miscarriage in pregnancies shortly before or after disease onset. An increased risk for preeclampsia was also documented in the same cohort, but only in the context of multiple autoimmune disorders in the mother (Nour, Nakashima et al. 2016). A mouse model of

maternal to foetal transfer of aquaporin-4 antibodies revealed inflammatory cell infiltration into the placenta and placental necrosis in dams injected with these antibodies and human complement, which resulted in foetal death in a significant percentage of cases (Saadoun, Waters et al. 2013). Surviving pups, however, showed no gross histological signs of CNS disease, which might be due to the fact that this protein is only expressed in the CNS at a late stage in neurodevelopment (Fallier-Becker, Vollmer et al. 2014).

The foetal outcome in patients with autoimmune encephalitis has only been documented in a few case reports (Table 1-3). All cases (Ito, Abe et al. 2010, Kumar, Jain et al. 2010, Magley, Towner et al. 2012, McCarthy, Dineen et al. 2012, Jagota, Vincent et al. 2014, Lamale-Smith, Moore et al. 2015, Lu, Samson et al. 2015) reporting pregnancy at or after disease onset, and where the baby outcome was provided, were from patients with NMDAR encephalitis, as expected given the age distribution in these conditions. Eight patients were reported and mostly presented a short follow-up of the offspring (longest being 3 years). Most patients were diagnosed at an early stage of pregnancy and received intense immunotherapy. In fact, in 3 mothers the antibody titres were already negative at delivery and, surprisingly, 2 mothers had antibodies only detected in CSF, which might justify the offspring good outcome. In 2 cases, however, the offspring showed neurodevelopmental sequelae. The first with torticollis and strabismus and the second with global developmental delay, seizures and cortical dysplasia. Nevertheless, it is important to note that since a number of therapeutic drugs were given to the mothers during pregnancy, we cannot exclude these as the cause for the neurodevelopmental problems observed. Despite the insufficient information given by this small number of cases, they unambiguously show that NMDAR antibodies are transferred from mother to foetus, since they were always detected in the foetal cord blood / newborn serum if they were still present in the mother.

Table 1-3: Publications on pregnancy outcome in patients with NMDAR encephalitis

Publication	Start of symptoms	NMDAR antibody titres – mother	NMDAR antibody titres - infant	Immunotherapy in pregnancy	Outcome (baby)	Offspring follow-up
Ito, 2010*	17th GW	Positive (blood and csf): decreasing titres during pregnancy, negative at delivery.	Unknown	Steroids	Baby normal at birth	
Kumar, 2010†	17th GW	19th GW: serum 1:320; CSF 1:640; 5 months post-delivery: serum 1:80.	Not tested	Steroids	No developmental delay at 5 months.	5 months
Kumar, 2010†	14th GW	14th GW: serum negative, CSF 1:80; 21st GW: serum negative, CSF 1:10; 32nd GW: Serum and CSF negative	At birth: serum, CSF, and umbilical cord blood negative	IvIg; steroids and plasma exchange	No developmental delay at 2 months.	2 months
Magleyl, 2012	5 years prior to pregnancy (symptomatic at pregnancy)	Antibody-positive 2 years prior to pregnancy; unknown during pregnancy	Not tested	None during pregnancy	Prematurity (35 weeks). Torticollis and strabismus detected at 4 months.	6 months
McCarthy, 2012	8th GW	8th GW : positive; 13th GW to delivery: negative	Not tested	Steroids and plasma exchange	Baby normal at birth	None
Jagota, 2014	9th GW	Information not given (but clinically no response to therapy; maternal outcome: death)	At birth: serum 1:450; at 2 months: serum 1:150; at 1 year: serum negative	IvIg Steroids	Neonatal period: episodes of fine abnormal movements (spontaneous and sound-induced). At 2 years: GDD and generalized seizures. MRI: cortical dysplasia	3 years
Lu, 2015	Prior to gestation (undetermined)	4th GW: CSF 1:5; serum negative	Unknown	Steroids and plasma exchange	Baby normal at birth	None
Lamale-Smith, 2015	20th GW	After 20th GW: CSF 1:80; serum positive	Fetal cord blood 1:20 at time of delivery.	IvIg; steroids and plasma exchange	Low Apgar score at birth. Respiratory and neuromuscular depression. Withdrawal-like symptoms. Supraventricular tachycardia. At 1 year: developmentally appropriate.	1 year

Abbreviations – NMDAR: N-methyl-D-Aspartate; GW – gestational week; CSF – cerebrospinal fluid; IvIg – Intravenous immunoglobulins; GDD – Global developmental delay; *Article in Japanese, information taken from abstract; † 2 patients reported in same publication.

1.7 Neurodevelopmental disorders and *in utero* exposure to maternal immune dysfunction

The term neurodevelopmental disorder is applied to a group of neurological illnesses that are the consequence of dysfunction occurring during neurodevelopment that permanently affects brain function. They typically manifest in early childhood, although some might present later, such as schizophrenia, and lead to a specific neurologic dysfunction (like epilepsy, language deficits or learning disabilities) or to a broader cognitive dysfunction and mental retardation. The prevalence of neurodevelopmental disorders in England is estimated to be around 3-4% of children (Emerson 2012).

Autism spectrum disorder (ASD) is one of the most common neurodevelopmental disorders of childhood, with a reported prevalence of 1% of the child population in the UK (Baird, Simonoff et al. 2006, Baron-Cohen, Scott et al. 2009). Affected children often have a heterogeneous presentation with variable degrees of deficits in social interaction and communication, and restricted interests or repetitive behaviours (reviewed in (Lai, Lombardo et al. 2014)). Other common neurodevelopmental disorders are mental retardation and speech and language disorders. Aetiology is currently unknown for the majority of these disorders but both genetic and environmental factors are believed to play a role (reviewed in (van Loo and Martens 2007)). Among the diverse risk factors, *in utero* or early life exposure to an abnormal immune response is supported by several lines of evidence from different fields, including epidemiology, immunogenetics and immunology, particularly in autism (Estes and McAllister 2015). This is the basis for the "immune theory" of neurodevelopmental disorders, which postulates that a genetically predisposed individual, if exposed to an immune system stressor (such as environmental toxins, infections or maternal immune molecules) during the prenatal or early postnatal period, will suffer irreversible neural circuit changes that could lead to a neurodevelopmental disorder (Figure 1-6) (reviewed in (Gottfried, Bambini-Junior et al. 2015)).

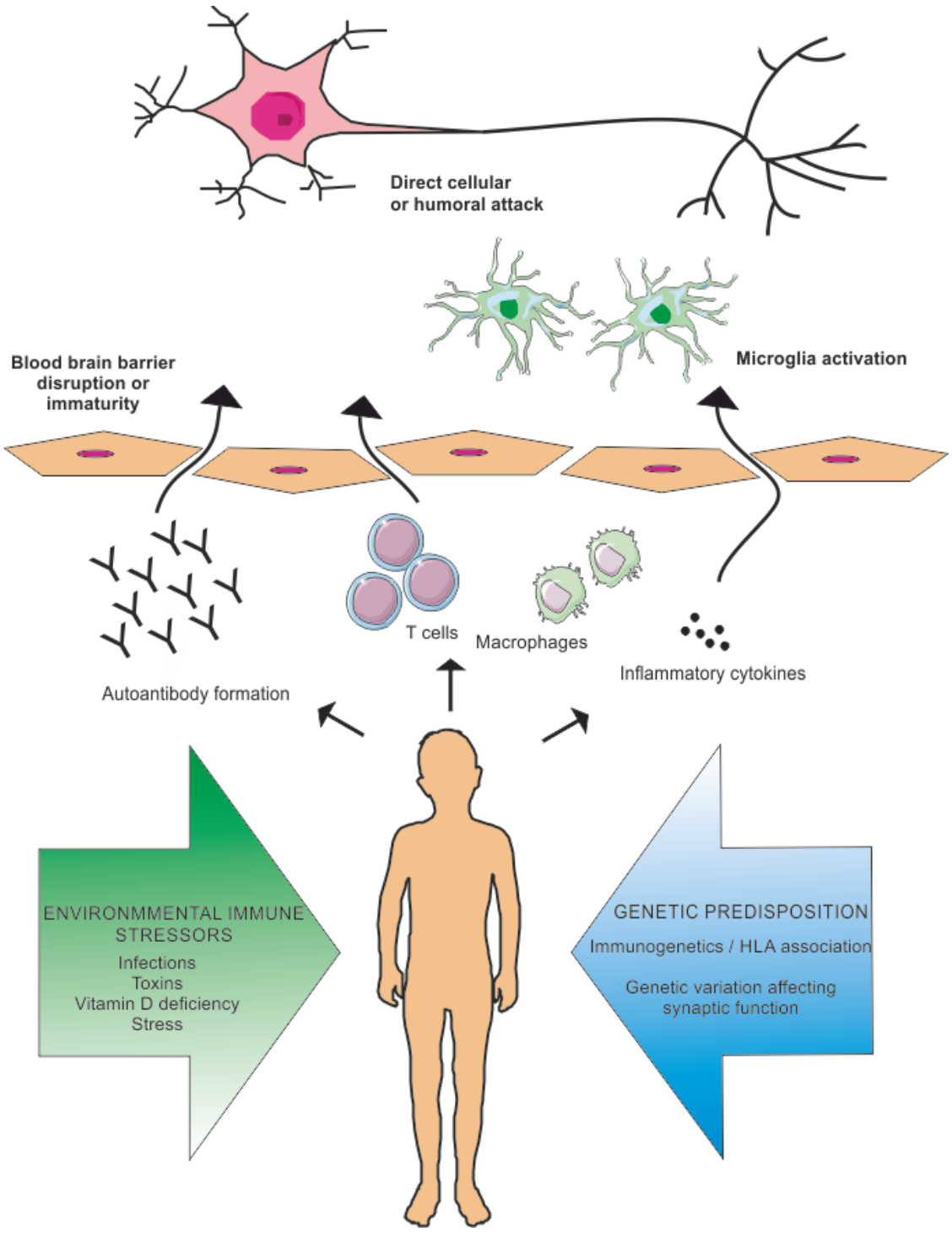


Figure 1-6: Immune theory of neurodevelopmental disorders

Environmental immune stressors acting on a genetically predisposed individual would lead to immune system activation and consequent humoral or cellular attack on neuronal cells. HLA, human leukocyte antigen

1.7.1 Epidemiology

Autoimmune disorders often associate within families (Cardenas-Roldan, Rojas-Villarraga et al. 2013). This familial association is also true for some neurodevelopmental disorders and autoimmunity. An association between a maternal history of celiac disease or rheumatoid arthritis, or a familial diagnosis of type 1 diabetes, and ASD was found in one large epidemiological study (Atladottir, Pedersen et al. 2009). Other studies found similar familial associations of ASD with maternal lupus or rheumatoid arthritis (Brimberg, Sadiq et al. 2013), or maternal diabetes (Xu, Jing et al. 2014), while others found an association with allergies and several autoimmune disorders in the patients themselves (Kohane, McMurry et al. 2012, Zerbo, Leong et al. 2015). A similar association was also found in patients with attention deficit and hyperactivity disorder (ADHD) (Chen, Su et al. 2013), suggesting that there might be a contribution of the immune system in other neurodevelopmental disorders as well.

1.7.2 Immunogenetics

Among the genetic risk factors for several neuropsychiatric disorders, multiple genes affecting the immune function have been described, particularly those in the MHC region. In ASD, an association with MHC class I and II genes, particularly the HLA A2 and HLA DR4 genes was found (Needleman and McAllister 2012). Recognition of expression of MHC class I molecules in the CNS (reviewed in (Boulanger 2004)), particularly during neurodevelopment, might explain this genetic association. It is possible that they have an important role via direct effects on brain development, as well as through immune mechanisms. Likewise, the MHC III genes encoding for complement proteins, particularly C4, have also been linked to ASD, with several studies showing an increased incidence of the C4b null allele (Warren, Singh et al. 1991, Odell, Maciulis et al. 2005, Mostafa and Shehab 2010). A very recent study in schizophrenia also showed an association with complement component 4 (C4) alleles (Sekar, Bialas et al. 2016). This

study implicated excess C4 activity as a pathophysiological mechanism in schizophrenia. It is unknown, however, if this is a mechanism shared by other neuropsychiatric / neurodevelopmental conditions. Other proteins in the classical complement cascade, particularly C3, have been implicated in synaptic pruning (reviewed in (Stephan, Barres et al. 2012)).It is possible that the pathophysiology of different neurodevelopmental disorders converge in complement-dependent aberrant synapse elimination during normal brain development (reviewed below).

1.7.3 Maternal immune activation

A strong body of epidemiological evidence shows a link between exposure to infectious agents during pregnancy and increased risk of neurodevelopmental disorders in the progeny (Patterson 2002, Knuesel, Chicha et al. 2014). This is supported by the “winter baby” phenomenon, reported in autism (Zerbo, Iosif et al. 2011) and other neurodevelopmental disorders (Liederman and Flannery 1994), which describes an increased risk of these disorders in children conceived in the colder months. There is no association with a particular infectious agent; infections by virus, bacteria or parasites have all been associated to these disorders, which likely indicates a dysfunction caused by the maternal immune activation and not the infection *per se*. It is postulated that this immune activation *in utero* might result in chronic dysfunction in the progeny, since neuropathological studies revealed increased microglial activation in ASD patients (Rodriguez and Kern 2011) and equally increased pro-inflammatory markers in the serum or CSF (Chez, Dowling et al. 2007, Ashwood, Krakowiak et al. 2011, Zerbo, Yoshida et al. 2014), when compared to controls, and many years after disease diagnosis. Animal models support this link between maternal infections / immune activation and structural and behavioural anomalies in the offspring. Existing models are based on maternal exposure to the infectious agent (e.g. human influenza virus), an infectious mimic (e.g. polyriboinosinic-polyribocytidilic acid [Poly(I:C)] or

lipopolysaccharide [LPS]) or the immune mediators themselves (inflammatory cytokines) (Meyer, Feldon et al. 2009).

1.7.4 The case for an antibody mediated form of autism

The idea of maternal pathogenic antibodies as culprits in ASD goes back to the 1990s when a first, small cohort study identified antibodies targeting paternal lymphocyte epitopes in the sera of mothers of children with autism (Warren, Cole et al. 1990). These findings were largely forgotten, and in fact never replicated, until two studies of maternal to foetal transfer of serum from mothers of autistic or dyslexic children in a mouse model were published a decade later (Vincent, Deacon et al. 2002, Dalton, Deacon et al. 2003). In the first study (Vincent, Deacon et al. 2002), dams injected with sera from one mother of two consecutive dyslexic children, but not dams injected with control sera, had pups that displayed deficits in neuromotor coordination that correlated with changes in cerebellar metabolites, assessed by magnetic resonance spectroscopy. The second study (Dalton, Deacon et al. 2003) showed partly similar findings in dams injected with sera from one mother with one autistic child and one with a language disorder. Importantly, this maternal serum was shown to bind to Purkinje neurons and other brainstem neurons, as well as to the cell surface of a neuroblastoma cell line, suggesting a membrane antigen. Since then, several publications assessed the presence of antibodies towards foetal antigens in the serum of mothers of autistic children (Zimmerman, Connors et al. 2007, Braunschweig, Ashwood et al. 2008, Croen, Braunschweig et al. 2008, Singer, Morris et al. 2008, Braunschweig, Duncanson et al. 2012, Braunschweig, Krakowiak et al. 2013, Brimberg, Sadiq et al. 2013, Nordahl, Braunschweig et al. 2013, Rossi, Fuentes et al. 2013, Piras, Haapanen et al. 2014). Western blots of mammalian brain extracts were used as the method of antibody detection in these studies and protein bands were identified by maternal serum IgG in cases but not in controls. However, it is important to note that the use of western blotting

for antibody-detection does not distinguish intra or extracellular epitopes or respect the three-dimensional structure of the protein, as mentioned above. As a consequence, the epitopes identified by this method are often intracellular proteins (lactate dehydrogenase, stress-induced phosphoprotein 1, cypin and collapsing response mediator protein 1 and 2) (Braunschweig, Krakowiak et al. 2013), making it unlikely that they are truly pathogenic antibodies. Despite this, these and other groups have observed behaviour and neuromotor deficits in the offspring of dams injected with purified IgG from mothers of autistic children, but not controls, in mouse (Singer, Morris et al. 2009, Braunschweig, Golub et al. 2012, Kadam, French et al. 2013) and primate (Martin, Ashwood et al. 2008, Bauman, Iosif et al. 2013) models of maternal to foetal transfer. In recent studies (Camacho, Jones et al. 2014, Martinez-Cerdeno, Camacho et al. 2014), the purified human IgG was directly injected intraventricularly in the embryos and pathological correlates for the observed behavioural changes were investigated. These studies showed increased stem cell proliferation in the subventricular zone of the embryonic neocortex, increased adult brain size and weight, and increased size of adult cortical neurons in those embryos exposed to IgG from mothers of autistic children. However, no study has identified a specific, neuronal surface antigenic target for the antibodies present in the maternal serum or presented evidence for the mechanism of CNS damage. Table 1-4 and Table 1-5 summarize the studies on maternal antibodies in autism and the maternal to foetal transfer animal models published to date, respectively.

The study of autoantibodies in the plasma of autistic patients themselves has produced contradictory findings and there is no evidence of clinical relevance (Wills, Cabanlit et al. 2007, Morris, Zimmerman et al. 2009, Rossi, Van de Water et al. 2011). However, autistic features can exist in children with known antibody-mediated encephalopathies (Creten, van der Zwaan et al. 2011, Hacohen, Wright et al. 2012, Scott, Richer et al. 2014), particularly in children with regressive forms of autism.

Table 1-4: Publications on maternal antibodies in autism and ASD

Publication	Antigen	Method of detection	Population
Warren, 1990	Paternal PBMC	LMC test	11 mothers of children with autism
Dalton, 2003	Rodent Purkinje cells and other neurons	IHC	1 mother of a child with autism and of a second child with a language disorder
Braunschweig, 2008	Human foetal and adult brain proteins (reactivity to bands at 37 and 73kDa)	WB	61 mothers of children with ASD; 62 mothers of TD children;40 mothers of children with non-ASD developmental delays
Croen, 2008	Human foetal brain proteins (reactivity to bands at 39 kDa and 73 kDa)	WB	84 mothers of children with ASD; 49 mothers of children with mental retardation or developmental delay; 160 mothers of children with TD
Singer, 2008	Human and rodent foetal and adult brain proteins (reactivity to bands at 36 kDa and 61 kDa)	WB	100 mothers of children with ASD; 100 mothers of children with TD
Braunschweig, 2011	Human and rhesus macaque foetal proteins (reactivity to bands at 37 and 73 Kda)	WB	560 mothers of children with full autism; 71 mothers of children with ASD; 183 mothers of children with TD
Goines, 2011	Human and rhesus macaque cerebellum proteins (reactivity to bands at 37, 39 and 73 Kda)	WB	194 mothers of children with autism, 65 mothers of children with ASD and 180 mothers of children with TD
Braunschweig, 2012	Foetal Human and rhesus macaque brain proteins (reactivity to bands at 37 and 73 Kda)	WB	204 mothers of children with autism; 71 mothers of children with ASD; 102 mothers of children with developmental delay; 183 mothers of children with TD
Nordahl, 2013	Foetal rhesus macaque brain proteins (reactivity to bands at 37 and 73 Kda)	WB	131 mothers of children with autism or ASD and 50 mothers of children with TD
Rossi, 2013	Adult and foetal rhesus macaque cerebellum proteins (reactivity to bands at 37, 39 and 73 Kda)	WB	37 mothers of children with ASD and 37 mothers of children with TD
Braunschweig, 2013	Foetal rhesus macaque brain proteins (reactivity to bands at 37 and 73 Kda). Antigens identified by mass spectrometry: LDH, YBX1, cypin, STIP1, CRMP1 and CRMP2	WB	246 mothers of children with autism or ASD and 149 mothers of children with TD
Brimberg, 2013	Adult and foetal mouse brain antigens (frontal cortex, hippocampus and cerebellum)	IHC (and WB for confirmation)	2749 mothers of children with ASD; 653 unselected controls
Piras, 2014	Human and rhesus macaque foetal proteins (reactivity to bands at 37, 39 and 73 Kda)	WB	333 mothers of children with ASD; no control population for the maternal samples

Abbreviations: LMC – Lymphocyte mediated cytotoxicity; WB – Western blot; IHC – Immunohistochemistry; ASD – Autism spectrum disorders; TD – Typical development

Table 1-5: Maternal to foetal transfer animal models in autism

Publication	Species	Type of model and administration	Injected material	Behavioural outcome	Histological outcome
Dalton, 2003	Mice	Passive transfer; intraperitoneal injection (dam)	Sera of MAC and sera from controls	Altered exploration and motor coordination	Not assessed (reductions in the concentrations of choline and creatine relative to water in MRS of the mice cerebella)
Singer, 2009	Mice	Passive transfer; intraperitoneal injection (dam)	IgG purified from MAC with western blot reactivity to foetal brain proteins and controls	MAC juvenile mice showed increased activity in a novel open field environment. MAC adult mice showed anxiety-like behaviours, increased response to startle and decreased sociability	Microglia activation in MAC mice (qualitative assessment)
Martin, 2008	Rhesus monkeys	Passive transfer; endovenous injection (dam)	IgG purified from MAC with western blot reactivity to foetal brain proteins and controls	More stereotypies and higher levels of motor activity	Not assessed
Braunschweig, 2012	Mice	Passive transfer; endovenous injection (dam)	IgG purified from MAC with western blot reactivity to foetal brain proteins and controls	Slower motor and sensory development during neonatal period; increase anxiety and marginal social interaction deficits in adult offspring	No differences in the mean number of CA1 pyramidal Neurons
Kadam, 2013	Mice	Passive transfer; intraperitoneal injection (dam)	IgG purified from MAC with western blot reactivity to foetal brain proteins and controls	Not assessed	Increased cell proliferation in the subventricular and subgranular zones and reduced postnatal neurogenesis
Bauman, 2013	Rhesus monkeys	Passive transfer; endovenous injection (dam)	IgG purified from MAC with western blot reactivity to foetal brain proteins and controls	Maternal heightened protectiveness during early development and changes in social interaction during adult life	Male IgG-ASD offspring had enlarged brain volume (white matter), particularly in the frontal lobe
Camacho, 2014	Mice	Passive transfer; intraventricular injection (embryos)	IgG purified from MAC with western blot reactivity to foetal brain proteins and controls	Increased grooming activity, decreased social interaction and increase marble burying activity	Not assessed
Martinez-Cerdeno, 2014	Mice	Passive transfer; intraventricular injection (embryos)	IgG purified from MAC with western blot reactivity to foetal brain proteins and controls	Not assessed	Increased cellular proliferation in the subventricular zone of the embryonic neocortex, increased adult brain size and weight, and increased size of adult cortical neurons

Abbreviations: IgG – Immunoglobulin G; MAC – mothers of autistic children; MRS - magnetic resonance spectroscopy; ASD – Autism spectrum disorders

1.7.5 Other neurodevelopmental disorders

The presence of antibodies in mothers of children with other neurodevelopmental disorders, including intellectual disability, have not been extensively investigated in large cohorts. However, some of the studies published on ASD (Braunschweig, Ashwood et al. 2008, Croen, Braunschweig et al. 2008, Singer, Morris et al. 2008, Braunschweig, Duncanson et al. 2012) have used mothers of children with other developmental disorders (usually mixed groups) as controls, but no brain-targeting antibodies were detected.

1.8 The developing central nervous system

The normal development of the neocortex, perhaps the structure of the nervous system better studied by developmental biologists, is a highly complex process that depends on a tightly orchestrated sequence of events, including neurogenesis, migration of neurons to their final position in the structure of the neocortex, acquisition of distinct neuronal identities, and formation of precise synaptic connections, continuously refined as a consequence of neuronal activity (reviewed in (Stiles and Jernigan 2010).

1.8.1 Neurogenesis, neuronal migration and cerebral cortical development

After closure of the neural tube, which in humans occurs at embryonic day 30 and in mice at embryonic day 9.5-10, the telencephalic vesicles are formed. The germinative area lies in the ventricular surface of the telencephalon and is designated the ventricular zone (VZ). Cells from the pseudostratified epithelium at the margin of the embryonic cerebral ventricles initially undergo symmetric cell division and give rise to two progenitor cells. As neurogenesis ensues, cells begin to undergo asymmetric cell division. Some cells begin to differentiate into neurons, while others remain as progenitor cells. As cortical development progresses, the proportion of cells differentiating into neurons

gradually increases leading to depletion of progenitor cells (Caviness and Takahashi 1995). This gives rise to a second proliferative zone, immediately above the VZ, called the subventricular zone (SVZ). After differentiation, neurons must migrate to their final position in the cortex. The first neurons to migrate from the VZ (around E11 in mice and E42 in humans) give rise to the preplate; a structure that is composed of neurons organized in an inside-out sequence, whereby the most recent neurons lie underneath the older ones. Between the VZ and the preplate is an area composed of axonal processes called the intermediate zone, which will later develop into the subcortical white matter. The preplate then divides into two layers; the outer layer, designated marginal zone, which contains the Cajal-Retzius cells, and the inner layer, designated subplate. The marginal zone is the outermost layer in the developing cortex (which in the adult corresponds to the molecular layer or layer I). The Cajal-Retzius cells are transient neurons in development that are a major source for reelin, a protein essential for the formation of the laminar cortical pattern, as evidenced by the cortical layering disorganization in the reeler mouse (a mouse that has a spontaneous mutation in the reelin gene) (reviewed in (Martinez-Cerdeno and Noctor 2014)). The subplate is also a transient structure in cortical development. It is composed of a heterogeneous population of neurons with crucial roles in establishing intra- and extra-cortical circuits (reviewed in (Hoerder-Suabedissen and Molnar 2015). The subplate neurons also seem to have a transient protein secretion function and these secreted proteins, such as neuroserpin and NTX1, are likely to have a role in synaptic development and maturation (Kondo, Al-Hasani et al. 2015). Between marginal zone and subplate is formed the cortical plate, which will give rise to the cortical layers II through VI.

Two neuronal migration routes have been identified in the forebrain and are classified according to their direction: radial migration and tangential migration. Radial migration is the predominant route for cortical glutamatergic neurons. This process depends on a system of radial glial fibres. The glial cell body is in the VZ and its cell processes are

elongated across the full thickness of the cortical wall and reach the pial surface. This arrangement provides a guideline for the locomotion of neurons, appropriately designated glial-guided neuronal locomotion (Rakic 1972). However, during early corticogenesis, radial neurons can migrate in a glia-independent way by a process of somal translocation (Nadarajah, Brunstrom et al. 2001). Interestingly, those neurons are generated from radial glial progenitors (Noctor, Flint et al. 2001).

The inhibitory interneurons and a smaller population of subcortical neurons, on the other hand, are generated in the medial or caudal ganglionic eminences at the ventral forebrain. These neurons move tangentially across the plane of the glial fibre system, descending towards the subventricular zone before finally migrating radially to their specific laminar position (reviewed in (Tanaka and Nakajima 2012)). Interestingly, radial migrating neurons and interneurons migrating tangentially share the same laminar position if born at the same time. Importantly, neurons born at later stages in development will be located in more peripheral, outer layers, while the earliest neurons will form the deeper cortical layers.

1.8.2 Synaptic pruning and circuit refinement

Synaptic density is already high in neonatal brains, but during early infancy it increases even further before decreasing to the stable densities of adulthood (Huttenlocher 1979). So, as the nervous system develops and the brain wiring is established, a process of synapse elimination seems to occur during adolescence and early adult life (Petanjek, Judas et al. 2011). Microglia are key cellular players in this process of synaptic refinement and this process is mediated by classic complement cascade proteins, and the selection of synapses for elimination seems to be activity-dependent. C1q and C3 knockout mice exhibited defects in synaptic refinement and elimination in the developing visual system (Stevens, Allen et al. 2007). Likewise, a C1q knockout mice showed enhanced excitatory synaptic connectivity, suggesting an identical role for the

complement in synapse elimination in the neocortex (Chu, Jin et al. 2010). These complement-tagged synapses are then engulfed and removed by microglia, a process that is partly mediated by the microglial C3 receptor (Paolicelli, Bolasco et al. 2011) (Schafer, Lehrman et al. 2012).

Importantly, many antigenic targets identified in patients with autoimmune encephalitis have important roles in neurodevelopment. The importance of the most important CNS antigenic targets in neurodevelopment is reviewed next.

1.9 Importance of known CNS antigenic targets during neurodevelopment

1.9.1 CASPR2

In humans, myelination does not occur until the 28th week of gestation and in mice it is only achieved in the brain at postnatal day 45-60 (Baumann and Pham-Dinh 2001). On the other hand, CASPR2 expression during human brain development is already high in the frontal lobes, striatum and dorsal thalamus, while in mice it starts around embryonic day 14 and is broadly expressed in the developing cortex, hippocampus and the ganglionic eminences (Rodenas-Cuadrado, Ho et al. 2014). Given the timeline of CASPR2 expression, it is postulated that it has other functions, beyond the juxtaparanode, and with relevance for neurodevelopment. In fact, CASPR2 has been implicated in neuronal migration and neuronal network formation (Penagarikano, Abrahams et al. 2011). Patients with homozygous mutations of CNTNAP2, the gene encoding CASPR2, have histological abnormalities of the brain, consisting of macroscopic areas of cortical thickness, blurring of white/ gray matter delineation and microscopical evidence of neuronal disorganization, aberrant morphology, astrogliosis and reduced CASPR2 expression (Strauss, Puffenberger et al. 2006). The *Cntnap2*

knockout mouse model (Penagarikano, Abrahams et al. 2011) recapitulated some of these abnormalities, displaying ectopic neurons in the corpus callosum, mislocalization of neurons within the cytoarchitecture of the somatosensory cortex and reduction of inhibitory gabaergic interneurons. *In vitro* knockdown of CASPR2 using sh-RNA showed suppression of neural network activity and an additional role in controlling arborisation of dendrites and maturation of dendritic spines (Anderson, Galfin et al. 2012). Additionally, CASPR2 was shown to be important in the correct trafficking of AMPAR in cultured cortical pyramidal neurons (Varea, Martin-de-Saavedra et al. 2015). Consistent with the function of CASPR2 during neurodevelopment, patients with homozygous mutations of CNTNAP2 suffer from the syndrome of cortical dysplasia-focal epilepsy (CDFE), and have frequent seizures, motor and intellectual delay, and autistic-like behaviour (Strauss, Puffenberger et al. 2006). Other disruptions of CNTNAP2 have been associated with several neurodevelopmental disorders, from ASD to intellectual disability, schizophrenia, epilepsy, ADHD and speech delay (Rodenas-Cuadrado, Ho et al. 2014). Interestingly, the *Cntnap2* knockout mouse (Penagarikano, Abrahams et al. 2011) also exhibits parallels with the human condition, with the animals displaying ASD core deficits, hyperactivity and epilepsy.

1.9.2 LGI1

During mouse early development, LGI1 expression follows a pattern of a dorsal to ventral spread. At early embryonic development, high levels of LGI1 expression were found in the surface ectoderm, the neuroepithelium, mesenchymal connective tissue, and sensory organs, but in postnatal development the expression is highest in the hippocampus and choroid plexus (Silva, Wang et al. 2011). Interestingly, LGI1-expressing cells co-express neuronal proteins important for neuronal migration (nestin, doublecortin and beta 3 tubulin) (Silva, Wang et al. 2011). The pattern of expression and this association points towards an important role of LGI1 in neurodevelopment. In fact, it

was shown, in a study using transgenic mice expressing a mutant LGI1 protein, that LGI1 regulates the maturation and pruning of glutamatergic synapses during early postnatal development. It showed that the mutant LGI1 inhibited dendritic pruning leading to an increase in the excitatory synaptic transmission, and ultimately epileptogenesis (Zhou, Lee et al. 2009).

1.9.3 NMDAR

The NMDAR is essential at several levels of brain development. Considering that NR1 is the obligatory subunit, it is the developmental expression pattern of the NR2 subunits that suggests a role in the fine-tuning at neural population and neural circuitry level. Supporting this perspective is the fact that NR2 subunits have very distinct electrophysiological properties, although the exact functional significance of each subunit is still largely unknown (Ewald and Cline 2009). However, transgenic mice with deletion of individual subunits have normal gross brain morphology, showing a more subtle effect at the level of the synapse and neural circuits. NR1 and NR2B knockout animals (Forrest, Yuzaki et al. 1994, Kutsuwada, Sakimura et al. 1996) die shortly after birth, while NR2A and NR2C null mice (Kadotani, Hirano et al. 1996) develop and mate normally, which is in accordance with their temporal pattern of expression.

Focusing on the NR1 subunit, the putative target of antibodies in NMDAR encephalitis (Gleichman, Spruce et al. 2012), mice expressing only 5% of this subunit survive until adulthood but develop behavioural abnormalities, including increased motor activity, deficits in social interaction and stereotypies akin to the manifestations seen in schizophrenic patients (Mohn, Gainetdinov et al. 1999). It is also interesting that NMDAR antagonists (e.g. ketamine, phencyclidine) can also induce schizophrenia-like symptoms in healthy volunteers and have been used in several animal models of this disease (Moghaddam and Krystal 2012). This, and the fact that many schizophrenia related

genes converge on glutamate and NMDA receptors (Harrison and West 2006), supports the glutamatergic theory for this disease. However, an NMDAR dysfunction has also been implicated in the pathophysiology of many other neurodevelopmental disorders, particularly ASD (Lee, Choi et al. 2015), and also intellectual disability or epilepsy (Burnashev and Szepetowski 2015, Lemke, Geider et al. 2016).

1.10 Potential modulating factors in antibody-mediated neurodevelopmental disorders

The known CNS antigenic targets, particularly CASPR2, LGI1 and NMDAR, are good candidates for an antibody-mediated neurodevelopment disorder, given their relevance in normal brain development. However, it is unlikely that exposure to a CNS targeting antibody during development would be the only factor responsible for the clinical outcome. Individual genetic predisposition, function and maturation of the foetal blood brain barrier, expression levels of the nFcR, timing of the antibodies in relation to the maximum potential damage to the developing baby, and presence of other inflammatory/immunologic mediators are likely to be contributing factors, although none of these were studied in the context of an antibody-mediated neurodevelopmental disorder.

As mentioned in section 1.7.2, several neurodevelopmental disorders have common genetic risk factors, including genetic variation in the MHC region and different alleles of C4. It is possible that immune/neurodevelopmental processes regulated by these genes are activated differently in individuals with different genetic susceptibility after exposure to a potentially pathogenic antibody. Other potential factors might relate, for instance, to blood brain barrier permeability. In adult patients with NMDAR antibodies, clinical significance was correlated with perturbations of blood-brain barrier function, such as birth complications (Hammer, Stepniak et al. 2013). The relevance of

neurodevelopmental stage at antibody exposure in the occurrence of a neurodevelopmental disorder is largely unknown, but it is easy to postulate that an antibody attack at different developmental ages might interfere with different processes in brain development that might be more or less susceptible to permanent sequelae.

1.11 Aims of this DPhil

The aim of this DPhil was to investigate the role of the maternal immune system in the pathophysiology of neuropsychiatric diseases, and in particular to assess the significance of autoantibodies in the aetiology of psychosis, autism and other neurodevelopmental disorders. The initial focus was on the effects of autoantibodies during adult life, in mothers who developed a first psychotic episode after pregnancy, but the results shifted the focus to the effects of maternal antibodies during foetal neurodevelopment and the relationship with neurodevelopmental and neuropsychiatric disorders.

A dual approach was undertaken to answer the questions dealt with in this thesis. First, the effect of mother-to-foetal transfer of antibodies was studied in patient cohorts, including patients with psychosis, autism, mental retardation and other disorders of psychological development. This was possible through a collaboration with the Danish biobank and the Danish registers, allowing us access to pregnancy serum samples from mothers of children later diagnosed with a neurodevelopmental disorder and from mothers of children with typical development. Established methods for detection of antibodies targeting neuronal surface proteins, using live cells, were used for this purpose (results in Chapter 3). Secondly, the effects of pathogenic neuronal surface antibodies in the foetal brain and long-term behaviour of the offspring were assessed in a mouse maternal-to-foetal model. Several known pathogenic antibodies, such as NMDAR, CASPR2 and LGI1 antibodies, were used for the first set of experiments, but

the main study was done with CASPR2 antibodies. In this animal model, the first aim was to establish whether the known CNS antibodies are effectively and efficiently transferred to the embryonic circulation and whether they reach foetal brain parenchyma and cause gross morphological abnormalities in the foetus (results in Chapter 4). Next, the effects of the antibody-transfer in the neonatal and adult behaviour of the offspring were assessed (results in Chapter 5). Finally, the long-term neuropathological sequelae of the antibody exposure were examined, by assessing general morphological and morphometric parameters, cortical lamination patterns, microglial and neuronal densities, and number of glutamatergic synaptic profiles (results in Chapter 6).

The ultimate goal was the identification of pathogenic antibodies in mothers of children who later developed neuropsychiatric disorders, as this could lead to different treatment approaches before irreversible neural circuit damage is established, and would allow us to better understand the pathophysiology of these diseases.

Chapter 2: Materials and Methods

2.1 Autoantibody screening in human cohorts

Research work performed on human samples was possible through collaboration with the Danish registries and the Statens Serum Institut. Initial work was performed on a maternal psychosis cohort, aiming at assessing the importance of antibodies in postpartum psychosis. In this cohort an association between maternal antibodies and mental retardation and other disorders of psychological development in the offspring was found. To validate this unexpected finding, 2 other cohorts of mother of children with neurodevelopmental disorders were also studied, as detailed in chapter 3.

2.1.1 Overview of the Danish data sources

2.1.1.1 The Danish Civil Registration System (CRS)

The CRS provides information on the identity and vital status of all residents in Denmark and links to their first-degree relatives (Pedersen, Gotzsche et al. 2006, Pedersen 2011). The register was established in 1968, at which time all people alive and living in Denmark were registered. All persons registered in the CRS are assigned a unique personal identification number, called the CPR ("Central Person Register") number. The CPR number ensures accurate linkage between all registers and biobanks. Among many other variables, it provides information on date of birth, gender, continuously updated information on vital status, and CPR number of parents, siblings and progeny.

2.1.1.2 The Danish Psychiatric Central Register

The Danish Psychiatric Central Register contains data on all admissions to Danish psychiatric in-patient facilities since April 1, 1969. As of January 1, 1995 registration of

psychiatric outpatient and emergency room activities has been included. On 1 February 2010 a total of 747,176 persons were registered with a virtually complete coverage. The information is updated monthly. There are no private psychiatric in-patient facilities in Denmark, and all in- and out-patient treatment is provided free of charge, ensuring that all psychiatric admissions are represented in the register. Furthermore, the input of data is mandatory for the psychiatric departments. However, cases with mild to moderate mental disorders are diagnosed and treated by general practitioners and those are not included in the register. Diagnostic information in the psychiatric registers was based on the International Classification of Diseases, 8th Revision (ICD-8) until 1993. From 1994, the diagnostic system used is the Danish modification of the ICD-10 (Mors, Perto et al. 2011).

2.1.1.3 Statens Serum Institut

The “Gravid screening” biobank at the Statens Serum Institut, is a clinical biobank approved by the Danish Data Protection Agency (journal nummer 2001-14-0025), and contains stored serum from pregnant women (maternal second-trimester sera in 20%) and blood-heel samples from virtually all new-borns after 1981 (Statens Serum Institut. Afdeling for klinisk biokemi og immunologi mod. [online]. Available at: http://www.ssi.dk/Service/OmSSI/Kontakt.aspx).

2.1.1.4 Patients’ clinical information

General clinical information from the maternal psychosis cohort (age at diagnosis, autoimmune co-morbidities and parity) was obtained from the Registries.

2.1.2 Human cohorts

2.1.2.1 Maternal psychosis cohort

The study cohort contains 33.386 women giving birth in five counties in Denmark from May 15, 1992 to January 15, 1995. A total of 166 women had first diagnosis of schizophrenia spectrum disorder (ICD-8 codes 295, 297, 298.39, 301.83; ICD-10 codes F20-F29) after delivery (cases). Of these, serum samples from the pregnancy of 95 women were found. Each of these 95 were individually age and parity-matched to women in the study cohort without a history of schizophrenia spectrum disorder (controls). After autoantibody screening of these samples, information regarding the samples (date of collection), mothers (age at delivery, history of autoimmune diseases, date of postpartum psychosis diagnosis) and children (diagnosis of psychiatric disorders or neurodevelopmental diseases - F20-F39, F60, F61, F70-79, F80-89, F90-98 from ICD-10 of the index child and the siblings) was collected (ICD-10 codes and diagnoses are detailed in the appendix).

2.1.2.2 Autism cohort

Cases and controls were selected in a cohort of singletons born in Denmark between 1994 and 1996 who were alive and living in Denmark at their 1-year birthday and where the mother had a serum sample collected between 10 and 220 days before the date of birth of the child with a gestational age at the time of serum sample collection between 14 and 18 weeks (study cohort). A total of 100 children (cases) in the cohort had a diagnosis in the autism spectrum (ICD-10: F84.0, F84.1, F84.5, F84.8, F84.9) in the Danish Psychiatric Central Research Register given at age 1 year or later (ICD-10 codes and diagnoses are detailed in the appendix). Each case was matched to a same-sex random control in the study cohort, who were not a sibling to the case, who were alive

and living in Denmark and without a diagnosis in the autism spectrum at the age where the case became a case, and where the mothers had the same parity as the mother of the case and the age of the mothers at the time of delivery were within 365 days.

At Statens Serum Institut 95 serum samples of the mother for 95 cases (19 girls and 76 boys) were found. For each of the 95 cases a serum sample for the mother of an appropriate control was retrieved.

2.1.2.3 Mental retardation and disorders of psychological development (MR/DPD) cohorts

Cases and controls were selected in a cohort of singletons born in Denmark between 1994 and 1996 who were alive and living in Denmark at their 1-year birthday and where the mother had a serum sample collected between 14 and 18 weeks of gestation (study cohort). Cases were selected from this cohort if a diagnosis of mild mental retardation or mental retardation without specification (ICD-10: F70; F79) or a diagnosis of disorder of psychological development, except autism (ICD-10: F80-F83.9. F88-F89.9) was found in the Danish Psychiatric Central Research Register (ICD-10 codes and diagnoses are detailed in the appendix). Each case was matched to a same-sex random control in the study cohort, who were not a sibling to the case, who were alive and living in Denmark and without a diagnosis in the above categories, and where the mothers had the same parity as the mother of the case and the age of the mothers at the time of delivery were within 365 days. Two subgroups were also created in this cohort: group 1 – mental retardation and group 2 – disorders of psychological development, with controls matched for each group. A case with both disorders would be placed in the case subgroup with the earliest first date of diagnosis. Persons with date of onset of both diseases on the same day were placed in a third subgroup. Considering that it is quite possible that individuals with one initial diagnosis could later be classified with a second diagnosis,

most of the analysis was conducted considering the entire group of cases and ignoring the subgroups.

2.1.3 Methods

2.1.3.1 Cell based assay (CBA)

Proteins that were likely relevant in the pathophysiology of the disease, and that are expressed on the neuronal surface, were chosen for screening, using human embryonic kidney (HEK) 293T cells transfected with the plasmid encoding the protein of interest. These live cells were incubated with patient's serum and staining with an immunofluorescent anti-human immunoglobulin G (IgG) antibody was performed to detect cell-surface antibody binding. The CBAs used were established in our laboratory previous to the start of my research work and are routinely performed as part of the Oxford diagnostic service.

HEK 293 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma D6429) supplemented with 10% Fetal Calf Serum (FCS; Biowest S181B) and 1% penicillin G/streptomycin/amphotericin (PSA; Life technologies 15240-062) in flasks kept in a humidified, 5% CO2 incubator. For screening purposes, those cells grown in flasks were transferred to 6-well plates with 13 mm poly-l-lysine coated glass coverslips, by gentle detachment with 1% trypsin in phosphate buffer saline (PBS) and re-seeding in DMEM/10% FCS/1% PSA at a concentration of 2.5 x 10^5 cells/mL, in a total volume of 2 mL/well. The following day, cells were transfected using 1.5 μl polyethylenimine (PEI) with 3 μg of the cDNA plasmid of interest per well. After 14-16 hours, medium was replaced with fresh DMEM/10% FCS/1% PSA. In the particular case of the NMDAR CBA, to prevent cytotoxicity as a result of glutamate in the medium activating the NMDARs, the medium was supplemented with 500 μM of ketamine. Immunocytochemistry (ICC)

was performed on the fourth day. Up until then, all procedures were conducted in sterile conditions in a class II tissue culture hood. For ICC, coverslips were transferred to individual wells in a 24-well plate. Patient serum samples were added to DMEM supplemented with bovine serum albumin (BSA) (1% w/v) and 200 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma H3375) at a 1:20 or 1:100 dilution ratio, to make a total volume of 250 µl/sample. Positive (known positive patients) and healthy controls were added in each assay. Diluted sera were incubated with the live cells on coverslips at room temperature for 1 hour, after which the supernatant was aspirated and washed 3 times in DMEM/HEPES. Cells were fixed in 4% formaldehyde in PBS and incubated for 10 minutes at RT and then washed 3 times in DMEM or PBS. Afterwards, cultures were incubated for 45 minutes in the dark, at room temperature, with goat anti-human IgG, Alexa Fluor 568 (Invitrogen; A21090; 1:750) in DMEM+HEPES+BSA. The coverslips were washed 4 times in PBS and mounted using fluorescent mounting media (Dako, S3023), containing 4',6'-diamidino-2-phenylindole (DAPI; Sigma D9542; 1:1000). After drying, cells were viewed with a Leica DM 2500 immunofluorescent microscope. Binding was then scored on a scale of 0-4 according to the intensity of the immunofluorescence (0= no binding, 1= weak binding, 2= moderate binding, 3=strong binding, 4=very strong binding). Any sample scoring 1 or above was repeated and double scored by a second, independent observer. If there was disagreement between observers, the sample would be retested. Positive samples (score ≥1) were then titrated and end-point titration determined (last titration at which a score=1 was still observed). Case and control samples were screened blinded to clinical status.

2.1.3.2 Co-localization with an anti-NR1 commercial antibody

A cell-based assay was performed as described in the above section, except for the fact that no EGFP plasmid was used during transfection. After incubation with the secondary

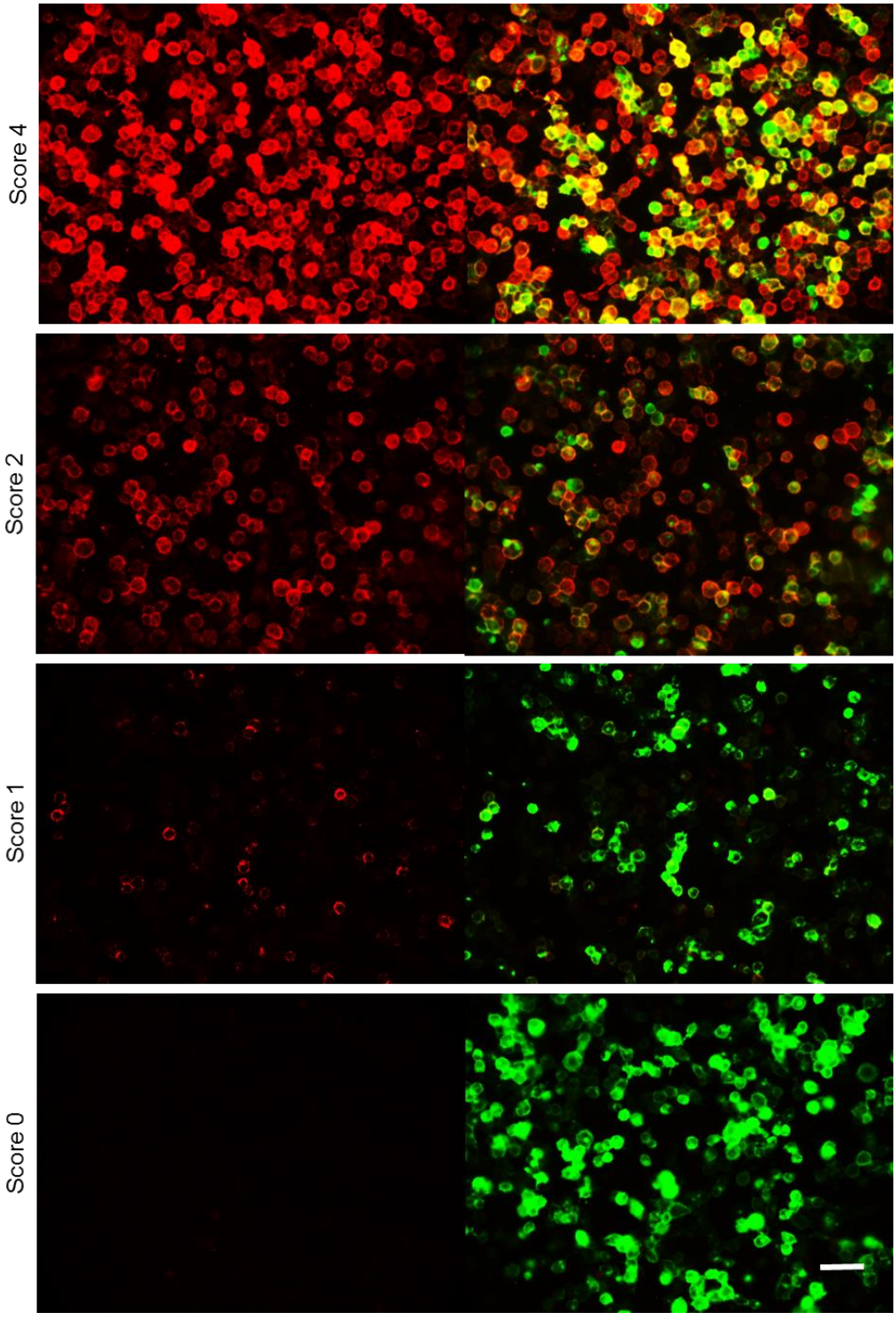


Figure 2-1: CBA scoring example

Scale bar: 100μm

anti-human IgG, cells were washed and again incubated for an hour with a mouse anti-NR1 monoclonal antibody (Chemicon, MAB363; 1:200) during 1 hour at room temperature. Cells were then washed in DMEM/HEPES and incubated with goat anti-mouse IgG, Alexa fluor 488 (1:750) for 45 minutes. After washing the coverslips 4 times in PBS, coverslips were mounted using fluorescent mounting media containing DAPI (1:1000). Samples were considered positive if all the cells with anti-human IgG Alexa fluor 568 signal co-localized with the anti-mouse IgG Alexa fluor 488 signal.

2.1.3.3 CBA with anti-Fc IgG

A cell-based assay was performed as described in section 2.1.3.1, but a goat anti-human Fc IgG secondary (Thermo Scientific; 31125; 1:750) was used instead. After incubating the secondary, coverslips were washed 3 times in DMEM/HEPES and further incubated with a mouse anti-goat IgG, Alexa fluor 568 (1:750) for 45 minutes at room temperature.

2.1.3.4 Pre-adsorption against NR1-transfected and non-transfected cells

For adsorption of NMDAR-antibodies from one serum sample, HEK 293 cells grown in coverslips were left untransfected or transfected with an expression vector encoding NR1 in DMEM/10% FCS/1% PSA. After 36 hours, coverslips with both transfected and blank cells were incubated with serum, diluted 1:10 for one hour at room temperature. This procedure was repeated in 10 coverslips, sequentially. The adsorbed samples were re-tested on the cell based assay (at a 1:20 dilution) to confirm complete adsorption.

2.1.3.5 Primary neuronal cultures

Live rodent neuronal cultures can be used as a confirmatory assay when a specific neuronal surface-antibody is detected using a CBA (although a less sensitive assay, particularly if low antibody titres are detected using a CBA, as discussed in chapter 3) or

as a method to detect neuronal surface-antibodies to unknown antigenic targets. This assay was performed in the CBA-positive samples from the maternal psychosis cohort as an extra confirmatory step and in all samples of the autism and MR/DPD cohorts to search for unknown antigenic targets.

2.1.3.5.1 Primary hippocampal neuronal cultures

Hippocampal neuronal cultures were carried out following a protocol adapted from (Kaech and Banker 2006). Sprague-Dawley rat pups, at postnatal day 0 (P0), or embryos at gestational day 19 (E19), were sacrificed by decapitation and the heads stored on ice. After removal of the brain from the skull, the brain was placed into a petri dish containing ice cold Hank's balanced salt solution (HBSS; Invitrogen, 14170-070). Hippocampi were dissected out of the brain and any meninges carefully removed. Dissected hippocampi were placed into fresh HBSS and the following procedures were conducted in sterile conditions in a class II tissue culture hood. The hippocampi were dissociated in 1% trypsin-HBSS solution for 20 minutes at 37˚C. The trypsin was aspirated and replaced with complete Minimal Essential Medium (MEM, GIBCO, 22561-021, supplemented with 10% FCS and 1% PSA). After 3 changes of MEM, cells were gently triturated until no visible tissue remained. The cell suspension was centrifuged at 1000 rpm for 5 minutes, the supernatant aspirated and the cell pellet re-suspended in complete MEM medium. A total volume of 3 mL with 2.5-3 x 10^5 cells (approximately 1 hippocampus/ well) were plated on to 5 PLL-glass coverslips on a 6-well plate and placed in the humidified 37˚C, 5% CO2 incubator. After four hours, the media was aspirated and replaced with 3 mL of Neurobasal medium (NB; Invitrogen, 21103049) supplemented with 1% B-27 serum free supplement (Life technologies; 17504-044), 200mM L-glutamine (Life technologies; 25030-081) and 1% PSA. One third of the culture medium was replaced every 3 days during the first week and every 2 days during the second week with fresh supplemented NB media. ICC was performed at day *in vitro* (DIV) 14-18.

2.1.3.5.2 Primary cerebellar granule neurons culture

Cerebellar granule neurons cultures were carried out using postnatal day 6 (P6) Sprague-Dawley rat pups using a protocol adapted from (Bilimoria and Bonni 2008). The pups were sacrificed by decapitation and heads stored in ice. Dissected cerebellum were placed in ice-cold HHGN dissection solution (1X HBSS with 2.5 mM HEPES, 35 mM glucose and 4 mM sodium bicarbonate, pH 7.4) and meninges carefully removed. Afterwards, in the tissue culture hood, cerebella were washed in fresh HHGN solution 3 times and then placed in HHGN with 1% trypsin, supplemented with 250 μL of DNAse I (Roche, 10104159001; 2 mg/mL) for 10 minutes at 37ºC. After this, the cerebella were washed again 3 times in fresh HHGN and placed in a solution containing 250 μL of DNAse (2 mg/mL) and Basal Medium Eagle (Invitrogen; 41010026) containing 25 mM KCl, 10% hyclone cosmic calf serum (Thermo scientific, SH30087.03) and PSA (BME solution) for titration. The suspension was then centrifuged at 300g for 5 minutes, the supernatant aspirated and the cell pellet re-suspended in BME solution. A total volume of 3 mL with 170-200x10^5 cells (approximately 2 cerebella/ plate) were plated on to 5 PLL-glass coverslips of a 6-well plate and placed in the humidified 37˚C, 5% CO2 incubator. Cytosine-1-β-D-arabinofuranoside (AraC, Sigma C1768) solution was added 24 h after plating cells to a final concentration of 10 μM and glucose was added on DIV3 to a final concentration of 25 mM. Neurons were stained at DIV12-14.

2.1.3.5.3 Detection of antibodies against the surface of live primary neuronal cultures

Coverslips were transferred to individual wells of a 24-well culture plate containing patient serum diluted 1:100 in conditioned NB medium (for hippocampal cultures) or 1:200 in conditioned BME medium (for granule cells cultures) supplemented with 2% bovine serum albumin (NB+/BSA) and incubated at room temperature for one hour. Coverslips were washed three times in NB/BME medium, fixed for 15 minutes in 3% formaldehyde in PBS and then re-washed three times before incubating with goat-anti

human IgG 488 Alexa Fluor (Life technologies, A11013) diluted 1:1000 for 45 minutes. Coverslips were washed three times in PBS before being permeabilised with 0.25% Triton-X-100 in PBS (PBS-TX100) for 15 minutes and then incubated with mouse monoclonal anti-MAP2 antibody (Sigma, M9942) for hippocampal cultures, or anti-β3 tubulin antibody (Sigma T8660) for granule cells cultures, diluted 1:1000 PBS/2%BSA, to identify the neuronal cells in the preparation. After one hour, coverslips were washed three times in PBS and incubated with the appropriate secondary antibody for 45 minutes, washed 3 times in PBS, and mounted and visualised as outlined in the CBA protocol.

2.2 Maternal to foetal transfer animal model

All *in vivo* experiments were performed in accordance with the United Kingdom Home Office Animals in Scientific Procedures Act (1986) and in accordance with institutional guidelines, under the following licences: PIL number 30/10344 (Dr Ester Coutinho), PPL number 40/3581 (Dr Bethan Lang).

2.2.1 Animals

MF1 outbred female mice (20-25gr) and adult male breeders of the same strain were purchased from a licensed breeding establishment (Charles River). The animals were housed and mated in the Biomedical Services Unit at the John Radcliffe Hospital under standard laboratory conditions (ad libitum access to food and water; 12:12 light:dark cycle, with lights on at 7:00). Establishment of pregnancy was done by detection of vaginal coagulant plugs in the morning after mating. E0.5 was defined as the day on which the plug was detected and P0 as the day of birth.

2.2.2 Preparation of purified human IgG

Purification of human IgG was done using the ammonium sulphate precipitation method (M. Page 1996), to avoid damage to the Fc region, as it is likely to occur after using the acidic elution buffers required for column purification. Saturated ammonium sulphate solution was added to the human plasma to produce a 45% final saturation and left to stir at 4°C overnight. The following day, this solution was centrifuged at 12000 rpm (16000g) for 2 hours. The supernatant was discarded and the precipitate was re-suspended in 10% of the original volume in Hartmann's solution (Viaflo, FKE 232Y). After homogenization, the solution was dialysed against a minimum of three changes of Hartmann's buffer (2 L per change). IgG concentration was determined using a commercially available human IgG radial immunodiffusion kit (BINDARID human IgG NL RID kit; Binding site, RIN055). Purified IgGs were tested for binding to mouse hippocampal neuronal cultures and brain sections in wildtype MF1 mice and *Cntnap2* knockout mice, and titres determined by cell-based assay, as previously described.

Intraperitoneal injections

Three cohorts of dams were injected IP with purified human IgG or saline. These cohorts were used for 3 separate experiments. Experiment 1 aimed at the detection of total human IgG and specific antibodies transfer throughout gestation. Dams were injected daily, intraperitoneally, with purified IgG (from NMDAR, LGI1 and CASPR2 encephalitis patients) from E12.5 until the time of death or until E18.5 (IgG concentration varied: 2.5 to 20 mg/day, volume:1-2 mL/day). Experiment 2 assessed the effects of human IgG transfer during embryonic development. Dams were injected daily from E12.5 to E17.5 with purified human IgG (NMDAR and CASPR2 encephalitis patients or healthy controls) or Hartmann's solution (IgG concentration: 20 mg/day; volume 1-2 mL/day). Experiment 3 assessed the effects of human IgG transfer post-delivery, during the neonatal and adult life of the offspring. Dams were injected daily from E12.5 to E18.5 with purified human

IgG (CASPR2 encephalitis patients and healthy controls) or with Hartmann's solution (IgG concentration: 20 mg/day; volume 1-2 mL/day). Further details provided in results.

2.2.3 Human IgG quantification in mice sera (experiment 1)

Blood was collected from dams by cardiac puncture, after CO2 anaesthesia. Pups were sacrificed by decapitation and trunk blood collected at that moment. Blood was pooled from the pups of each litter. The levels of total human IgG in mice sera from experiment 1 were determined by quantitative western blotting. Human IgG standards of known concentration (BINDARID human IgG NL RID kit; Binding site) and mouse sera from the different gestational time points were diluted in deionised water (dilutions were established for each experiment in order to fit the samples into the calibration curve). The diluted samples and standards were mixed with appropriate amounts of NuPAGE sample reducing agent (10x; Invitrogen, NP0009) and LDS sample buffer (4x; Invitrogen,) and the mixed solution was boiled for 5 minutes at 90ºC. 10μL of the mixed sera and calibrators and 10μL of SeeBlue Plus2 pre-stained standard molecular weight ladder (Life Technologies, LC5925) were loaded into a 4-12% NuPAGE Bis-Tris SDS polyacrylamine gel (Invitrogen, NP0322). The gel was placed in a gel electrophoresis tank (XCell Sure Lock gel tank, Invitrogen) containing 1x MES SDS Running buffer (NuPage, Invitrogen, NP0002) and electrophoresed at 150mV for 1-1.5 hours. After electrophoresis, gels were removed and assembled in an X Cell II Blotting Module (Invitrogen, UK) and proteins transferred at 50V for 90 minutes in a 10% methanol transfer buffer (NuPage, Invitrogen, NP0006-1) into a nitrocellulose membrane. The membrane was then blocked in 5% BSA in PBS/0.1% Tween for 30 minutes and then incubated with rabbit anti-human IgG HRP (Dako, P0214; 1:2000) for 1 hour at room temperature. Next, the blot was washed 3 times in PBS/0.1% tween and incubated with ECL (Pierce, 32106) for 2 minutes. The membrane was exposed to photographic film in the dark until signal was visualized. The photographic film was then scanned and

analysed with Image J. Mean gray values for each band were measured and the optical density calculated. To estimate the specific protein density, the optical density readings were corrected for non-specific background density, measured in an area of the blot with no reactivity. A calibration curve was then created on Graphpad software (San Diego, California, USA) using the specific optical density readings of the known standards and the mice sera samples' concentration inferred.

2.2.4 Antibody titres in mice sera (experiment 1-3)

Dams and foetus/pups sera from all experiments were diluted from 1:5 to 1:400 and tested for specific antibodies. Antibody titres were established by end-point titration on a cell based assay, as previously described.

2.2.5 Histology of embryonic tissue (experiment 2)

2.2.5.1 Tissue processing

After decapitation, the head of E18.5 embryos was placed in a plastic mold and embedded in Tissue Tek OCT (Sakura, 4583). Next, the mold was snap-frozen in ice-cold isopentane. On a cryostat, 12 μm sagittal sections were sampled throughout the entire head and collected on superfrost slides (4 consecutive sections per slide), dried overnight at room temperature and then stored at -20ºC. Immunohistochemistry (IHC) was done on every tenth slide in order to provide a full representation of the brain.

2.2.5.2 Cresyl violet staining for visualization of the Nissl substance

Tissue sections were lightly stained with a cresyl violet solution (Sigma C5042) that stains Nissl bodies (granular endoplasmic reticulum and ribosomes) and allows observation of neuronal soma. After allowing the sections to dry at room temperature,

they were fixed with 4% paraformaldehyde for 10 minutes, rinsed twice in PBS for 5 minutes and once in deionized water, followed by immersion in cresyl violet solution for 10 minutes. Slides were dehydrated in graded alcohols, cleared with xylene and coversliped with DPX mounting medium. Photomicrographs were scanned with the Aperio AT2 slide scanner and analysed with the e-pathology Aperio Imagescope image analysis system from Leica Biosystems.

2.2.5.3 Human IgG IHC and mean fluorescent intensity measurements in the embryonic tissue

Brain sections (1 in 10 series) from one embryo per litter were stained for total human IgG. In the same slide containing 4 consecutive sections, the bottom sections (2) were gently washed 3 times in PBS, while the upper sections (2) were not washed. Afterwards, all sections were fixed with 4% PFA for 10 minutes. After 3 washes in PBS, sections were incubated with CF488A anti-human IgG (Biotium, 20022) at 1:500 overnight at 4ºC. The following day, sections were washed 3 times in PBS and incubated with rabbit anti-von Willebrand factor (VWF) (Millipore, AB7356, 1:250) for 3 hours at room temperature, followed by incubation with goat anti-rabbit Alexa fluor 568 secondary for 1h at RT. After washing 3 times in PBS, coverslips were mounted using fluorescent mounting media containing DAPI (1:1000). Sections were visualized using a Leica DM 2500 immunofluorescent microscope. For a quantitative analysis of the mean fluorescent intensity, 3 photomicrographs were taken from the isocortex in 3 different sections from 3 embryos / treatment group (27 photomicrographs/treatment group). Mean fluorescent intensity was then assessed for each photomicrograph using ImageJ. Results were plotted for both individual photomicrographs and for the mean results from each pup.

2.2.6 Behavioural testing (experiment 3)

At P1, 6 pups were randomly selected from each litter and individually tattooed, in order to provide a form of identification that was persistent throughout the entire period of behavioural testing (Figure 2-2 shows the tattoo system used). While the pups were tattooed, the dam was left in the home cage and placed in the testing room for habituation. The remaining pups from that litter were culled and blood collected for antibody testing, as previously described

2.2.6.1 Neonatal period

Testing was done daily, from P1 to P21, in the dark phase (lights off at 7pm). Pups were separated from the dam and placed in a petri dish with home cage nesting material at the beginning of testing. All testing was done with the pups placed in a heating pad and, for the first five postnatal days, under a lamp with a 60 watt bulb. Pups were returned to the dam after testing. Care was taken to extensively rub the examiners hands and the pups in the home cage shavings in order to avoid maternal rejection. All material was disposed of or washed with tepid water between litters.

2.2.6.1.1 Modified battery of Fox

The modified battery of Fox (Hill J. Neuromethods, vol 39) (Figure 2-3) allows a comprehensive evaluation of neonatal developmental milestones. The battery includes maturation readouts describing physical development (individual weight, pinna detachment and eye opening), neurodevelopmental measures based on neurological reflexes (surface righting, negative geotaxis, cliff aversion, rooting, ear twitch and air righting) and tests of neuromotor coordination (forelimb grasping and open field traversal). Tests were repeated daily until the pup met the test criterion for 2 consecutive days. Tests were performed by the same examiner daily and a second examiner would

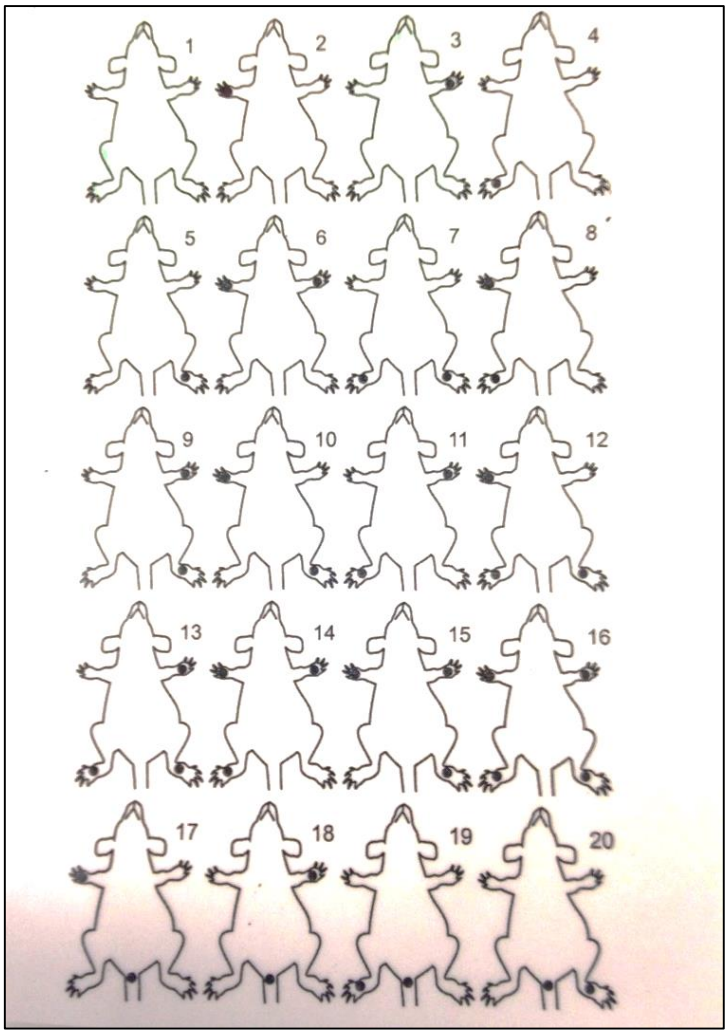


Figure 2-2: AIM (Animal Identification and Marking) tattoo system

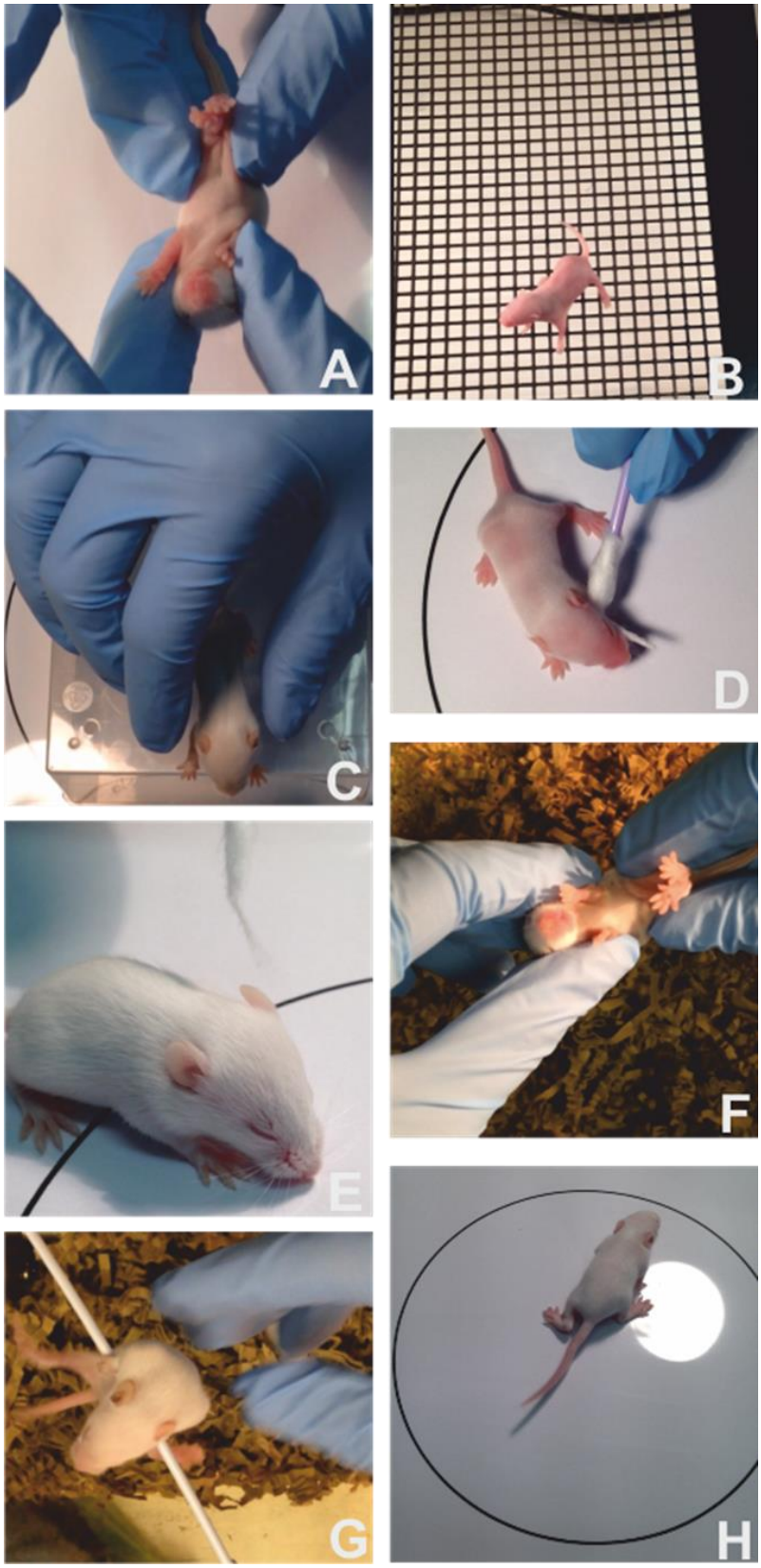


Figure 2-3: Modified Battery of Fox

(A) Surface righting; (B) Negative geotaxis; (C) Cliff aversion; (D) Rooting; (E) Ear twitch; (F) Air righting; (G) Forelimb grasping; (H) Open field traversal

record the time of performance with a stopwatch. Results were registered in the individual recording sheet. If a pup did not reach criterion by the end of the specified testing days, the day following the last test day was used for statistical analysis.

Weight measurements

Weight was measured daily, from P1 to P21, for each individual pup, prior to the testing.

Eye opening and pinna detachment

The mouse pup was examined daily, from P1 to P21, to determine the first day when both eyes were opened or both ears detached from the skull.

Surface righting

The mouse pup was held on its back, supported on both sides of the head and on the hind quarters, and released onto a smooth surface. The time for the pup to flip to its abdomen and touch the surface with all four paws was measured in seconds. If the pup could not reach that position within 30 seconds, the test was terminated. The test was performed from P1 to P13 or until the pup could reach that position in less than 1 second for two consecutive days (Figure 2-3A).

Negative geotaxis

The pup was placed in a screen at a 45º angle with its head facing down. The time for the pup to turn 180º, so the head faced up, was measured in seconds. If the pup fell, the test was repeated once. If the pup could not reach that position within 30 seconds, the

test was terminated. The test was performed from P1 to P14 or until the pup could reach that position in less than 30 seconds for two consecutive days (Figure 2-3B).

Cliff aversion

The mouse pup was positioned on the top of a box with the forepaws and snout placed on the edge of the box. Time for the pup to crawl away from the edge of the box was measured in seconds. If the pup fell, the test was repeated once. If the pup could not reach that position within 30 seconds, the test was terminated. The test was performed from P1 to P14 or until the pup could reach that position in less than 30 seconds for two consecutive days (Figure 2-3C).

Rooting

A cotton filament was used to stroke three times the pup's head, laterally, from front to back. A rooting response was considered to occur if the pup moved the head towards the filament. If no response occurred, the other side of the head was stroked in the same way. The test was performed from P1 to P12 or until a response was elicited for two consecutive days (Figure 2-3D).

Ear twitch

A cotton filament was used to stroke three times the pup's ear tip. A response was considered to occur if the mouse would flatten the ear against the side of the head. If no response occurred, the other ear was stroked in the same way. The test was performed from P7 to P15 or until a response was elicited for two consecutive days (Figure 2-3E).

Air righting

The mouse pup was held on its back, supported on both sides of the head and on the hind quarters, and released approximately 10cm above a clean cage padded with nesting material. The day in which the pup would flip to its abdomen and land on the surface with all four paws was recorded. The test was performed from P8 to P21 or until the pup could reach that position for two consecutive days (Figure 2-3F).

Forelimb grasping

The pup was held supported on both sides of its trunk and the forepaws placed on a small rod (approximately 5mm in diameter) suspended over a clean cage padded with nesting material. The pup was then released and the amount of time the mouse remained gripping the rod was measured. If the mouse fell immediately after release, the test was repeated once. The test was performed from P4 to P14 or until the mouse could hold the grip for 1 second or more for two consecutive days (Figure 2-3G).

Open field traversal

The pup was placed in the centre of a circle with a 13cm diameter printed on a sheet of paper. The amount of time the mouse would take to move outside the circle with all four paws was recorded. If the mouse failed to leave the circle in 30 seconds, the test was terminated. The test was performed from P8 to P21 or until the mouse would reach criterion in less than 30 seconds in two consecutive days (Figure 2-3H).

2.2.6.1.2 Ultrasonic vocalizations in separated pups

A standard isolation test protocol was used to record ultrasonic vocalisations (USVs) in separated pups (Hofer, Shair et al. 2002). On each day of testing, the dam was removed

from the home cage and placed in a separate cage 10-15 minutes before the recording session. The home cage containing the litter was placed on a heating pad and moved to a room without adult mice. Three pups of each litter were tested at postnatal days 6 and 12. The test pup was placed into a plastic container (diameter, 50 cm; height 20 cm) with 1 cm of shavings covering the bottom, and assessed for USVs during a three minute test. Care was taken to pick the pup by gentle pressure on the side of its shoulders and not by grasping a fold of its skin. At the beginning and end of the recording session, each pup had its axillary temperature measured by gentle insertion of the thermal probe in the skin pocket between upper foreleg and chest of the animal for about 30 seconds. After the recording session, the tested pup was placed in another cage as not to disturb the littermates. A clean test chamber (cleaned with tepid water and completely dried) was used for each pup.

USVs were recorded with a Batbox duet USV detector (Batbox Ltd.) in frequency division mode. The microphone was placed 15 cm above the pup in its plastic box. The temperature of the room was maintained at 23ºC. Recording settings included sampling rate at 22.05 kHz; format 16 bit. For acoustical analysis, recordings were done with Avisoft SASLab Pro software (Avisoft Bioacoustics) and a fast Fourier transformation (FFT) was conducted. A FFT-length of 256 points and a time window overlap of 75% (100% Frame, Hamming window) were used to generate spectrograms. The spectrogram was produced at a frequency resolution of 488 Hz and a time resolution of 1 ms. A lower cut-off frequency was established at 1.5 kHz. Call detection was provided by an automatic threshold-based algorithm and a hold-time mechanism (hold time: 0.01 s).

2.2.6.1.3 Maternal retrieval test

From each litter, 3 pups were isolated from the dam and littermates for 2 minutes. After this period, the dam was isolated in a clean cage and the 3 pups returned to the home

cage approximately 20 cm from the nest. The dam was then returned to the box, at the opposite corner from the 3 pups, facing away. Time in seconds was recorded until retrieval of the first pup (latency to retrieval) and until retrieval of the 3 pups to the nest (time to retrieval). Total test duration was 5 minutes. If the dam failed to retrieve the 3 pups at the end of this period, 300 seconds was used for statistical purposes. Test was performed at P6.

2.2.6.2 Adult period

The pups were weaned from the dam at P21 and separated according to sex. To avoid isolation, if a litter only had one male or one female, that animal would be placed with a different litter. Animals were housed in groups of two to five. Behavioural testing was done from 4 to 10 months of age during the light phase. Unless indicated otherwise, behaviour tests were performed in half of the cohort (12 animals per treatment group), randomly selected.

2.2.6.2.1 Locomotor activity

To assess locomotor activity, mice were transferred to a novel cage (42cm long x 22cm wide x 20cm high) placed within a photobeam frame (San Diego instruments, San Diego, CA, USA) and activity was quantified as number of beam breaks during a 2-hour session. Data was divided in 24 five minutes bins and analysed. This test was done in all mice (except 1 HC IgG-exposed mouse, deceased due to a non-experimental).

2.2.6.2.2 Accelerating rotarod

Neuromotor coordination was assessed using the accelerating rotarod (Deacon 2013). The animals were brought to the experimental room approximately 15 minutes before testing. The mouse was placed using a dowel on the rotating rod at an angle of 30

degrees (head down), facing away from the direction of rotation. Steady starting speed of 4 rotations per minute was maintained during 10 seconds. Afterwards, an acceleration rate of 20 rotations per minute ensued. Time to fall was recorded. If the mouse had fallen off during the initial 10 seconds it would have another try, to a maximum of 3 trials.

2.2.6.2.3 Elevated Plus-maze (EPM)

Anxiety was assessed using the EPM. The apparatus is in the shape of a plus sign, made up of two opposite 27 x 8 cm x 30 cm enclosed black arms, a 12.5 cm x 6 cm black central rectangular area and two opposite 29 x 4 x 0.5 cm open white arms, and is elevated 50 cm from the floor on a stand. Mice were brought to the experimental room approximately 15 minutes before testing. The mouse was placed at the central rectangular area facing the open arms. Behaviour was then video recorded for 5 min and analysed using the Ethovision software. Recorded parameters were: total distance travelled, latencies to enter four paws into an open and closed arm, total time spent in enclosed and open arms; number of entries into enclosed and open arms and number of faecal boli.

2.2.6.2.4 Light-dark (LD) box

The LD box test was also used to assess anxiety. The apparatus consists of an open white compartment 30 x 20 x 20 cm joined by a 3 x 3 cm opening to a dark box 15 x 20 x 20 cm. The white side of the box was transparent allowing detection of the mouse movement and was further illuminated by a 60W lamp. The mouse was placed in the middle of the dark side facing away from the opening and a count-down timer for 5 min started. The latency to cross with all four feet to the light side, the total time spent on the light side and the number of transitions through the opening were registered for the test

duration. The number of faecal boli and the presence/absence of urination was also recorded

2.2.6.2.5 Hyponeophagia test

The hyponeophagia test is another test to measure anxiety (Deacon 2011). It is conducted after placing the animals in overnight food deprivation. The following morning, animals were separated into different cages 5 to 15 minutes before the test. The test animal was placed under a small receptacle facing away from a well containing a mixture of condensed milk and water (1:1). The latency to contact the well (sniff or touch), as well as the latency to drink (drinking continuously for at least 2 sec) was recorded. Each mouse was given 120 seconds to drink the condensed milk. If the mouse failed to drink, it was placed in the holding cage and retested for a maximum of 3 trials.

2.2.6.2.6 T-maze spontaneous alternation test

The T maze spontaneous alternation test is based on the natural tendency of rodents to alternate their choice arm, as a manifestation of the animal's motivation to explore its environment (Deacon and Rawlins 2006). It assesses working memory and perseveration of behaviour. The T-maze consists of a T-shaped apparatus (30cm long x 10 cm wide x 29 cm high) with one start arm and 2 goal arms. It further has a removable central partition extending 7 cm into the start arm from the back of the T, restricting access to only one goal arm at a time and three guillotine doors that can restrict animal to either goal arm or the first 22.5 cm of the start arm. The floor of the apparatus was covered with a layer of soiled bedding from cages holding animals unknown to the test mouse, providing a stimulus for exploration. The bedding was mixed between animals and changed between trials. The test consists of a sampling and a choice phase. During the sample phase, the animal was placed at the end of the start arm facing away from

goal arms. During this phase, the central partition was in place. Once the mouse entered a goal arm, the guillotine door on that side was gently closed and the animal was allowed to explore for 30 seconds. The animal was then removed from the goal arm, the central partition removed and the guillotine doors opened, allowing free choice of goal arm for second exploration. The mouse was then returned to the start arm and the choice recorded. Latency to choice was also recorded. The test was run in pairs of trials, once in the morning and once in the afternoon for 5 consecutive days. This test was done in all mice (except for the above mentioned HC IgG-exposed mouse and 2 saline-exposed mice also deceased due to non-experimental causes).

2.2.6.2.7 Three-chamber social interaction test

The three-chamber social interaction test is an automated method to measure non-reciprocal social interaction (Nadler, Moy et al. 2004). The test has 3 phases: a habituation period, a sociability phase (when the test mouse chooses to interact with an empty cage or a stimulus mouse) and a social memory phase (when the test mouse chooses to interact with the known stimulus mouse or with an unknown stimulus mouse).

The three-chamber apparatus is a rectangular Plexiglas box (60 x 30cm for whole arena). The whole arena is divided into three equal parts by red-prespex slider walls (each chamber is 30x20cm; walls are 15cm high), that control the access to the lateral chambers. Stimulus mice (gender-matched CD1 outbreds) were extensively habituated to the enclosure (a wire cage) previously to the test. Stimulus mice were separated into 2 groups and housed in different cages in order to have distinct smells. Habituation protocol for the stimulus mice included: free exploration of the apparatus (same-cage animals would explore the chamber at the same time) for five minutes, enclosure of a single mouse during free exploration (first session for 40 seconds; second session for 1 minute; each stimulus mouse enclosed for 3 times in each session), direct enclosure of a single mouse (first session for 2 minutes, second session for 10 minutes; each stimulus

mouse enclosed 2 times in each session), and direct enclosure of the stimulus mouse with a second mouse (from a different group) freely-exploring for 5 minutes. Each stimulus mouse was used 2 times per test day, at maximum. In the test session, a test mouse would be placed in the middle chamber and allowed to explore for 5 minutes. Afterwards, the slide doors would be opened and the mouse would explore the entire empty chamber for 10 minutes. The mouse would then be enclosed in the middle chamber, briefly, while an empty cage and a cage with a novel stimulus mouse were placed in opposite sides of the apparatus. The test mouse would then be released, by opening of the sliding doors, and allowed to explore the entire apparatus. The object and novel mouse position alternated across subjects. Next, the test mouse was again enclosed in the central chamber, and the empty enclosure replaced by an enclosure with a new stimulus mouse (from a different group). The test mouse was again released and allowed to explore the apparatus for 10 minutes. The novel and old mouse position alternated across subjects. The test session was video recorded and analysed by the Any-Maze tracking software (Stoelting, USA). Parameters analysed included: distance travelled, freezing and immobility times in the habituation phases, and in the sociability and social memory phases, absolute time in interaction zone (2cm area around enclosure), time spent in each chamber, number of entries into interaction zone. The test was run in the dark, with two desk lamps facing away from the arena on each side and using red-light; background noise was masked with a white noise amplifier. The apparatus and enclosures were cleaned with 70% alcohol and water between subjects.

2.2.6.2.8 Reciprocal social interaction test

The reciprocal social interaction test is another social interaction test where two mice of the same treatment group, unknown to each other, are allowed to interact freely (Barkus, Feyder et al. 2012). The apparatus consisted of a clean cage (41.5 × 25.5 ×11.5 cm) with bedding sufficient to cover the bottom of the cage. Each mouse was individually

habituated to the testing arena on 5 consecutive days, 10 minutes per day. Habituation sessions were video-recorded and analysed with the Any-maze software (distance travelled, freezing and immobility times) to ensure equal exploratory activity between animals. On the sixth day, each mouse was exposed for 10 minutes to a mouse from the same treatment group but housed in a different cage. The animals were sex-matched and tightly matched for weight (within 5% difference). This was repeated on the seventh day with new pairs. No animal was tested more than twice. The test was video recorded and scored offline for time spent in social and non-social behaviours and number of social and non-social events. Social behaviours included sniffing, grooming and following closely; non-social behaviours included self-grooming and digging. Testing room conditions were the same as for the three-chamber social interaction test. The apparatus was cleaned between tests with 70% ethanol and water.

2.2.6.2.9 Nesting test

Nesting is a species-typical behaviour, relevant to home-cage social interaction (Deacon 2006). Approximately 1 hour before the dark phase, the animals were transferred to individual cages with wood-chip bedding and without any other environmental enrichment. A cotton nestlet weighing 2.5 grams was placed in each cage. The following morning, the nests were scored according to (Deacon 2006) (Figure 2-4) and any unshredded material was weighed.

2.2.6.2.10 Olfaction test

An olfaction test was performed in order to assess olfaction deficits that could interfere with the results from the social interaction tests. The apparatus from the three chamber social interaction test was used in order to benefit from previous habituation. A small glass container with an odour (1 mL of banana or peppermint food flavouring) was placed

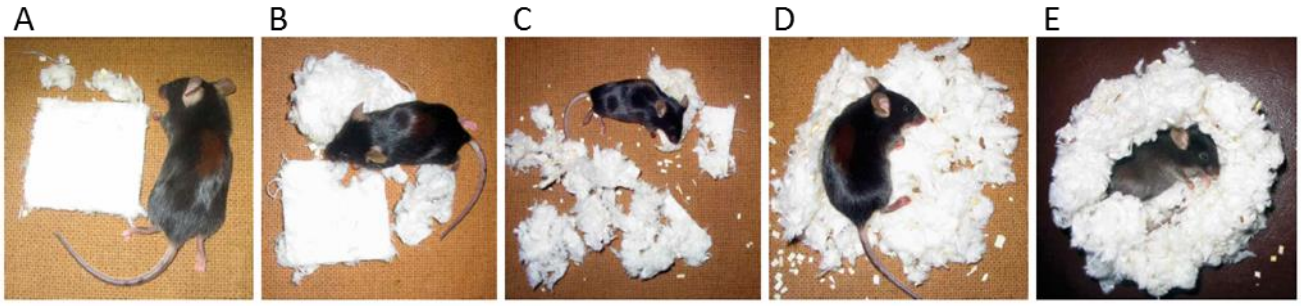


Figure 2-4: Nesting test.

Scoring of the nesting test from 1 to 5 (A to E), after overnight exposure to bedding material. Figure from (Deacon 2006).

at each corner. The test comprises 3 phases: a sample phase (15 minutes), a delay period (15 minutes) and a test phase (3 minutes). During the first phase, the same odour is placed in both containers (half of the mice for treatment group would smell one odour, while the other half would smell the other). After this, the mice would return to the home cage for 15 minutes. During the test phase, a container with the alternate (new) odour was placed in one of the corners. The location of the new odour was counterbalanced across treatment groups and gender. Testing room conditions were the same as for the social interaction tests. The test was video recorded and analysed using the Any-maze software. The same parameters as in the 3-chamber social interaction test were analysed.

2.2.7 Histological analysis of adult offspring (experiment 3)

I received substantial help from Dr David Menassa for the histological characterization of the adult offspring. I conducted all tissue processing, the Ñissl staining in embryonic and adult offspring, most of the morphometric analysis and the immunofluorescence staining for GFAP/NeuN and CD68. Dr Menassa did all the confocal imaging, the staining for cortical layer markers, calbindin and PSD-95, and the cell counts. Dr Steve West has done the automated quantification of PSD-95 puncta.

2.2.7.1 Tissue processing

Six animals per treatment group (3 males and 3 females) were randomly selected for histological analysis at 12 months. Mice were deeply anaesthetised with Isofluorane followed by pentobarbital injection (100 mg kg-1 i.p) and transcardially perfused with 100 mL of PBS followed by 50 ml of ice cold 4% paraformaldehyde in 0.1M phosphate buffer. Brains were removed and post-fixed for 24 hours at room temperature before transferring

to 30% sucrose in PBS. Using a freezing stage sledge microtome, free-floating coronal sections were collected in 15 series at a thickness of 50 µm.

2.2.7.2 Morphological and morphometric analysis

Morphology and morphometry were assessed in one series of Nissl stained (staining protocol as described in section 2.2.5.2) sections. Gross morphology of the entire brain and of specific regions (cortex, hippocampus, corpus callosum and cerebellum) was assessed. Sections were scanned using the Aperio AT2-scanner and analysed with the e-pathology Aperio Imagescope image analysis system from Leica Biosystems, which allowed for thickness and area measurements.

2.2.7.2.1 Dorsoventral thickness measurements

Total cortical thicknesses were measured across the prelimbic, infralimbic and anterior cingulate areas. Total and specific layers (layer I, II-IV and V-VI) thicknesses were measured across the somatosensory cortex. The thickness of the corpus callosum was measured at the level where the genu was thickest (Bregma: + 0.98 mm). Anatomical boundaries were determined based on the Allen Mouse Brain Atlas (available from: http://mouse.brain-map.org).

2.2.7.2.2 Volumetric measurements

To obtain total brain volume or specific structure volume (corpus callosum, hippocampus and cerebellum), the Cavalieri's Principle was applied, which states that ‘the volume (V) of an arbitrary shaped object can be estimated in an unbiased manner from the product of the distance between planes (D) and the sum of the areas (*A*) on systematic random parallel sections through the object’ (Mandarim-de-Lacerda 2003). The equation applied

was: $V = D \times A$, where *D* is the number of sections in series (i.e. 15 series) times the thickness of the sections (i.e. 50 µm)

Total brain volume

For total brain volume, the Cavalieri's Principle was applied to at least 14 sections sampled anteroposteriorly across the entire brain in the coronal plain, from the appearance of the frontal pole cortex (Bregma: +3.20 mm) to the cerebellum at its most posterior disappearance (Bregma: -7.92).

Corpus callosum

Anatomical boundaries were visible from the start of the structure at the level of the forceps minor (Bregma: +1.98 mm) to its most posterior at the level of forceps major (Bregma: -3.08 mm). At least 8 sections spanning the entire corpus callosum anteroposteriorly were used to measure this structure's area.

Hippocampus

Anatomical boundaries were visible from the start of the structure at the level of the field CA3 (Bregma: -0.94mm) to the most posterior area of the dentate gyrus (Bregma:-4.04 mm). At least 4 sections were used for measurements. Both hippocampus were measured and averaged.

Cerebellum

Anatomical boundaries were visible from the start of the structure at the most anterior level of the lobules 4/5 of the cerebellar vermis (Bregma: -5.02 mm) to the most posterior level of lobule 9 of the cerebellar vermis (Bregma: -7.92 mm). At least 4 sections were used for measurements.

2.2.7.3 Neuronal and astrocytic immunohistochemistry

Neurons and astrocytes were identified by immunofluorescence using a polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) antibody (Dako, Z0334; 1:500) and mouse anti-NeuN (Chemicon, MAB377; 1:500) in free-floating sections. Sections were fixed with 4% formaldehyde, washed with PBS then blocked with 10% normal goat serum (Vector laboratories, S-1000) in PBS-Triton-X-100 (0.3%) for an hour then incubated overnight with primary antibodies at 4ºC. The sections were washed the next day with PBS then incubated for two hours at room temperature with goat anti-rabbit (568) and goat anti-mouse (488) Alexa-fluor secondary antibodies from life technologies at 1:1000 dilutions in the dark. Sections were subsequently washed in PBS, mounted on slides after a brief TNS wash (pH = 7.4) and counterstained with DAPI mounting medium from Vector Laboratories (UK), left to dry for an hour then sealed and stored protected from the light at 4ºC for confocal imaging

2.2.7.3.1 Corpus callosum

Qualitative analysis

Morphology of the indusium griseum, abnormalities in glial morphology and alignment of oligodendrocytes as well as the presence of neuronal clusters in the white matter were examined

Quantitation of ectopic neurons in the corpus callosum

Ectopic neurons in the corpus callosum were counted in the coronal slice where the corpus callosum genu was thickest. At least 12 images (353.9 µm x 353.9 µm) were taken per case using a Zeiss confocal microscope in one plane (depth = 7.5 µm) in NeuN/GFAP/DAPI fluorescently-tagged 50 µm coronal sections. At least 80 neurons were counted using Fiji cell counter and an average density (cells/mm^2) per case was obtained.

2.2.7.3.2 Hippocampus

Qualitative analysis

The hippocampus was qualitatively assessed for signs of astrogliosis in the CA4 region and neuronal loss in this region and the CA3 and CA1 regions. Gross morphology was also assessed.

Hippocampal CA4 GFAP/NeuN quantitation

Neuronal and astrocyte cell densities were determined in the CA4 region of the hippocampus (Bregma: - 1.46 mm) in 50 µm sections. Confocal images were taken across the z-plane in three locations spanning the entire CA4 region in all cases in both hemispheres. The guard zone was 5 µm from the top and bottom of the z-plane and astrocyte and neuronal counts were determined in at least 8 optical sections spanning the CA4. The z-step used was 5 µm and cells were counted in every third image in the same stack. A point density was calculated per stack as the total number of cells per volume (with area = 353.9 µm x 353.9 µm, depth = 40 µm) and an average density was obtained from three stacks across the CA4 (cells/mm3) in both hemispheres. The

corresponding astrocyte to neuron ratio was also calculated per case. At least 120 neurons and 220 astrocytes were counted in each case.

2.2.7.4 Purkinje and granular cell immunohistochemistry

Purkinje and granular cells were identified by immunofluorescence using a rabbit anti-Calbindin D28K antibody (Millipore, AB1778; 1:500) and mouse anti-NeuN (Chemicon, MAB377; 1:500) in free-floating sections. Sections were fixed with 4% formaldehyde, washed with PBS then blocked with 10% normal goat serum in PBS-Triton-X-100 (0.3%) for an hour then incubated overnight with primary antibodies at 4ºC. The sections were washed the next day with PBS then incubated for two hours at room temperature with goat anti-rabbit (555) and goat anti-mouse (488) Alexa-fluor secondary antibodies from life technologies at 1:1000 dilutions in the dark. Sections were subsequently washed in PBS, mounted on slides after a brief TNS wash (pH = 7.4) and counterstained with DAPI mounting medium from Vector Laboratories (UK), left to dry for an hour then sealed and stored protected from the light at 4ºC for confocal imaging.

2.2.7.4.1 Qualitative analysis of the cerebellum

Alignment of the Purkinje cells in a monolayer was assessed visually. GFAP sections were used to also assess for signs of Bergmann gliosis that usually underlies Purkinje cell loss (Love, Budka et al. 2015)

2.2.7.4.2 Purkinje cell densities

Confocal images were taken across the z-plane in 5 locations spanning the cerebellar lobules (Bregma: - 6.0 mm). The guard zone was 5 µm from the top and bottom of the z-plane and at least 8 optical sections spanning the CA4. The z-step was 10 µm and CB+ cells were counted in two optical sections in the same stack usually sufficient to reveal

to Purkinje cell layers within a 50-µm section. A point density was calculated per stack as the total number of cells per volume (area = 707.8 µm x 707.8 µm, depth = 40 µm) and an average density was obtained from 5 stacks across the cerebellum (cells/mm3) in both hemispheres. At least 350 Purkinje cells were counted in each case.

2.2.7.5 Cortical layers immunohistochemistry

Deep layers (layers V and VI) of the neocortex were identified by immunofluorescence using a goat anti-FOXP2 antibody (SantaCruz (sc-21069); 1:50) and the middle layers (layers II/III) using a rabbit anti-CUX-1 antibody (SantaCruz (sc-13024); 1:200) in free-floating sections, following the previously described laminar-specific genes in the mouse neocortex (Molyneaux, Arlotta et al. 2007). Sections were fixed with 4% formaldehyde, washed with PBS then blocked with 10% normal donkey serum in PBS-Triton-X-100 (0.3%) for an hour then incubated for 48 hours with primary antibodies at 4ºC. The sections were washed the next day with PBS then incubated for three hours at room temperature with donkey anti-rabbit (568) and donkey anti-goat (488) Alexa-fluor secondary antibodies from life technologies at 1:500 dilutions and kept in the dark. Sections were subsequently washed in PBS, mounted on slides after a brief TNS wash (pH = 7.4) and counterstained with DAPI mounting medium from Vector Laboratories (UK), left to dry for an hour then sealed and stored protected from the light at 4ºC for confocal imaging.

2.2.7.5.1 Qualitative assessment of CUX-1 and FOXP2 distribution

Laminar distribution of CUX-1 and FOXP2-positive cells was assessed in the prefrontal and somatosensory cortex.

2.2.7.5.2 Quantitation of CUX-1 and NeuN cell densities in layers V and VI of the somatosensory cortex

CUX-1 positive cells in the somatosensory cortex were counted in the coronal slice at Bregma – 1.46 mm. 16 images (418.4 µm x 418.4 µm) were taken per case using a Zeiss confocal microscope in one plane (depth = 5 µm) in fluorescently tagged 50 µm coronal sections. At least 350 cells were counted per case using Fiji cell counter and an average density (cells/mm2) per case was obtained. Neuronal densities were quantified by layer in the coronal slice at Bregma – 1.46 mm. The z-stack area was 174.3 µm x 174.3 µm for the prefrontal areas and 111.15 µm x 111.15 µm for the somatosensory areas. The z-step/interval was 5 µm and NeuN cells were counted within 8 optical sections through the stack with a guard zone of 5 µm at the top and bottom of the section (depth = 40 µm). 4 z-stacks per layer were imaged in the infralimbic and prelimbic areas and in the somatosensory area. An average density was obtained (cells/mm^3) in both hemispheres. For layer I, at least 100 cells were counted, for the remaining layers at least 400 cells/ layer were counted.

2.2.7.6 Activated microglia immunohistochemistry

Reactive microglial cells were identified by immunofluorescence using a rat anti-CD68 antibody (BioRad, MCA1957; 1:400) in free-floating sections. Sections were fixed with 4% formaldehyde, washed with PBS then blocked with 10% normal donkey serum in PBS-Triton-X-100 (0.3%) for an hour then incubated for 48 hours with primary antibodies at 4ºC. The sections were washed the next day with PBS then incubated for three hours at room temperature with goat anti-rat (488) Alexa-fluor secondary antibody from life technologies at 1:500 dilution and kept in the dark. Sections were subsequently washed in PBS, mounted on slides after a brief TNS wash (pH = 7.4) and counterstained with DAPI mounting medium from Vector Laboratories (UK), left to dry for an hour then sealed and stored protected from the light at 4ºC for confocal imaging.

2.2.7.6.1 Quantitation of activated microglia in the prefrontal and the somatosensory cortices

Microglial cells were counted in layers I, II/III and V-VI in the infralimbic and prelimbic areas (Bregma: + 1.42 mm) and in layers I, II-IV and IV-VI in the somatosensory cortex (Bregma: - 1.46 mm). The z-stack area was 174.3 µm x 174.3 µm for the prefrontal areas and 111.15 µm x 111.15 µm for the somatosensory areas. The z-step/interval was 2 µm and microglial cells were counted within 10 µm (every 2nd section) optical sections through the stack with a guard zone of 5 µm at the top and bottom of the section (depth = 40 µm). 4 z-stacks per layer were imaged in the infralimbic and prelimbic areas and 6 z-stacks per layer in the somatosensory area. An average density was obtained (cells/mm^3) in both hemispheres. At least 100 cells per region were counted.

2.2.7.7 Synaptic proteins immunohistochemistry

A guinea pig anti-synaptophysin antibody (Frontiers institute, Syn-GP-Af300; 1:200) and a rabbit anti-PSD-95 antibody (Frontiers institute, PSD-95-Rb-Af628; 1:200) were used to identify the presynaptic zone and the post-synaptic density of asymmetric glutamatergic synapses respectively. 50 µm sections were air-dried for 48 hours, rehydrated in PBS for 15 min then placed in a solution of 50% ethanol: PBS-Azide (0.01%) for 30 min at room temperature. Sections were subsequently dipped in double distilled H_20 to wash off the ethanol then in citrate-EDTA buffer (10 mM citric acid, 2 mM EDTA, 0.05% Tween-20 at pH = 6.2) prior to a heat-mediated antigen retrieval step in citrate buffer for 10 min. Proteinase K treatment (4 µg/mL) for 10 min followed by a 10 min pepsin treatment (1 mg/mL) at 37ºC in 0.2M HCl solution were required to unmask antigenic epitopes (Fukaya and Watanabe 2000, Polgar, Watanabe et al. 2008). Sections were then incubated with primary antibodies diluted in PBS-Azide-Triton-X-100 (0.3%) for three days at room temperature, washed then incubated overnight with 1:500

dilutions of an anti-guinea pig (1:500), anti-rabbit (1:500) secondary antibodies from Life Technologies conjugated to Alexa-fluor 555 and 488 fluorophores, respectively, and concentrated DAPI at 1:50000 in PBS. Sections were subsequently washed; mounting medium added and sections were sealed and stored at -20ºC prior to confocal imaging.

2.2.7.7.1 Confocal imaging and analysis of synapses

Two z-stacks at an interval of 0.1 µm were taken in each layer of the infralimbic, the prelimbic and the somatosensory cortices in both hemispheres. This resulted in 60 optical sections available to quantify the number of PSD-95 and synaptophysin puncta and at least 2000 puncta per layer quantified.

All image processing and analysis was performed in ImageJ. To process images for quantification of synaptic profiles, first z-stacks were deconvolved using the WPL deconvolution algorithm in the Parallel Iterative Deconvolution ImageJ plugin (Wendykier and Nagy 2010) based on the Iterative Deconvolve 3D algorithm (Robert 2005), utilising a theoretical 3D point spread function generated in PSF Lab (Nasse and Woehl 2010). Image stacks were then filtered with a 3x3 median filter and thresholded using the OTSU method (Otsu, 1979), to obtain accurate binary representations of PSD95+ puncta. To assess synapse profiles, individual z-slices separated by 0.4µm were isolated and PSD95+ synaptic puncta profiles were analysed using the Analyze Particles algorithm in ImageJ. Puncta profile counts in each layer were averaged over two sections per animal, and plot as profile counts per 100µm^2.

2.2.8 Statistical analysis

Results were analysed by Student's t-test, One-way ANOVA, Two-way ANOVA or Kruskal-wallis one-way analysis depending on number of groups and data distribution. Specific test used is detailed for each experiment in the results chapters. Analysis was

performed in IBM SPSS statistics version 21.0 (SPSS Inc., Chicago, USA). Graphs were plotted using Graph Pad prism version 6 (GraphPad software, San Diego, California, USA). Data is presented as mean ± standard error of the mean, unless otherwise stated.

Chapter 3: Autoantibody screening in neuropsychiatric and neurodevelopmental disorders

3.1 Introduction

The rapidly expanding field of neuroimmunology and, particularly, the field of antibody-mediated diseases, has affected the way patients with neurological disorders are assessed and investigated in day to day practice. This is due to the expanding clinical phenotype associated with neuronal surface antibodies, which is now proposed to encompass limited forms of disease in almost every field of neurology, including dementia, epilepsy and movement disorders. As reviewed in the introductory chapter of this thesis, this expansion extended beyond what are usually defined as the borders of neurology and is moving towards the field of psychiatry, thus forming new bridges between similar specialities that have suffered a divide in their evolution (Price, Adams et al. 2000). However, as is common in any rapidly growing field, grey areas are starting to come to light, questioning the importance of those autoantibodies in the pathophysiology of these diseases (Coutinho, Harrison et al. 2014). The detection of CNS-targeting antibodies in a small percentage of the healthy population (Dahm, Ott et al. 2014) or in a few patients with definite non-autoimmune disorders (Rossi, Mead et al. 2015) makes their meaning less clear, and has led to new questions regarding the role of the blood brain barrier (Hammer, Stepniak et al. 2013, Hammer, Zerche et al. 2014, Castillo-Gomez, Kastner et al. 2015), but also the possibility of these antibodies occurring as a secondary phenomenon (Rossi, Mead et al. 2015). Further complicating this picture are the often blurred divisions across diagnostic entities in psychiatry and the different methodological methods for antibody testing.

With these questions and limitations in mind, the purpose of this study was to examine the association between autoantibodies to neuronal surface proteins found in the serum

of pregnant women and the risk of neuropsychiatric disorders, first in the mother and then in the progeny. Our hypothesis was that serum autoantibodies to extracellular epitopes of CNS proteins will be found in some women during pregnancy and will be more common in women who then develop postpartum psychosis or in those with progeny diagnosed with a neurodevelopmental disorder.

This was achieved through the combination of information from the Danish National Registries with biological samples from the “Gravid screening” biobank, which is a collection of pregnancy samples available at the State Serum Institute in Copenhagen. In Denmark, all residents are assigned a personal identification number (Central Person Register number, CPR). The CPR number assures linkage between registries and biobanks, and further links to information on first degree relatives. For the purpose of this study, the gestational serum samples from the “Gravid screening” biobank were matched to information available in the registries on psychiatric diagnoses of the mother and progeny. Serum samples were screened using a live CBA, to ensure only potentially relevant antibodies targeting extracellular epitopes were identified. The link between the Danish National Registries and the State Serum Institute is depicted in Figure 3-1. Further details on patients’ cohorts and methods of screening are available in Chapter 2.

3.2 Maternal psychosis cohort

Autoantibodies to cell-surface neuronal proteins were screened in 189 pregnancy serum samples from mothers who subsequently developed postpartum psychosis (n=95) or did not develop psychiatric disease (n=94). Matching of cases and controls was done for mean maternal age and maternal parity (Table 3-1)

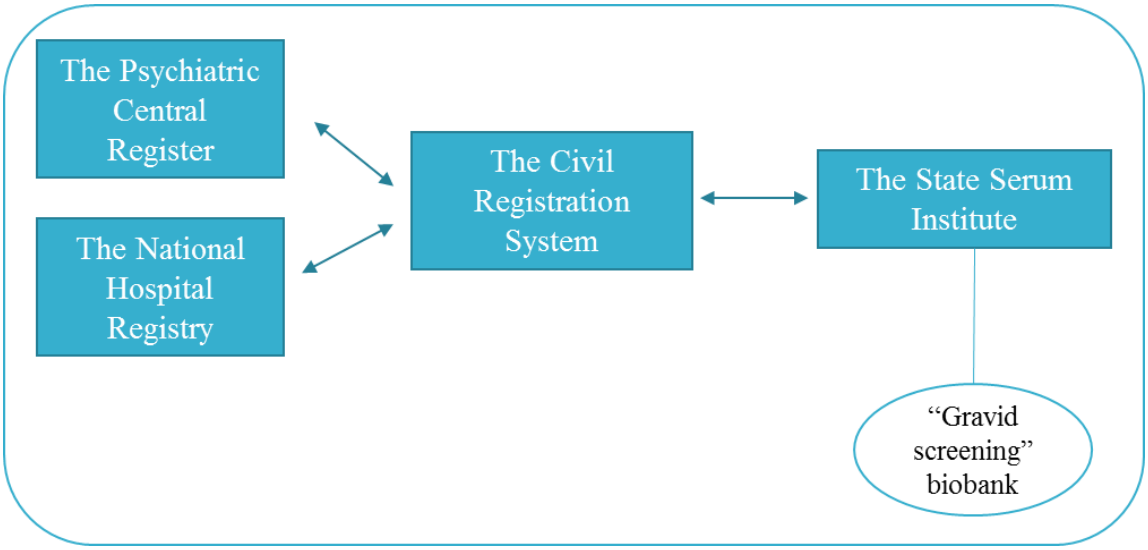


Figure 3-1: Overview of Danish data sources and linkage

The Civil Registration service provides information on all residents in Denmark and links to their first-degree relatives. A CPR number is attributed to each individual and this is used to link the information contained in each registry and the serum samples stored at the State Serum Institute, where a cohort of gestational samples ("Gravid screening biobank") collected from 1992-1995 is available.

3.2.1 Time to psychiatric diagnosis

Although we had requested samples from mothers with “postpartum” psychosis, only 3.2% (n=3) of the cases had received a diagnosis of psychosis within the first month and 6.3% (n=6) within 6 months of delivery. Thus, almost 90% were given the diagnosis more than 1 year after delivery (Table 3-2).

3.2.2 Autoantibody screening for known antigenic targets using CBA

The 189 samples (95 cases and 94 controls) from this cohort were screened by CBA for NMDAR, CASPR2, LGI1, AMPAR and GABABR antibodies, the known neuronal-surface antibodies at the time. Positive samples were found only in the NMDAR and CASPR2 CBAs: 18 and 3 samples, respectively (Figure 3-2A). Titres were in general low: NMDAR 1:20 to 1:320 and CASPR2 1:100 (Figure 3-2 B/C) and there was no association between antibody positivity and clinical status of the mother ($p=0.64$, Fisher’s exact test (two-tailed)) (Figure 3-2D). After decoding of the cohort, further analysis was performed in order to assess whether other variables could have influenced CBA positivity. No association of antibody-positivity with age at delivery ($p=0.83$; independent samples T test (two-tailed)), parity ($p=0.37$; Mann-Whitney U test (two-tailed)) or maternal history of autoimmunity ($p=0.68$; Fisher’s exact test (two-tailed)) was found (Figure 3-3).

3.2.3 Neuropsychiatric disorders in the progeny

A post-hoc analysis was done concerning the diagnosis of psychiatric or neurodevelopmental diseases in the index child (serum sample collected during that pregnancy) and the siblings (ICD-10 codes searched in the database: F20-F39, F60, F61, F70-79, F80-89, F90-98). Figure 3-4 provides a breakdown for the diagnoses in the index children. There was a strong trend for mothers with a diagnosis of psychosis to

Table 3-1: Maternal psychosis cohort and matching of cases and controls

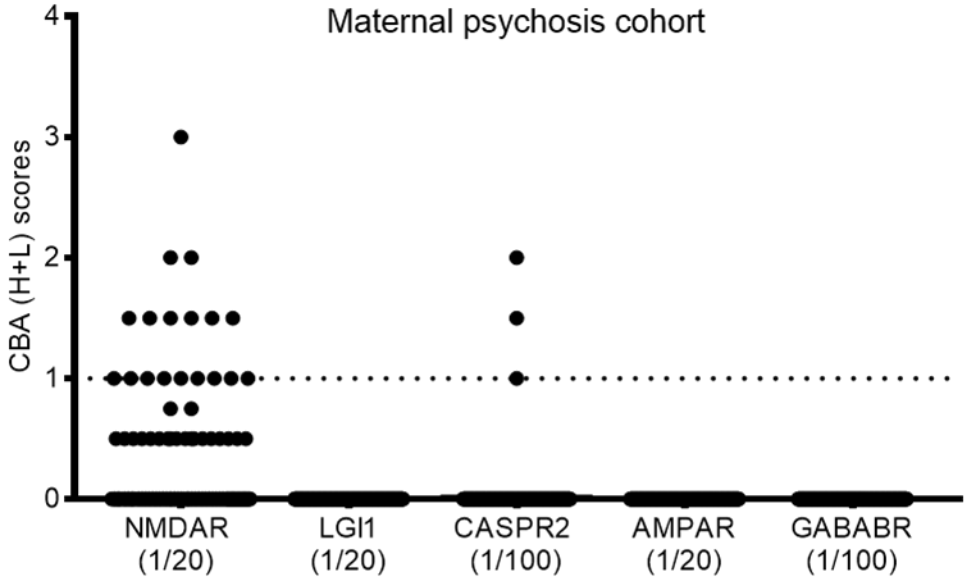
Maternal psychosis cohort			
	Cases	Controls	p-value
n	95	94	
Mean maternal age (±SD)	29.1 (±5.2)	29.1 (±5.4)	n.s.*
Mean maternal parity (±SD)	1.77 (±0.9)	1.78 (±0.9)	n.s.**

* Student's t test; ** Mann-Whitney u test; n.s. non-significant (p>0.05)

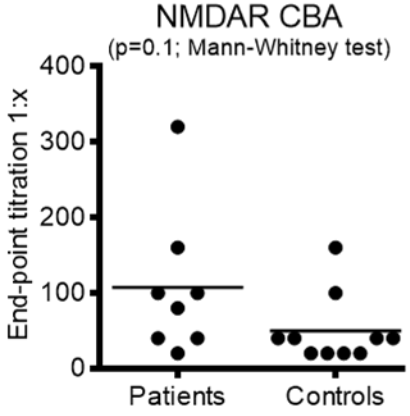
Table 3-2: Time to psychiatric diagnosis after delivery in the maternal psychosis cohort

Months (M) to psychiatric diagnosis after delivery	Frequency	Cumulative percent
< 1 M	3	3.2
1M-6 M	3	6.3
6M-12M	4	10.5
12M-36M	18	29.5
> 36M	67	100

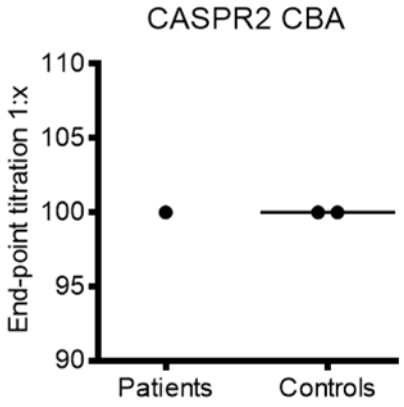
A



B



C



D

CBA results	Cases	Controls	Total
NMDAR positive	8	10	18
CASPR2 positive	1	2	3
Negative (all assays)	85	83	168
Total	95	94	189

Figure 3-2: Antibody screening results in the maternal psychosis cohort

(A) CBA results for the 5 antigenic targets screened in the whole Danish maternal psychosis cohort and breakdown of positive results with end-point titration in the NMDAR CBA (B) and CASPR2 CBA (C) according to maternal clinical status post-umblinding. (D) Total numbers are presented in the table.

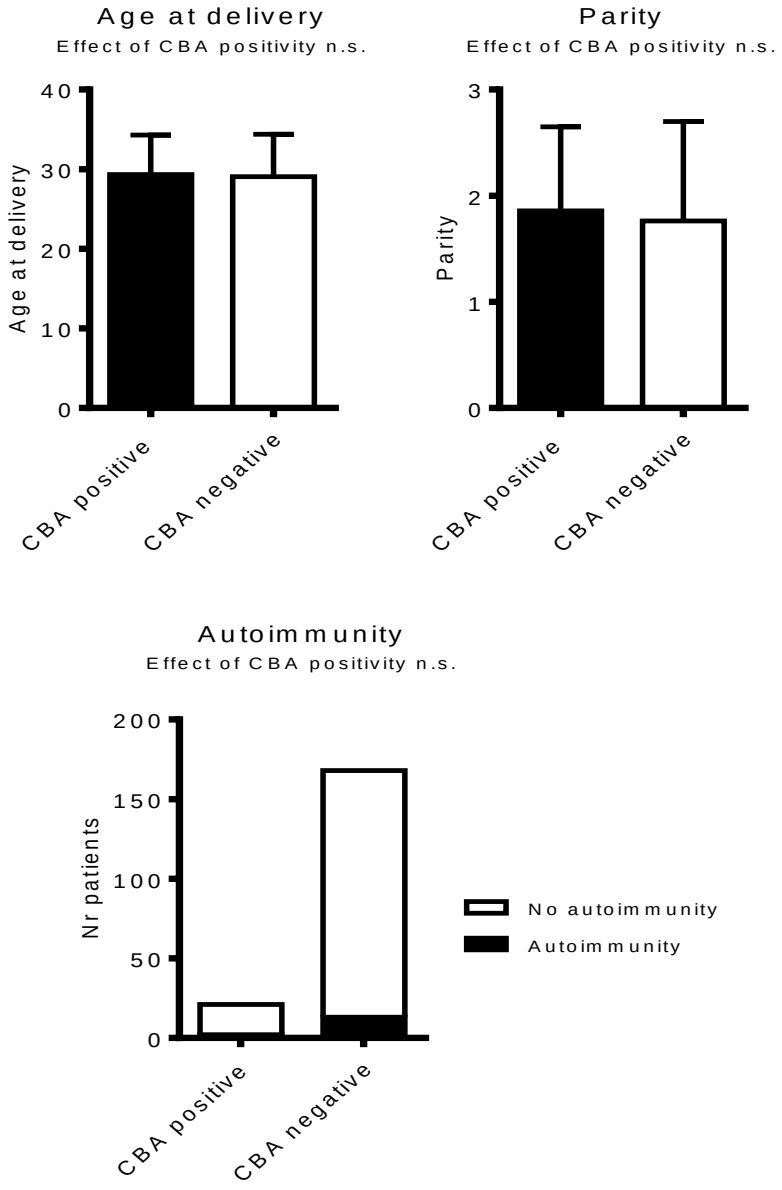


Figure 3-3: Influence of age at delivery, parity and maternal autoimmunity on CBA positivity

No association of antibody-positivity with maternal age at delivery, parity or maternal history of autoimmunity was found in the maernal psychosis cohort.

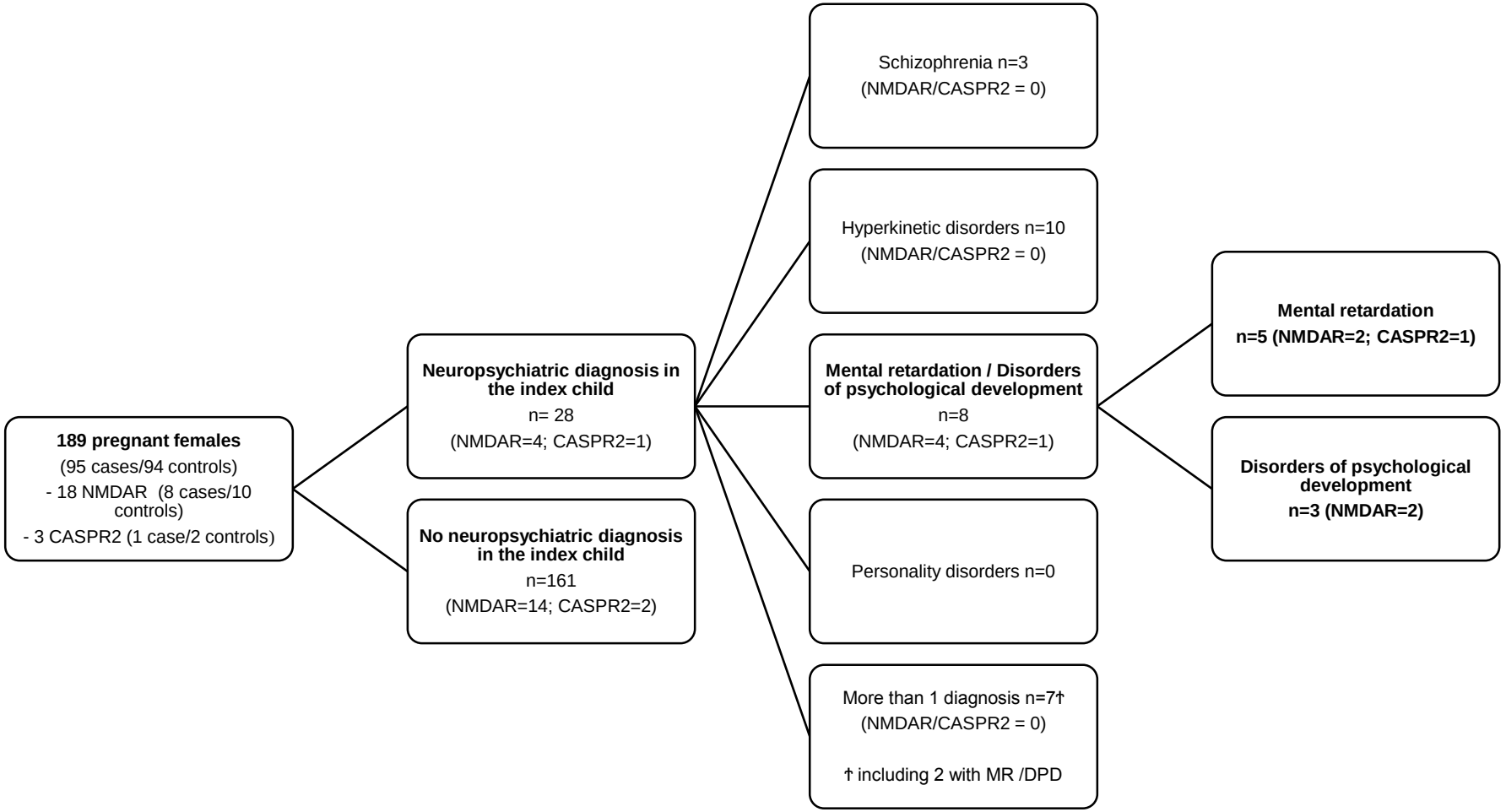


Figure 3-4: Neuropsychiatric diagnoses in the index children of mothers with and without psychosis after delivery

have children with a diagnosis of neurodevelopmental disorders (p=0.06, Fisher's exact test (two-tailed); Table 3-3), which could represent the genetic overlap of these conditions or a common antibody-mediated aetiology. Despite this, there was no association between the diagnosis of a neurodevelopmental disorder in the progeny with CBA positivity (p=0.21, Fisher's exact test (two-tailed), Table 3-4). However, ten index children (including 2 with multiple diagnoses) received a diagnosis in the category of mental retardation/ disorders of psychological development (MR/DPD). Five of these ten were born from mothers with autoantibodies (four with NMDAR antibodies, one with CASPR2). By contrast, in the 161 mothers of children without any psychiatric or neurodevelopmental disorder, only sixteen (10%) had autoantibodies (p=0.003, Fisher's exact test (two-tailed), Table 3-5). Exact ICD-10 codes of the offspring (from positive mothers) are given in Table 3-6.

Neuropsychiatric diagnoses in the siblings of each index child were also assessed, given the fact that some autoantibodies can be in circulation many years before any clinical symptoms (Leite, Coutinho et al. 2012). In the whole cohort, three siblings had MR/DPD, one from a mother with NMDAR antibodies, although the index child from that mother did not have a MR/DPD diagnosis (Table 3-7).

3.2.4 Validation of positive results

At the time experiments were conducted, the existence of CNS-targeting autoantibodies among healthy controls had not been published by other groups (Rhoads, Guirgis et al. 2011, Zandi, Irani et al. 2011, Haussleiter, Emons et al. 2012). A series of experiments was done to confirm the positive results, including screening of positive samples on primary hippocampal cultures, assessment of co-localization with a commercial antibody targeting the extra-cellular epitope (in the NMDAR-positive cases), use of a secondary antibody specific for the Fc portion of IgG (to avoid cross-reactivity with IgM antibodies,

Table 3-3: Association between maternal status and index child status.

		Neurodevelopmental disorder in the index child Yes : No (% positives)
Mother status	Psychosis (n=95)	19 : 76 (20%)
	No Psychosis (n=94)	9 : 85 (9.6%)
	Fisher's exact test, p=0.06 (two tailed)	

Table 3-4: Association between maternal antibody-positivity and neurodevelopmental disorders in the offspring.

		CASPR2 and NMDAR CBA (H+L) Positive : Negative (% positives)
Index child status	Developmental disorder (n=28)	5: 23 (17.9%)
	No neurodevelopmental disorder (n=161)	16:145 (9.9%)
	Fisher's exact test, p=0.21 (two tailed)	

Table 3-5: Association between antibody-positivity and MR/DPD diagnosis in the offspring.

		CASPR2 and NMDAR CBA(H+L) Positive : Negative (% positives)
Index child status	MR/DPD (n=10)	5 : 5 (50%)
	No neurodevelopmental disorder (n=161)	16 : 145 (9.9%)
	Fisher's exact test, p=0.003 (two tailed)	

Table 3-6: ICD-10 diagnoses of the index children from antibody-positive mothers.

ICD-10 code	Diagnosis
F70.9	Mild mental retardation without mention of impairment of behaviour
F79.0	Unspecified mental retardation
F79.9	Unspecified mental retardation without mention of impairment of behaviour
F81.0	Specific developmental disorders of scholastic skills
F88.9	Other disorders of psychological development
F89.9	Unspecified disorders of psychological development

*2 children with both MR and DPD

Table 3-7: Neuropsychiatric diagnoses in the subsequent siblings in the maternal psychosis cohort.

Neuropsychiatric diagnosis	CBA result			Total
	Negative	NMDAR positive	CASPR2 positive	
F20-39: schizophrenia	4	0	0	4
F70-89: MR / DPD	0	1	0	1
F90-98: Hyperkinetic disorders	7	0	0	7
None of the above	150	17	3	170
More than one diagnosis	7	0	0	7
Total	168	18	3	189

as explained below) and pre-adsorption of antibodies against the antigenic protein. Patients and controls in the following experiments are defined according to the status of the mother, not the child, although the full data are provided below (see Table 3-8).

3.2.4.1 Screening with primary neuronal hippocampal cultures

Twenty-one out of 22 positive samples were screened using hippocampal neuronal cultures; it was impossible to do further tests in one sample due to insufficient serum. Eight samples out of 17 NMDAR-positives contained antibodies that bound to the neuronal cultures. These corresponded to 3 patients and 5 controls. Out of the 3 CASPR2-positive samples, one control was positive in the neuronal cultures (Figure 3-5). However, one must note that hippocampal neuronal staining is less sensitive for detection of low levels of antibodies than the CBA, which likely relates to the levels of expression of the target protein in each assay and the lower serum concentration used in this assay (neurons are screened using a 1:100 serum dilution). This could explain why some CBA-positive samples were negative on hippocampal neurons.

.

3.2.4.2 Co-localization with commercial antibody

Co-localization of patient's IgG with the NMDAR/NR1 subunit was assessed using a mouse commercial monoclonal antibody towards an extracellular portion of the NR1. Seventeen NMDAR-positive samples were available for screening. Eight samples were also positive in this assay: 5 out of 7 cases and 3 out of 10 controls (Figure 3-6). A strict criterion was used for this assay, since only those samples with all positive cells showing co-localization of both antibodies were considered positive. Care was taken to choose a commercial antibody with an extracellular epitope different from the suspected epitope in patients with NMDAR encephalitis (Gleichman, Spruce et al. 2012). However, it is

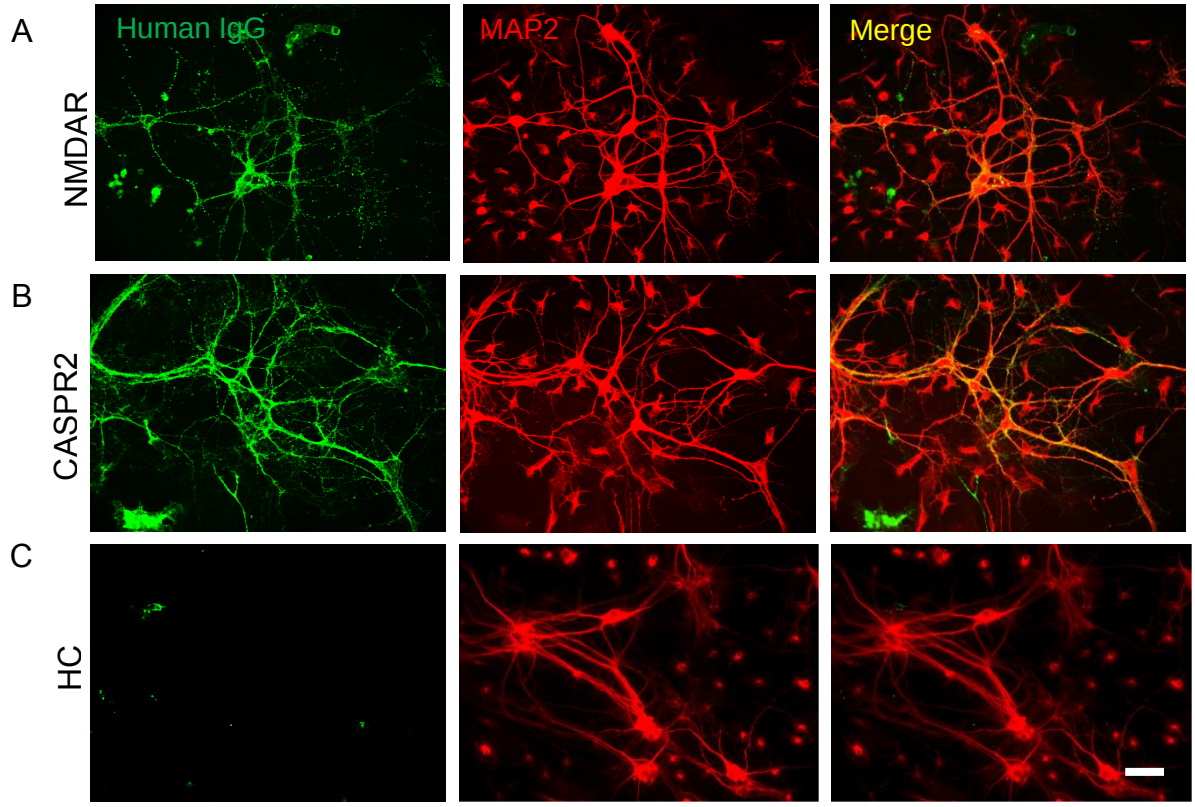


Figure 3-5: Representative images of neuronal surface staining.

(A) NMDAR-positive sample; (B) CASPR2-positive sample and (C) negattive healthy control (HC) from the maternal psychosis cohort. Scale bar: 50 μm

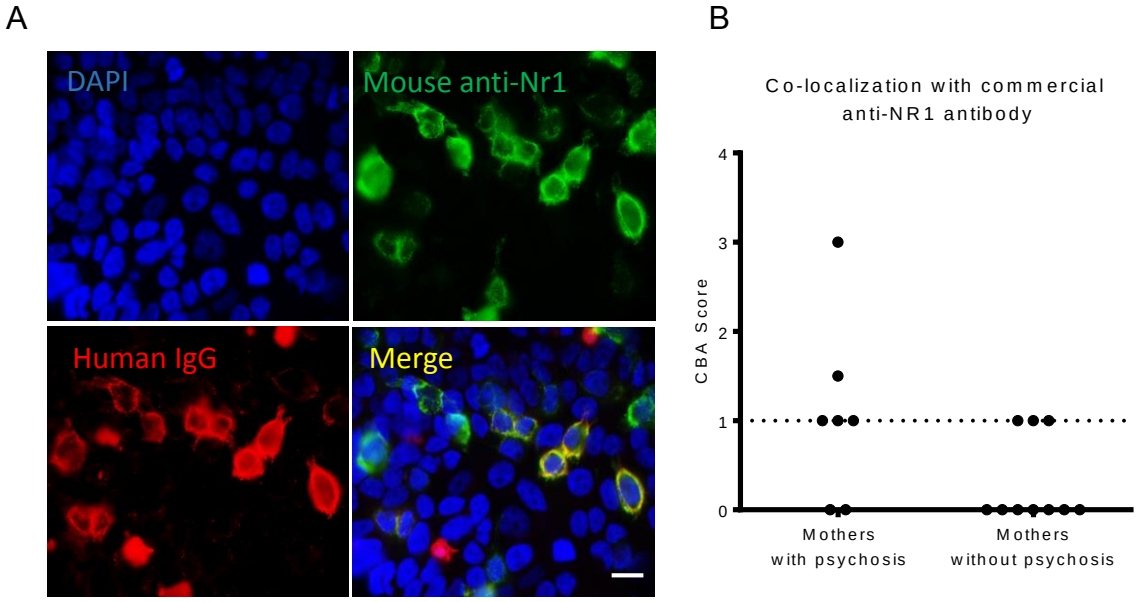


Figure 3-6: NR1 commercial co-localization assay on CBA.

(A) Representative image (scale bar: 20μm) and (B) score results.

impossible to be sure there was no antibody competition for the same epitopes, which would have reduced the sensitivity of this method.

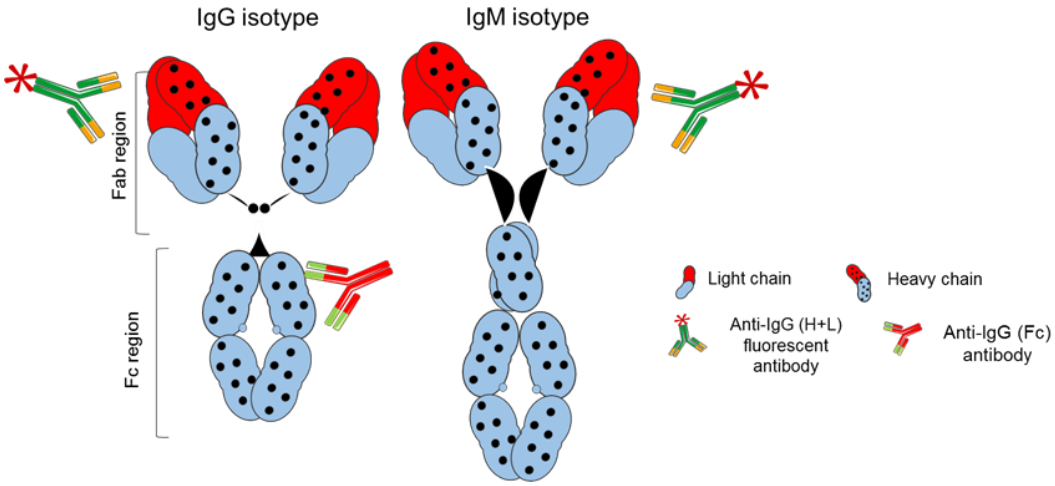
3.2.4.3 Screening with anti-FC IgG antibody

The CBA used to screen this cohort uses a fluorescently tagged secondary antibody that reacts with the human IgG heavy chains and the light chains of all classes of immunoglobulin (referred in this thesis as CBA (H+L)). This could lead to unexpected reactivity with human IgM (scheme provided in Figure 3-7A). Thus, all positive samples were also screened using a secondary antibody targeting the Fc region of IgG specifically (referred as CBA (Fc)). This was impossible to perform in 2 samples due to lack of serum, so these were excluded from analysis. Five samples previously positive on the CBA (H+L) were negative on the CBA (Fc); none of these samples were from mothers with psychosis or from mothers of children with neurodevelopmental disorders. There was still no association between antibody positivity on this assay and clinical status of the mother (Figure 3-7B) but there was an association between the diagnosis of a neurodevelopmental disorder in the progeny with CBA (Fc) positivity ($p=0.04$, Fisher's exact test (two-tailed); Figure 3-7C) and between the specific diagnoses of MR/DPD and CBA (Fc) positivity ($p=0.0004$, Fisher's exact test (two-tailed); Figure 3-7D).

3.2.4.4 Pre-adsorption against NMDAR

To further clarify the existence of CNS-surface antibodies in healthy controls, one positive control sample (NMDAR-positive at 1:160) was pre-adsorbed against NMDAR-transfected cells and against non-transfected HEK cells. Pre-adsorption of this serum against NMDAR-transfect cells, but not non-transfected HEK cells, abrogated IgG staining, thus confirming the antigenic specificity in this particular sample (Figure 3-8)

A



B

Association between antibody-positivity and maternal status		CASPR2 and NMDAR CBA(Fc) Positive : Negative
Mother status	Psychosis (n=94)	8 : 86 (8.5%)
	No Psychosis (n=93)	6 : 87 (6.5%)
	Fisher's exact test, p=0.78 (two tailed)	

C

Association between antibody-positivity and neurodevelopmental disorders in the offspring		CASPR2 and NMDAR CBA(Fc) Positive : Negative
Index child status	Developmental disorder (n=28)	5: 23 (17.9%)
	No neurodevelopmental disorder (n=159)	9:150 (5.7%)
	Fisher's exact test, p=0.04 (two tailed)	

D

Association between antibody-positivity and MR/DPD diagnosis in the offspring		CASPR2 and NMDAR CBA (Fc) Positive : Negative
Index child status	MR/DPD (n=10)	5 : 5 (50%)
	No neurodevelopmental disorder (n=159)	9 : 150 (5.7%)
	Fisher's exact test, p=0.0004 (two tailed)	

Figure 3-7: CBA (Fc) assay.

Schematic representation of antibody binding in the CBA (H+L) and CBA (Fc) assay (A) and contingency tables for association between antibody-positivity in the CBA (FC) and maternal status (B), neurodevelopmental disorders (C) and MR/DPD in the offspring (D).

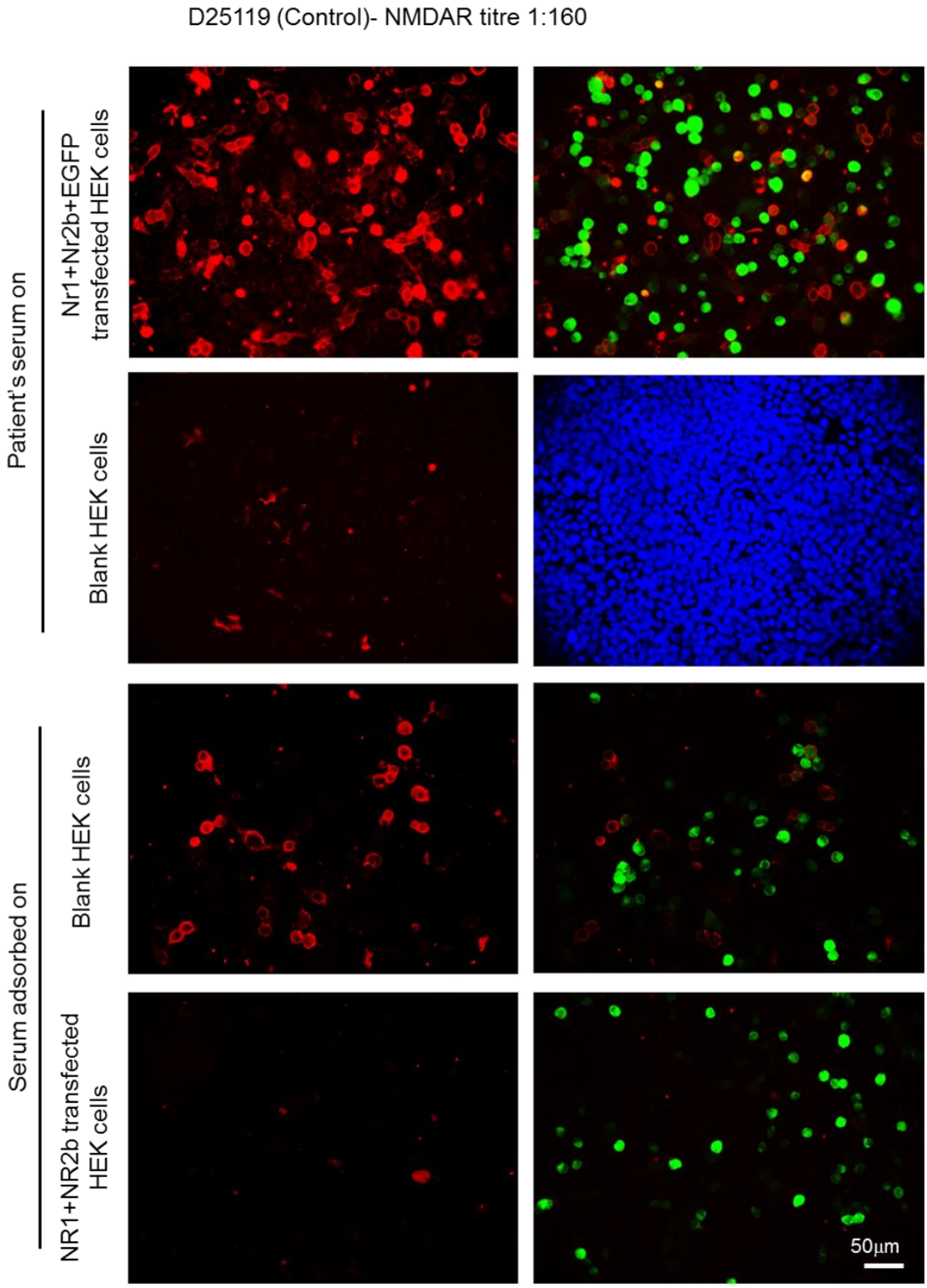


Figure 3-8: Pre-adsorption against NMDAR-transfected and non-transfected HEK cells.

3.2.4.5 Comparison of different IgG staining methods

All the different IgG staining methods used have different sensitivities and specificities. However, they allow us to conclude that at least a few controls have antibodies towards NMDAR and CASPR2, even if we use a stricter criteria, demanding positivity in 2 or 3 of these different methods. Table 3-8 gives an overview of the results of all of the tests performed for each sample together with the diagnostic status of the mother and the progeny.

Shortly after termination of this study, a confirmatory CBA (Fc) became routine for all positive samples on the CASPR2 and NMDAR CBA (H+L) in the diagnostic laboratory. Other validation studies performed in the laboratory concluded that the CBA(Fc), done as an extra confirmatory step, would result in higher specificity of the antibody test. Thus, the following cohorts were screened with a CBA (H+L) and confirmed on a CBA (Fc).

3.3 Autism and MR/DPD cohorts

To confirm the results obtained with the previous cohort, two other cohorts were requested from the “Gravid screening collection” at the Danish State Serum Institute. The first comprised gestational sera from mothers of children with a diagnosis of autism (cases, n=95) and mothers of children without neurodevelopmental disorders (controls, n=95) and will be referred to as Autism cohort. The second comprised gestational sera from mothers of children with a diagnosis of mental retardation and/or disorders of psychological development (MR/DPD) except autism (cases, n=171), and mothers of children without neurodevelopmental disorders (controls, n=171). This cohort is referred in this thesis as MR/DPD cohort. Matching of cases and controls in both cohorts was done for gender distribution, mean maternal age and maternal parity (Full description of the cohorts is provided in chapter 2. As described above, the autism and MR/DPD cohorts were screened first using the anti-human IgG (H+L) secondary and all the

positives were then screened using the anti-human Fc IgG secondary. Samples were only considered positive if confirmed in the CBA (Fc). All samples from these cohorts were also screened using primary neuronal cultures.

3.3.1 Autism cohort

3.3.1.1 Screening for known antigenic targets

Samples were screened for NMDAR, CASPR2, LGI1 and glycine receptor alpha2 antibodies. Positive samples were found only for NMDAR and CASPR2 antibodies (Table 3-10). Five samples (2 patients and 3 controls) were NMDAR antibody-positive (titre 1:50-1:250) (Fisher's exact test: p=1.0) and 3 samples (1 patient and 2 controls) were CASPR2 antibody-positive (titre 1:100-1:250) (Fisher's exact test: p=1.0), thus revealing no association of these maternal antibodies with a diagnosis of autism in the progeny.

3.3.1.2 Screening for unknown antigenic targets

Hippocampal neuronal cultures and cerebellar granule cell neuronal cultures were used to assess for binding to other, yet unknown, antigenic targets. Seven samples (4 cases and 3 controls; p=1, Fisher's exact test) showed binding to the surface of hippocampal neurons and 9 samples (6 cases and 3 controls; p=0.5; Fisher's exact test) showed binding to the surface of cerebellar granule cells neurons (Table 3-10 and Figure 3-9). Two samples showed binding to both primary cultures, these samples were both from mothers of autistic children.

Table 3-9).

Table 3-8: Clinical association of all positive screening tests in the maternal psychosis cohort

	CBA (H+L)	CBA (Fc)	CBA Nr1 comercial	Neuronal staining	Mother	Index child	Sibling
1	NMDAR 1:80	Positive	Negative	Positive	Psychosis	**MR**	No NPsych
2	NMDAR 1:40	Negative	Negative	Negative	No Psychosis	No NPsych	No NPsych
3	NMDAR 1:40	Negative	Negative	Positive	No Psychosis	No NPsych	No NPsych
4	NMDAR 1:40	No serum	Negative	Negative	No Psychosis	No NPsych	No NPsych
5	NMDAR 1:40	Positive	Positive	Positive	No psychosis	No NPsych	**DPD**
6	NMDAR 1:40	Positive	Positive	Positive	Psychosis	No NPsych	No NPsych
7	NMDAR 1:40	Positive	Negative	Negative	Psychosis	**MR**	No NPsych
8	NMDAR 1:320	Positive	Positive	Negative	Psychosis	No NPsych	No NPsych
9	NMDAR 1:20	Negative	Negative	Positive	No psychosis	No NPsych	No NPsych
10	NMDAR 1:20	Negative	Negative	Negative	No psychosis	No NPsych	No NPsych
11	NMDAR 1:20	Negative	Negative	Negative	No psychosis	No NPsych	No NPsych
12	NMDAR 1:20	No serum	No serum	No serum	Psychosis	No NPsych	No NPsych
13	NMDAR 1:20	Positive	Negative	Positive	No psychosis	No NPsych	No NPsych
14	NMDAR 1:160	Positive	Positive	Negative	Psychosis	No NPsych	No NPsych
15	NMDAR 1:160	Positive	Positive	Positive	No psychosis	No NPsych	No NPsych
16	NMDAR 1:100	Positive	Positive	Negative	Psychosis	No NPsych	No NPsych
17	NMDAR 1:100	Positive	Positive	Positive	Psychosis	**DPD**	No NPsych
18	NMDAR 1:100	Positive	Positive	Negative	No psychosis	**DPD**	No NPsych
19	CASPR2 1:100	Positive	-	Positive	No psychosis	No NPsych	No NPsych
20	CASPR2 1:100	Positive	-	Negative	Psychosis	No NPsych	No NPsych
21	CASPR2 1:100	Positive	-	Negative	No psychosis	**MR**	No NPsych

Full description of the cohorts is provided in chapter 2. As described above, the autism and MR/DPD cohorts were screened first using the anti-human IgG (H+L) secondary and all the positives were then screened using the anti-human Fc IgG secondary. Samples were only considered positive if confirmed in the CBA (Fc). All samples from these cohorts were also screened using primary neuronal cultures.

3.3.2 Autism cohort

3.3.2.1 Screening for known antigenic targets

Samples were screened for NMDAR, CASPR2, LGI1 and glycine receptor alpha2 antibodies. Positive samples were found only for NMDAR and CASPR2 antibodies (Table 3-10). Five samples (2 patients and 3 controls) were NMDAR antibody-positive (titre 1:50-1:250) (Fisher's exact test: p=1.0) and 3 samples (1 patient and 2 controls) were CASPR2 antibody-positive (titre 1:100-1:250) (Fisher's exact test: p=1.0), thus revealing no association of these maternal antibodies with a diagnosis of autism in the progeny.

3.3.2.2 Screening for unknown antigenic targets

Hippocampal neuronal cultures and cerebellar granule cell neuronal cultures were used to assess for binding to other, yet unknown, antigenic targets. Seven samples (4 cases and 3 controls; p=1, Fisher's exact test) showed binding to the surface of hippocampal neurons and 9 samples (6 cases and 3 controls; p=0.5; Fisher's exact test) showed binding to the surface of cerebellar granule cells neurons (Table 3-10 and Figure 3-9). Two samples showed binding to both primary cultures, these samples were both from mothers of autistic children.

Table 3-9: Autism and MR/DPD cohorts and matching of cases and controls

Autism cohort			
	Cases	Controls	p-value
N	95	95	
Gender distribution	76M/19F (4:1)	76M/19F (4:1)	
Mean maternal age (±SD)	28.8 (±4.33)	28.74 (±4.32)	n.s.*
Mean maternal parity (±SD)	1.87 (±0.82)	1.87 (±0.82)	n.s.**

* Student's t test; ** Mann-Whitney u test; n.s. non-significant (p>0.05)

Mental retardation and other disorders of psychological development			
	Cases	Controls	p-value
N	171	171	
Gender distribution	112M/59F (4:1)	112M/59F (1.9:1)	
Mean maternal age (±SD)	28.29(±4.33)	28.26 (±4.96)	n.s.*
Mean maternal parity (±SD)	1.9(±0.95)	1.9 (±0.92)	n.s.**

* Student's t test; ** Mann-Whitney u test; n.s. non-significant (p>0.05)

3.3.3 MR/DPD cohort

3.3.3.1 Screening for known antigenic targets

All samples were screened for CASPR2 and NMDAR antibodies. There was a trend towards an association between maternal CASPR2 antibodies (titres 1:100-1:200) and MR/DPD in this cohort but it did not reach statistical significance: eight samples were positive for CASPR2 antibodies, (7 cases and 1 control; p=0.067; Fisher's exact test (two-tailed)). Nineteen samples were positive for NMDAR antibodies (titres 1:20-1:500), but 10 samples were from cases and 9 samples from controls (p=1.0; Fisher's exact test) (Table 3-11).

3.3.3.2 Screening for unknown antigenic targets

All samples were tested on primary hippocampal cultures. Six were positive, 5 cases and 1 control (p=0.21; Fisher's exact test). One of the positive cases was also positive for CASPR2 antibodies (Table 3-11 and Figure 3-10).

3.4 All cohorts

The results above indicate that a small number of samples with CBA-identified neuronal surface antibodies is found, but both in controls and in patients. Nevertheless, an association between the presence of neuronal surface antibodies and the specific diagnosis of MR/DPD was detected in the first cohort and a trend towards association of CASPR2 antibodies with the same diagnosis was detected in the MR/DPD cohort. To give more power to the study, the data from the 3 cohorts were combined and 3 groups established: children with MR/DPD, children without neurodevelopmental disorders (our healthy control group), and children with other neurodevelopmental disorders (our disease control group). For consistency, only the results obtained in the CBA (Fc) were used in this analysis.

	CASPR2 CBA(Fc) Positive : Negative	**NMDAR CBA(Fc)** Positive : Negative	**Hippocampal neurons** Positive* : Negative	**Cerebellar granule cells** Positive† : Negative
Cases (n=95)	1 : 94 (1.1%)	2:93 (2.2%)	4:91 (4.4%)	6 :89 (6.7%)
Controls (n=95)	2: 93 (1.1%)	3:92 (3.3%)	3:92 (3.3%)	3 : 92 (3.3%)
Fisher's exact test (two-tailed)	p=1.0	p=1.0	p=1.0	p=0.5
*All CBA negative; † 2 cases also positive on hippocampal cultures				

Table 3-10: Screening results in the autism cohort

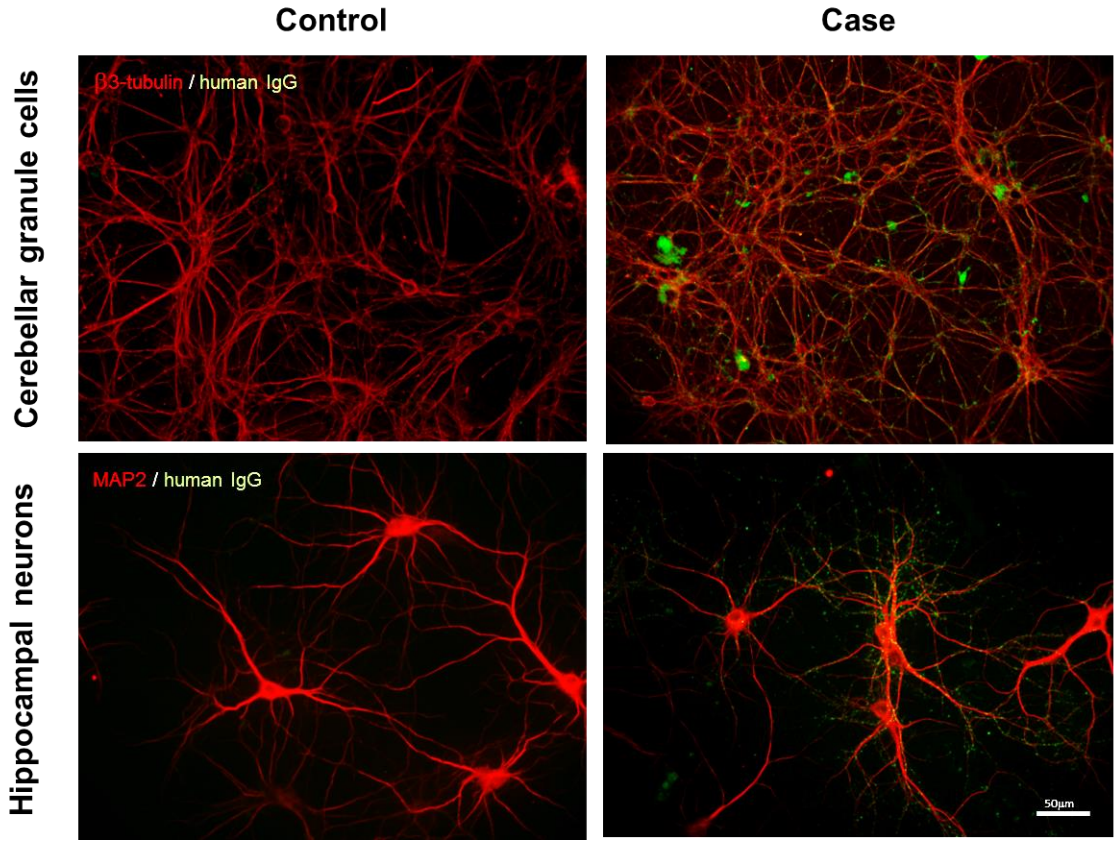


Figure 3-9: Neuronal surface staining in the Autism cohort

Representative images of neuronal surface staining from a case and a control from the autism cohort. Scale bar: 50 µm

Table 3-11: Screening results in the MR/DPD cohort

	CASPR2 CBA(Fc) Positive : Negative	**NMDAR CBA(Fc)** Positive : Negative	**Hippocampal neurons** Positive* : Negative
Cases (n=171)	7:164 (4.3%)	10:161 (6.2%)	5:166 (3.0%)
Controls (n=171)	1: 170 (0.6%)	9:162 (5.6%)	1:170 (0.6%)
Fisher's exact test (two-tailed)	p=0.067	p=1.0	p=0.21
*1 case CASPR2 positive			

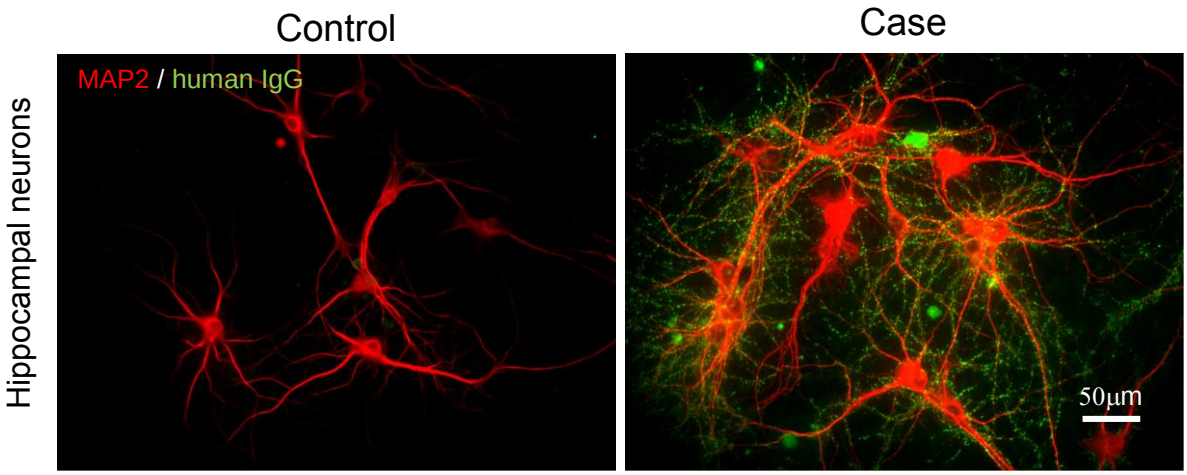


Figure 3-10: Neuronal surface staining in the MR/DPD cohort

Representative images of neuronal surface staining from a case and a control from the MR/DPD cohort. Scale bar: 50 µm

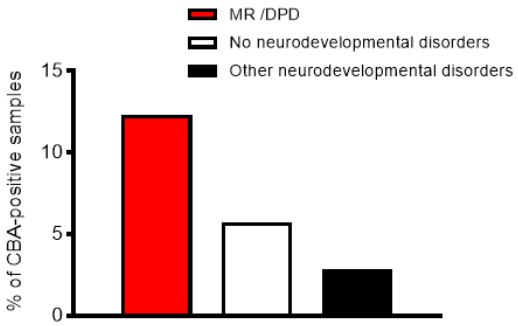
A positive CBA (for CASPR2 or NMDAR antibodies) was found in 12.2% of samples from mothers of children with MR/DPD, while only 5.6% and 2.7% were found in mothers of children with no neurodevelopmental disorders or with other neurodevelopmental disorders, respectively (p=0.002; Pearson Chi-square; Figure 3-11A). CASPR2 antibodies were found in 4.4% of samples from mothers of children with MR/DPD, in 1.2% of samples from mothers of children with no neurodevelopmental disorders and in 0.9% of samples from mothers of children with other neurodevelopmental disorders (p=0.02; Pearson Chi-square; Figure 3-11B). There was only a trend for an association of maternal NMDAR antibodies with MR/DPD in the progeny: 7.7% vs 4.5% vs 1.8% in mothers of children with MR/DPD, no neurodevelopmental disorders or other developmental disorders, respectively (p=0.057; Pearson Chi-square; Figure 3-11C).

3.5 Discussion

In this study, the presence of antibodies targeting extracellular epitopes of synaptic proteins was investigated in the gestational serum of mothers of children with neurodevelopmental disorders and mothers of healthy children. It is the first study to use a live cell-based assay for this purpose; this is a method of screening which specifically detects extracellular-targeting antibodies, thus focusing on those antibodies that are more likely to be directly pathogenic. Additionally, our collaboration with the Danish State Serum Institute and Registries gave us access to gestational samples, allowing us to investigate the presence of antibodies directly during the period of neurodevelopment. This is undoubtedly a strength of this study in comparison to other studies where mothers were investigated (sometimes many years) after delivery (Zimmerman, Connors et al. 2007, Braunschweig, Ashwood et al. 2008, Singer, Morris et al. 2008, Braunschweig, Duncanson et al. 2012, Braunschweig, Krakowiak et al. 2013, Brimberg, Sadiq et al. 2013, Nordahl, Braunschweig et al. 2013, Rossi, Fuentes et al. 2013, Piras, Haapanen et al. 2014).

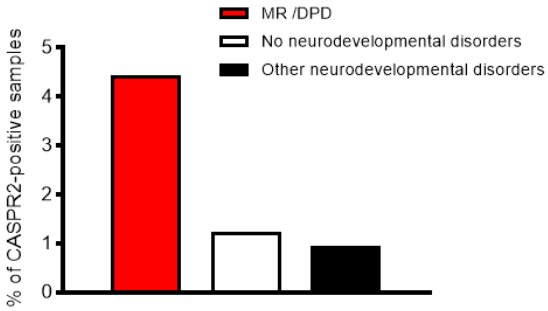
A

	CASPR2 and NMDAR CBA(Fc) Positive : Negative
MR/DPD (n=181)	22 : 159 (12.2%)
No neurodevelopmental disorders (n=425)	24 : 401 (5.6%)
Other neurodevelopmental disorder (n=113)	3 : 110 (2.7%)
Pearson $\chi^2(2) = 12.1$, $p = 0.002$ (two-tailed)	



B

	CASPR2 CBA(Fc) Positive : Negative
MR/DPD (n=181)	8 : 173 (4.4%)
No neurodevelopmental disorders (n=425)	5 : 420 (1.2%)
Other neurodevelopmental disorder (n=113)	1 : 112 (0.9%)
Pearson $\chi^2(2) = 7.8$, $p = 0.02$ (two-tailed)	



C

	NMDAR CBA(Fc) Positive : Negative
MR/DPD (n=181)	14 : 167 (7.7%)
No neurodevelopmental disorders (n=425)	19 : 406 (4.5%)
Other neurodevelopmental disorder (n=113)	2 : 111 (1.8%)
Pearson $\chi^2(2) = 5.7$, $p = 0.057$ (two-tailed)	

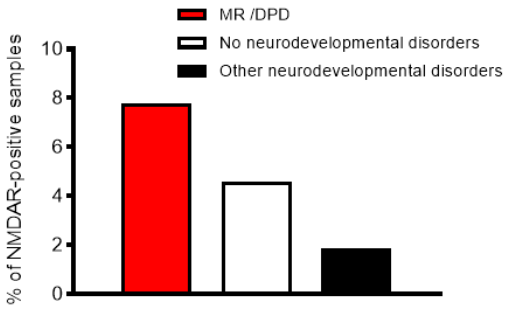


Figure 3-11: Analysis of all Danish cohorts.

CBA (A), CASPR2 (B) and NMDAR (C) positivity among mothers of children with MR/DPD, with no neurodevelopmental disorders and with other neurodevelopmental disorders.

Autoantibodies towards NMDAR / CASPR2 were found to be present at low levels in a small percentage of the population. The presence of neuronal surface antibodies during pregnancy was not associated with maternal psychosis after delivery, although the number of samples from true postpartum psychosis was too small to allow for a definite conclusion. However, when pooling the data from all cohorts, the presence of neuronal surface antibodies during pregnancy in the maternal circulation, particularly those against CASPR2, was associated with an increased risk of MR/DPD diagnosis in the progeny. Considering that 8 out of 181 children with MR/DPD versus 5 out of 425 children without neurodevelopmental disorders were exposed to maternal CASPR2 antibodies, children exposed to maternal CASPR2 antibodies had 2.1 times the risk of developing MR/DPD than children not exposed to those antibodies (RR=2.1; 95% CI: 1.35-3.3).

There are evident limitations. The levels of antibodies were often low (maximum titre 1:500), but IgG antibodies tend to be low in the mother during pregnancy due to the physiological increase in plasma volume (Hytten 1985) and transfer of IgG to the foetus (Roopenian and Akilesh 2007). Both NMDAR and CASPR2 antibodies were also present in our controls, in 4.5% and 1.2% of mothers of children with no neurodevelopmental disorders, respectively. The seroprevalence of NMDAR and CASPR2 antibodies in previous publications (Hammer, Stepniak et al. 2013, Dahm, Ott et al. 2014) was approximately 10 and 1%, respectively. These numbers include all immunoglobulin isotypes, and the percentages of specific IgG antibodies were much lower, namely 1% and 0.3% for NMDAR and CASPR2 antibodies respectively. One must note, however, that the previous publications (Hammer, Stepniak et al. 2013, Dahm, Ott et al. 2014) used a commercial assay which, although reliably used by many laboratories for antibody screening, could be less sensitive than our live-cells research assay, since the cells expressing the proteins of interest need to be fixed for transport. This difference in methods could account for the higher seroprevalence detected in our population. For another antigen, aquaporin 4, this difference in sensitivity was already documented

(Waters, McKeon et al. 2012). Several methods were explored in this thesis to validate the positive results. Importantly, it was not possible to detect antibody binding to neuronal cultures in several samples that were positive in the CBA. This limitation is most likely the result of the low antibody titres in the positive samples, the use of a lower antibody concentration in this method of screening, and the lower target protein expression found in rodent neurons in comparison to the transfected HEK cells. However, an assay using a specific anti-Fc IgG antibody, thus eliminating the potential detection of IgM or IgA antibodies, was found to be more specific than our previous assay.

The association of MR/DPD in the progeny with the presence of antibodies in the maternal serum is a very interesting result and potentially very important for the diagnosis and treatment of this subgroup of children. However, as previously noted, NMDAR/CASPR2 antibodies were also detected in mothers of children with, to the best of our knowledge, normal neurodevelopment. This points towards the importance of understanding other contributing factors to the pathophysiology of the disease, such as individual genetic predisposition, function and maturation of the foetal blood brain barrier, expression levels of the nFcR that controls IgG transfer, timing of the antibodies in relation to the maximum potential damage to the developing baby, and the existence of additional pro-inflammatory cytokines or other predisposing factors, such as infections. Also, it is important to note that the percentage of children who might have a maternal antibody-mediated neurodevelopmental disorder is very small, certainly less than 10% of the entire group. Indeed, a high rate of maternal antibodies in any category was not anticipated since neurodevelopmental disorders are heterogeneous, with genetic and environmental causes. Ideally, a detailed clinical characterisation of this subgroup of children would allow us to compare the phenotype of those with a putative antibody-mediated form of disease with the remaining cohort, thus providing specific clinical criteria that might point to the former diagnosis. Unfortunately, this was impossible to do from a Registry perspective.

Interestingly, the diagnosis of autism, the most widely studied neurodevelopmental disorder in the context of maternal antibodies (reviewed in (Estes and McAllister 2015)), was not associated with the antibodies screened in this study and less than 9% of cases had antibodies towards neuronal-surface proteins, a percentage that was not significantly different than that found in mothers of children without this disorder. Two samples from mothers of autistic children (none in controls) had reactivity against both hippocampal and granular neurons, perhaps suggesting a more widespread reactivity against neuronal surface antigens in these two mothers. The importance of this finding, however, is difficult to establish without further studies on the antigenic target of these samples. It was, unfortunately, impossible to pursue these findings during the period of my research project. Given the fact that many proteins identified as genetic targets in ASDs are important membrane synaptic proteins and receptors (reviewed in (Chen, Yu et al. 2014)), there are multiple antigenic targets to explore.

As previously discussed, our only diagnostic information was provided by registers based on the ICD-10 classification and this has resulted in some limitations. Knowing that the distinction between the spectrum of “normality” and discrete categories of neurodevelopmental disorders is often blurred, it is possible that some individuals classified as controls in our study might have a subclinical or undiagnosed neuropsychiatric disorder. Likewise, other diagnoses with potential relevance in the context of maternal autoimmunity, such as epilepsy or cerebral palsy, were not included. Nevertheless, this potential bias in our controls, most likely reinforces our findings. Furthermore, one must note that there is considerable phenotypic overlap between mental retardation, autism and other disorders of psychological development, making it difficult to establish strict divisions.

Another potential limitation comes from the transversal nature of the study. Autoantibody reactivity was only detected at a single time point during gestation and additional sampling could give us further information regarding the antibody titres profile among

patients and controls. Likewise, antibodies formed at a different time point in gestation could be present in other mothers of children with neurodevelopmental disorders. Also, the combined results showed an increase in the number of CASPR2 antibodies in mothers of children with MR/DPD, but this association was not found in the first cohort where only the NMDAR antibodies were increased. It is possible that NMDAR antibodies are more common in those women who subsequently develop psychosis and thus play a greater role in the neurodevelopmental disorders in their children. Further studies are necessary to explore these findings.

Finally, an important question remains on whether the antibodies identified in this study represent a primary or secondary phenomenon in the pathophysiology of MR/DPD. This study focused on antibodies towards extracellular epitopes and, as such, with the potential to be directly pathogenic. Nevertheless, previous reports have identified similar antibodies in a few patients suffering from definite non-immune conditions, such as Creutzfeldt-Jakob disease (Fujita, Yuasa et al. 2012, Mackay, Ahmad et al. 2012, Rossi, Mead et al. 2015), or as a consequence of a previous non-autoimmune condition (Hacohen, Deiva et al. 2013). It remains possible that the CASPR2 antibodies detected reflect a widespread immune activation, perhaps secondary to maternal infection, and that antibodies themselves were not the pathogenic entity in the patients. An answer to this question would only be possible if a clinical trial was to take place and the antibody insult was removed (through immunosuppression or plasma exchange) during gestation and the critical period of brain development. Another way to attempt to answer this question is by establishing an animal model of gestational antibody transfer, followed by assessment of the offspring.

In conclusion the work described in this chapter linked a gestational serum biobank and a patient register to show that maternal antibodies to a specific neuronal antigen, CASPR2, were associated with MR/DPD disorders in the progeny. Our results are the

first to identify a specific target for potentially foeto-pathogenic antibodies in a large human gestational cohort

Next, the aim was to assess the pathogenicity of neuronal surface antibodies, particularly CASPR2, in a mouse model of maternal to foetal transfer of antibodies. The results of such animal model are described in the following chapters.

Chapter 4: Mouse model of maternal to foetal transfer of neuronal surface antibodies

4.1 Introduction

Reproduction of a disease phenotype in an animal model through passive immunization or induction of the disease in a neonate through gestational transfer are established criteria for antibody pathogenicity, as reviewed in chapter 1, section 1.1. To investigate further whether maternal neuronal surface antibodies can be directly pathogenic during the neurodevelopment of the progeny, a mouse model of maternal to foetal transfer was established and the long-term effects from *in utero* exposure to these antibodies studied in the offspring. A similar experimental protocol to that published by our group for the study of maternal to foetal transfer of AChR antibodies in AMC was used (Jacobson, Polizzi et al. 1998, Jacobson, Polizzi et al. 1999), with the adaptations necessary for the study of CNS targeting antibodies.

First, we aimed to establish whether the known CNS antibodies are effectively and efficiently transferred to the embryonic circulation. Once the maternal antibodies are detected in the embryos, an important question is whether they reach the antigenic target. The blood-brain barrier would prevent this from happening in normal circumstances. In fact, it is known that both blood-brain barrier and brain-CSF barrier are present even at very early foetal stages (Saunders, Liddelow et al. 2012). However, it is possible that specific transporters exist during development or that there is a change in permeability in disease-states, since the mouse developing brain is partially permeable to IgG until E17.5 (Braniste, Al-Asmakh et al. 2014). Reaching the antigenic target, the autoantibody can block ligand-receptor interactions, activate complement, mediate cell cytotoxicity, alter signal transduction or change protein expression (Brimberg, Mader et al. 2015). It is also possible that the antibody itself does not interfere directly with the

expression or function of the antigenic target but that its presence in the CNS leads to an inflammatory reaction that is detrimental to the neural circuitry assembly. Any of these events could lead to permanent sequelae in the exposed offspring.

The following chapters of this thesis describe a mouse model of maternal to foetal transfer of neuronal surface targeting antibodies, particularly CASPR2 antibodies. In this chapter, transfer of human IgG and of specific CNS-targeting antibodies from mother to foetus is assessed, as well as the IgG deposition in the foetal brain.

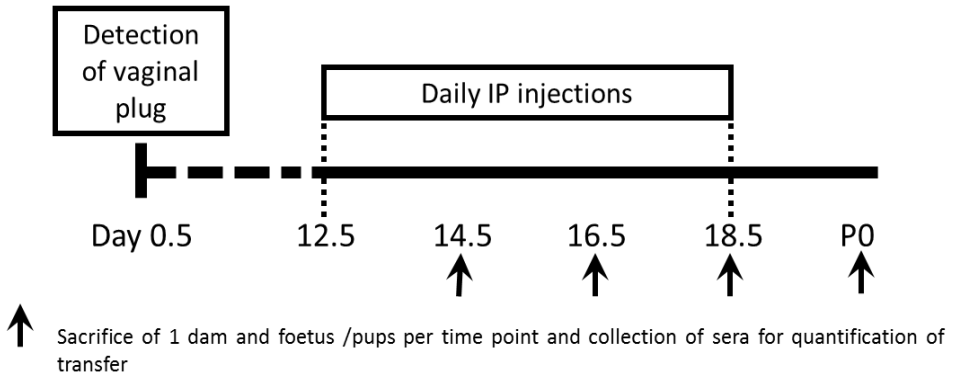
4.2 Mouse model experimental design

The animal model established consisted of 3 main experiments (see Figure 4-1). Experiment 1 quantified human IgG and specific antibody transfer throughout gestation (results in this chapter). Experiment 2 assessed the histological effects of human IgG transfer in the embryonic development (results in this chapter). Experiment 3 assessed the behavioural and histological effects of human IgG transfer post-delivery, during the neonatal and adult life of the offspring (results in Chapter 5 and 6). Experiments 2 and 3 were conducted blinded for the treatment groups. A full description of the methods is provided in Chapter 2.

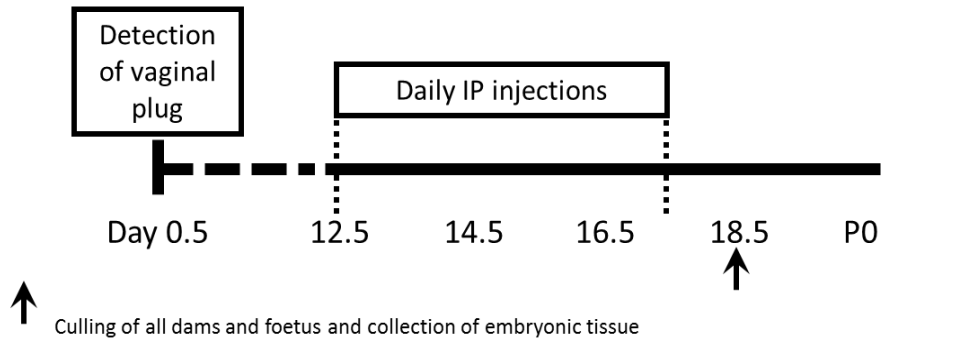
Experiment 1 – Detection of total human IgG and specific antibodies transfer throughout gestation

Dams were injected daily, intraperitoneally, with purified IgG from E12.5 until the time of death or until E18.5 (E0.5 defined as day of vaginal plug detection). In a first trial, a low dose of human IgG purified from the plasmas of one patient with NMDAR and one patient with LGI1 encephalitis were each given to four dams (2.5-3.5 mg/day; volume 1-2 mL/day). In a second trial, higher IgG concentrations purified from plasmas of patients with CASPR2, LGI1 and NMDAR encephalitis (one patient each) were injected (15-20

Experiment 1 - Human IgG and specific antibody transfer throughout gestation



Experiment 2 - Effect of human IgG transfer during embryonic development



Experiment 3 - Effect of human IgG in the offspring neonatal / adult period

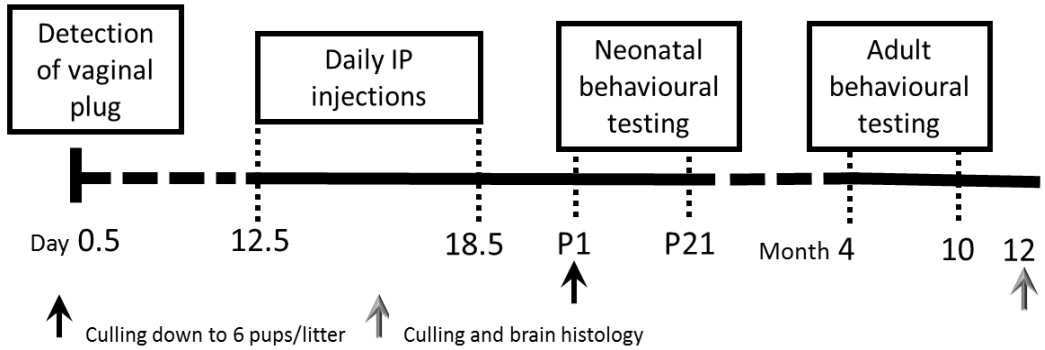


Figure 4-1: Animal model experimental design

Experiment 1: MF1 pregnant females were injected intraperitoneally (IP) with purified human IgG (CASPR2, NMDAR and LGI1 patients) daily from gestational day E12.5 until the day before sacrifice. One dam and offspring were sacrificed at each time point (E14.5, E16.5, E18.5 and P0) and sera collected for human IgG quantification and CBA testing. **Experiment 2** – MF1 pregnant females (n=3/treatment group) were injected IP with purified human IgG (CAPR2 and NMDAR patients and Healthy controls) or equal volume of saline from E12.5 until E17.5. All dams and offspring were sacrificed at E18.5 and embryonic tissue collected for analysis. **Experiment 3** - MF1 pregnant females (n=4/treatment group) were injected IP with 20mg of purified human IgG (CASPR2 patients and healthy controls) or equal volume of saline from E12.5 until E18.5. At postnatal day 1, litters were reduced to 6 pups. Neonatal behavioural assessment was conducted from P1 to P21 and adult behavioural assessment conducted from 4 to 10 months of age. At 12 months, animals were sacrificed and brains dissected for histological analysis.

mg/day; volume 1-2 mL/day) into four dams per treatment group. One dam per treatment group was sacrificed at E14.5, E16.5, E18.5 and P0 (defined as day of delivery) by CO2 anaesthesia, followed by cardiac puncture and dislocation of the neck. The dams were then dissected and the uteri exposed and removed. Foetuses were dissected out of their membranes, separated from the placenta and washed in saline. Foetuses/pups were sacrificed by decapitation and trunk blood collected at that time. Blood was pooled from the foetuses/pups of each litter. Serum was separated from the blood collected from the dam or the foetuses/pups and stored at -20ºC for subsequent quantification of human IgG and specific antibodies.

Experiment 2 – Assessment of the effects of human IgG transfer during embryonic development

Dams were injected daily from E12.5 to E17.5 with purified IgG from patients with NMDAR encephalitis and CASPR2 encephalitis, healthy controls and Hartmann's solution (3 dams per treatment group; IgG concentration: 20 mg/day; volume 1-2 mL/day). Plasma from 3 patients with NMDAR encephalitis, 1 patient with CASPR2 encephalitis and 3 healthy controls was used in this experiment. The dams were sacrificed at E18.5 as stated above. The number and appearance of the foetuses were recorded, as well as the collective weight of the litter. Dissected foetuses were decapitated or flash frozen in ice cold isopentane. Tissue was processed for histology and western blotting.

Experiment 3 – Assessment of the effects of human IgG transfer post-delivery, during the neonatal and adult life of the offspring

Dams were injected daily from E12.5 to E18.5 with purified IgG from two patients with CASPR2 encephalitis, two healthy controls and with Hartmann's solution (4 dams per treatment group; IgG concentration: 20 mg/day; volume 1-2 mL/day) and allowed to deliver. At P0 (usually the morning following delivery), the number of live and dead pups

was counted and the litter left undisturbed with the dam for 24h. At P1, 6 pups were randomly selected from each litter and individually tattooed for behavioural testing (described in chapter 5). During these procedures, the dam was left in the home cage which was placed in the testing room for habituation. The remaining pups from that litter were culled by decapitation and blood was pooled for antibody testing.

4.3 Purified IgG from human plasma

Plasma obtained from different patients undergoing plasma exchange during the course of their disease was used in this animal model. Human IgG from patients' plasma and healthy controls' sera was purified using the ammonium sulphate precipitation method, to avoid damage to the Fc region (which likely occurs after using the acidic elution buffers required for column purification), and concentrations measured using a commercially available Human IgG radial immunodiffusion kit (details in Chapter 2). For the purpose of this thesis, purified IgG from patients with NMDAR, LGI1, and CASPR2 encephalitis, and from healthy controls will be designated NMDAR IgG, LGI1 IgG, CASPR2 IgG and HC IgG, respectively. A summary description of each patient and plasma antibody titres are provided in Table 4-1. Number of injected dams and injected material description for each experiment are provided in Table 4-2. Plasma reactivity and antibody titres were assessed in patient and healthy control plasmas and IgG preparations. All patients showed reactivity to a single antigen, except for one patient who had reactivity to CASPR2 and LGI1 transfected cells (CASPR2 – patient 2). However, given the very low LGI1 and very high CASPR2 antibody titres detected, and the fact that LGI1 antibodies were shown not to transfer from dam to foetus (described below), this patient is referred to as a CASPR2 patient for the purpose of this thesis. Titres were overall lower in the plasmas of patients with NMDAR or LGI1 encephalitis available at the time. Healthy controls were negative for all antigenic targets.

Table 4-1: Patients' clinical information and plasma antibody titres

Antigenic target (Patient)	Clinical information	Plasma CBA results
LGI1 (1)	F.L. Female. Typical limbic encephalitis. Plasma exchanged in June 2007.	LGI1 CBA titre: 1:500
NMDAR (1)	R.G. Female. NMDAR encephalitis, non-paraneoplastic. First episode of encephalitis at the age of 16 (psychiatric manifestations, seizures, movement disorder and disturbance of consciousness), diagnosed as viral encephalitis (PCR negative). Relapse 3 years later with neuropsychiatric symptoms, during which was diagnosed with NMDAR encephalitis and plasma exchanged.	NMDAR CBA titre: 1:100
NMDAR (2)	J.B. Female. NMDAR encephalitis, non-paraneoplastic. At the age of 13, behavioural changes, hallucinations, aphasia and choreiform movement disorder. Positive response to plasma exchange but ongoing memory problems.	NMDAR CBA titre: 1:50
NMDAR (3)	S.C. Male Unknown information	NMDAR CBA titre: 1:100
CASPR2 (1)	C.B. Male. Morvan's syndrome secondary to high VGKC antibodies / CASPR2 antibodies. Paraneoplastic cause excluded. At the age of 70, progressive, disabling neuropathic pain in his feet and hands, memory complaints (short and long term memory, and particularly word-finding difficulties) and short episodes of lack of perception with "goosebumps" suggestive of temporal seizures. Partial response to plasma exchange and immunosuppressive therapy.	CASPR2 CBA titre: 1:4000
CASPR2 (2)	Male. Morvan's syndrome secondary to high VGKC antibodies / CASPR2 antibodies. Pulmonary adenocarcinoma. At the age of 76, symptoms suggestive of neuromyotonia, excessive sweating and salivation, small joint pain and weight loss. Progressive deterioration with sleep disturbance, confusion and hallucinations. Initial response to plasma exchange but posteriorly further deterioration and death. Full clinical report in Liguori, Vincent et al. 2001.	CASPR2 CBA titre: 1:8000 LGI1 CBA titre: 1:20

Table 4-2: Number of injected dams and injected material description for each experiment

	Patients (antigenic target and case number)						Controls			
	LGI1 (1)	NMDAR (1)	NMDAR (2)	NMDAR (3)	CASPR2 (1)	CASPR2 (2)	HC (1)	HC (2)	HC (3)	Saline
Experiment 1										
Injected dams	2/4*	5/4*			4					
IgG										
Conc. (mg/mL)	3.5/20	2.5/20			15					
CBA titre	1:1000	1:1000 -1:1500			1:6400					
Experiment 2										
Injected dams		1	1	1	3		1	1	1	3
IgG										
Conc. (mg/mL)		20	20	20	20		20	20	20	NA
CBA titre		1:500	1:500	1:500	1:12500		neg	neg	neg	NA
Experiment 3										
Injected dams					4	4	4	4		4
IgG										
Conc. (mg/mL)					20	20	20	20		NA
CBA titre					1:12500	1:27000	neg	neg		NA

* Two experiments conducted, one using low and the other using high IgG dosage. Two different IgG preparations from patients NMDAR (1) and CASPR2 (1) were used.

4.3.1 Detection of human IgG binding to mouse hippocampal cultures

Binding of the purified IgG to mouse hippocampal cultures was tested for each patient and healthy control (Figure 4-2). Purified IgG from CASPR2, NMDAR and LGI1 patients stained the neuronal surface of hippocampal neurons. None of the healthy controls used showed binding to live hippocampal neurons.

4.3.2 CASPR2 IgG binding to *Cntnap2* knockout neuronal tissue

To further establish that the purified IgG from our CASPR2 patients had no additional antibodies targeting proteins in the neuronal surface, binding to hippocampal cultures and whole-brain sections from a *Cntnap2* knockout mouse (a kind gift from Professor David Bennet and Dr John Dawes) was assessed. CASPR2 IgG from patient 1 showed no reactivity to other neuronal targets, since there was no binding to hippocampal neurons from the *Cntnap2* knockout mouse. CASPR2 IgG from patient 2 still showed mild reactivity against hippocampal cultures, which was likely due to the presence of the much weaker LGI1 antibodies. Binding to whole-brain sections was only assessed for patient 1. There was binding to wildtype mouse tissue but no binding to *Cntnap2* knockout mouse tissue (Figure 4-3).

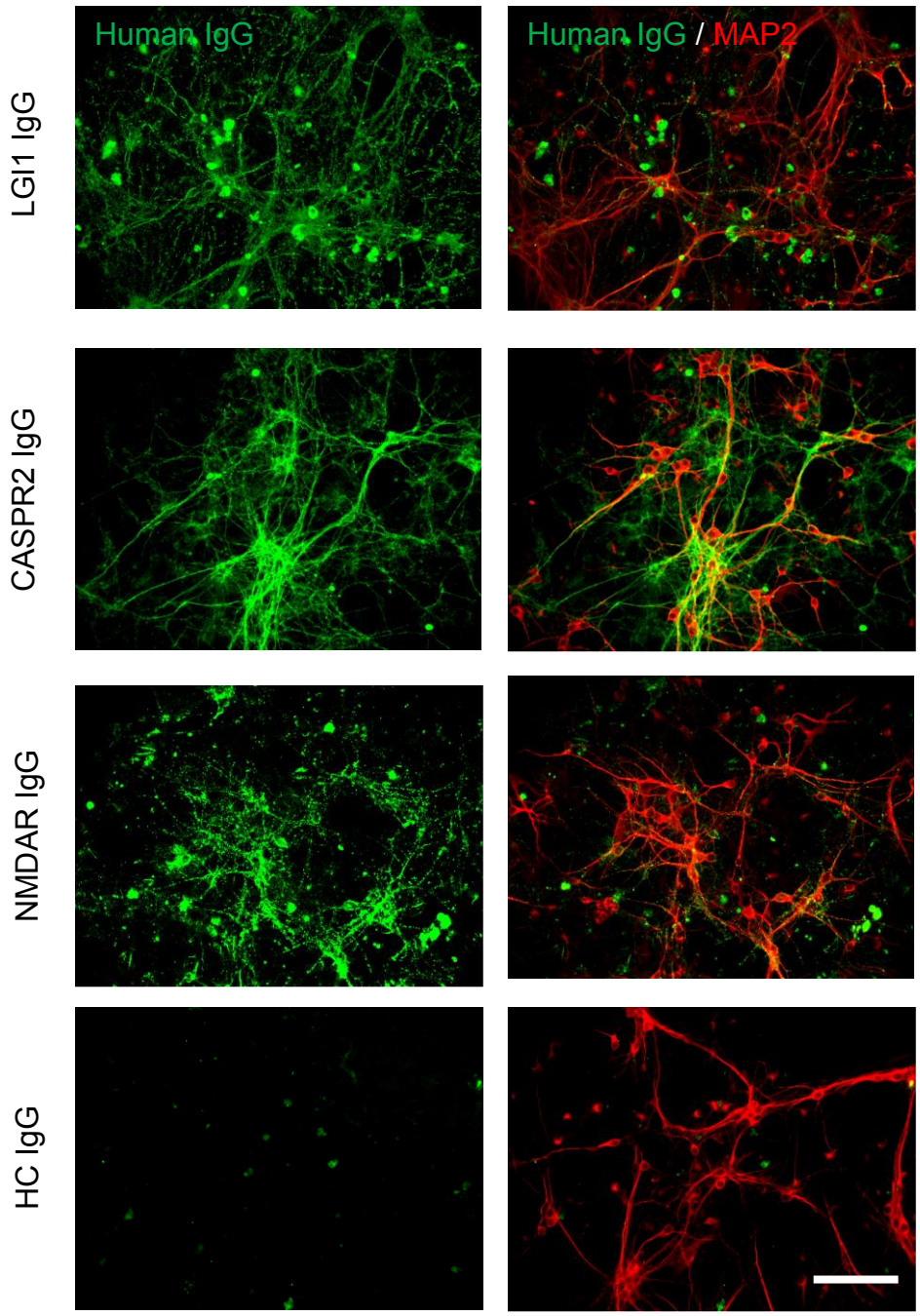


Figure 4-2: Representative photomicrographs of purified IgG binding to MF1 wild-type hippocampal neurons

Scale bar: 100 μm

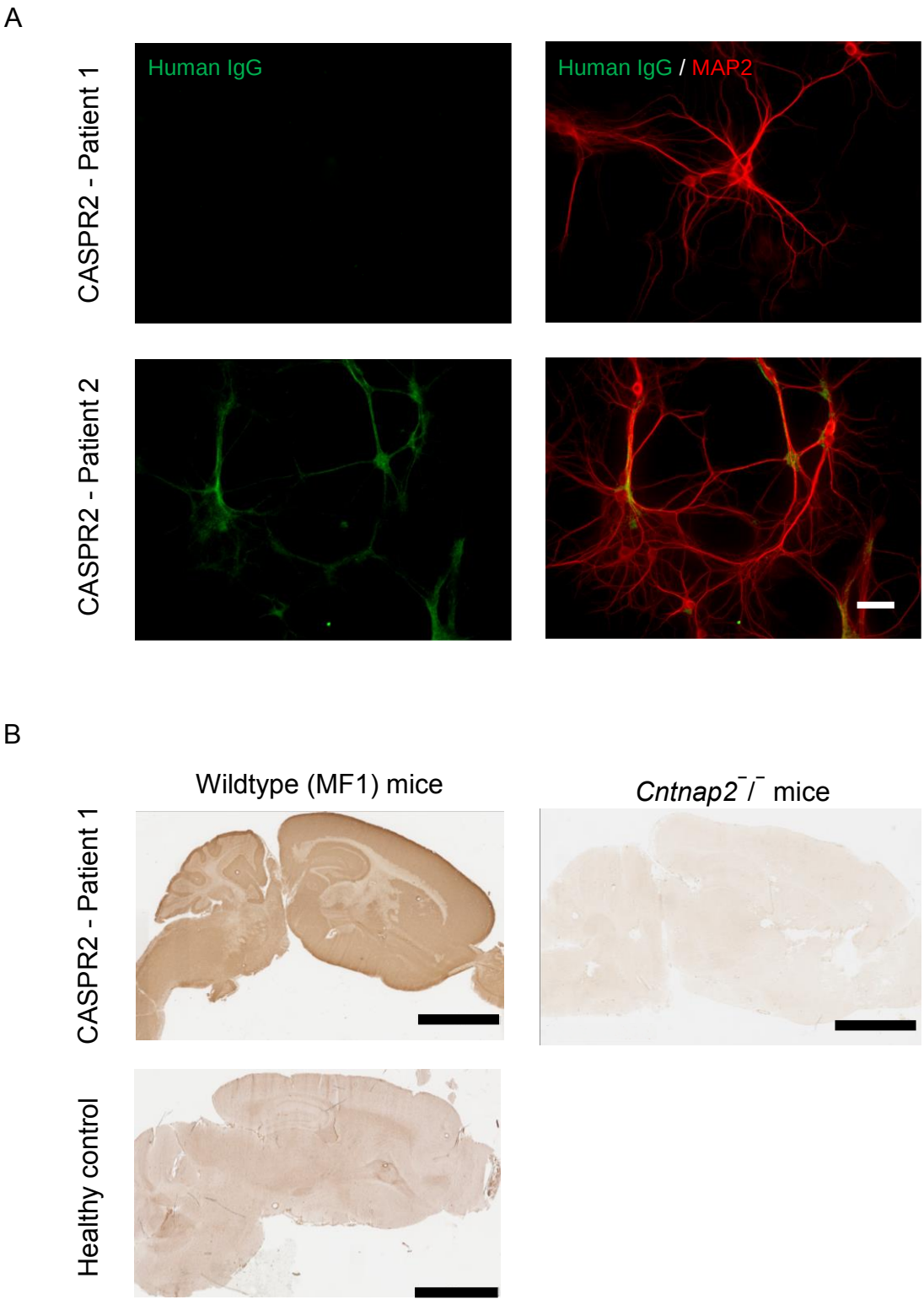


Figure 4-3: CASPR2 purified IgG binding to *Cntnap2* knockout mouse tissue.

(A) CASPR2 purified IgG binding to hippocampal neurons from *Cntnap2* knockout mouse. Scale bar: 50μm. (B) Comparison between CASPR2 and HC IgG staining of tissue sections from a wildtype MF1 mouse, and CASPR2 IgG staining in the *Cntnap2* knockout mouse. Scale bar: 3 mm.

4.4 Maternal-to-foetal transfer of total human IgG and specific antibodies throughout gestation

4.4.1 Assessment of transfer with administration of purified human IgG at a low concentration

Initial experiments were performed with a low dosage of purified human IgG, 2.5 mg/day (NMDAR CBA titre 1:1000), from one patient with NMDAR encephalitis. In this first experiment, total human IgG was quantified by western blotting in the dam and foetus / pups at E12.5 (only dam), E14.5, E16.5, E18.5 and P0. The level of human IgG rose steadily in the foetal circulation until birth (Figure 4-4A, left panel), with higher total human IgG concentrations detected in the embryos /pups than in the dam from E18.5 onwards. Measurement of NMDAR antibodies was done by CBA, from E14.5 to P0 in dams and embryos / pups. NMDAR antibodies were detected, using a serum dilution of 1:5, in the dams at P0 and in the pups, but not at any other embryonic stage (Figure 4-4A, right panel).

A smaller experiment was also conducted with purified IgG from a patient with LGI1 encephalitis, where a low dosage of 3.5 mg/day (LGI1 CBA titre 1:1000) was injected into 2 dams, daily, from E12.5 and quantified at E18.5 and P0. Total human IgG rose in the foetal and maternal circulation from E18.5 to P0 in a manner similar to the previous experiment with NMDAR IgG (Figure 4-4B, left panel), but LGI1 antibodies were only detected in the dam at P0 at a serum dilution of 1:5, and not in the foetuses/pups (Figure 4-4B, right panel). At this stage, the low dose of purified IgG used was assumed to be the cause of lack of transfer to the offspring.

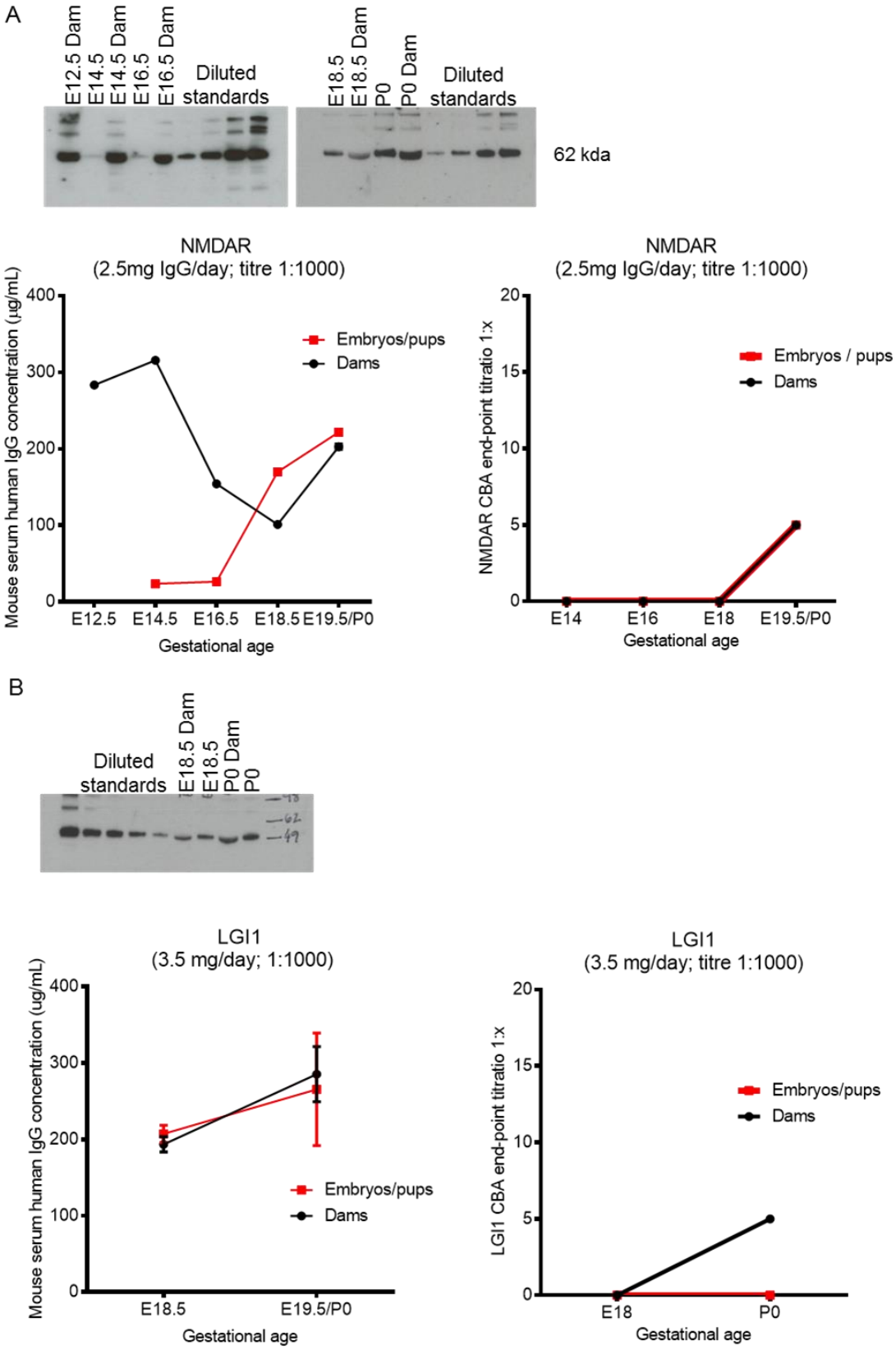


Figure 4-4: Total human IgG and specific antibodies determination in dams and embryos after exposure to low-dose purified human IgG from NMDAR and LGI1 patients.

Total human IgG results are given as mean ± SD from 3 western blot replicates.

4.4.2 Assessment of transfer with administration of purified human IgG at a high concentration

Aiming at achieving higher antibody titres in the foetal circulation, a higher dose of purified IgG from one patient with NMDAR (20 mg/day; CBA titre 1:1500), LGI1 (20mg/day; CBA titre 1:1000) and CASPR2 (15 mg/day; CBA titre 1:6400) encephalitis was injected in the dams. Quantification of total human IgG and CBA titres was determined at E14.5 (dam only), E16.5, E18.5 and P0. At this higher dosage, the levels detected in the foetal circulation were slightly higher, but relatively constant throughout gestation and did not reach the much higher concentrations detected in the dam (Figure 4-5), possibly representing saturation of the nFcR by the high dose of injected material. A comparison done across experiments at gestational day E18.5 (an end of gestation time point less variable than P0) shows that in the experiments using lower IgG concentration, the human IgG concentration detected in the foetuses was 6-7% of the concentration in the injected material, while in the dam it was 4-6%. Injecting higher dosages led to an increase in the dams (12-30% of injected material) but a relative decrease in the embryos (1-3% of the injected material) (Table 4-3). In fact, a small experiment where the same NMDAR IgG preparation was injected at different concentrations show this plateau effect in the antibody transfer (Figure 4-6). These results highlight the fact that in an ideal situation, a low concentration of purified IgG with a high titre of specific antibodies would be best.

CASPR2 CBA titres at the end of gestation reached 1:40 in the foetuses in experiment 1 (Figure 4-5), and 1:100-1:200 for experiments 2 and 3 (data not shown). Interestingly, LGI1 injected foetuses/pups had no detectable LGI1 antibodies in circulation (determined at E16 and P0 but not measured at E18 due to pregnancy loss) despite the fact that the injected material used had similar IgG concentration and antibody titre to the NMDAR IgG preparation, which was detected at 1:10 (Figure 4-5).

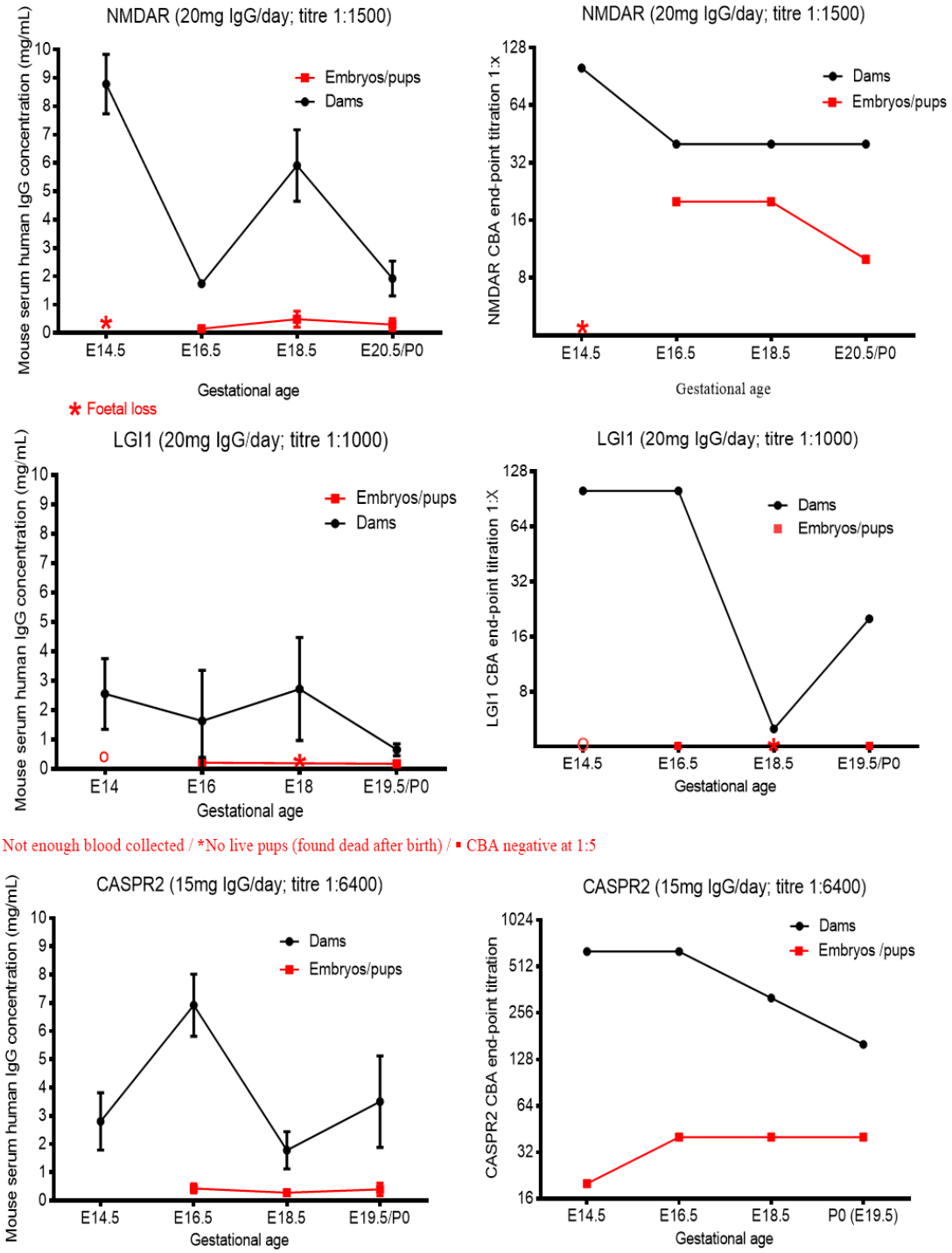


Figure 4-5: Total human IgG and specific antibody determination in dams and embryos after exposure to high-dose purified human IgG from NMDAR, LGI1 and CASPR2 patients.

Total human IgG results are given as mean ± SD from 3 western blot replicates.

Table 4-3: Comparison of antibody transfer across low and high dosage experiments

Percentage of initial total human IgG detected in the dams and embryos at E18.5			
	Injected material IgG dosage (mg/day)	IgG concentration (mg/mL) embryo (E18.5) serum (% original material)	IgG concentration (mg/mL) dam (E18.5) serum (% original material)
NMDAR	2.5mg/day	0.17 mg/mL (6.8%)	0.1mg/mL (4%)
LGI1	3.5 mg/day	0.21 mg/mL (5.9%)	0.19 mg/mL (5.5%)
NMDAR	20 mg/day	0.5 mg/mL (2.5%)	5.91 mg/mL (29.5%)
LGI1	20 mg/day	0.2 mg/mL (1%)	2.71 mg/mL (13.5%)
CASPR2	15 mg/day	0.3 mg/mL (2%)	1.8 mg/mL (12%)
Dilution of initial specific antibody titres detected in the dams and embryos at E18.5			
	Injected material CBA titre	CBA titre embryo (E18.5) serum (dilution factor from original)	CBA titre dam (E18.5) serum (dilution factor from original)
NMDAR	1:1000	Neg (at 1:5)	Neg (at 1:5)
LGI1	1:1000	Neg (at 1:5)	Neg (at 1:5)
NMDAR	1:1500	1:20 (75X)	1:40 (37.5X)
LGI1	1:1000	Neg (at 1:5)	1:5 (200X)
CASPR2	1:6400	1:40 (160X)	1:320 (20X)

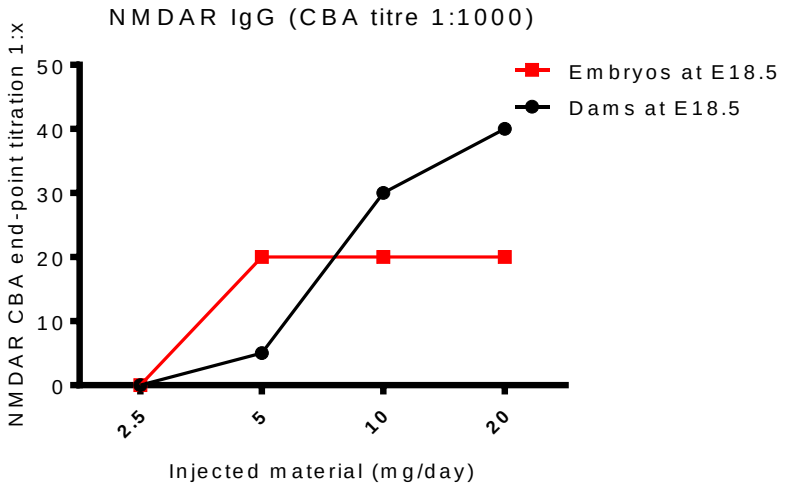


Figure 4-6: NMDAR antibody titres in dams and embryos at E18.5 after exposure to different concentrations of the same IgG preparation.

4.4.3 Determination of purified IgG subclass composition

A possible explanation for the absence of LGI1 antibodies in foetal circulation, is a different affinity of the mouse FC neonatal receptor for different human IgG subclasses, since NMDAR antibodies were previously reported to be mainly of IgG1 subclass and LGI1 antibodies to be mainly IgG4 subclass.

To assess whether human IgG transfer in the mouse is selective to IgG subclass, the IgG subclass composition of the plasmas used in these experiments was determined (Figure 4-7). NMDAR IgG was mainly of IgG1 and IgG2 composition, while LGI1 IgG was almost entirely of IgG4 subclass. CASPR2 IgG was equally IgG1 and IgG4, and IgG2 to a lesser extent. This subclass distribution is in accordance with the possibility that different human IgG subclasses are transferred differently by the mouse nFcR.

4.4.4 IgG1 and IgG4 subclasses in CASPR2 exposed dams and embryos

The IgG subclass of the CASPR2 antibodies detected in the dam and embryos' sera at E18.5 was determined by CBA using 1:20 and 1:100 dilutions. The dam serum was still highly positive for both IgG1 and IgG4 at 1:100. In the embryos' pooled sera, IgG1 was positive at 1:20 but not IgG4 (Figure 4-8).

4.4.5 Assessment of AChR and MuSK antibodies transfer

To extend this observation, an experiment was conducted with purified IgG from patients with AChR and MuSK myasthenia gravis. In these disorders, it is known that AChR antibodies are mainly IgG1 subclass and MuSK antibodies are mainly IgG4 subclass. This type of distribution was also observed in our purified IgG preparations (Figure 4-9A). An approximate concentration of 12.5mg/day (CBA titre - AChR 1:1000 / MuSK 1:800; RIA – AChR 16085 / MuSK 14353 cpm) was injected into two dams per treatment group at E12.5 (Figure 4-9B). In each treatment group, one dam was culled at E16.5 and one

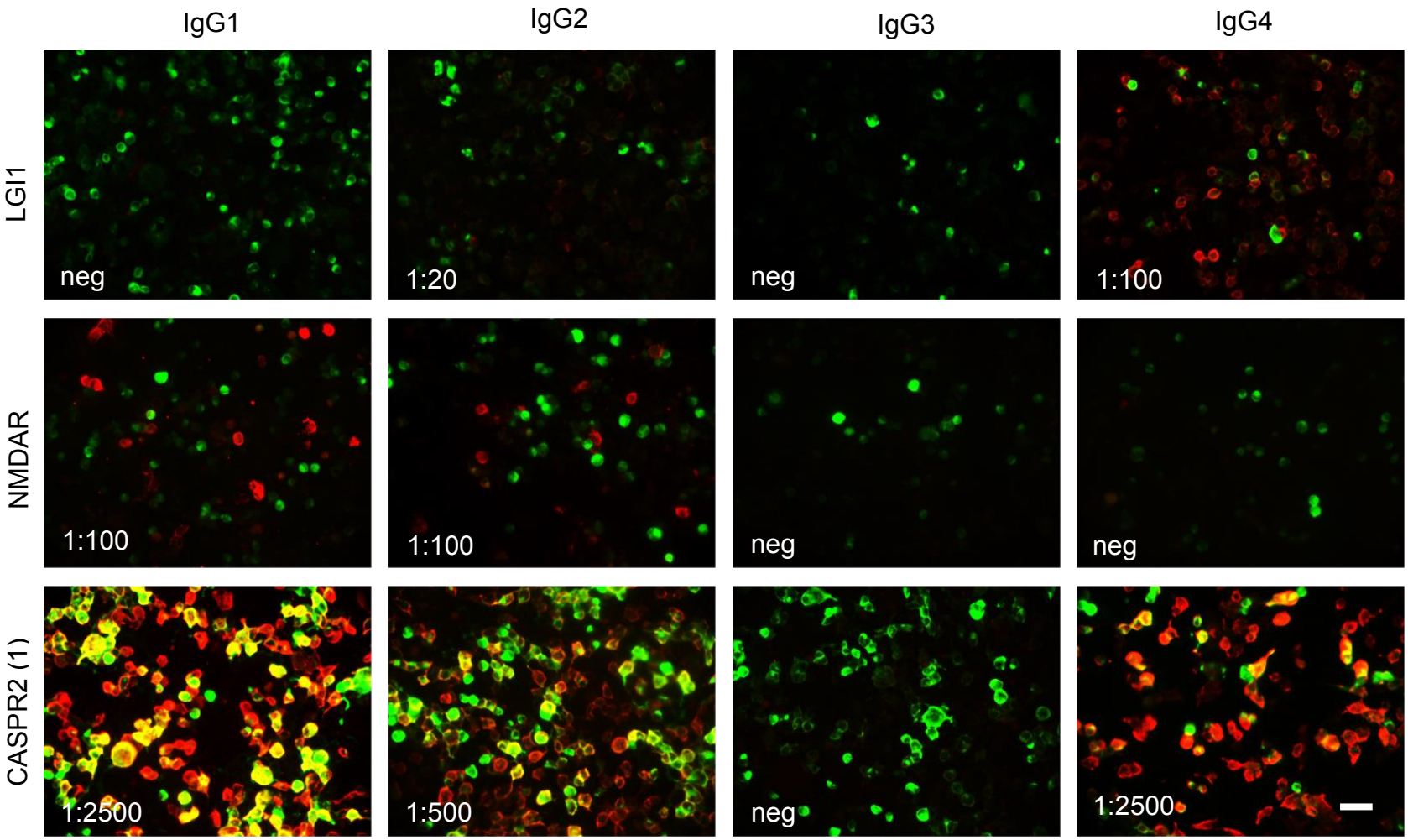


Figure 4-7: Specific IgG subclasses antibodies in the LGI1, NMDAR and CASPR2 plasmas used in experiment 1.

End-point CBA titrations are given for each subclass in the left lower corner of the photomicrographs. Scale bar: 100 μm.

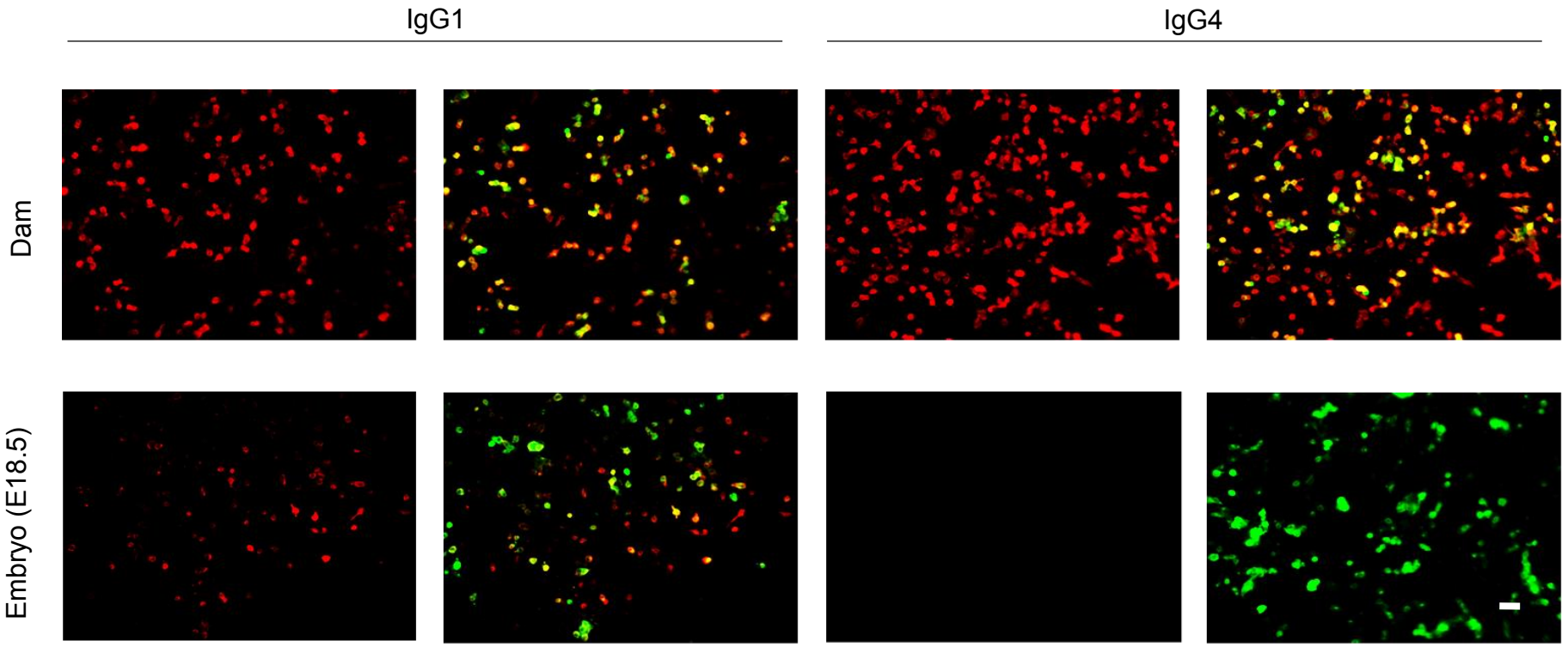
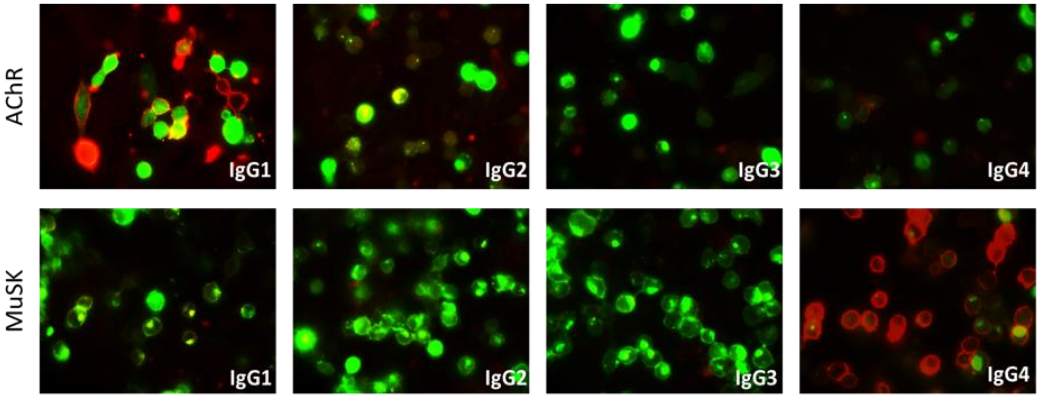


Figure 4-8: IgG1 and IgG4 CASPR2 antibodies in dam and embryos sera at E18.5

Scale bar: 50 μm

A



B

	CBA titre	IgG concentration (mg/mL)	RIA (cpm; nmol/L)
AChR purified IgG	1 in 1000	23.6 (injected 0.5mL/day)	16085; 10.7
MuSK purified IgG	1 in 800	26.1 (injected 0.5mL/day)	14353; 0.78

C

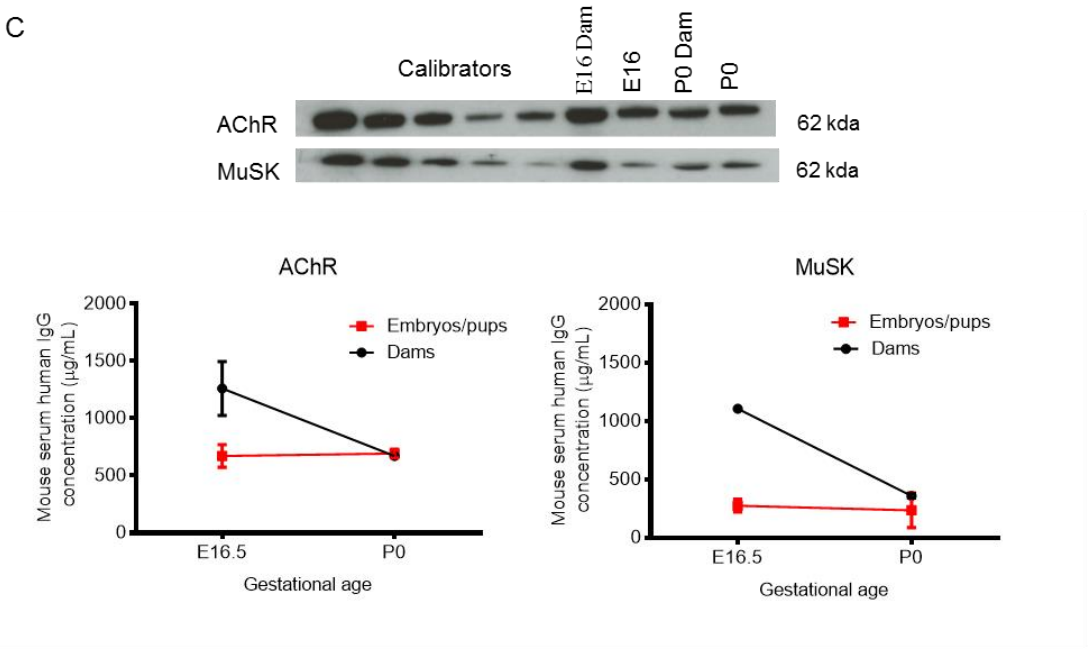
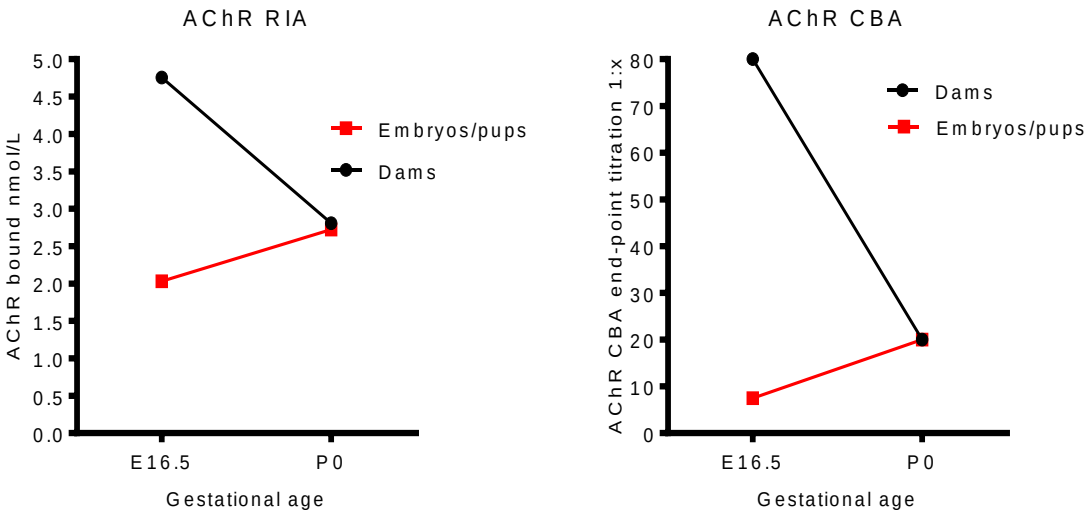


Figure 4-9: Transfer of AChR and MuSK antibodies from dam to embryos/pups.

(A) IgG subclasses of the injected material. (B) Characterization of the total IgG and specific antibodies in the human IgG preparations. (C) Total human IgG quantification in dams and embryos/pups.

D



E

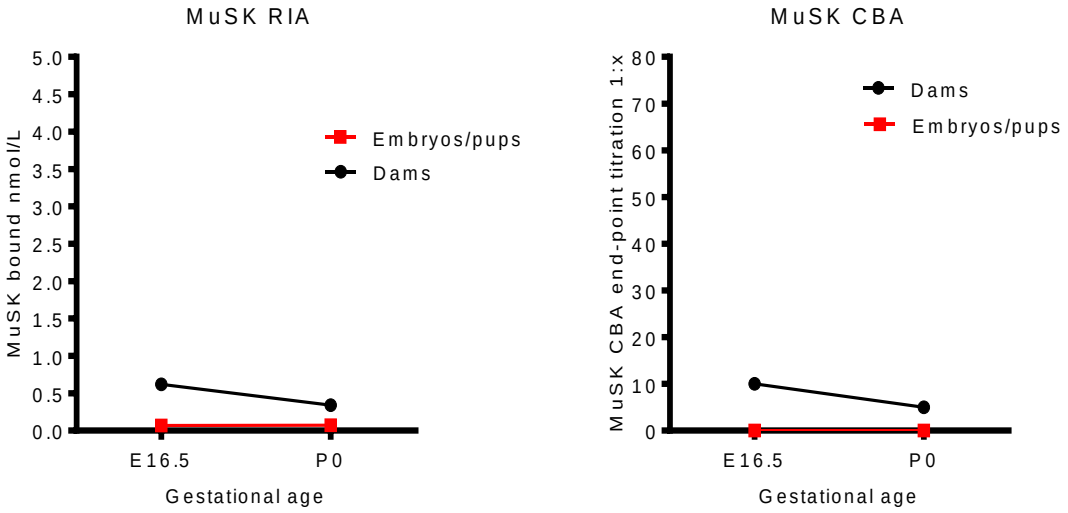


Figure 4-9 (cont): Transfer of AChR and MuSK antibodies from dam to embryos/pups.

dam at P0. Total human IgG western blot quantification revealed a similar pattern of transfer, with IgG concentration approximately 6 and 2% of the injected material in the AChR and MuSK-exposed pups, respectively (Figure 4-9C). Two different methods were used to determine AChR and MuSK antibodies in the foetal circulation, CBA and RIA. In both methods, AChR antibodies were detected at E16 and at P0, but MuSK antibodies were absent at these gestational ages (Figure 4-9 D and E), thus confirming that human IgG4 subclass, present in a preparation of total human IgG, was not transferred in the mouse.

4.5 Human IgG deposition in the foetal brain

To assess human IgG deposition in the foetal brain, pregnant dams (4/treatment group) were injected with NMDAR (20mg/day; CBA titre 1:1500), CASPR2 (20mg/day; CBA titre 1:6400), HC IgG (20mg/day) and Saline from E12.5 to E17.5. The foetuses were harvested and decapitated at E18.5. Snap-frozen, cryostat-cut 12μm sections of the head of one foetus per litter were tested with anti-human IgG. In the first instance, the sections were PFA-fixed, in order to preserve any unbound IgG. This staining revealed intense deposition of human IgG in the foetal vessels, choroid plexus and the periventricular zones in all experimental groups except saline (Figure 4-10). A detailed observation of the peri-vascular area revealed deposition of human IgG beyond the vessels much more prominently in the NMDAR and CASPR2 injected embryos (Figure 4-11).

A second staining was then performed on adjacent sections which were gently rinsed before fixation, in order to wash away any unbound human IgG and to preserve human IgG bound to the tissue. Qualitatively, the bound human IgG was higher in the NMDAR and CASPR2 IgG-exposed embryos, compared to HC IgG or saline-exposed embryos (Figure 4-12). A quantification of the mean fluorescence intensity was performed in the

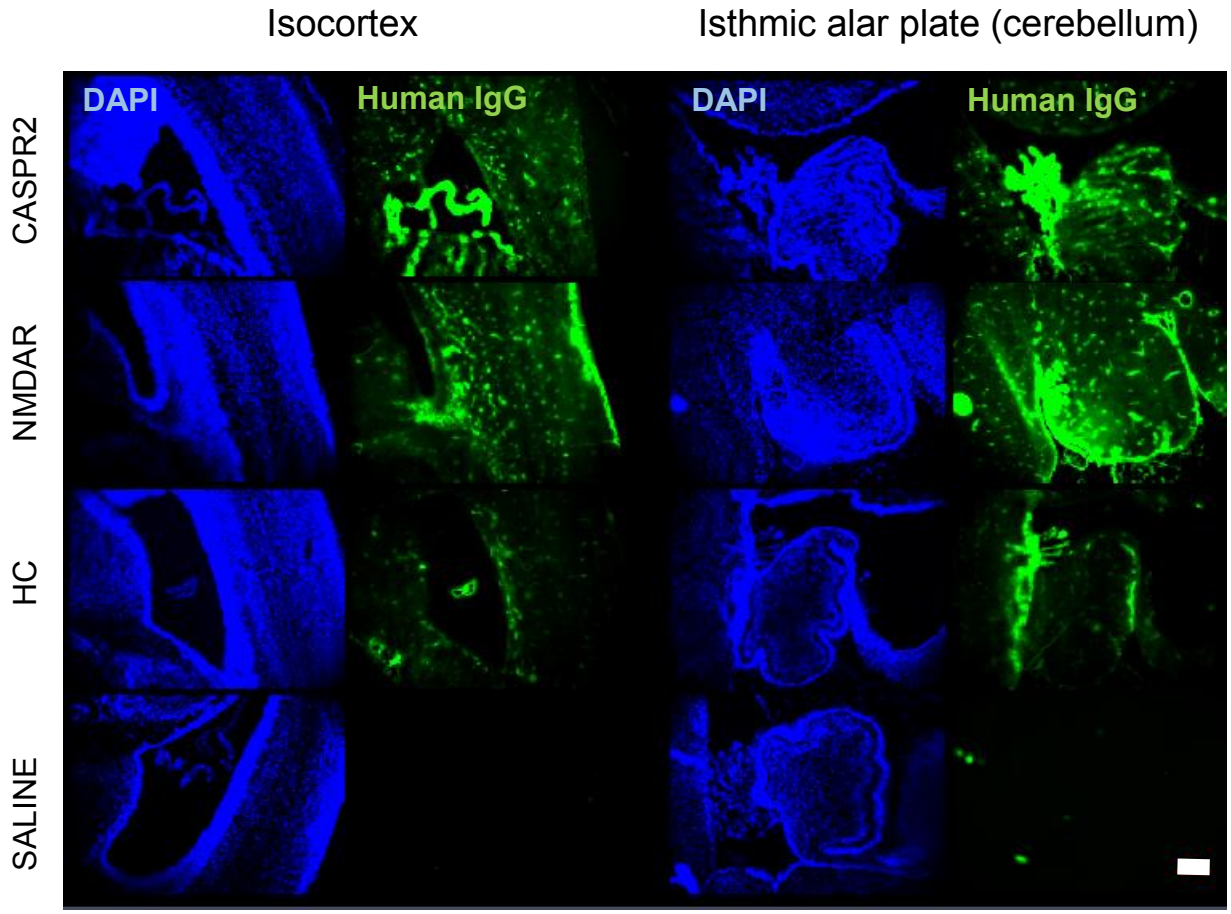


Figure 4-10: Human IgG deposition in the foetal isocortex and isthmic allar plate (cerebellum)

Representative photomicrographs of the isocortex and cerebellum showing deposition of human IgG (green) in the foetal vessels, choroid plexus and the periventricular zones in the CASPR2, NMDAR and HC IgG but not in saline-exposed foetus. Scale bar: 200μm

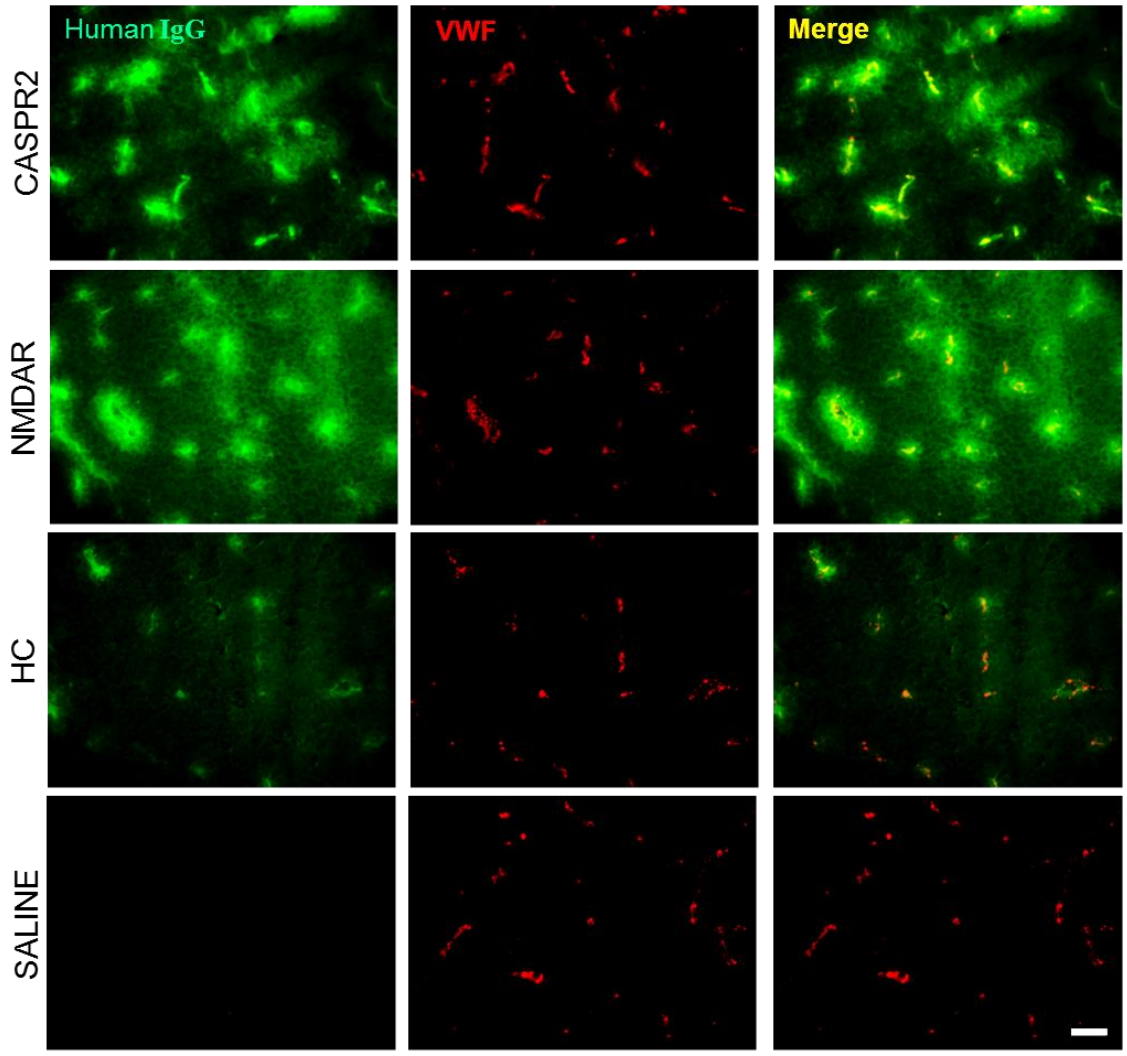


Figure 4-11: Total human IgG deposition in the foetal vessels and brain parenchyma at E18.5 in fixed sections of the isocortex

Total human IgG, in fixed sections of embryonic brain (isocortex), show co-localization with a vessel marker, Von Willebrand factor (VWF), in NMDAR, CASPR2 and HC IgG but not in saline injected embryos. Total human IgG is also visible beyond the vasculature in the parenchyma. Scale bar: 50 μm.

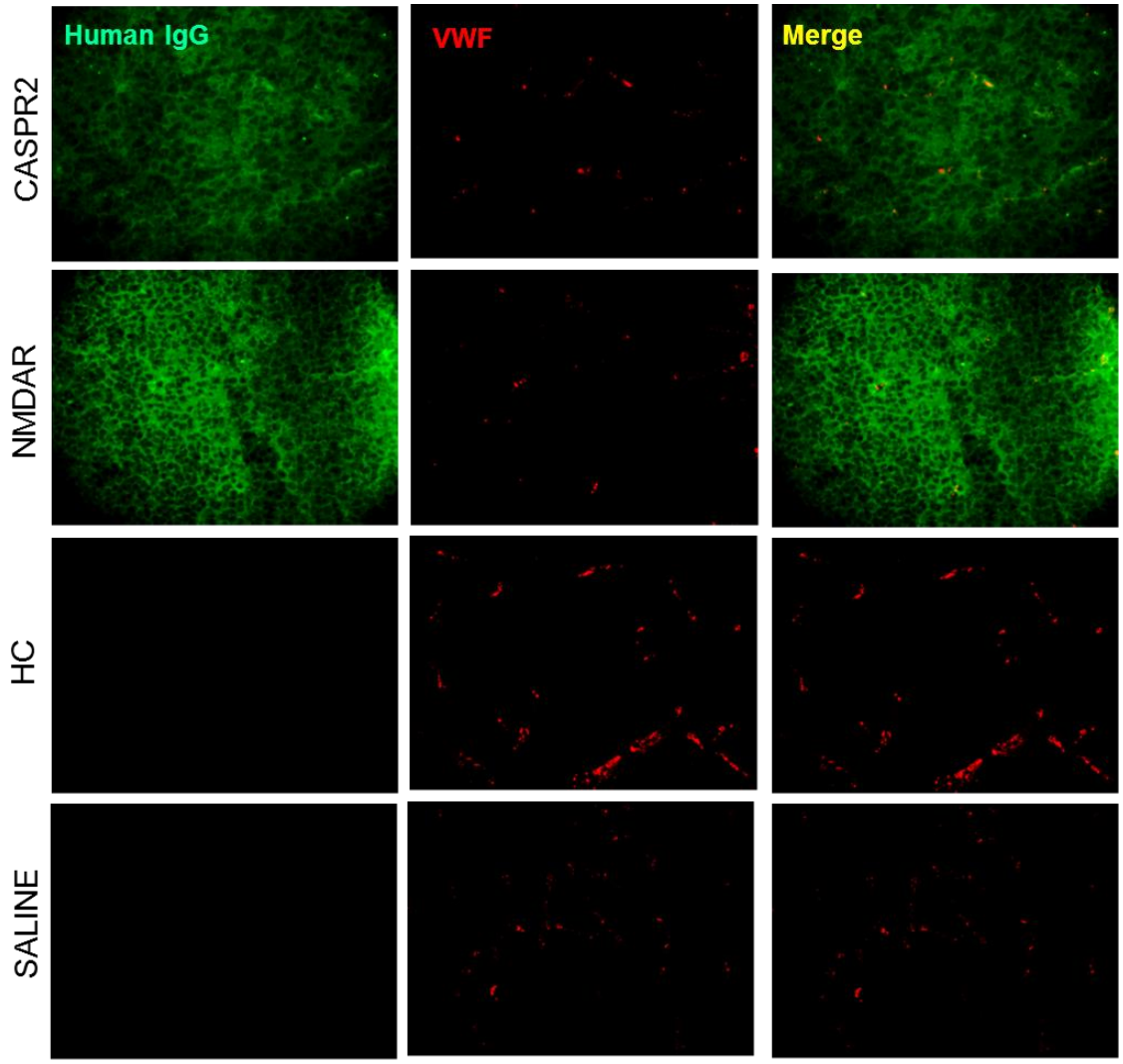


Figure 4-12: Total human IgG deposition in the foetal vessels and brain parenchyma at E18.5 in gently washed sections of the isocortex.

After gentle wash of unbound IgG, total human IgG is still visualized in the embryonic parenchyma of NMDAR and CASPR2 IgG-exposed embryos, but not in the HC IgG-exposed embryos. Scale bar: 50 μm.

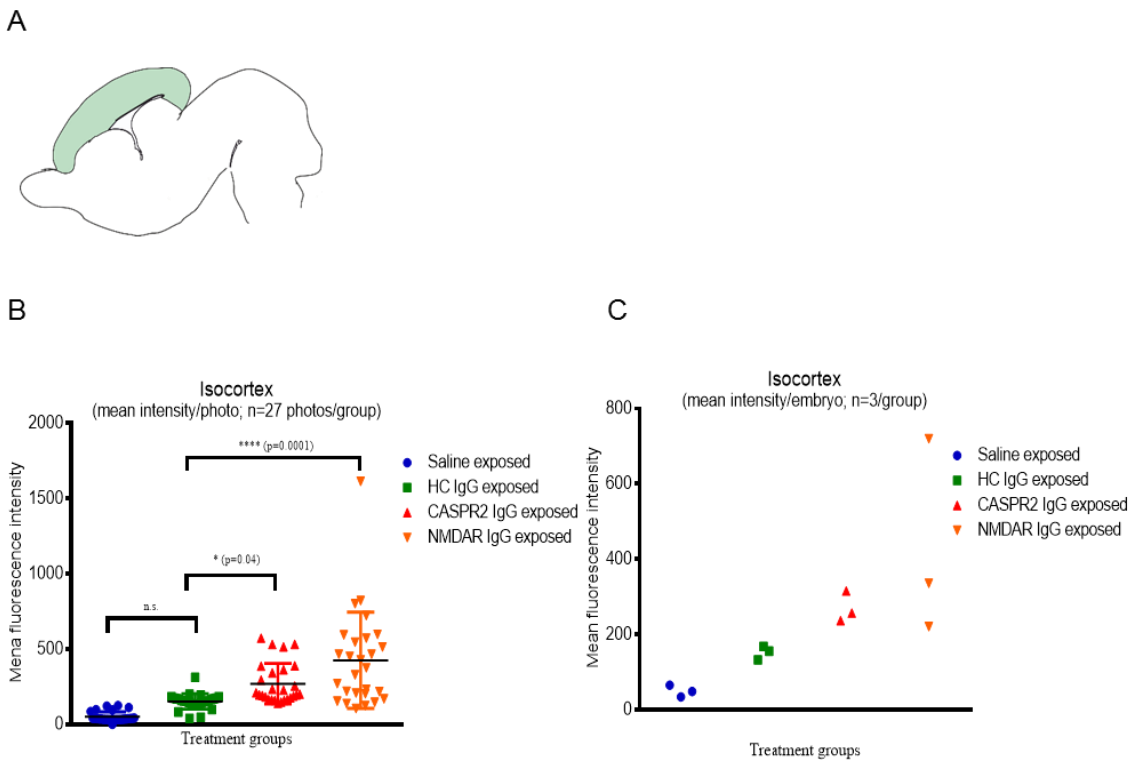


Figure 4-13: Quantification of bound human IgG in the embryonic isocortex by mean fluorescence intensity determination.

(A) Adapted Allen developing mouse brain atlas diagram representative of the photographed area (in green). (B) Mean fluorescent intensity measurements of all photos (n=27 photos/treatment group); (C) Average of mean fluorescent intensity measurements per embryo (n=3 embryos/treatment group).

isocortex (Figure 4-13A). Measurements were made in 3 photomicrographs per section in 3 sections per embryo, 3 embryos per treatment group, each embryo from a different litter (Figure 4-13B). There was higher fluorescence intensity in the NMDAR IgG-exposed embryos than in the HC IgG–exposed embryos ($p=0.0001$, ANOVA with Dunnet post-hoc correction) and a similar, although not so pronounced, increase in the CASPR2 IgG-exposed versus HC IgG-exposed embryos ($p=0.04$, ANOVA with Dunnet post-hoc correction), thus suggesting that the IgG bound in the tissue is higher in those embryos exposed to antibodies targeting neuronal surface proteins. The average of mean fluorescent intensity per embryo (Figure 4-13C) illustrates this difference, although with the small numbers it does not reach statistical significance. It is possible that differences between CASPR2 and NMDAR IgG-exposed embryos reflect different levels of protein expression at this stage or the embryo's variability in the transfer of IgG or passage of antibodies through the embryonic blood brain barrier.

4.6 Embryonic gross morphology

A visual inspection of all the embryos was done prior to culling. There were no gross malformations in the litters of any treatment group (Figure 4-14A).

A Nissl staining was conducted in 1 embryo per litter from experiment 2 (4 embryos per treatment group) in order to assess gross morphological abnormalities. Staining was done on every tenth slide in order to provide a full representation of the brain. No gross morphological differences were found across treatment groups in the embryonic (E18.5) brain of the offspring (Figure 4-14B).

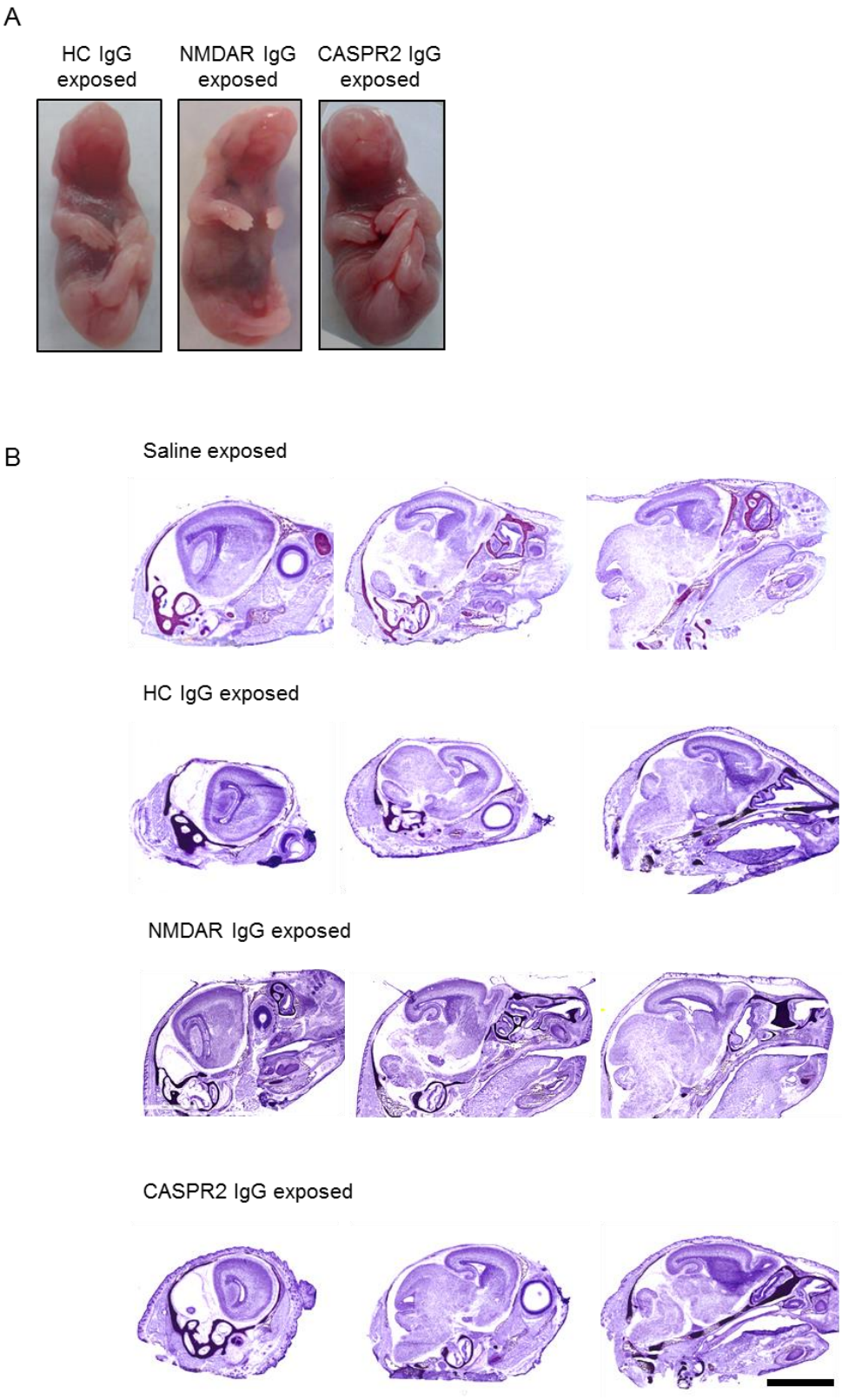


Figure 4-14: Embryonic offspring (E18.5) global and brain morphology

(A) Representative photographs from HC, NMDAR and CASPR2 IgG-exposed E18.5 embryos (B) Representative images of the Nissl staining performed in every 10th sagital section for each animal (n=4/treatment group). Scale bar: 3mm.

4.7 Discussion

A mouse model of maternal to foetal transfer of CNS-targeting antibodies was established by injecting dams with purified IgG from patients with antibody-mediated CNS diseases and healthy controls, throughout gestation, following the protocol previously established for AChR antibodies transfer in our laboratory (Jacobson, Polizzi et al. 1998, Jacobson, Polizzi et al. 1999).

Following the results obtained from the Danish cohorts screening, transfer of CASPR2 and NMDAR antibodies was attempted. Plasmas from two patients with CASPR2 encephalitis were available in sufficient amounts to establish this passive transfer animal model. Available plasmas from NMDAR encephalitis patients had low antibody titres and thus further experiments concentrated on the effects of CASPR2. Ideally, the samples from CASPR2 positive mothers of children with MR/DPD and antibody negative-mothers of children with typical neurodevelopment from the Danish cohorts should have been used as injected material. This was not possible due to the limited amounts of serum available and the low antibody titres in these samples. Also, one must note that using human IgG is associated with specific limitations that are common to any passive transfer model. IgG was precipitated, dialysed and filter-sterilized from the patients' plasma but it is possible, nevertheless, that other proteins with potential pathogenic effects had remained in the preparation. This is even more problematic given the need to use the ammonium sulphate method (due to the risk of damage to the Fc region by the acidic elution buffers required for column purification) to precipitate the human IgG, given that this method only yields an approximately 50% pure preparation (M. Page 1996). An alternative could have been the use of a purified human monoclonal recombinant antibody. However, the generation of these antibodies is in itself a laborious and expensive procedure, and would defeat the conceptual purpose of assessing the results of exposure to antibodies that are found in patients.

The assessment of maternal to foetal transfer allowed us to conclude that only a small percentage of human IgG is transferred to the mouse embryo at the end of gestation. This is likely due to the competitive effect between human and mouse IgG for the nFcR binding, and due to the low gestational transfer that occurs in mice (contrary to the much more efficient transfer via lactation). Previous animal models in the autism field (Singer, Morris et al. 2009, Braunschweig, Golub et al. 2012, Kadam, French et al. 2013) have used much lower concentrations of purified human IgG, sometimes as low as 1.5μg/g weight (approximately 90μg/day), which likely results in a very low transfer of any specific antibody to the mouse embryo. In this model, a much higher dosage was used from the start, but even 2.5-3.5 mg of purified IgG per day was not sufficient to attain significant levels of specific antibodies in the embryonic circulation. However, a higher dosage did not lead to higher transfer of total human IgG, which could be explained by saturation of the nFcR in the yolk sac endoderm. In fact, Rogers Brambell, the pioneer in the field of maternal transmission of immunity, had already postulated a mechanism of receptor saturation and non-bound IgG degradation in situations of hypergammaglobulinemia (Brambell 1966). Interestingly, this phenomenon was also documented in humans. In a study comparing total IgG and specific anti-measles antibodies transfer in German and Nigerian populations (Hartter, Oyedele et al. 2000), higher total maternal human IgG levels were associated with lower proportion of transfer of total IgG and specific antibodies to the newborn. A way to circumvent this limitation would be the use of purified IgG preparations with high titres of specific antibodies. As previously mentioned, at the time of this study, only plasmas from CASPR2 patients with appropriately high antibody titres were available. With these materials, antibody titres of 1:100-1:200 were achieved in the offspring. Importantly, these titres are similar to the antibody titres found in the CASPR2-positive maternal samples in the Danish cohorts.

The mouse gestational transfer was found to be selective for human IgG subclass and specific antibodies of the IgG4 subclass, such as LGI1, MuSK and CASPR2 IgG4

antibodies, were not found in the embryo circulation. This is a novel finding as there are no *in vivo* studies assessing the transfer of specific human subclasses in the mouse and there are no maternal to foetal transfer mouse models of an IgG4-mediated disease. However, one *in vitro* study (Neuber, Frese et al. 2014) showed that human IgG4 binds with high affinity to the mouse nFcR at pH 6 and with low affinity at pH 7.2, thus making it plausible that it is transposed by the mouse nFcR. The reasons for this apparent contradiction are not clear. IgG4 represents only 5% of the total IgG pool and is in direct competition with more abundant IgG subclasses for nFcR binding. It is possible that the amount transferred is too negligible to be detected by the methods employed. The use of exclusively IgG4 subclass preparations would allow full understanding of this finding, but this was not pursued further.

Finally, and most importantly, human IgG was detected in the embryonic brain vasculature and brain parenchyma, confirming that the mouse developing brain is partially permeable to IgG. Furthermore, the presence of bound human IgG in the brain parenchyma was higher in the embryos exposed to CNS-targeting antibodies than those exposed to IgG preparations from healthy controls (confirmed not to bind to the neuronal surface *in vitro*). This finding suggests that those antibodies might be binding the target proteins in the tissue, although only co-localization of the human IgG with a commercial antibody to the targeted protein, adsorption studies or use of knockout animals, would allow a definite conclusion. Unfortunately, NMDAR and CASPR2 commercial antibodies tested did not produce a reliable staining in the embryonic tissue, it was impractical to adsorb the CASPR2 IgG (given the high titres of the preparation) and it was not possible to do further experiments using *Cntnap2* knockout dams.

In conclusion, the experimental work described in this chapter demonstrated that human IgG and specifically CASPR2 antibodies are transferred from the dam to the foetal circulation. It further showed that CASPR2 IgG is detected in the foetal brain parenchyma

suggesting that specific CASPR2 antibodies are bound to the protein target in the foetal brain.

Having established an efficient transfer of human CASPR2 antibodies from the dam to the mouse embryo, next experiments aimed at assessing the consequences of this exposure in the offspring.

Chapter 5: Effects of antibody transfer in the neonatal and adult offspring

5.1 Overview of the experimental design and behavioural testing

Behavioural testing was performed in the offspring of dams injected with purified IgG from two patients with CASPR2 encephalitis, purified IgG from two healthy controls and saline from E12.5 to E17.5 (4 dams per treatment group; 20mg/day; Volume 1-2mL). During the neonatal period (from P1 to P21), a comprehensive evaluation of neonatal developmental milestones was done, using the modified battery of Fox. The battery includes readouts of physical development, neurological reflexes and tests of neuromotor coordination and was performed in all pups (24 per treatment group). A maternal retrieval test and a recording of neonatal ultrasonic vocalizations was also performed. During the adult period (from 4 to 10 months of age) further behavioural testing was performed, usually in half of the cohort (12 animals per treatment group, unless otherwise stated) assessing several domains: locomotor activity, anxiety, neuromotor coordination, working memory and perseverance, social interaction, species typical behaviours and olfaction (Figure 5-1). Detailed description is provided in chapter 2. The same number of adult males and females was used in each experiment, unless otherwise stated, but given that there were no statistically significant gender-interactions (data not shown), results are presented for the entire group.

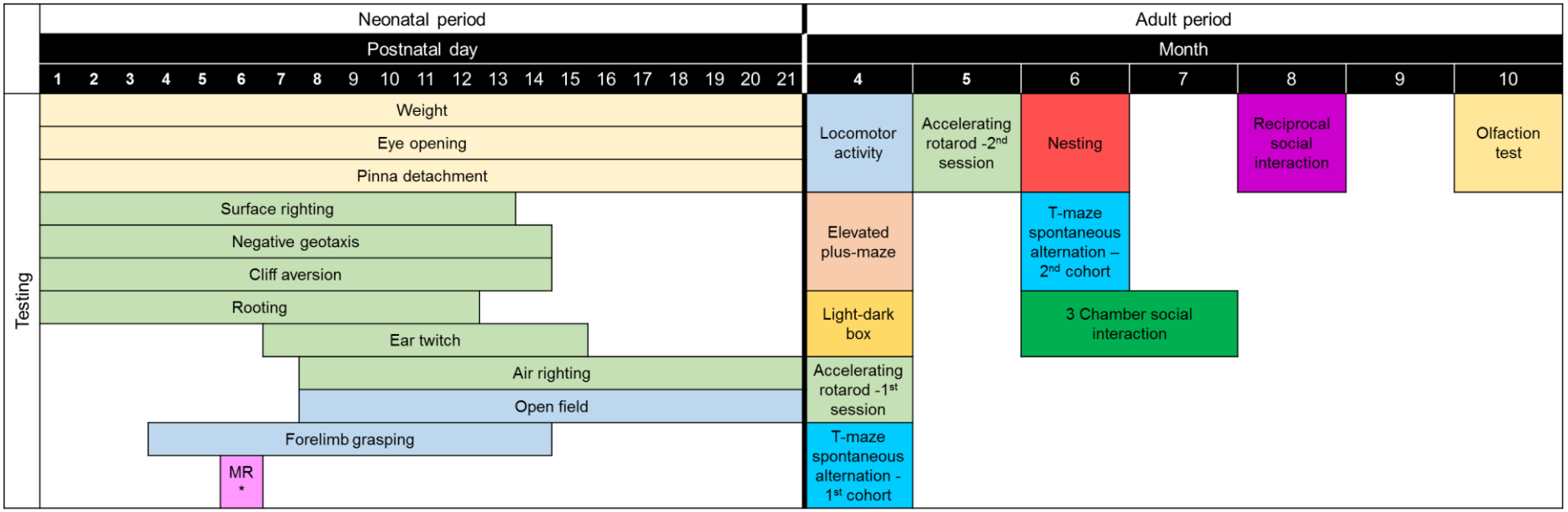


Figure 5-1: Schedule of behavioural assessment

Neonatal period: Neonatal neurodevelopmental assessment was done in all pups (n=24/treatment group). Testing days are indicated by the shaded areas and testing continued daily until the pup reached criterion in 2 consecutive days. The maternal retrieval test (Abbreviation: MR) was performed once on post-natal day 6. **Adult period:** Animal age in months at time of testing as indicated for each individual test. The majority of tests was performed in half of the cohort (12 animals/treatment group), with the exception of the locomotor activity and the T-maze spontaneous alternation test that was done in all animals (1 HC exposed mouse deceased due to non-experimental reasons), and the reciprocal social interaction test that was done in all pairs that met the weight-matching criteria.

5.2 Experimental controls: saline versus HC IgG

Two experimental control groups were kept in this animal model: a HC IgG-exposed group and a saline-exposed group. However, the true experimental control is the HC IgG-exposed group. This takes in account any confounding effect resulting from mouse exposure to human IgG and is the control group used in the majority of animal models published in the field of neuroimmunology (for instance, (Jacobson, Polizzi et al. 1998, Braunschweig, Golub et al. 2012, Planaguma, Leypoldt et al. 2015, Wright, Hashemi et al. 2015)). The information on the saline group was kept in the graphs presented in this chapter, but our statistics were only conducted comparing the CASPR2 IgG-exposed group with the HC IgG-exposed group. However, a full statistical comparison of HC IgG and Saline-exposed animals, for the main results, is provided in Table 5-1. There were no significant differences, except for day of eye opening and the percentage of time spent interacting with the mouse in the 3 chamber social interaction test. An effect of weight is possibly the reason for the latter difference (discussed below).

5.3 Dams weight during gestation and general effects on the dam

Dams weights per number of pups in the litter increased over time, as expected ($F(6, 54)=440.55$; $p<0.0005$), but there was no effect of treatment by time ($F(12,54)=0.39$; $p=0.96$) (Figure 5-2). No abnormal behaviour of the dams was detected after injection (observation of spontaneous activity before and after injection for a period of 15 minutes).

5.4 Offspring survival and gender distribution

Number of live pups per litter varied between 6 and 15. There were no statistical significant differences in the offspring survival between CASPR2 and HC IgG-exposed animals ($U=6$; $Z=-0.59$; $p=0.686$). However, the number of live pups was assessed at P0

Table 5-1: Results comparison between HC IgG-exposed and Saline-exposed controls

	HC IgG-exposed Mean (±SEM)	Saline-exposed Mean (±SEM)	p-value
GENERAL			
Dams gestational weight / number of pups (end of gestation weight)	5.16 (±0.47)	5.38 (±0.53)	n.s.
Nr pups per litter	11.5 (±2.55)	11.5 (±1.26)	n.s.
NEONATAL PERIOD			
Pups weight (end of neonatal period)	15.98 (±0.05)	16.5 (±0.68)	n.s.
Day of eye opening	13.5 (±0.04)	13.13 (±0.08)	**p=0.004**
Ear detachment	3.13 (±0.16)	3.04 (±0.04)	n.s.
Surface righting	9.42 (±0.39)	8.17 (±0.42)	n.s.
Negative geotaxis	5.63 (±0.82)	5.75 (±0.14)	n.s.
Cliff aversion	3.46 (±0.99)	3.21 (±0.44)	n.s.
Rooting	4.08 (±0.62)	4.74 (±0.77)	n.s.
Ear twitch	10.17 (±0.12)	9,88 (±0.25)	n.s.
Air righting	10.29 (±0.43)	10.58 (±0.26)	n.s.
Forelimb grasping	8.42 (±0.54)	8.21 (±0.08)	n.s.
Open field	9.17 (±0.18)	8.63 (±0.24)	n.s.
Maternal retrieval - latency to retrieval	24,6 (±11.7)	95.25 (±69.1)	n.s.
Maternal retrieval - time to retrieval	77.25 (±25.4)	123.5 (51.6)	n.s.
Neonatal ultrasonic vocalizations -Day 6	366.83 (±72.6)	453.17 (±83.3)	n.s.
Neonatal ultrasonic vocalizations -Day 12	466.8 (±124.45)	286.1 (±44.28)	n.s.
ADULT PERIOD			
Locomotor activity (mean nr beam breaks)	184.3 (±3.09)	211.53 (±11.01)	n.s.
Rotarod (mean time to fall)	53.8 (±6.03)	50.25 (±6.03)	n.s.
Light-dark box - time spent in the light	145.58 (±30.72)	133.75 (±30.22)	n.s.
Light-dark box - latency to the light	71.41 (±16.91)	50.33 (±13.47)	n.s.
Light-dark box - entries in the light	3.58 (±1.03)	4.58 (±1.03)	n.s.
Elevated plus-maze - total distance	1278.21 (±119)	1426.6 (±116.6)	n.s.
Elevated plus-maze - time in open arm	26.75 (±11.96)	41.62 (±8.2)	n.s.
Elevated plus-maze - entries in open arm	9.25 (±2.2)	10.83 (±1.17)	n.s.
Hyponeophagia - latency to contact	3.33 (±0.83)	2.67 (±0.56)	n.s.
Hyponeophagia - time to drink	23.17 (±12.75)	4.25 (±0.93)	n.s.
T-maze spontaneous alternation	74.09 (±3.82)	75.65 (±3.49)	n.s.
Nesting	4.5 (±0.15)	4.08 (±0.15)	n.s.
3-chamber social interaction - weights	56.38 (±1.4)	62.08 (±1.61)	**p=0.014**
3-chamber social interaction- % time_mouse	83.2% (±1.9)	72.3% (±2.4)	**p=0.002**
3-chamber social interaction- Nr entries_ mouse	26.08 (±3.17)	29.83 (±4.5)	n.s.
3-chamber social interaction- % time_ new mouse	64.9% (±3.8)	64.2% (±3.8)	n.s.
3-chamber social interaction- Nr entries_ new mouse	22.5 (±2.34)	19.83 (±2.33)	n.s.
Reciprocal interaction - time in social contacts	186.4 (±20.29)	180.74 (±14.53)	n.s.
Reciprocal interaction - Nr of social contacts	94.33 (±8.64)	95.7 (±5.29)	n.s.
Reciprocal interaction - time in non-social contacts	73.17 (±33.71)	80.7 (±19.5)	n.s.
Reciprocal interaction - Nr of non-social contacts	3.83 (±1.62)	4.0 (±1.18)	n.s.
Olfaction test - smell time (sampe phase)	63.63 (±5.02)	59.8 (±5.24)	n.s.
Olfaction test - smell entries (sample phase)	72.63 (±5.85)	75.13 (±3.92)	n.s.
Olfaction test - % time new smell (test phase)	64.9% (±7.6)	72% (±7.7)	n.s.
Olfaction test - % entries new smell (test phase)	65.1% (±5.3)	66% (±8.1)	n.s.

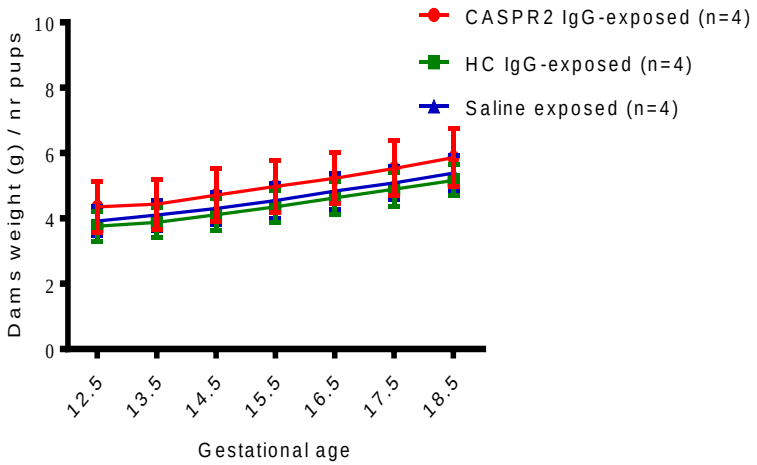


Figure 5-2: Dams weight increase per number of pups during injection period

Data presented as mean ±SEM.

(usually the morning after delivery) and might be underrepresented in case of paedophagia. At P1, six pups per litter were randomly selected and the rest of the litter culled. Gender was not determined in the culled animals, but there was no statistical difference in the gender distribution of the pups randomly selected for behavioural testing (Fisher's exact test: p=0.39) (Table 5-2).

5.5 Neonatal period

Behavioural testing of the neonatal pups consisted of an evaluation of neurodevelopmental reflexes using the modified battery of Fox, a maternal retrieval test and an analysis of the neonatal ultrasonic calls. To account for intra-litter variability and the effect of maternal-litter interaction, the litter was used as the experimental unit during the neonatal period.

5.5.1 Neurodevelopmental milestones (modified battery of Fox)

5.5.1.1 Physical development readouts

Pups body weight was assessed every day from P1 to P21 (figure 3A). Using a repeated measures ANOVA there was an effect of time ($F(2.22, 13.33)=2702.66$; $p<0.0005$), as expected, but no effect of treatment by time ($F(2.22, 13.33)=0.27$; $p=0.79$). There was no effect of treatment in day of eye opening ($t(3.3)=3.452$; $p=0.69$) or ear detachment ($t(6)=0.63$; $p=0.55$) (figure 3B). This reflects optimal standardization achieved by culling the litters to the same number of pups.

5.5.1.2 Neurodevelopmental reflexes

Tests of surface righting, negative geotaxis, cliff aversion, rooting, ear twitch and air righting were performed as indicated in Figure 5-1, to assess neurodevelopment. Tests

Table 5-2: Offspring survival and gender distribution of CASPR2 and HC IgG–exposed litters

	CASPR2 IgG exposed					HC IgG exposed					
	Survival										
Litter	1	2	3	4	Mean (±SD)	1	2	3	4	Mean (±SD)	p-value (2 tailed)
Live pups at birth	13	11	10	6	10 (±2.94)	11	15	11	9	11,5 (±2.55)	0.69†
Dead pups at birth	0	0	0	0		0	1	0	0		
	Gender distribution*										
Litter	1	2	3	4	Total	1	2	3	4	Total	p-value (2 tailed)
F/M	3/3	5/1	1/5	5/1	14/10	2/4	4/2	1/5	3/3	10/14	0.39†

* Gender distribution of studied pups (litters randomly culled to 6 animals / litter at P1);
† Mann-Whitney U test; † Fisher's exact test

were performed daily until the pup reached criterion for two consecutive days and the second day used for statistical analysis. There was no effect of treatment on day to reach criterion for any of the tests: surface righting ($t(6)=-0.535$; $p=0.612$), negative geotaxis ($t(6)=-0.697$; $p=0.512$), cliff aversion ($t(3.628)=0.32$; $p=0.766$), rooting ($t(6)=-2.037$; $p=0.088$), ear twitch ($t(4.271)=0.302$; $p=0.777$) or air righting ($t(6)=-1.101$; $p=0.313$) (Figure 5-4).

5.5.1.3 Neuromotor coordination

Neuromotor coordination was assessed by a forelimb grasping test and an open field traversal. There was no effect of treatment on day to reach criteria, either on the forelimb grasping ($t(6)=0.0$; $p=1$) or the open field traversal ($t(6)=0.38$; $p=0.717$) (Figure 5-5).

5.5.2 Maternal retrieval test

Maternal retrieval is a complex behaviour that describes the search of a female for an isolated pup and the action of pushing or carrying by mouth the pup to the nest. It results from the interaction of both mother and pup and behavioural changes in either or both, such as rate of ultrasonic calls from the pups or predisposition to maternal care, can affect this behaviour (Hahn and Lavooy 2005). There was high variability in both parameters but no effect of treatment in the latency to retrieval ($t(6)=-0.798$; $p=0.456$) or the time taken for the mother to return all pups to the nest ($t(6)=-1.165$; $p=0.288$) (Figure 5-6).

5.5.3 Neonatal ultrasonic vocalizations

Neonatal rodent ultrasonic vocalizations or calls (USVs) are sounds emitted by pups in the ultrasonic range. In neonatal mice, they occur between P2-P14, have a 30-50 kHz frequency and the rate can vary between 5-100 calls/minute (Hahn and Lavooy 2005).

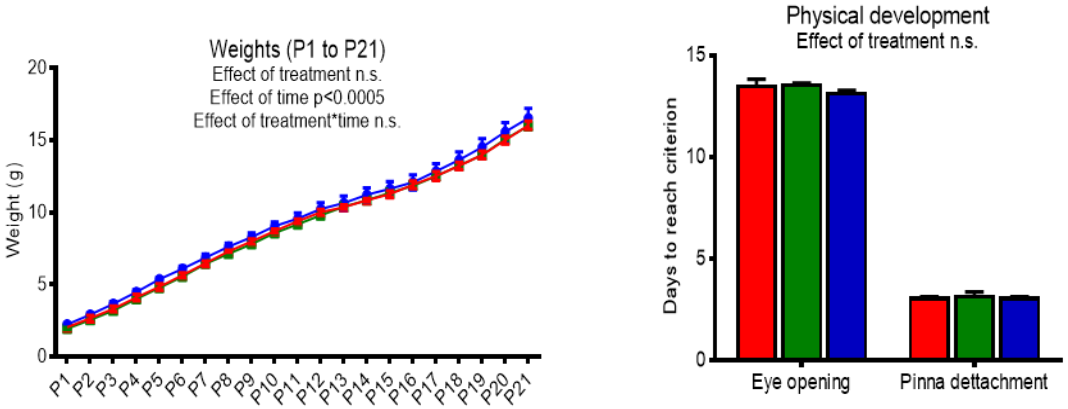


Figure 5-3: Physical maturation readouts of the offspring.

(A) weight increase and (B) eye opening and ear detachment. Data presented as mean±SEM.

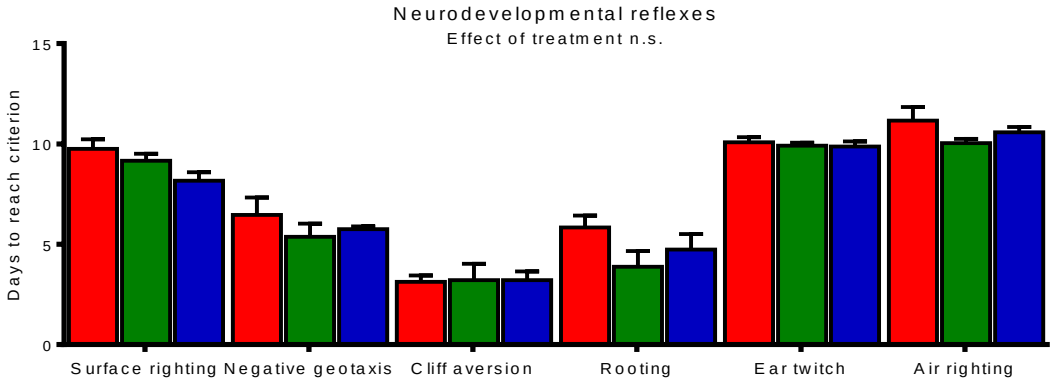


Figure 5-4: Neurodevelopmental reflexes during the neonatal period

Data presented as mean±SEM.

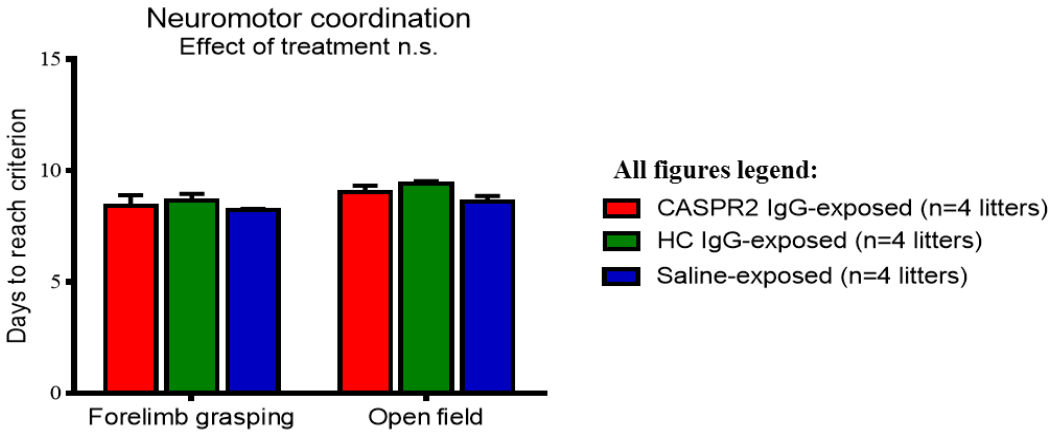


Figure 5-5: Neuromotor coordination tests during the neonatal period

Data presented as mean±SEM.

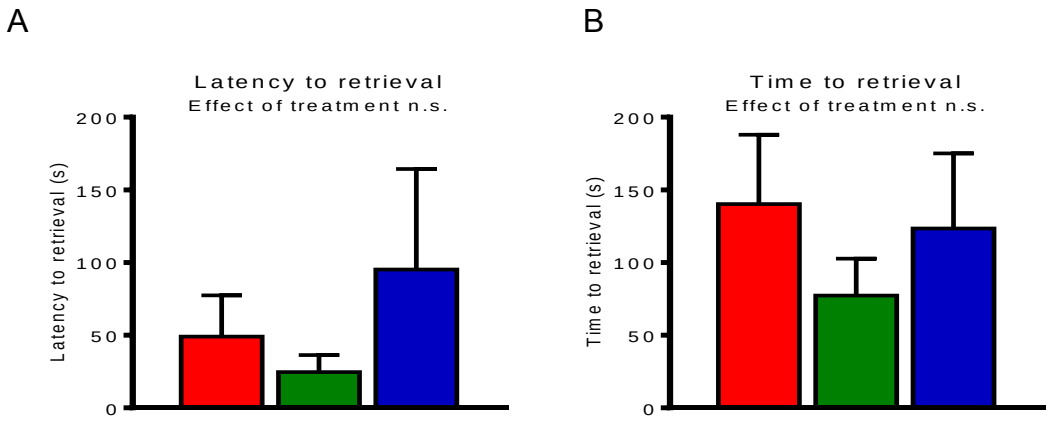


Figure 5-6: Maternal retrieval test

(A) latency to retrieval and (B) time to retrieval. Data presented as mean±SEM.

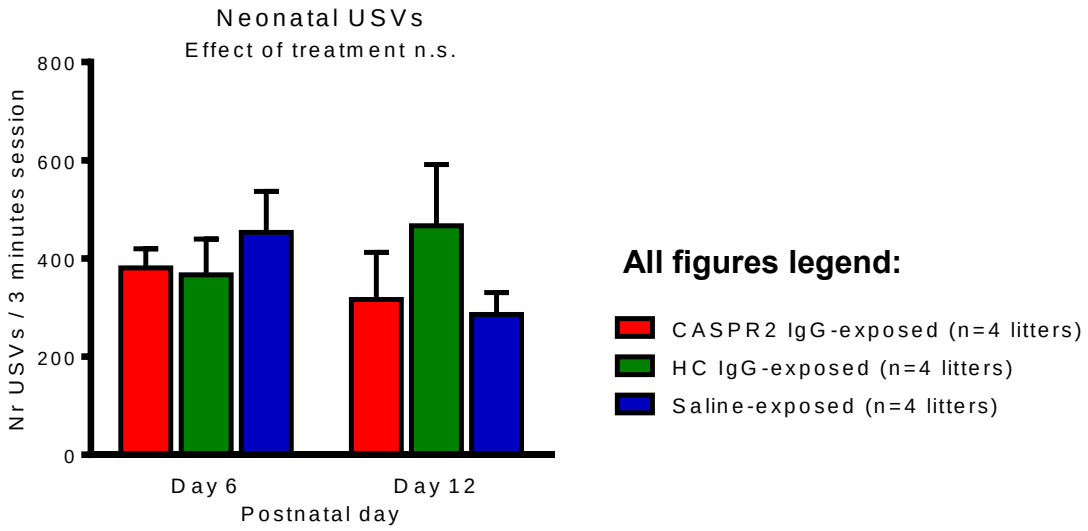


Figure 5-7: Neonatal ultrasonic vocalizations at postnatal day 6 and day 12.

Data presented as mean±SEM

A crude recording of ultrasonic calls was made during a 3-minute isolation test at P6 and P12. There was wide variation in the number of calls but no treatment effect on the number of USVs at P6 (U=7; Z=-0.289; p=0.888) or P12 (U=6; Z=-0.577; p=0.686). The mean number of calls per minute varied between 122 and 151 at day 6, and between 95 and 155 at day 12, which is slightly higher than expected and could result from contamination of the recording by other sounds (Figure 5-7).

5.6 Adult period

5.6.1 Weight increase

During the behavioural testing in adult life, weight increase was different across treatment groups (Figure 5-8). For reasons not understood, saline-exposed animals had higher weight increase across time than HC IgG-exposed animals ($F(12, 198)=2.608$; $p=0.004$), although there were no differences between CASPR2 IgG and HC IgG-exposed animals ($p=0.23$).

5.6.2 Tests of locomotor activity and motor coordination

Locomotor activity was assessed by an automated photobeam activity system during a 2 hour session and motor coordination was assessed by the accelerating rotarod (Figure 5-9). There was an effect of time on locomotor activity ($F(19,874)=21.145$; $p<0.0005$), which reflects a decrease in exploratory drive after habituation to the environment, but no effect of treatment group ($F(1,46)=1.472$; $p=0.231$) and no interaction of time with treatment group ($F(19,874)=0.604$; $p=0.905$).

Motor coordination was assessed during 2 sessions at months 4 and 5. There was an effect of rotarod session ($F(1,30)=6.305$, $p=0.018$), but no effect of treatment group

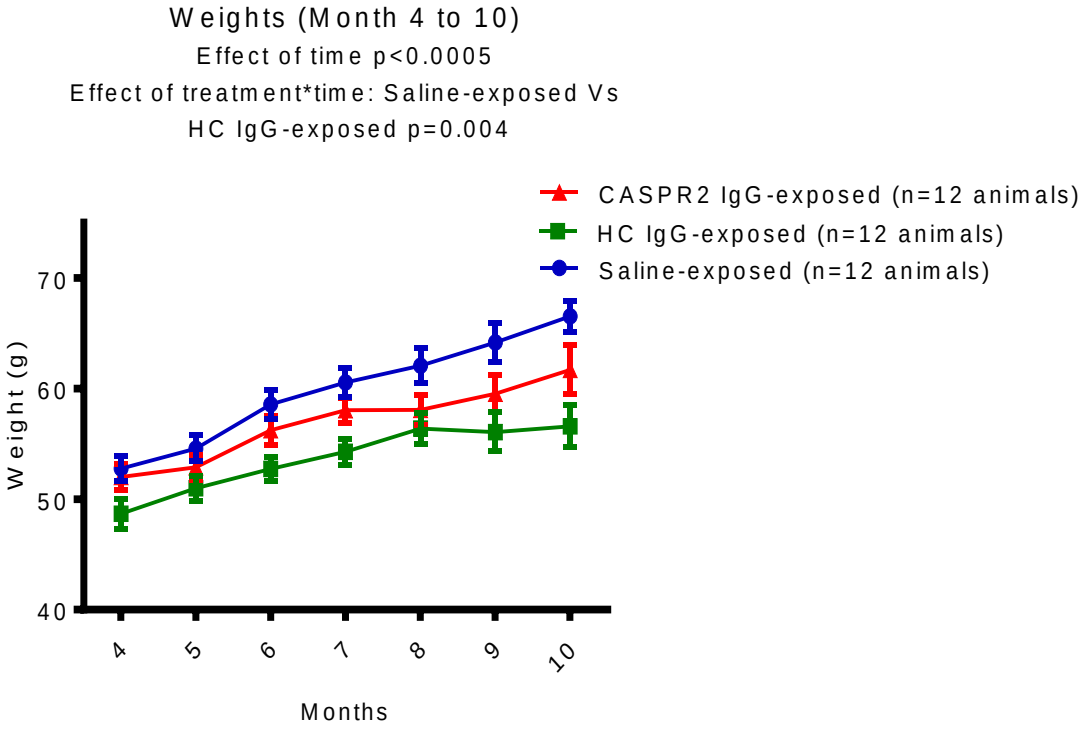
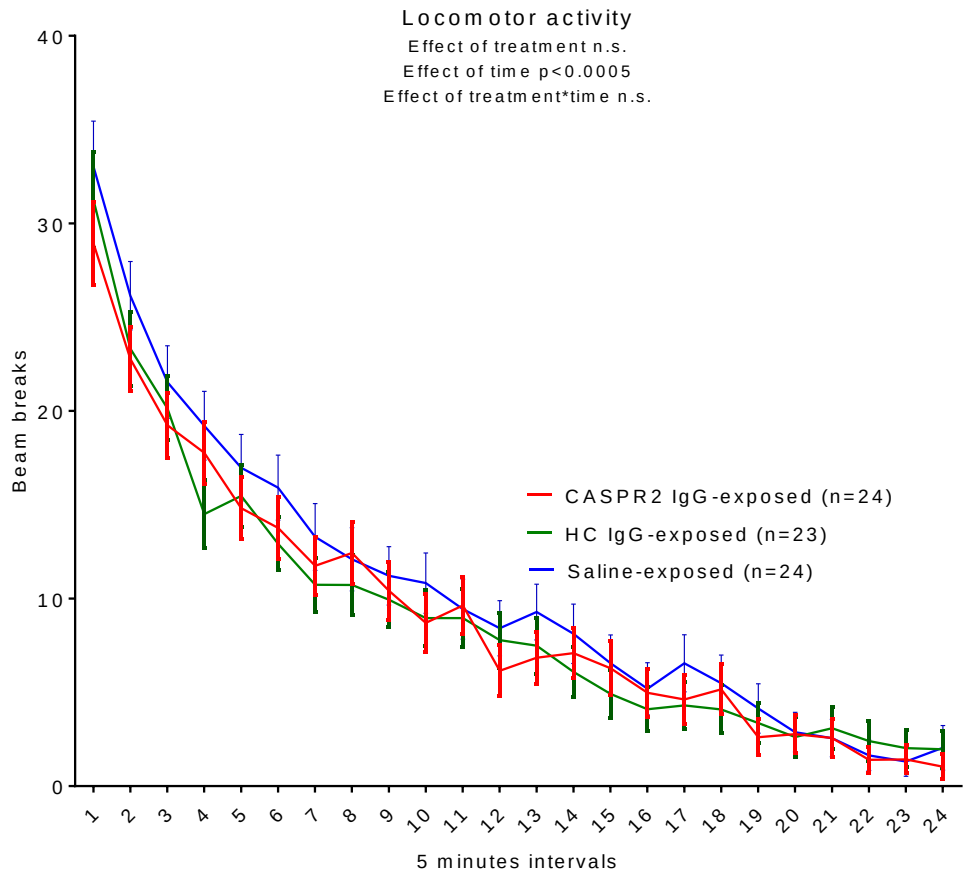


Figure 5-8: Weight increase during the adult period, in animals undergoing behavioural testing

Data presented as mean ±SEM.

A



B

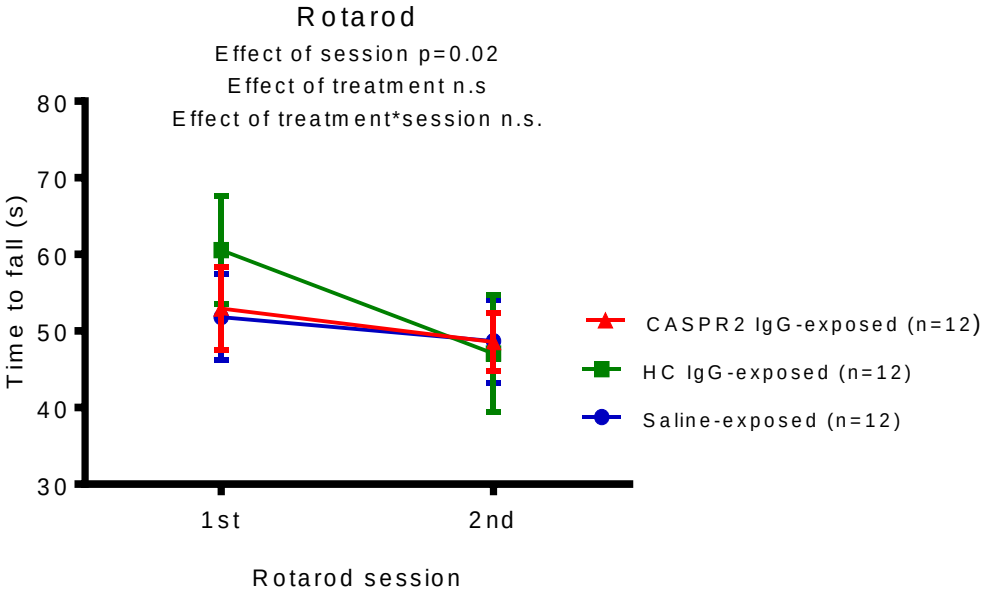


Figure 5-9: Locomotor activity (A) and neuromotor coordination (B)

Data presented as mean ±SEM.

(F(2,30)=1.507; p=0.876) and no interaction of session with treatment group (F(2,30)=1.374; p=0.269).

5.6.3 Tests of anxiety

Anxiety behaviours were measured in three different tests. The first test was the light-dark box, which explores the innate preference of mice to explore dark areas. There were no differences between groups, despite a trend towards less time spent in the light by the CASPR2 IgG-exposed animals, in all parameters analysed: time spent in the light box (t(22)=1.887; p=0.07), latency to enter the light box (t(22)=0.747; p=0.46) and total number of entries in the light box (U=60.5; Z=-0.676; p=0.514) (Figure 5-10). The second test was the elevated plus-maze, which explores the innate preference of mice to enclosed areas and avoidance of bright, open and elevated zones. There were no differences between groups in all parameters analysed: total distance travelled (t(22)=0.271; p=0.79), time spent in open arms (t(21)=-0.162; p=0.87) or entries in open arms (U=75; Z=0.174; p=0.89) (Figure 5-11). Finally, we also performed a hyponeophagia test, which relies on the reluctance of mice to eat a novel food. There were no differences between groups in the time to contact (t(22)=-1.03; p=0.31) or time to drink (t(22)=0.512; p=0.61) the new food (condensed milk) (Figure 5-12). However, the short latencies found, are suggestive of failure of this test in being sufficiently anxiogenic to detect any differences.

5.6.4 T-maze spontaneous alternation test

All mice were assessed for working memory and perseverance of behaviour using the T-maze spontaneous alternation test. Tested individuals were separated into 2 different cohorts for practical reasons, but there was no effect of cohort, so the results were combined for presentation. There was no statistically significant effect of treatment

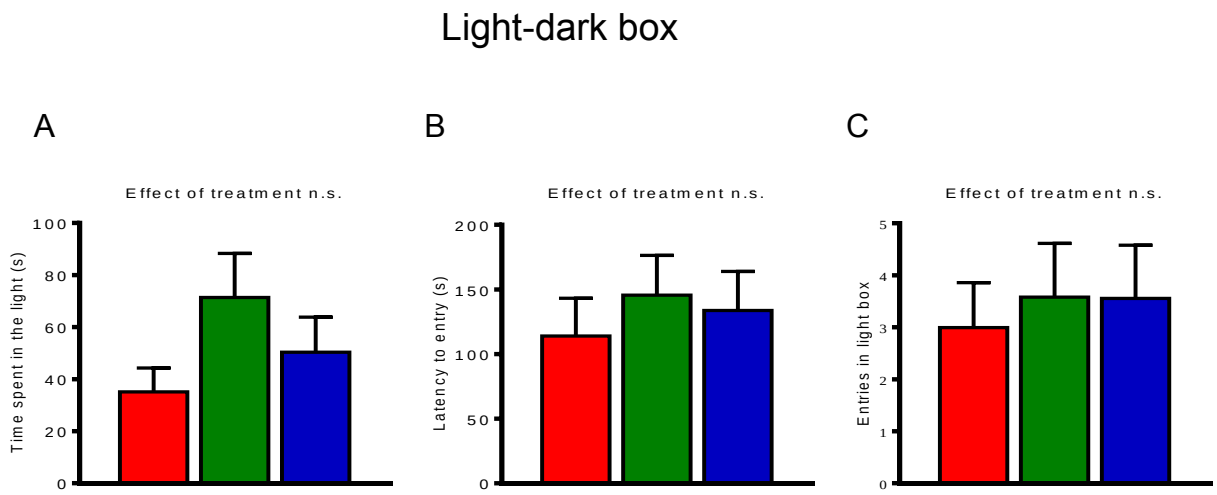


Figure 5-10: Light-dark box test

(A) Time spent in the light; (B) Latency to entry in the light; (C) Entries in light box. Data presented as mean ±SEM.

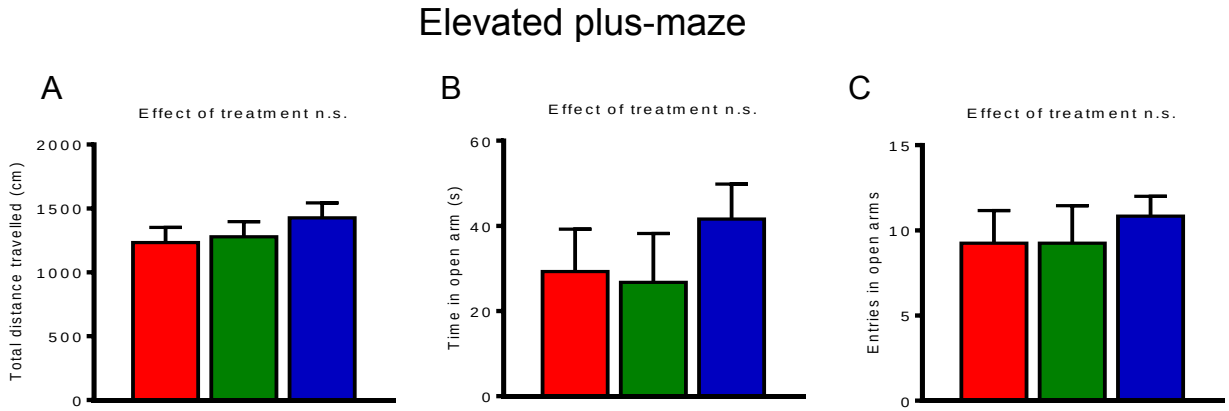


Figure 5-11: Elevated plus-maze test

(A) Total distance travelled; (B) time in open arms; (C) entries in open arms. Data presented as mean ±SEM.

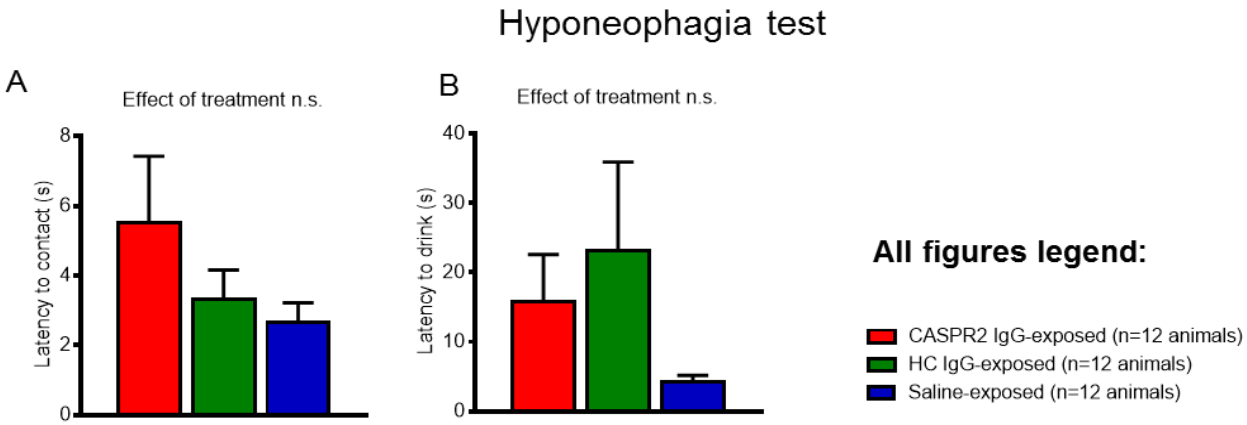


Figure 5-12: Hyponeophagia test

(A) Latency to contact and (B) Latency to drink new food. Data presented as mean ±SEM

group, although there was a trend towards less alternation by the CASPR2 exposed group (t(45)=-1.874;p=0.067) (Figure 5-13).

5.6.5 Nesting test

The nesting test explores a social, species-typical behaviour. There was a effect of treatment group, the CASPR2-IgG exposed group had lower mean ranks than the HC IgG-exposed group (U=39; Z=-2.126; p=0.034), suggesting a mild defect in this social behaviour (Figure 5-14).

5.6.6 Three-chamber social interaction test

The three-chamber social interaction test comprises 2 periods of habituation to the apparatus, a sociability test (that assesses non-reciprocal social interaction) and a social memory test. The habituation phases of the test are very important for interpretation of the following results. If one group displays less exploratory activity during the habituation phase, it is likely that this will lead to a decrease in interaction with the other mice in the following phases (as the animal will still be exploring the surroundings). There were no differences between CASPR2 IgG and HC IgG-exposed mice in the habituation parameters analysed. During habituation phase 1 there were no differences in distance travelled (t(21.5)=0.05; p=0.96), time immobile (t(22)=0.066; p=0.95) and time freezing (t(22)=0.033; p=0.97) (Figure 5-15A). The same was true for these parameters in habituation phase 2 (distance travelled: t(20.8)=1.29, p=0.21; time immobile: t(22)=0.39, p=0.7; time freezing: t(22)=0.163; p=0.87) (Figure 5-15B). The sociability phase of the test explores the mouse innate preference to interact with another animal rather than with an object, given that mice are highly-sociable animals. CASPR2 IgG-exposed mice still showed preference for interaction with another mouse than with an object. However, the percentage of time spent in the mouse interaction zone, was significantly lower than

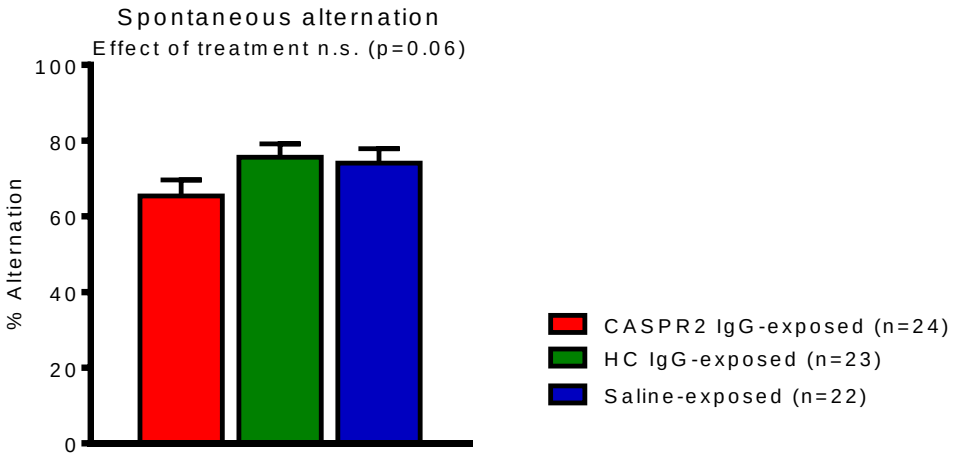


Figure 5-13: T-maze spontaneous alternation test

Data presented as mean ±SEM

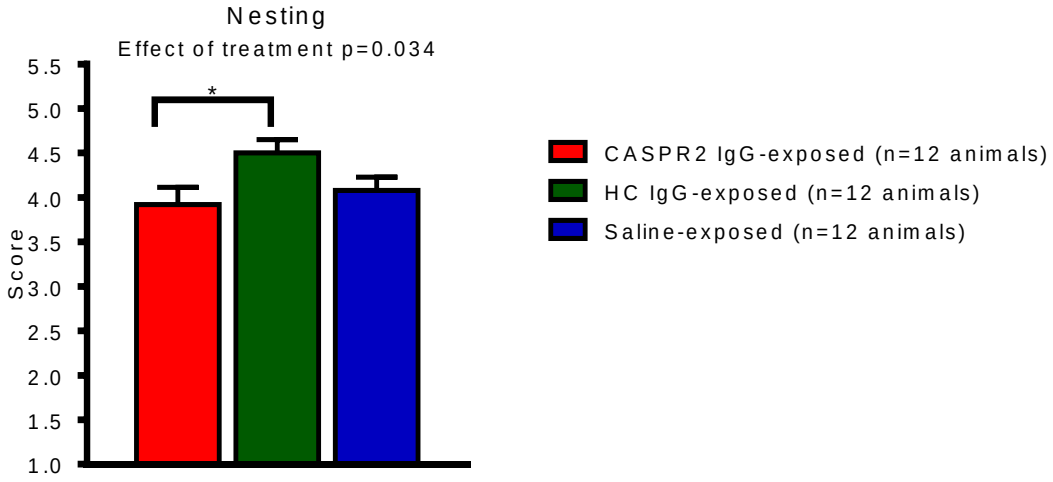


Figure 5-14: Nesting test

Data presented as mean ±SEM

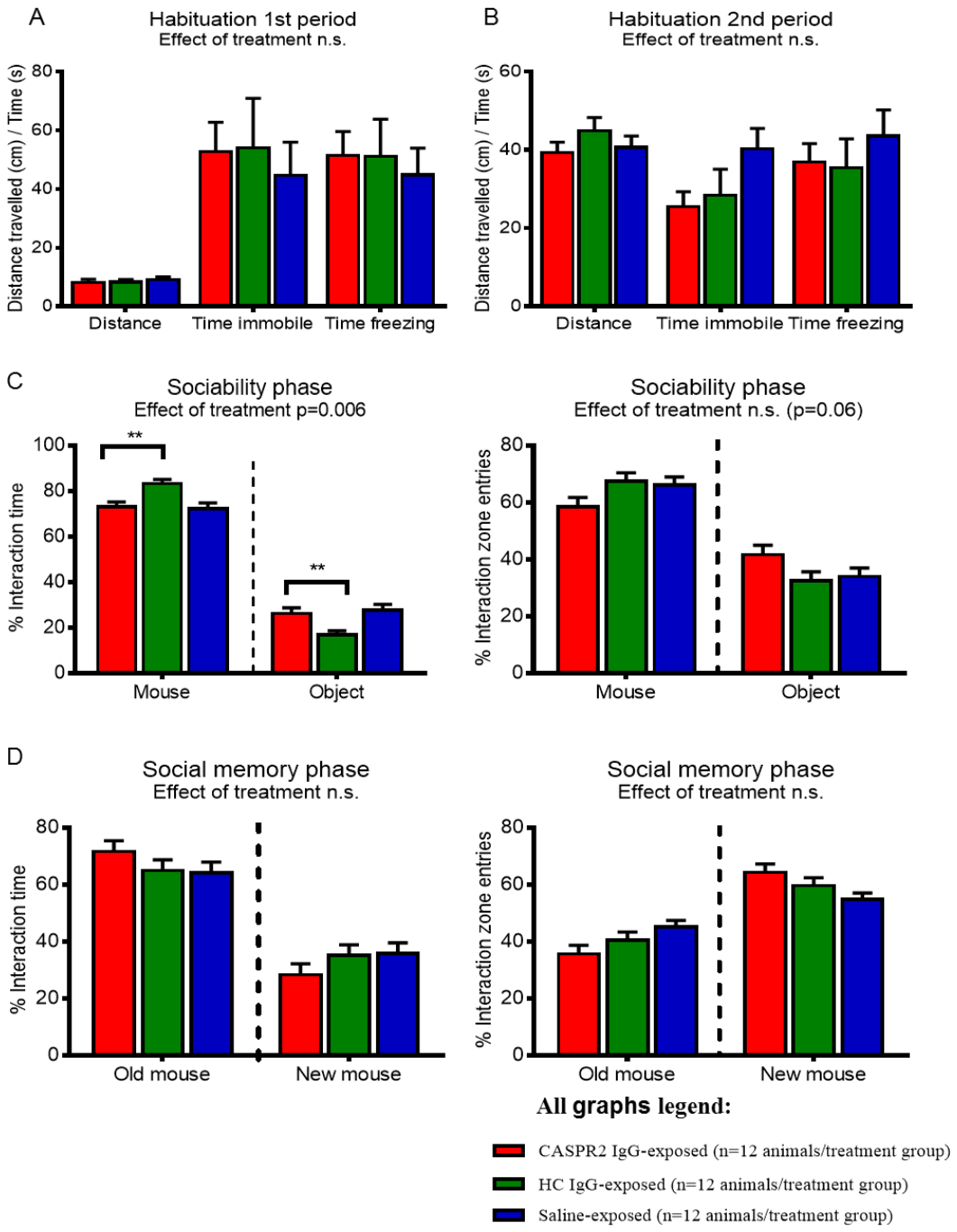


Figure 5-15: Three-chamber social interaction test

(A) First habituation period; (B) second habituation period; (C) Sociability phase and (D) Social memory phase. Data presented as mean ±SEM.

that for HC IgG-exposed mice (t(22)=3.068; p=0.006) and there was a trend towards a decrease in the number of entries in the mouse interaction zone (t(22)=1.984; p=0.06) (Figure 5-15C). During the social memory phase of the test, a choice is given to the mouse on whether to interact with the same mouse or with a new, unknown mouse. A normal mouse will preferentially interact with the unknown mouse. No differences between treatment groups were found during the social memory phase, either in the percentage of time the test mouse spent interacting with the new mouse (t(22)=1.254; p=0.22) or in the number of entries in the interaction zone (t(22)=0.267; p=0.792) (Figure 5-15D).Taken together, those findings suggest that the social defects found in the CASPR2 IgG-exposed mice are founded on a lack of social affiliation and motivation rather than a disturbance of social memory or social novelty.

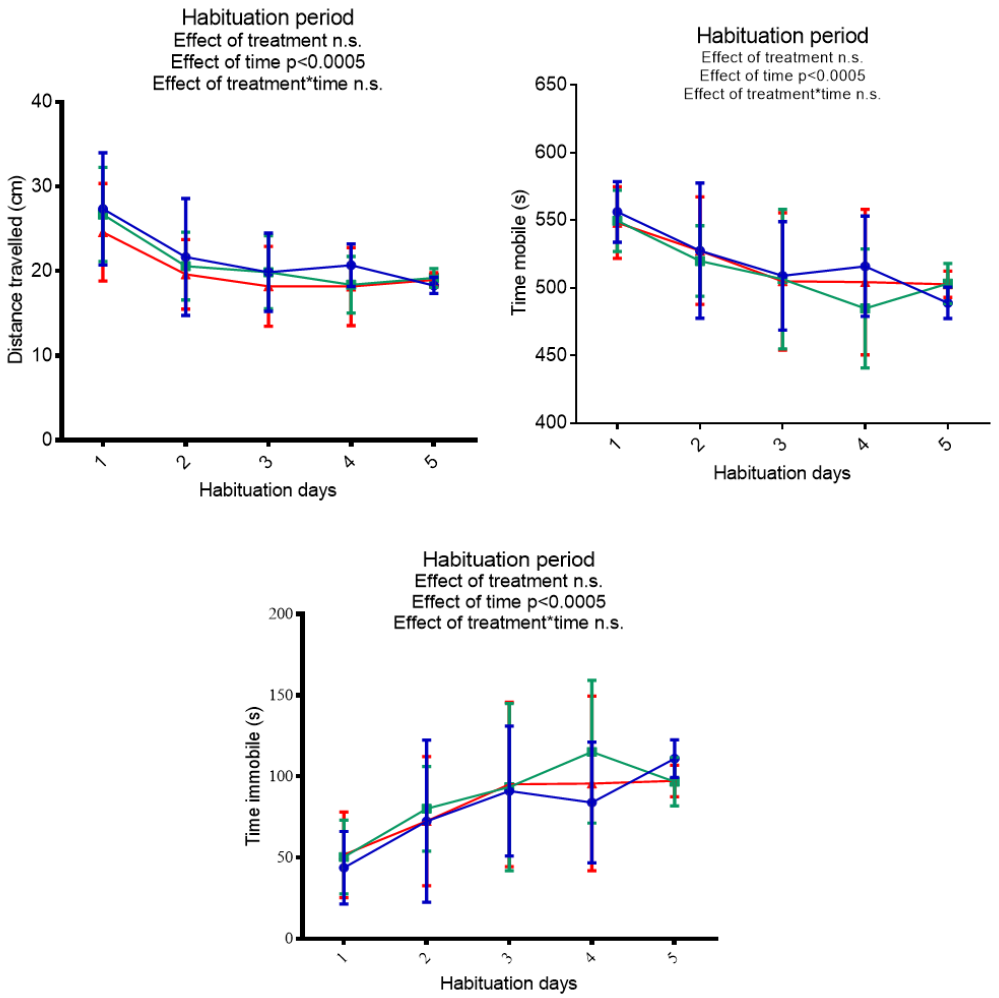
In this test, saline-exposed animals showed an atypical, unexpected behaviour. Interaction with the stranger mouse was also reduced in this group when compared to HC IgG-exposed animals (t(22)=3.51; p=0.002), and similar to the interaction results of the CASPR2 IgG-exposed mice (Figure 5-15C). One possible explanation for this finding lies in the differences in weight between groups. At this stage (and throughout behavioural testing as shown above), and for reasons not understood, saline-exposed animals' weights were in average higher than the weights in the other groups and this difference was statistically significant when compared to the weights of HC IgG-exposed animals (t(22)=2.66; p=0.014). We postulated that the heavier animals might have had a reduced exploratory activity or that this might have affected the interaction between the test and the stimulus animal. To confirm whether weight was interfering with the social behaviour in the saline-exposed group, we have used a different test, the reciprocal interaction test, where this factor is tightly controlled.

5.6.7 Reciprocal interaction test

The reciprocal interaction test assesses social behaviours in a pair of animals of the same group. The animals were tightly matched according to weight (within 5% of the weight), so any variation resulting from these factor was avoided. This led to a difference in the number of pairs in each group (CASPR2 IgG-exposed n= 15 pairs; HC IgG exposed n= 6 pairs; Saline expose n= 11 pairs), in order to avoid repeating the test in each animal more than twice. The test starts with a habituation protocol that lasts 5 days, to ensure equal exploratory activity between groups. There was no effect of treatment over time in the habituation parameters analysed: distance travelled in the apparatus ($F(4,132)=0.44$; $p=0.78$), time immobile ($F(4,132)=0.53$; $p=0.72$) and time freezing ($F(4,132)=0.26$; $p=0.18$) (Figure 5-16A). During the test phase, a pair of animals was allowed to interact freely, the interaction video recorded and both social (licking, sniffing, grooming and following closely the other animal) and non-social behaviours (digging, self-grooming) were scored offline. CASPR2 IgG-exposed pairs spent less time in social behaviours ($U=11$; $Z=-2.65$; $p=0.008$) and there was a trend for having less social contacts, although not statistically significant ($U=23.5$; $Z=-1.68$; $p=0.095$) than HC IgG-exposed animals (Figure 5-16B). Interestingly, these animals also showed more repetitive, non-social behaviours. CASPR2 IgG-exposed animals spent more time in non-social behaviours ($U=71$; $Z=2.02$; $p=0.043$) and there was a trend for more non-social events in this group than in the HC IgG-exposed animals ($U=69.5$; $Z=1.92$; $p=0.055$) (Figure 5-16C). An increased tendency for self-grooming had also been noted in juvenile CASPR2 IgG-exposed mice but this was not adequately quantified at the time. In one CASPR2-IgG exposed litter, all females showed extensive areas of hair loss in their snout and behind the ears at the time of culling, although this wasn't seen in other animals from the same treatment group.

Saline-exposed mice behaved in a similar way to HC IgG-exposed mice, thus suggesting that the social deficit found in the previous test might have been weight-related.

A



B

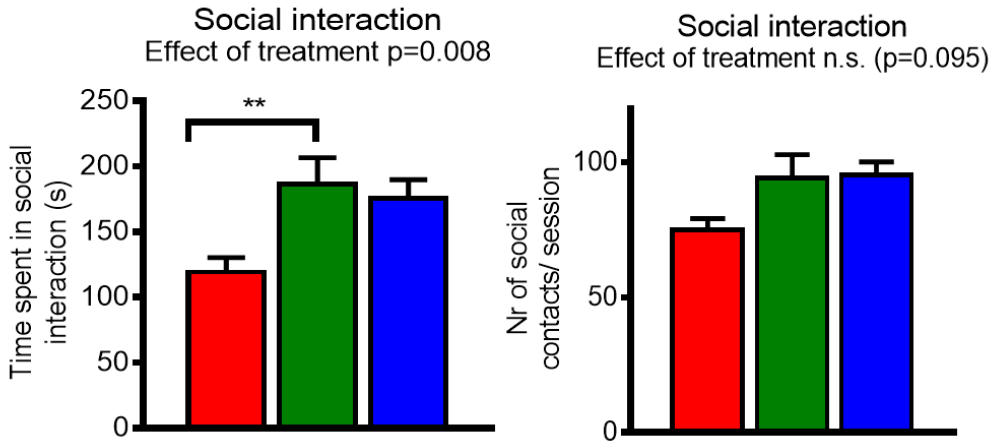


Figure 5-16: Reciprocal interaction test

(A) habituation phase; (B) social behaviours and (C) non-social behaviours across treatment groups. Data presented as mean±SEM.

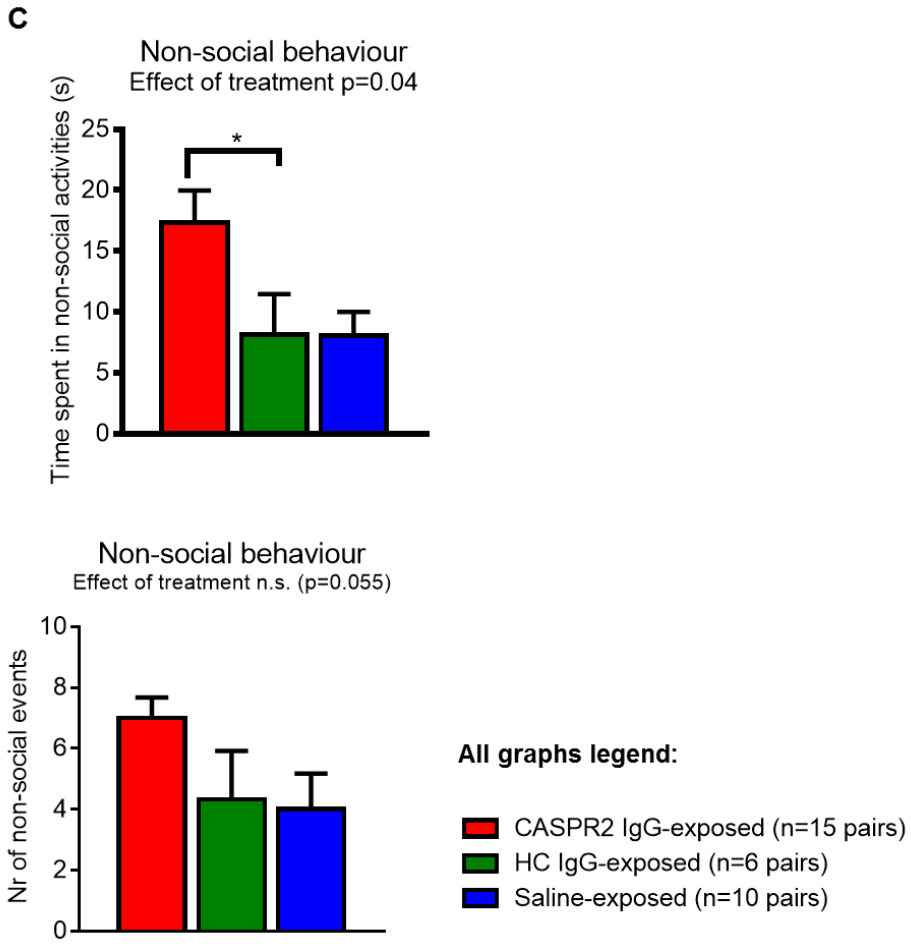


Figure 5-16 (cont): Reciprocal interaction test.

5.6.8 Olfaction test

An olfaction test was done as a control for the previous social interaction experiments, since mice rely heavily on smell for their social interactions. A small experiment (n=8 animals / treatment group) was done to assess olfaction. This experiment consisted of a sampling phase, where the same odour was placed in 2 containers and the test mouse allowed to freely explore, a delay period and a test phase, when the mouse was allowed to choose between the old or a novel odour. The mouse was expected to preferentially explore a novel odour, if its olfaction sense was intact. There was no effect of treatment group in the time spent exploring the odours ($t(14)=0.55$; $p=0.59$) or the number of times the mouse explores the odours ($U=34.5$; $Z=0.792$; $p=0.798$) during the sample phase (Figure 5-17A) and there was no effect of treatment group in the preference for the novel odour, as determined by the percentage of time spent with the old/new odour ($t(14)=0.29$; $p=0.78$) or the percentage of entries in the interaction zone of the new/old odour ($t(14)=0.07$; $p=0.95$) (Figure 5-17B).

5.7 Discussion

In this chapter, the behavioural outcome of mice exposed to CASPR2 IgG *in utero* is presented. Mice exposed to CASPR2 IgG *in utero* did not show any differences during the postnatal development or in tests of locomotor activity, coordination or anxiety during the adult period. However, CASPR2 IgG-exposed mice had deficits in social interaction and social activities, and increased repetitive, non-social behaviours, when compared to HC IgG-exposed mice.

The initial hypothesis was that exposure to CASPR2 antibodies in early development would lead to a disruption of the protein function and neural circuitry formation that would lead to permanent behavioural deficits. Given the difficulty in anticipating the specific deficits caused by this type of antibody-mediated injury to CASPR2, this is an exploratory

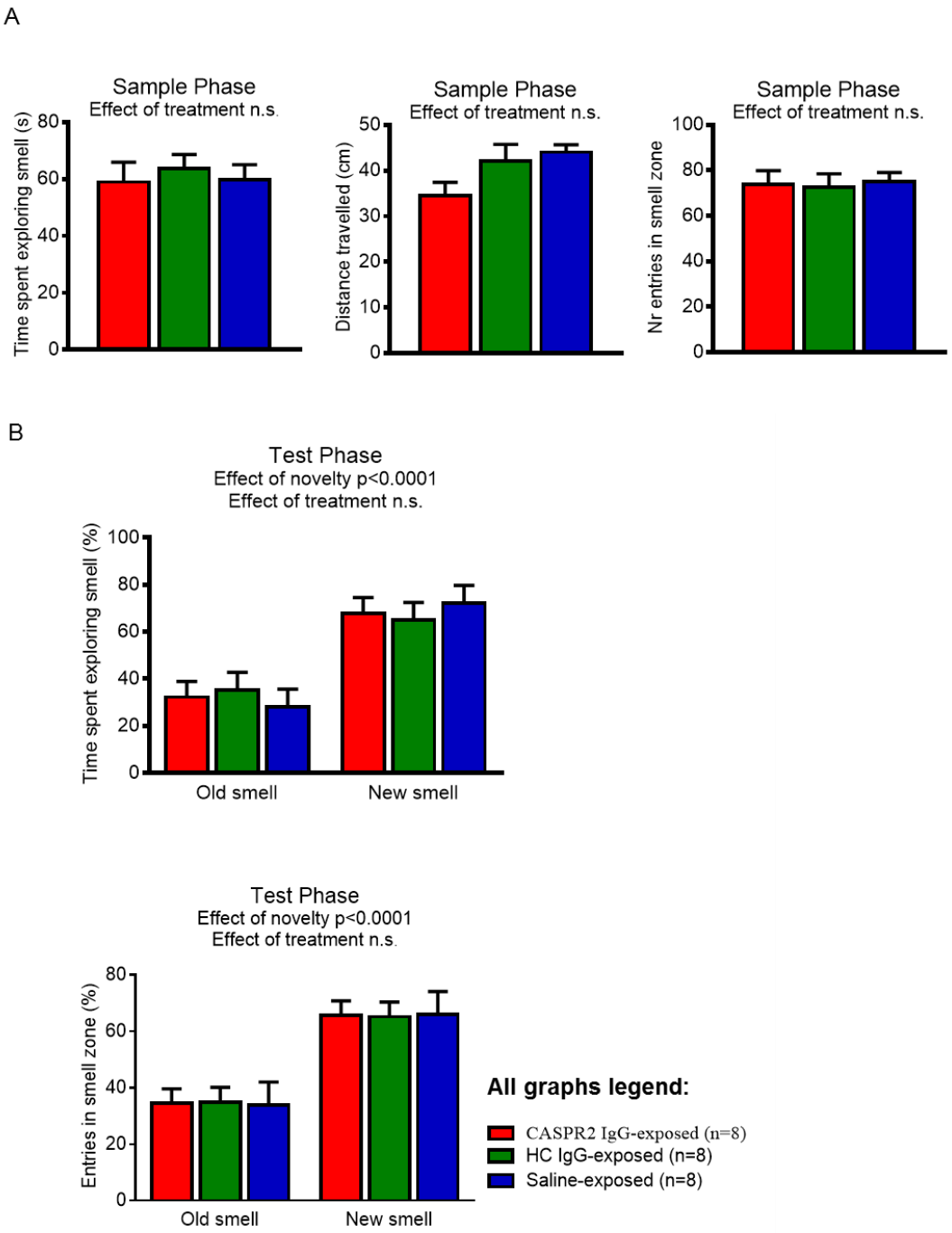


Figure 5-17: Olfaction test

(A) Sample phase and (B) Test phase results across treatment groups. Data presented as mean±SEM.

model, although influenced by the previously reported *Cntnap2* knockout mouse (Penagarikano, Abrahams et al. 2011), which exhibits hyperactivity, hyperreactivity to sensory stimuli, behaviour inflexibility, deficits in social interaction, increased stereotypic behaviour and epilepsy.

A detailed battery of tests was initially done during the neonatal period. During this period, mice are extremely dependent on their mothers and changes in their development could be the consequence of changes in maternal behaviour and attention. Likewise, peer interaction with littermates will also have consequences in future individual behaviour (Branchi 2009). To account for these complex interactions during the neonatal period, the female or the whole litter was used as the experimental unit, as is recommended for animal models of development (Festing 2006). Later, litter effects are expected to gradually disappear once the animals are weaned and are no longer dependent on maternal care. There were no differences between the CASPR2 and HC IgG exposed offspring during the neonatal period, which is a proxy measure for similar maternal care from HC and CASPR2 IgG-injected dams. This is particularly important in this model, since an effect of the human IgG on the dam's behaviour could have occurred. However, the adult blood brain barrier is, in normal circumstances, impermeable to IgG (Braniste, Al-Asmakh et al. 2014), which explains the different susceptibility of dam and foetus to an antibody-mediated injury. One must note, nevertheless, that given the need to consider the litter as the experimental unit, the assessment of the neonatal period is slightly underpowered, given that only 4 dams (4 litters) per treatment group were assessed. Unfortunately, this model is dependent on the availability of human material, thus limiting the possibility to inject a higher number of dams. Likewise, Home Office restrictions on the offspring numbers prevented the use of more litters.

Despite no obvious abnormalities in the neonatal period, mice exposed *in utero* to CASPR2 IgG showed permanent, long-term sequelae in their behaviour, when

compared to HC IgG-exposed mice. CASPR2 IgG exposed mice showed pronounced social interaction defects and increased tendency to non-social activities. Social interaction defects were demonstrated in two different experimental paradigms and were not a consequence of differences in exploratory behaviour/locomotor activity, in olfaction or in anxiety profile. Likewise, these animals showed not only a social interaction deficit but also defects in nesting, another social activity. Additionally, CASPR2 IgG-exposed animals showed an increase in non-social, repetitive behaviours such as grooming and digging. Importantly, this model used an outbred strain (MF1 mice), chosen due to their heightened maternal behaviour (given the risk of maternal rejection due to early experimental manipulation of the pups) and large litters, despite the obvious downside of increased phenotypical variability associated with the use of outbred animals.

It is always difficult to establish correlations between animal phenotypes with neuropsychiatric human disorders. Social interaction defects and increased stereotypies are clinical features of many neurodevelopmental disorders, such as autism, mental retardation or even psychotic disorders (Cervantes and Matson 2015). Unfortunately, this model has not explored extensively memory or cognitive deficits, which constitutes a limitation to our interpretation, but there was a strong trend for an impairment of working memory in CASPR2 IgG-exposed mice, when assessed for spontaneous alternation in the T-maze.

Interestingly, these findings further support the importance of CASPR2 during neurodevelopment and its involvement in a wide range of neuropsychiatric disorders (Rodenas-Cuadrado, Ho et al. 2014). Also interesting, this animal model reproduces partially the phenotype of *Cntnap2* knockout mice (Penagarikano, Abrahams et al. 2011). *Cntnap2* knockout mice were shown to have social deficits in the 3 chamber social interaction test, in the reciprocal social interaction test and sored lower in the nest-building task. However, *Cntnap2* knockout mice not only had social deficits , but also had

abnormal motor activity, cognitive deficits and seizures, as expected from a much more extensive disruption occurring from a genetic manipulation.

In conclusion, *in utero* exposure to a maternal pathogenic antibody, targeting an important neural-surface protein, CASPR2, was shown to affect offspring neurodevelopment and long-term behaviour. CASPR2 IgG-exposed mice developed similarly to HC IgG-exposed mice during the neonatal period, but in adult life these mice had deficits in social interaction and social activities, and increased repetitive, non-social behaviours.

Next, a detailed histological analysis on the brains of these animals exposed to CASPR2 IgG and HC IgG was conducted.

Chapter 6: Effects of antibody transfer on brain histology

A detailed analysis of the brain was conducted in a randomly selected subgroup of animals at 12 months of age, in order to provide a histological substrate for the behaviour abnormalities found in the CASPR2 IgG-exposed mice. It is important to note that the behavioural sequelae described in the previous chapter were found many months after these animals had been exposed to CASPR2 IgG *in utero*, suggesting that permanent changes had occurred in the brain development. A general morphological and morphometric study was conducted initially. Afterwards, a hypothesis-driven analysis looked for similarities with the histological abnormalities reported in the *Cntnap2* knockout model (Penagarikano, Abrahams et al. 2011), such as cortical migration defects, hippocampal gliosis or the presence of ectopic neurons in the corpus callosum. The remaining analysis focused on histological features associated with brain development, particularly microglia-dependent synaptic pruning (Paolicelli, Bolasco et al. 2011) (Schafer, Lehrman et al. 2012).

6.1 Overview of tissue processing and histological analysis

Six animals per treatment group (3 males and 3 females) were randomly selected for histological analysis after completion of the behaviour studies. Histological studies were performed on PFA-fixed, free-floating, 50 μm coronal sections of the brain. A Nissl staining was conducted on all 6 brains (staining done on every 15th section) to assess gross morphological abnormalities and to determine volume and/or thickness of total brain or specific brain regions (corpus callosum, hippocampus, cerebellum, prefrontal and somatosensory cortices). The remaining studies were done using

immunofluorescence and included determination of specific cell densities in regions of interest and quantification of synaptic proteins. These were performed in only 3 animals per treatment group, given the prolonged imaging times required for the analyses. Similarly to the behaviour tests, we present the data for the saline group but, as in the behavioural studies, the statistical analysis was conducted only for the CASPR2 and HC IgG-exposed groups. Due to time constraints, microglia densities and synaptic puncta analysis were not conducted in the saline-exposed group. All experiments were coded for treatment groups.

For this part of the project, I received the invaluable help of Dr David Menassa. I conducted all tissue processing, the Nissl staining in embryonic and adult offspring, most of the morphometric analysis and the immunofluorescence staining for GFAP/NeuN and CD68. Dr Menassa did all the confocal imaging, the staining for cortical layer markers, calbindin and PSD-95, and the cell counts. For each experiment, his contribution is highlighted in the figure legends. I am also very grateful to Professor David Bennett and Dr Steven West who have shared their expertise in the staining of synaptic proteins and have performed the quantification of PSD-95 profiles.

6.2 Results on the morphological and morphometric analysis

No gross morphological differences were found across treatment groups in the brain of the adult offspring (12 months; n=6 brains/group) (Figure 6-1). Special attention was given to the morphology of the cortex, hippocampus, corpus callosum and cerebellum, particularly looking for abnormalities in glial morphology or the presence of neuronal clusters in the white matter.

No differences were found across treatment groups in the total brain volume ($t(10)=0.44$, $p=0.67$) or in the volume of the corpus callosum ($t(10)=1.05$, $p=0.32$), hippocampus

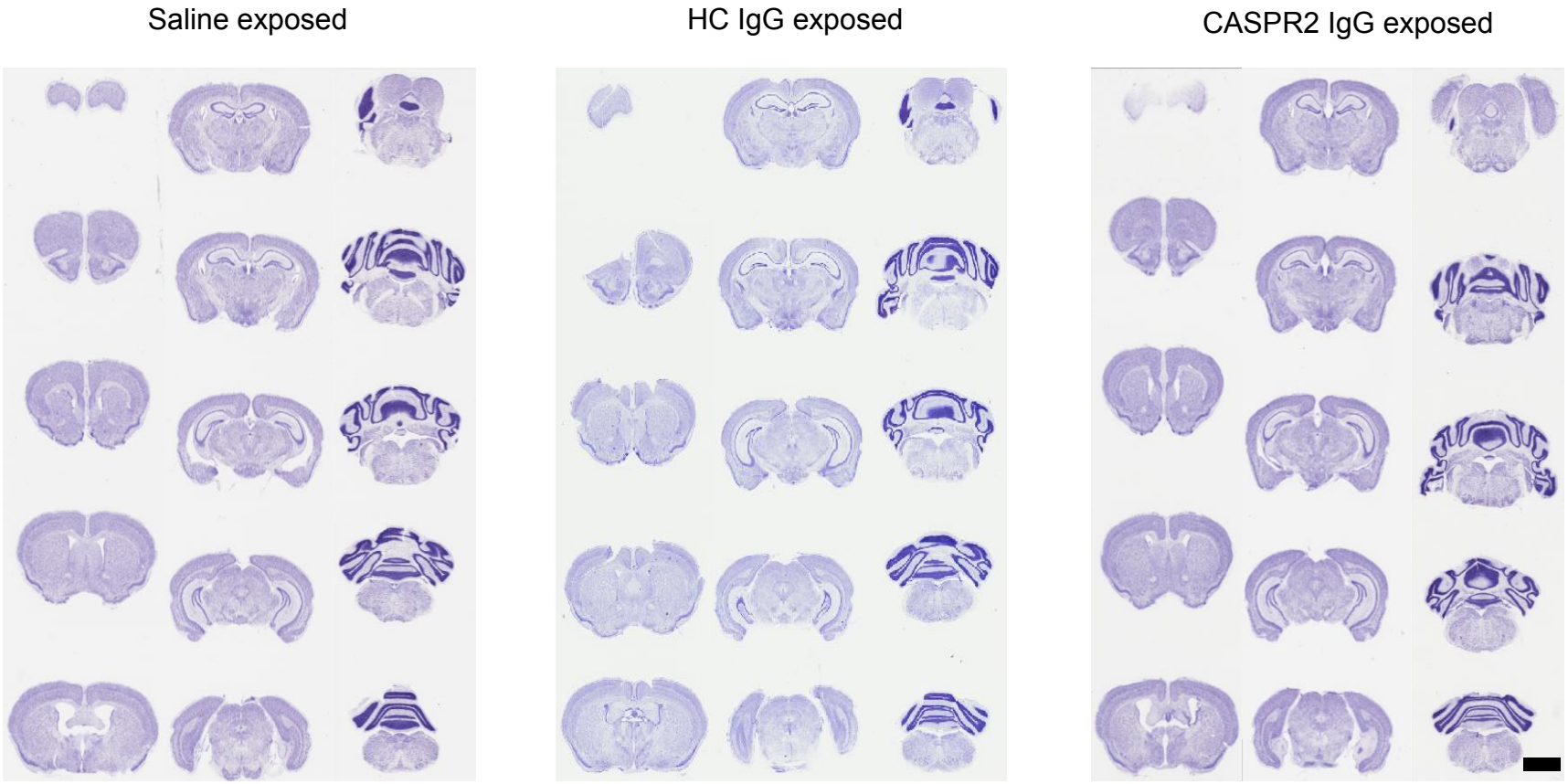


Figure 6-1: Adult offspring (12 months) gross brain morphology

Nissl staining performed in every 15th coronal section of the adult offspring brain (n=6 brains / treatment group). Scale bar: 2 mm.

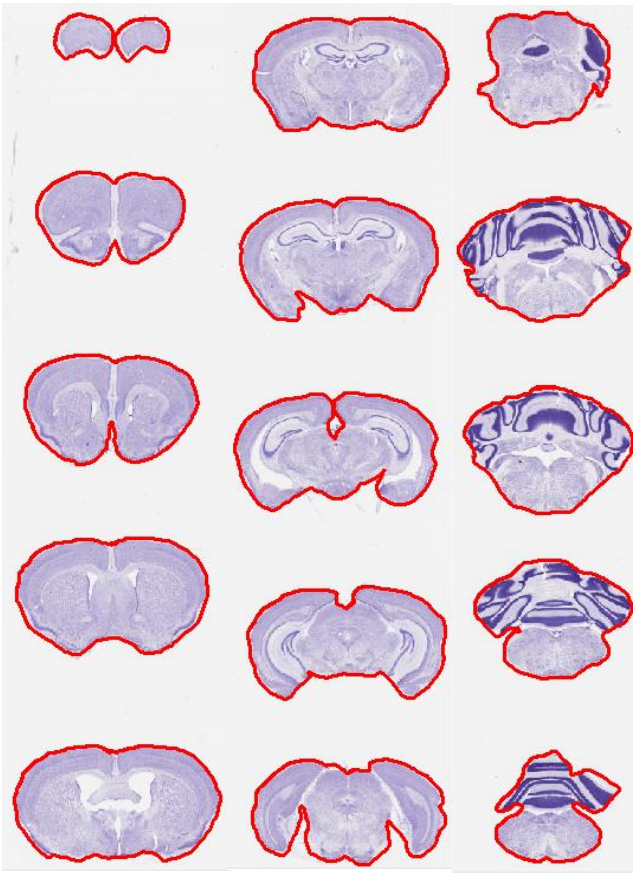
(t(7)=0.78, p=0.46; 2 CASPR2 and 1 HC IgG-exposed brains were excluded from analysis due to damage to sections containing the hippocampus) or cerebellum (t(10)=0.17, p=0.87) (Figure 6-2, Figure 6-3A, Figure 6-4 and Figure 6-5). Likewise, there were no differences in the thickness of corpus callosum (t(10)=0.26, p=0.8), in the thickness of prefrontal cortex, including prelimbic cortex (t(10)=1.71, p=0.12), infralimbic cortex (t(10)=1.45, p=0.18) or anterior cingulate cortex (t(10)=0.14, p=0.89), or in the thickness of somatosensory cortex (overall: t(10)= 0.21, p=0.84; layer I: t(10)=0.38, p=0.71; layer II-IV: t(10)=0.96, p=0.36; and layer V-VI: t(10)=1.12, p=0.29) (Figure 6-3B, Figure 6-6 and Figure 6-7). A detailed morphometric description across treatment groups is provided in Table 6-1. In summary, there were no gross morphological or morphometric differences between groups in any of the parameters analysed.

6.3 Analysis of neuronal ectopia / neuronal misalignments

An increase in the number of ectopic neurons in the corpus callosum and in deep cortical layers was reported in the *Cntnap2* knockout mice model (Penagarikano, Abrahams et al. 2011), as evidence for neuronal migration abnormalities in the mutant mice. However, in our model, neurons were found in the corpus callosum in all treatment groups, as assessed by NeuN (neuronal nuclei) staining, and there were no differences in neuronal densities in this region between CASPR2 and HC IgG-exposed animals (t(2.04)=0.56, p=0.63) (Figure 6-8).

Neuronal migration abnormalities were also assessed in the somatosensory cortex. Cortical layers were identified with CUX1 (marker for neurons in layers II-III) and FOXP2 (marker of neurons in layer VI). Laminar position patterns were analysed using those two markers (Figure 6-9). A qualitative assessment of the FOXP2-positive neurons did not reveal any differences between CASPR2 and HC IgG-exposed groups, but there were differences in the number of CUX1-positive cells found in the deeper layers; in one case

A



B

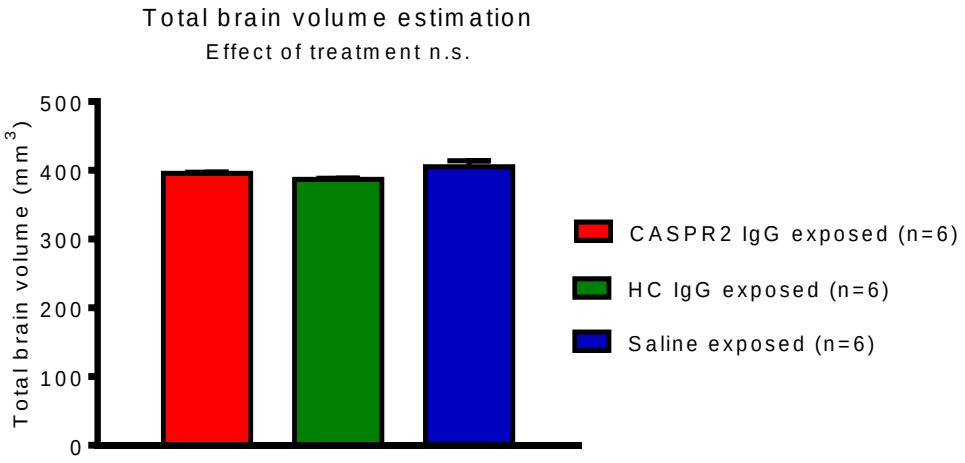
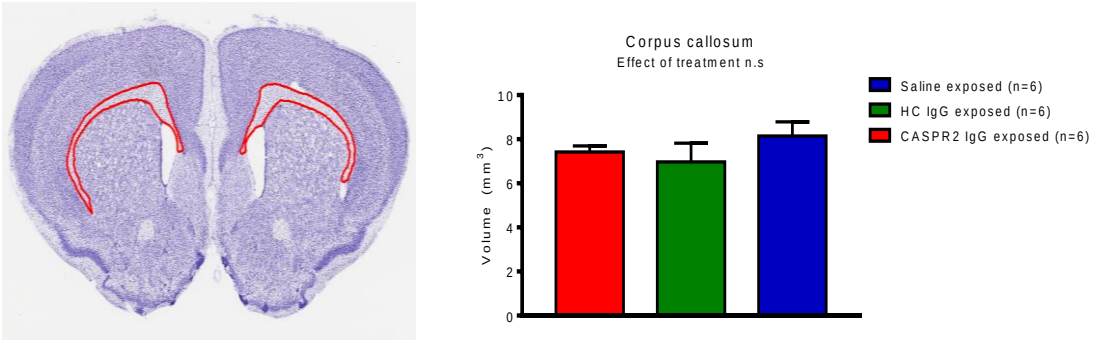


Figure 6-2: Total brain volume in adult offspring

(A) Representative image of total brain area quantification across one brain. Total brain area was then used for estimation of brain volume, through application of the Cavalieri's principle. Figure B: No differences found between CASPR2 and HC IgG-exposed animals in the total brain volume of the adult offspring (12 months), (n=6 brains/group). Data presented as mean±SEM.

A



B

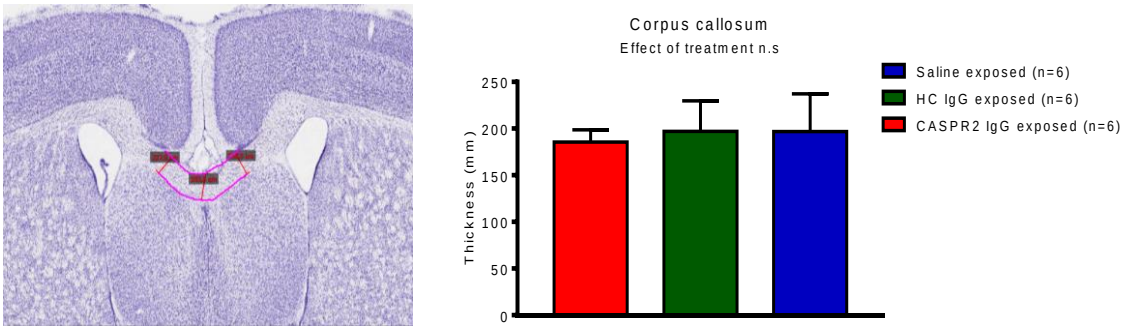


Figure 6-3: Corpus callosum volume and thickness in adult offspring

(A) Representative image of the histological boundaries used to measure corpus callosum area (left) and estimated corpus callosum volume of the adult offspring, (n=6 brains/group) (right). No differences found between CASPR2 and HC IgG-exposed animals. (B) Representative image of corpus callosum thickness measurements in the mid-sagittal area (left) and estimated corpus callosum thickness (n=6 brains/group) (right). No differences found between CASPR2 and HC IgG-exposed animals. Data presented as mean±SEM. Measurements performed by Dr Menassa.

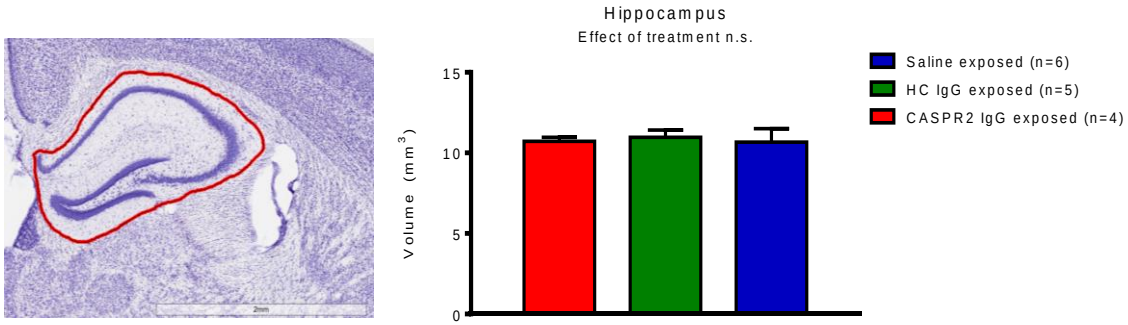


Figure 6-4: Hippocampal volume in adult offspring

Representative image of the histological boundaries used to measure hippocampal area (left) and estimated hippocampal volume of the adult offspring, (n=6 brains/group). No differences found between CASPR2 and HC IgG-exposed animals. Data presented as mean±SEM.

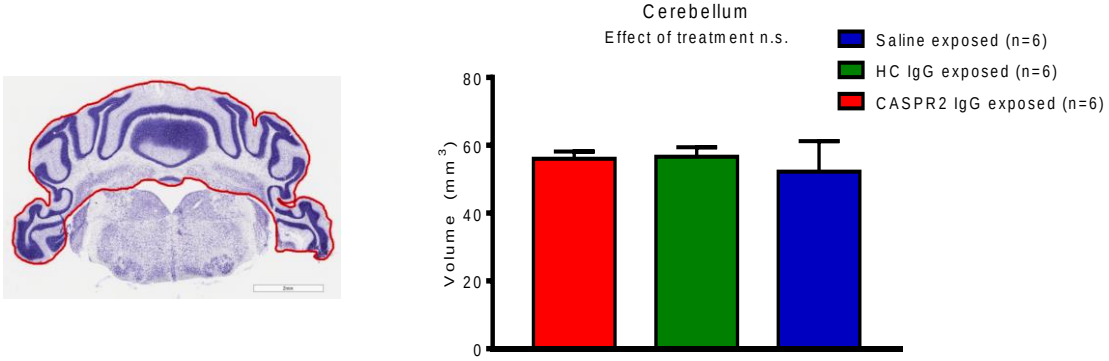


Figure 6-5: Cerebellum volume in adult offspring

Representative image of the histological boundaries used to measure cerebellar area (left) and estimated cerebellar volume of the adult offspring, (n=6 brains/group) (right). No differences found between CASPR2 and HC IgG-exposed animals. Data presented as mean±SEM.

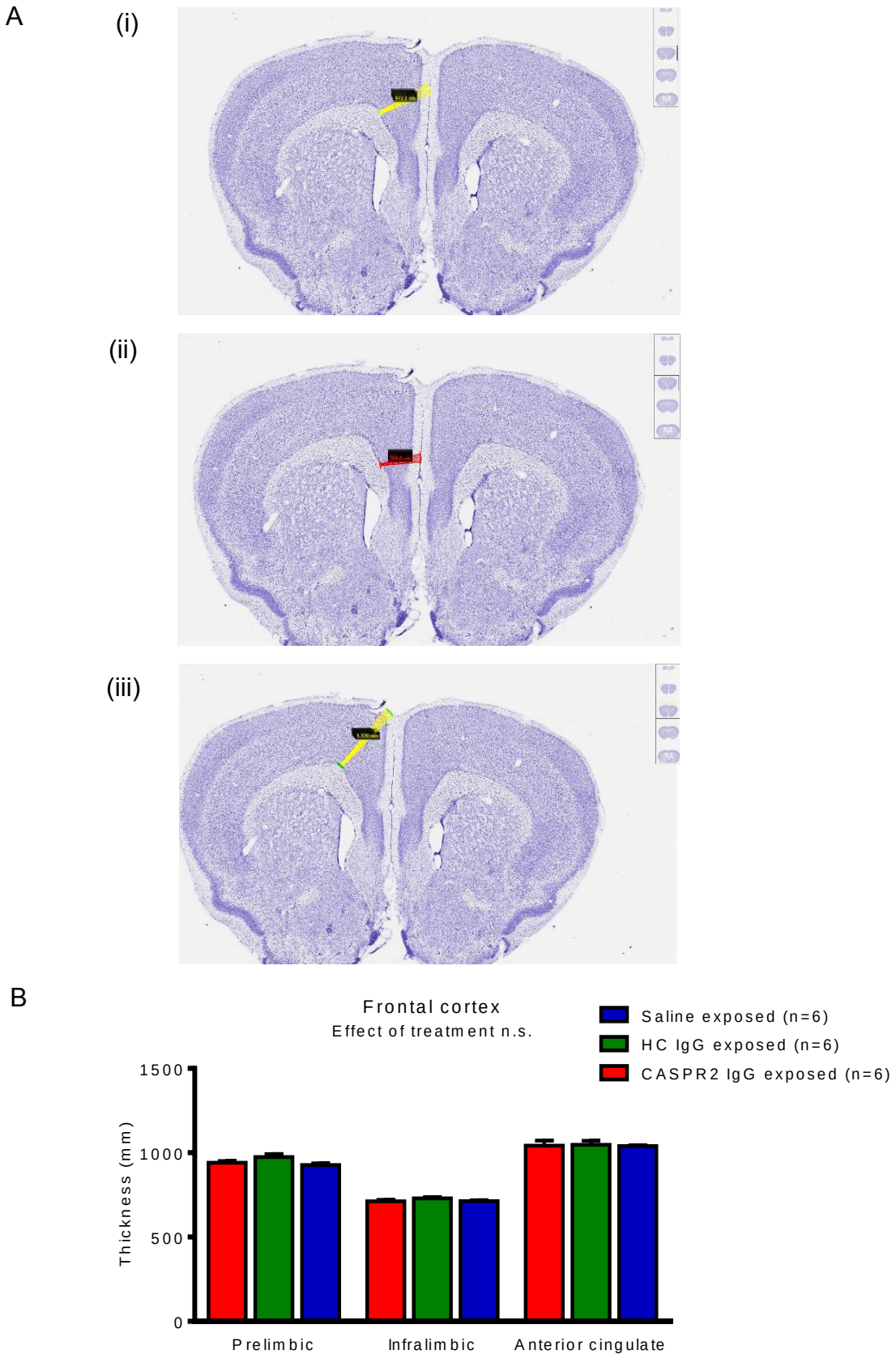
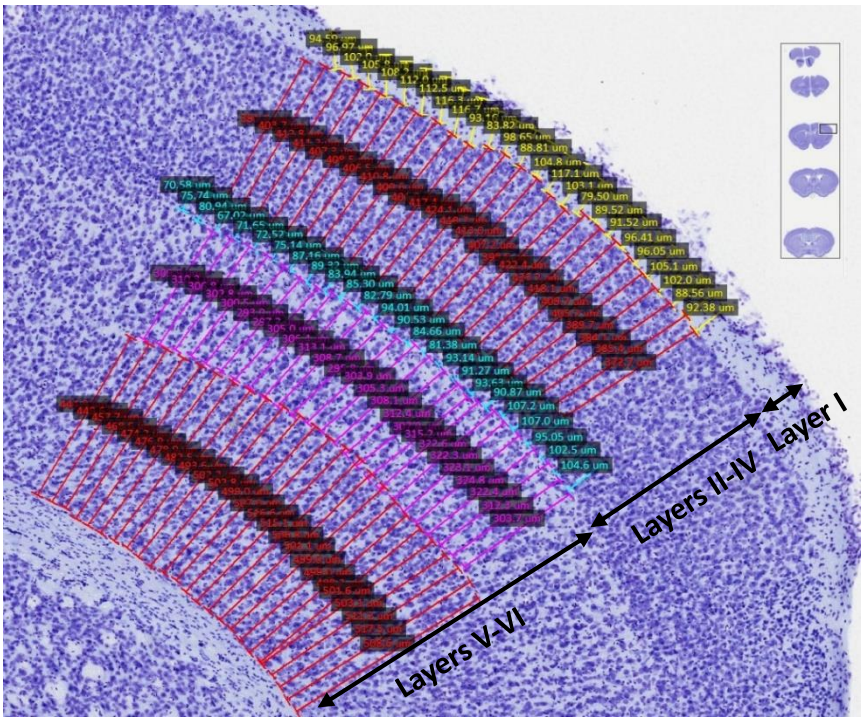


Figure 6-6: Prelimbic, infralimbic and anterior cingulate cortex thickness

(A) Representative image of the histological boundaries used to define the different prefrontal cortex areas: (i) prelimbic; (ii) infralimbic; (iii) anterior cingulate. (B) No differences found between CASPR2 and HC IgG-exposed animals in the thickness of total cortical layer in the prefrontal cortex areas (n=6 brains/group). Data presented as mean±SEM. Measurements performed by Dr Menassa.

A



B

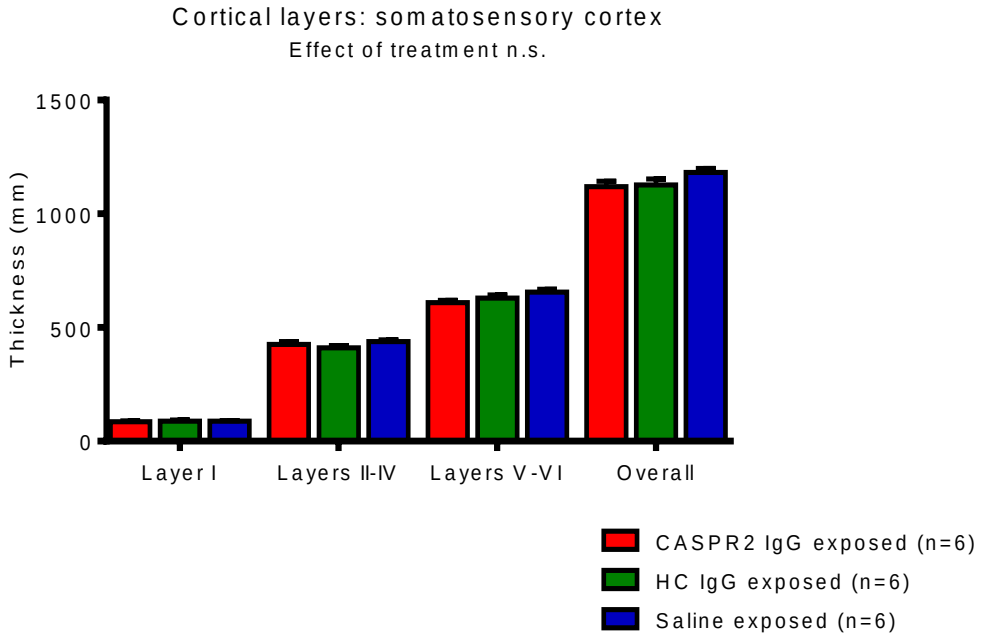


Figure 6-7: Cortical layers of the somatosensory cortex

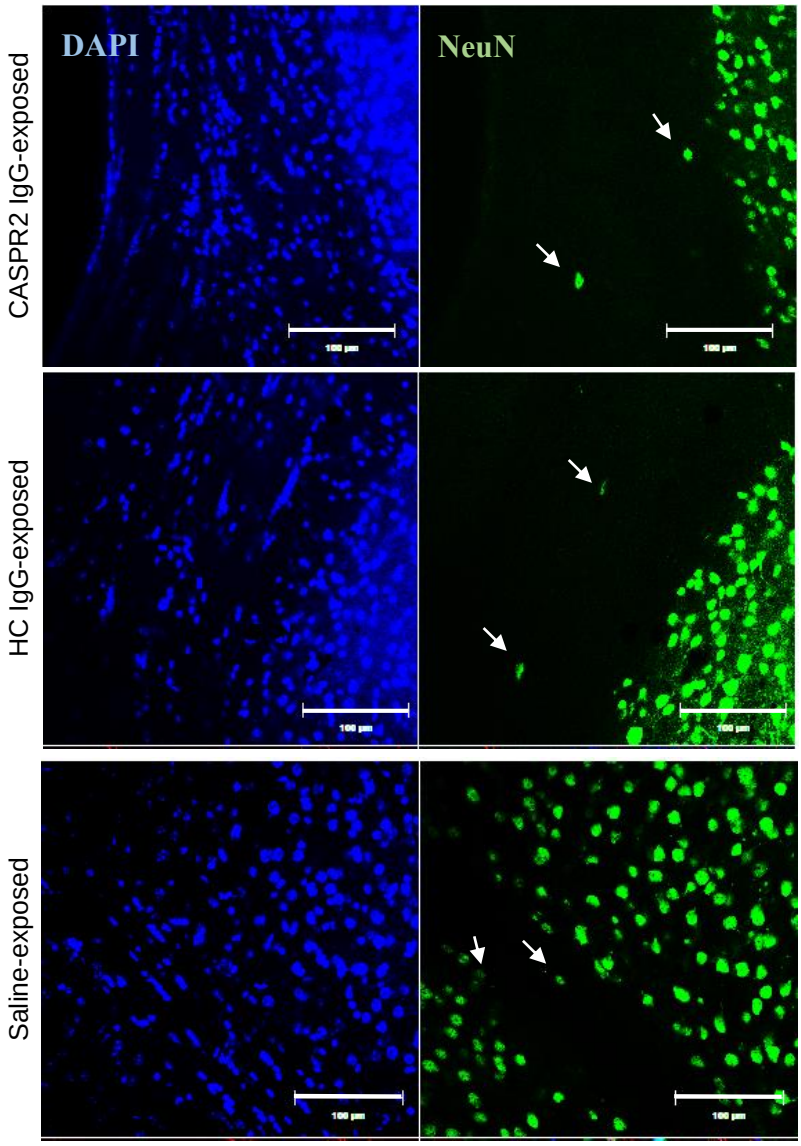
(A) Representative image of the histological boundaries used to define the different cortical layers. (B) No differences found between CASPR2 and HC IgG-exposed animals in the thickness of the cortical layers in the somatosensory cortex, (n=6 brains/group). Data presented as mean±SEM.

Table 6-1: Morphometric analysis across all treatment groups in the adult offspring (n=6 brains / group)

	CASPR2 IgG-exposed (mean ±SEM)	HC IgG-exposed (mean ±SEM)	Saline-exposed (mean ±SEM)	ANOVA p-value*
Total brain volume	385.6 (±7.0)	380.8 (±8.2)	397.6 (±7.1)	n.s.
Corpus callosum				
Volume	7.0 (±0.4)	7.3 (±0.3)	8.2 (±0.2)	n.s.
Thickness	185.5 (±13.2)	191.1 (±16.9)	212.3 (±10.1)	n.s.
Hippocampus				
Volume	8.8 (±1.8)	7.4 (±2.4)	10.7 (±0.2)	n.s.
Cerebellum				
Volume	56.0 (±2.1)	56.6 (±2.8)	52.2 (±3.7)	n.s.
Prefrontal cortex				
Prelimbic thickness	941.1 (±9.9)	974.6 (±16.9)	927.1 (±9.2)	n.s.
Infralimbic thickness	711.3 (±9.9)	729.5 (±7.7)	713.0 (±4.7)	n.s.
Anterior cingulate thickness	1041.9 (±30.1)	1047.3 (±22.9)	1038.8 (±5.4)	n.s.
Somatosensory cortex				
Layer I thickness	85.3 (±4.3)	87.8 (±5.2)	87.7 (±3.2)	n.s.
Layer II-IV thickness	425.6 (±12.3)	410.3 (±10.1)	438.2 (±7.4)	n.s.
Layer V-VI thickness	608.8 (±11.0)	628.8 (±14.1)	655.4 (±12.4)	n.s.
Overall	1119.6 (±22.9)	1127.0 (±26.3)	1181.2 (±18.4)	n.s.

All values mm (thicknesses) or mm^3 (volumes). *ANOVA with Bonferroni correction: non-significant (n.s.) if $p>0.05$

A



B

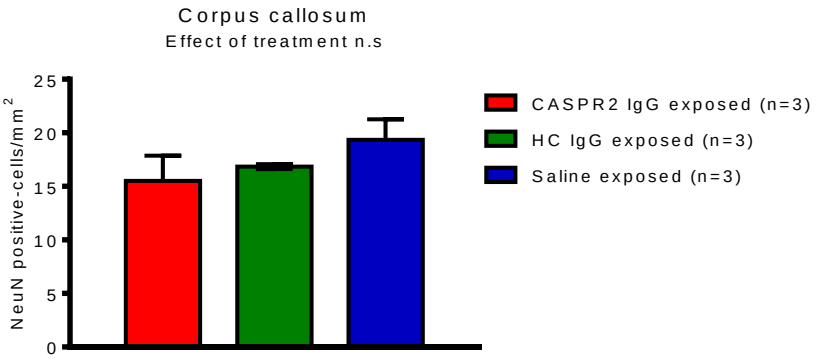


Figure 6-8: Quantification of ectopic neurons in the corpus callosum

(A) Representative images of the corpus callosum showing small number of ectopic neurons (arrows); DAPI, blue; Neuronal Nuclei (NeuN), green. Scale bar: 100 μm; (B) No differences found between CASPR2 and HC IgG in the neuronal densities in the corpus callosum (n=3 brains /treatment group). Data presented as mean±SEM. Confocal imaging and counts performed by Dr Menassa.

this seemed to correlate with an abnormal distribution of these cells in the upper layers (highlighted by white arrow in Figure 6-10A). Quantification of CUX1-positive cells in the deeper layers was then performed, revealing a significantly higher density of these cells in the deeper layers in CASPR2 IgG-exposed animals ($t(4)=3.63$, $p=0.02$), suggesting a neuronal migration defect (Figure 6-10B). The prefrontal cortex, in particular the anterior cingulate cortex, was also assessed for abnormalities of the cortical layers, but no differences were detected between CASPR2 and HC IgG-exposed mice upon visual inspection (data not shown). To determine what type of neurons where expressing CUX1 in layers V/VI, the gabaergic and glutamatergic markers GAD65/67 and Glutaminase were used. Most, but not all, CUX1-positive neurons were glutamatergic, both in CASPR2 and HC IgG-exposed animals. No CUX1-positive neuron was gabaergic (Figure 6-11).

For the cerebellum, Purkinje cell line alignment in a monolayer was assessed qualitatively across groups and no differences were found. Although Purkinje cells densities were slightly higher in the CASPR2 IgG-exposed group, the results were statistically non-significant ($t(4)=0.84$, $p=0.45$). GFAP-stained sections of the cerebellum were also used to assess for signs of Bergmann gliosis, which is usually reactive to Purkinje cells hypoplasia/loss (Love, Budka et al. 2015). There was no evidence for Bergmann gliosis in any of the brains analysed (Figure 6-12)

6.4 Histological analysis of the hippocampus

Cntnap2 knockout mice were shown to have reactive astrocytosis after the onset of seizures, which occur spontaneously in these mutant mice (Penagarikano, Abrahams et al. 2011). No seizures were observed in our CASPR2 IgG-exposed mice, however the same analysis of the hippocampus was conducted. The hippocampus was qualitatively assessed for signs of astrogliosis in the CA4 region and neuronal loss in this region and

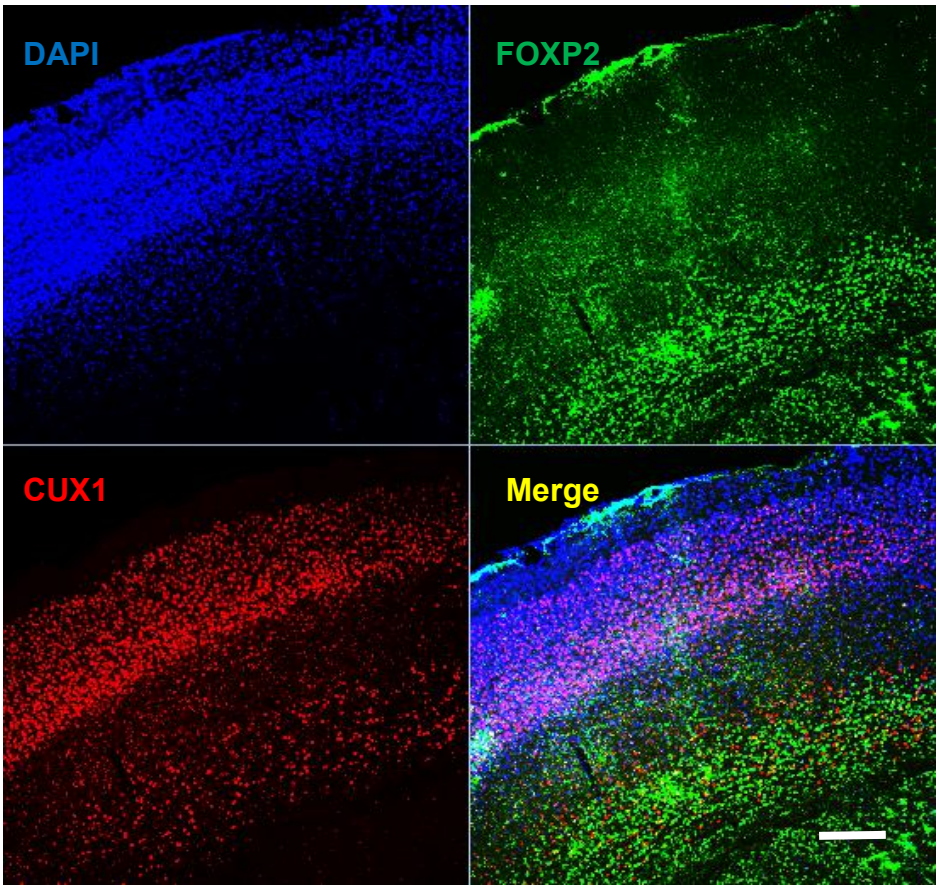


Figure 6-9: Laminar position assessment of cortical neurons

Representative images of the cortical levels demarcation using CUX1 (red) and FOXP2 (green) antibodies in a control animal. Scale bar:200 μm. Staining and confocal imaging done by Dr Menassa.

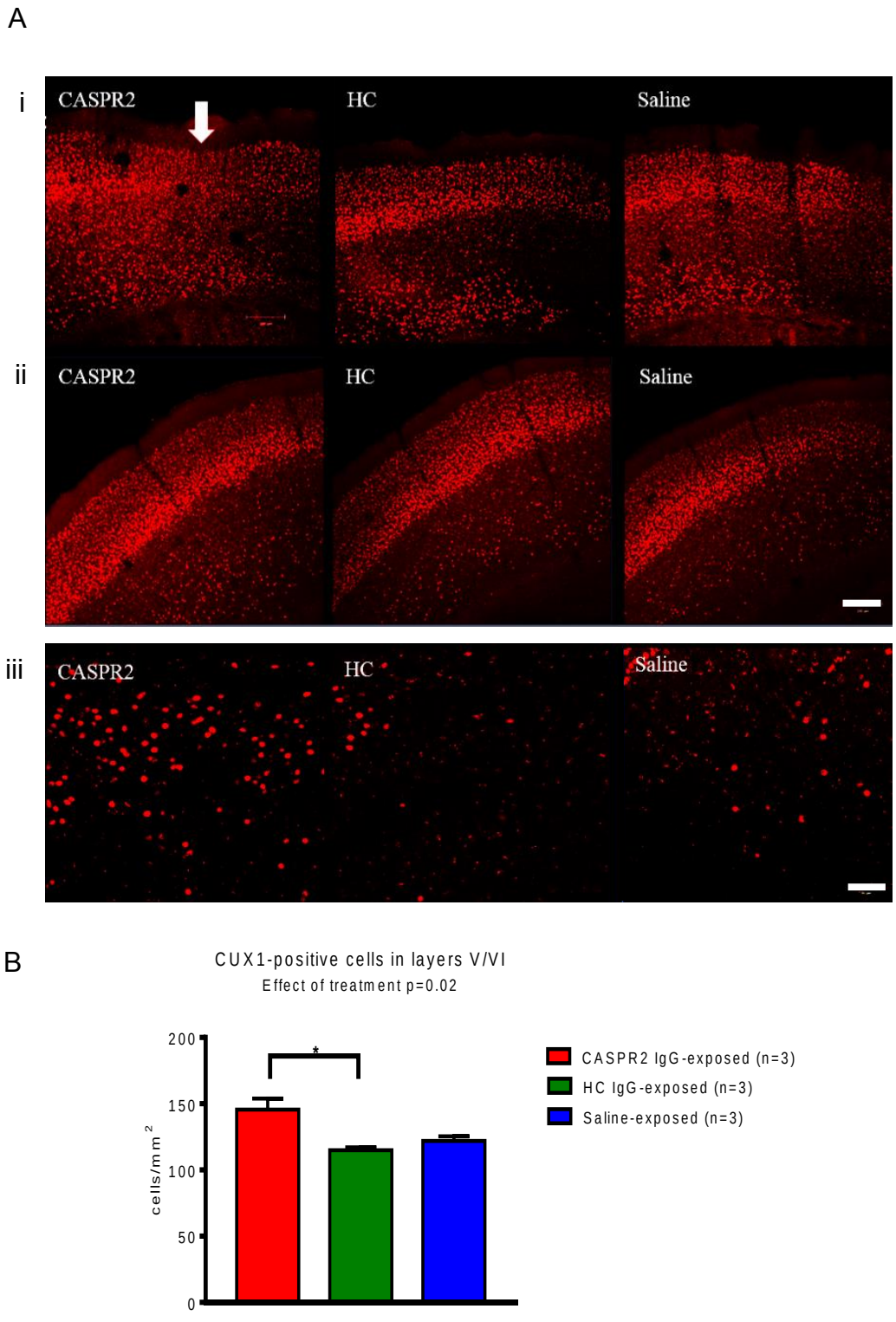


Figure 6-10: CUX1-positive cells in layers V/VI of the somatosensory cortex

(A) Representative images of the CUX1 staining across treatment groups at low (i; ii) and high magnification (iii), focusing on layers V/VI. White arrow in figure i highlights an area of abnormal distribution of CUX1-positive cells in layers II-IV in one CASPPR2 IgG-exposed animal. Scale bars: 200 μm (i; ii) and 50 μm (iii). (B) A statistically significant increase in CUX1-positive cells in layers V-VI was found in CASPR2 versus HC IgG-exposed animals (n=3 brains/group). Data presented as mean±SEM. Staining and confocal imaging performed by Dr Menassa. Cell counts done by Dr Menassa and myself.

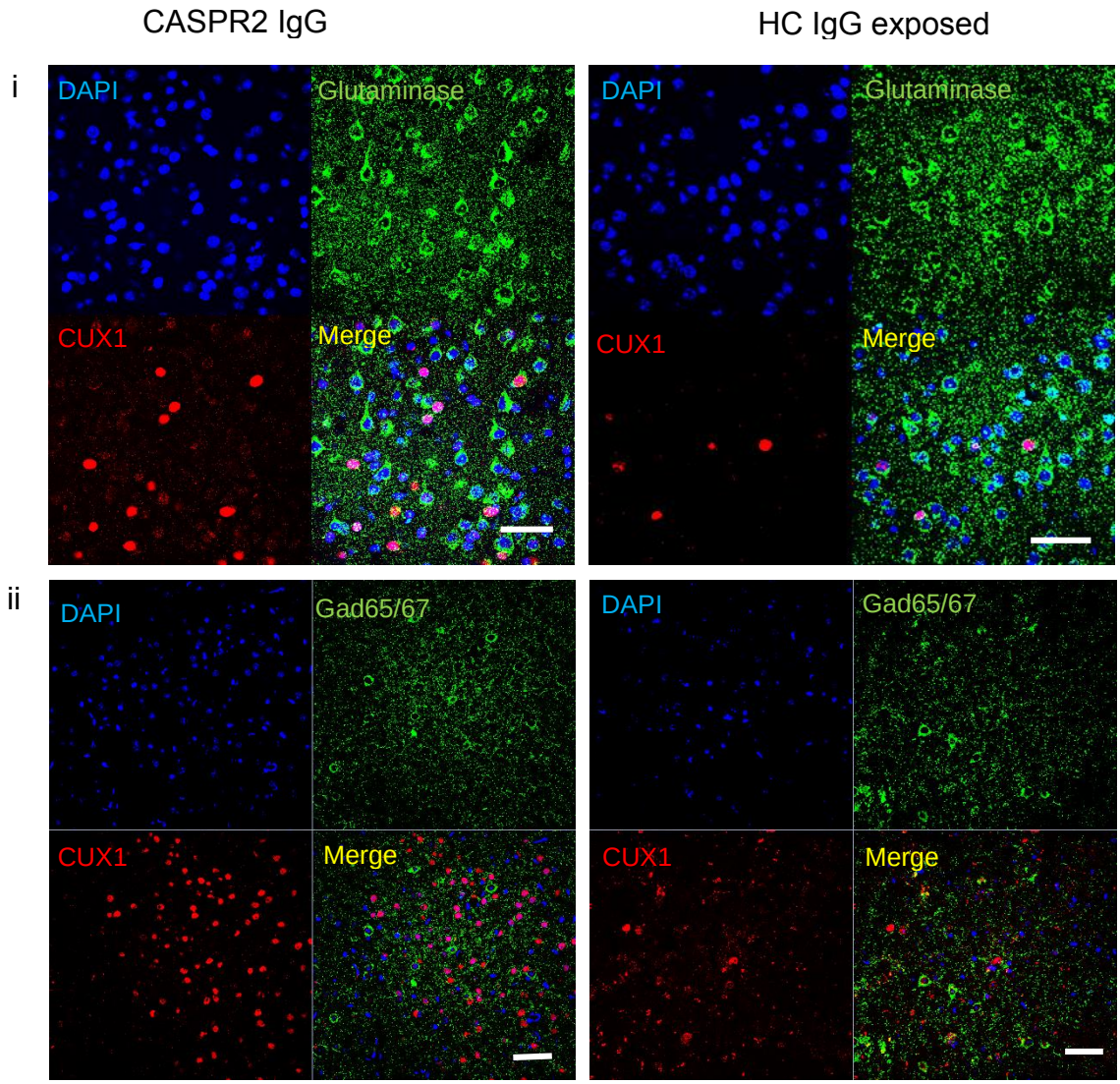


Figure 6-11: CUX1/Glutaminase and CUX1/GAD65_67 staining of layers V/VI neurons.

(A) Representative images of the CUX1 (green)/ glutaminase (red) staining of neurons in layers V-VI in the somatosensory cortex. Scale bar: 50 μm. Staining and confocal imaging performed by Dr Menassa. (B) Representative images of the CUX1 (red)/ gad 65/67 (green) staining of neurons in layers V-VI in the somatosensory cortex. Scale bar: 50 μm.

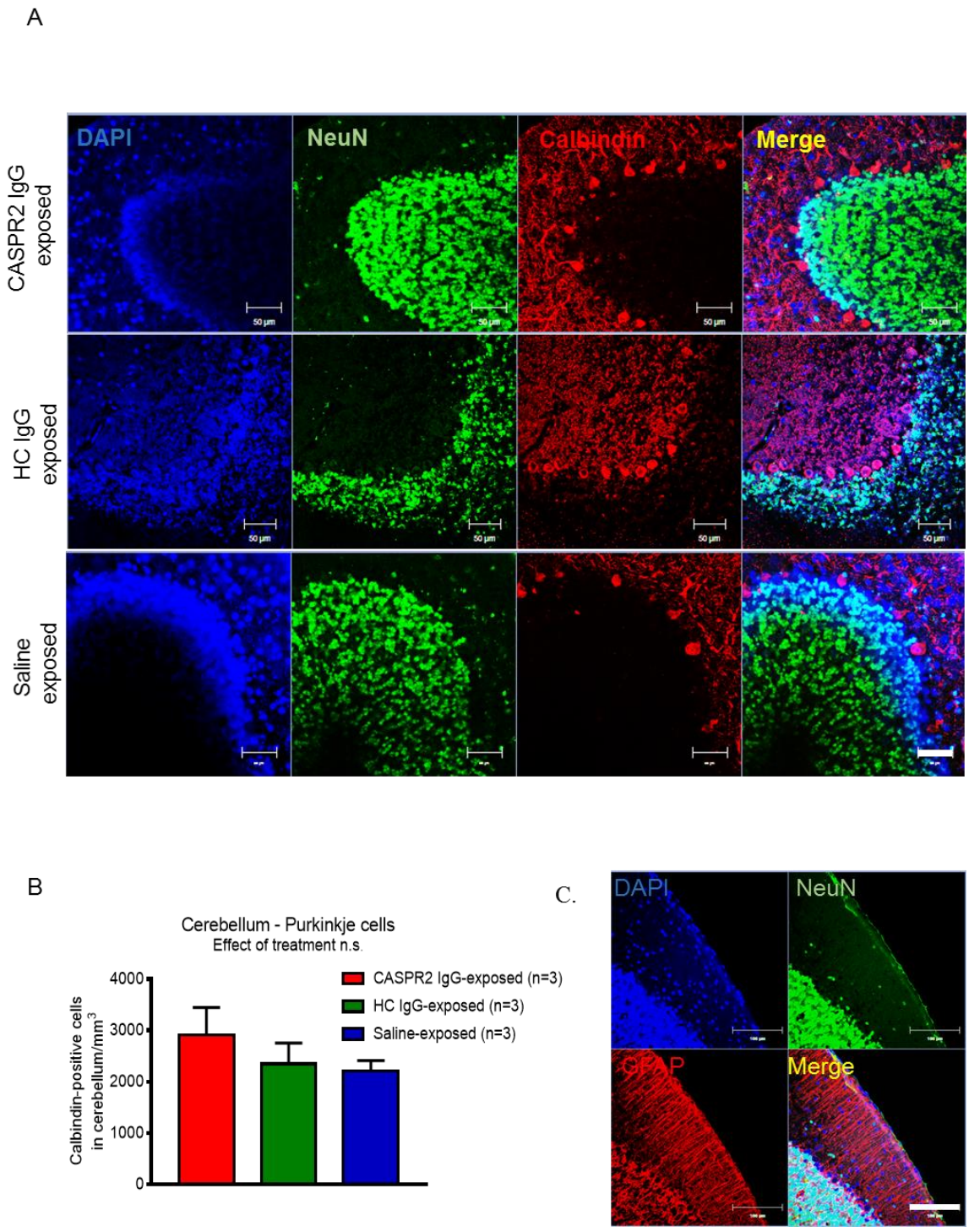


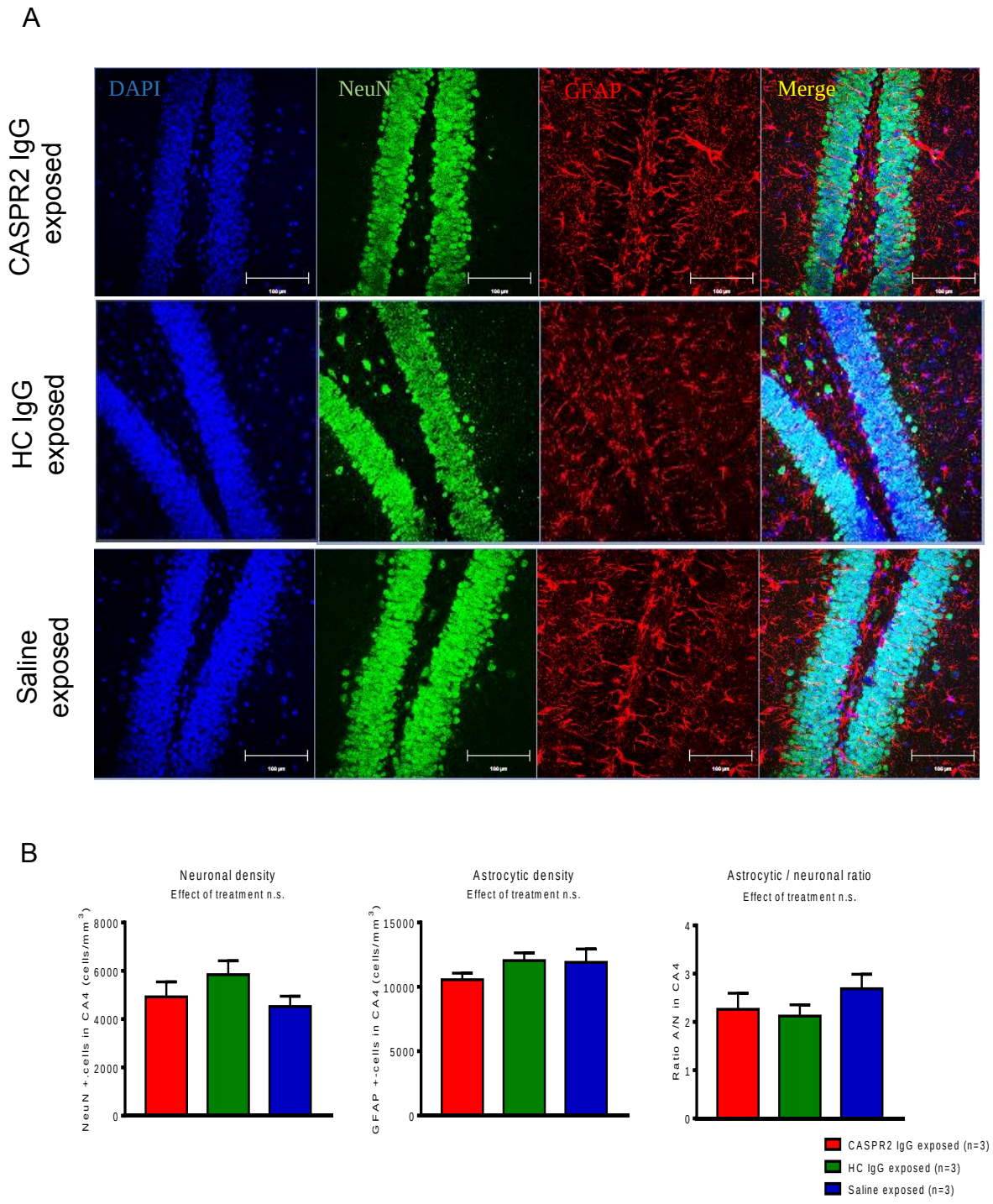
Figure 6-12: Histological characterization of the cerebellum

(A) Representative images showing normal Purkinje cell line in all treatment groups (DAPI, blue; Neuronal Nuclei (NeuN), green; Calbindin, red).Scale bar: 50 μm (B) No differences found across treatment groups in the Purkinje cells density (n=3 brains /group). Data presented as mean±SEM. (C) Representative image of normal Bergmann's glia (DAPI, blue; Neuronal Nuclei (NeuN), green; Glial-fibrillary acidic protein (GFAP), red) in a CASPR2 IgG exposed animal. Scale bar: 100 μm. NeuN/Calbindin staining, confocal imaging and cell counts done by Dr Menassa.

the CA3 and CA1 regions. Astrocytic and neuronal densities in this area were determined through quantification of GFAP and NeuN-positive cells, respectively. There were no differences in neuronal density ($t(4)=0.89$, $p=0.42$), astrocytic density ($t(4)=1.55$, $p=0.2$) or in the astrocyte to neuron ratio ($t(4)=0.28$, $p=0.8$) between CASPR2 and HC IgG-exposed animals (Figure 6-14).

6.5 Microglia analysis

Microglia are the resident macrophages of the CNS, have well-established functions in the event of disease or trauma to the brain, and are also critical regulators in normal brain development (Frost and Schafer 2016). To identify activated microglia in the brain, an anti-CD68 antibody was used and sections containing prefrontal and somatosensory cortex were stained. The presence of CD68-positive cells was quantified in the prelimbic, infralimbic and somatosensory cortices. There was a 15 to 43% increase in mean microglial density in the CASPR2 IgG-exposed animals, across all layers of the studied areas (Figure 6-14). In the prelimbic cortex, after Holm-Sidak correction for multiple comparisons, there was a statistically significant difference between CASPR2 and HC IgG-exposed animals in all layers (layer I: $t(12)=4.4$; adjusted $p=0.0016$; layers II-IV: $t(12)=3.9$; adjusted $p=0.0023$; layer V-VI: $t(12)=4.8$; adjusted $p=0.0012$). In the infralimbic, the difference was statistically significant after correction in layers V-VI only, although there was a strong trend in the other layers (layer I: $t(12)=2.5$; adjusted $p=0.056$; layers II-IV: $t(12)=2.1$; adjusted $p=0.061$; layer V-VI: $t(12)=3.6$; adjusted $p=0.012$). Finally, in the somatosensory cortex, the same trend was observed in all layers, which was statistically significant in layers II-IV (layer I: $t(12)=2.5$; adjusted $p=0.057$; layers II-IV: $t(12)=2.9$; adjusted $p=0.038$; layer V-VI: $t(12)=2.5$; adjusted $p=0.057$). In the studied areas, there was an increase in reactive microglia found in adulthood in the animals exposed to CASPR2 IgG during neurodevelopment, when compared to those exposed to HC IgG.



Fig

(A) Representative images of the CA4 area of the hippocampus showing neuronal and astrocytic populations; DAPI, blue; Neuronal Nuclei (NeuN), green; Glial-fibrillary acidic protein (GFAP), red. Scale bar: 100 μm. (B) No differences found across treatment groups in the neuronal density, astrocytic density or astrocytic/neuronal ratio in the CA4 area of the hippocampus (n=3 brains /group). Data presented as mean±SEM. Confocal imaging and cell counts done by Dr Menassa.

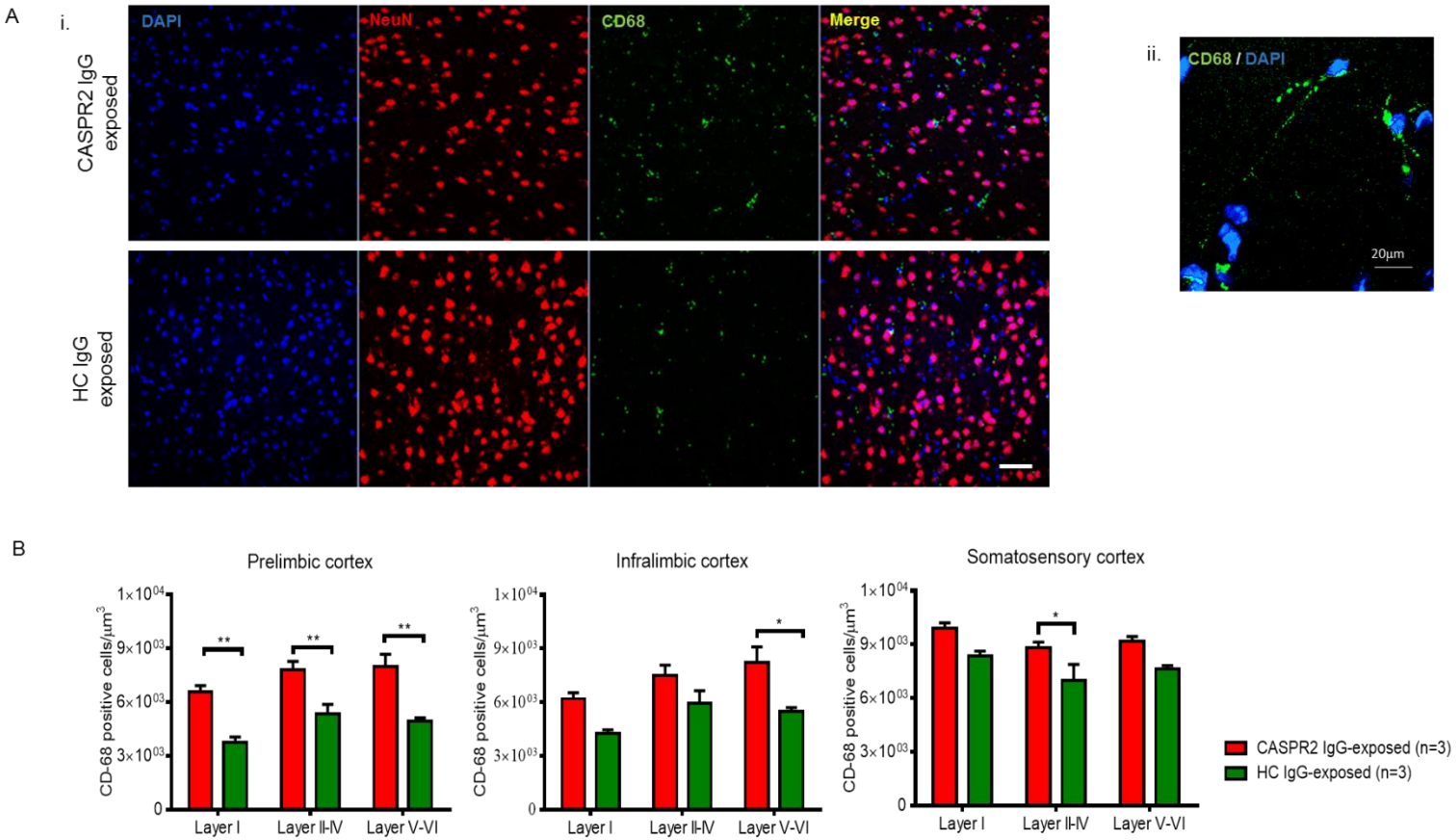


Figure 6-14: Reactive microglia densities in the prefrontal and somatosensory cortices

(A) i: Representative images of the somatosensory cortex showing reactive microglia; CD68, green; DAPI, blue; Neuronal Nuclei (NeuN), red in CASPR2 IgG and HC IgG-exposed brains. Scale bar: 50µm. ii: Morphological detail of CD68-stained microglia at higher magnification. Scale bar: 20 µm. (B) Statistically significant differences found in microglial densities between CASPR2 and HC IgG-exposed animals in the prelimbic, infralimbic and somatosensory cortex (n=3 brains /group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Data presented as mean±SEM. Confocal imaging and cell counts done by Dr Menassa.

6.6 Synaptic profiles

Considering the role of microglia in regulating the maturation and elimination of synapses both in the embryonic and in the postnatal brain (Roumier, Pascual et al. 2008, Paolicelli, Bolasco et al. 2011), an analysis of the synaptic profiles was conducted in the same areas where an increase of microglia densities was shown. Tissue was stained for PSD-95 and synaptophysin. PSD-95 is a protein located primarily in neural postsynaptic densities and is enriched in glutamatergic synapses (Keith and El-Husseini 2008), while synaptophysin is a membrane glycoprotein that occurs in the presynaptic vesicles. Quantification was done for the PSD-95 alone.

There was a 15 to 52% reduction in the number of PSD-95 positive synaptic profiles across all cortical layers of the prelimbic, infralimbic and somatosensory cortices in the CASPR2-exposed animals. In the prelimbic cortex, there was statistically significant difference between CASPR2 and HC IgG-exposed animals in layers I and II-III and a strong trend in the deep layers (layer I: $t(12)=7.0$; adjusted $p=0.00004$; layers II-III: $t(12)=4.9$; adjusted $p=0.0007$; layer V-VI: $t(12)=2.1$; adjusted $p=0.055$). In the infralimbic cortex there was a statistically significant decrease in all layers (layer I: $t(12)=5.1$; adjusted $p=0.0005$; layers II-III: $t(12)=7.3$; adjusted $p=0.00003$; layer V-VI: $t(12)=4.1$; adjusted $p=0.001$). In the somatosensory cortex, there was also a statistically significant decrease in all layers (layer I: $t(12)=3$; adjusted $p=0.02$; layers II-IV: $t(12)=4.5$; adjusted $p=0.002$; layer V-VI: $t(12)=2.2$; adjusted $p=0.049$) (Figure 6-15). In summary, we detected a decrease in synapses, in practice glutamatergic synapses, in the adult brains of CASPR2 IgG-exposed animals.

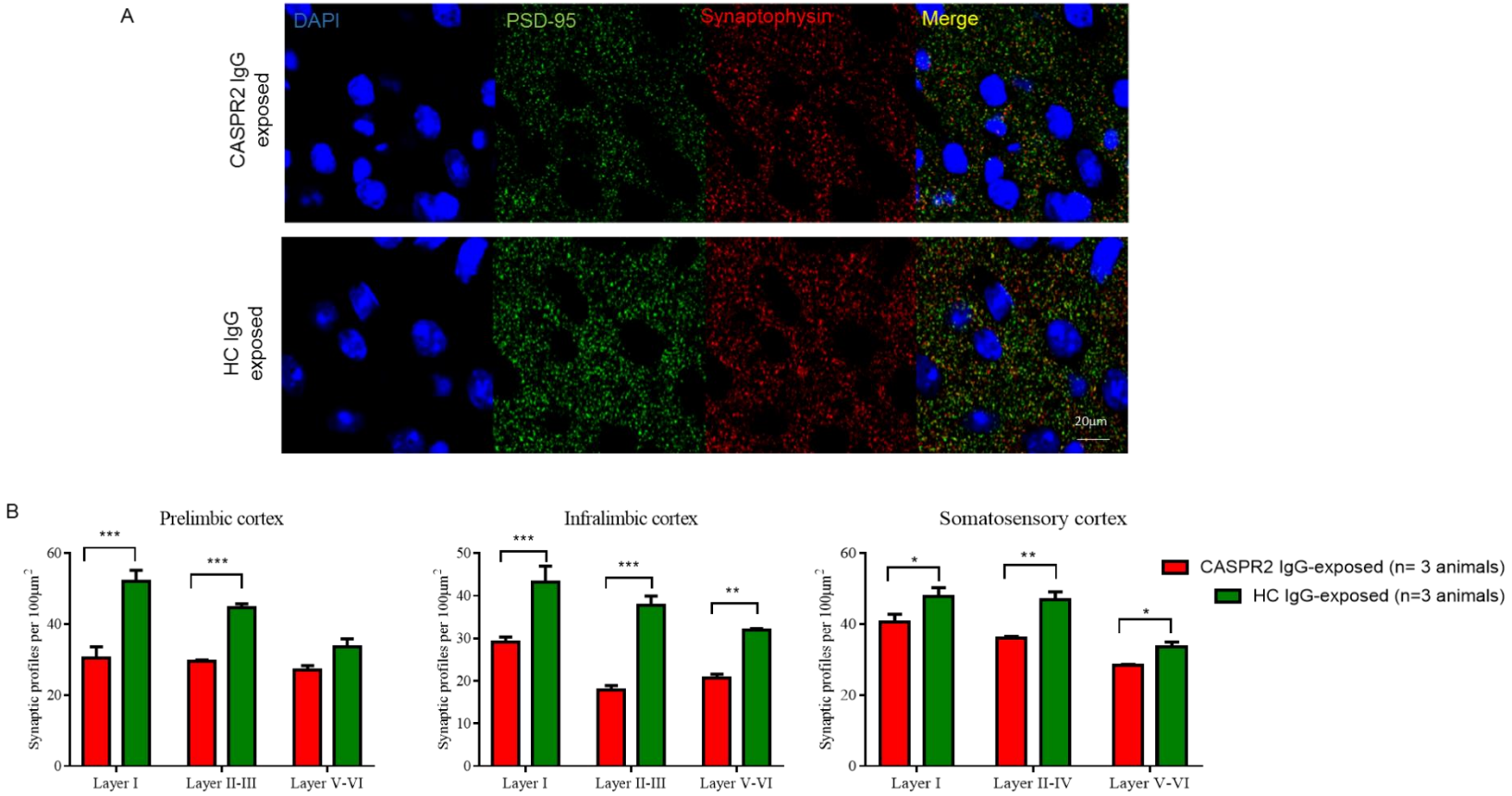


Figure 6-15: PSD-95 profiles in the prefrontal and somatosensory cortices

(A) Representative image of the staining for PSD-95 (green) and synaptophysin (red) in the somatosensory cortex of a CASPR2 and a HC IgG-exposed brain. Scale bar: 20µm. (B) Statistically significant differences found in the number of PSD-95 synaptic profiles between CASPR2 and HC IgG-exposed animals in the prelimbic, infralimbic and somatosensory cortex (n=3 brains /group). * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Data presented as mean±SEM. Staining and confocal imaging by Dr Menassa. Quantification analysis by Dr West.

6.7 Neuronal densities

To further explore whether the reduction in synaptic profiles detected in CASPR2 IgG-exposed mice was due to an effect on the synapses themselves or a consequence of a reduction in neuronal densities, NeuN positive-cells were quantified in the prelimbic, infralimbic and somatosensory cortex. There were no differences between CASPR2 and HC IgG-exposed animals in the medial prefrontal cortex. In the somatosensory cortex, there was a significant decrease in the neuronal densities in layer II-IV, but not in layer I or layers V-VI (layer I: adjusted p=0.8; layers II-IV: adjusted p=0.02; layer V-VI: adjusted p=0.1) (Figure 6-16).

6.8 Discussion

In this chapter, the results of a detailed analysis on the brains of mice exposed to CASPR2 IgG *in utero* are presented. Mice exposed to CASPR2 IgG in *utero* did not show any gross morphological or morphometric changes but had abnormalities in the distribution of neurons in cortical layers, increased reactive microglial densities and decreased number of glutamatergic synapses in the prefrontal and somatosensory cortex.

The importance of CASPR2 during brain development is still not fully understood, nor the mechanisms by which it contributes to neuronal organisation and function. It is known that patients with homozygous mutations of *CNTNAP2* have histological abnormalities

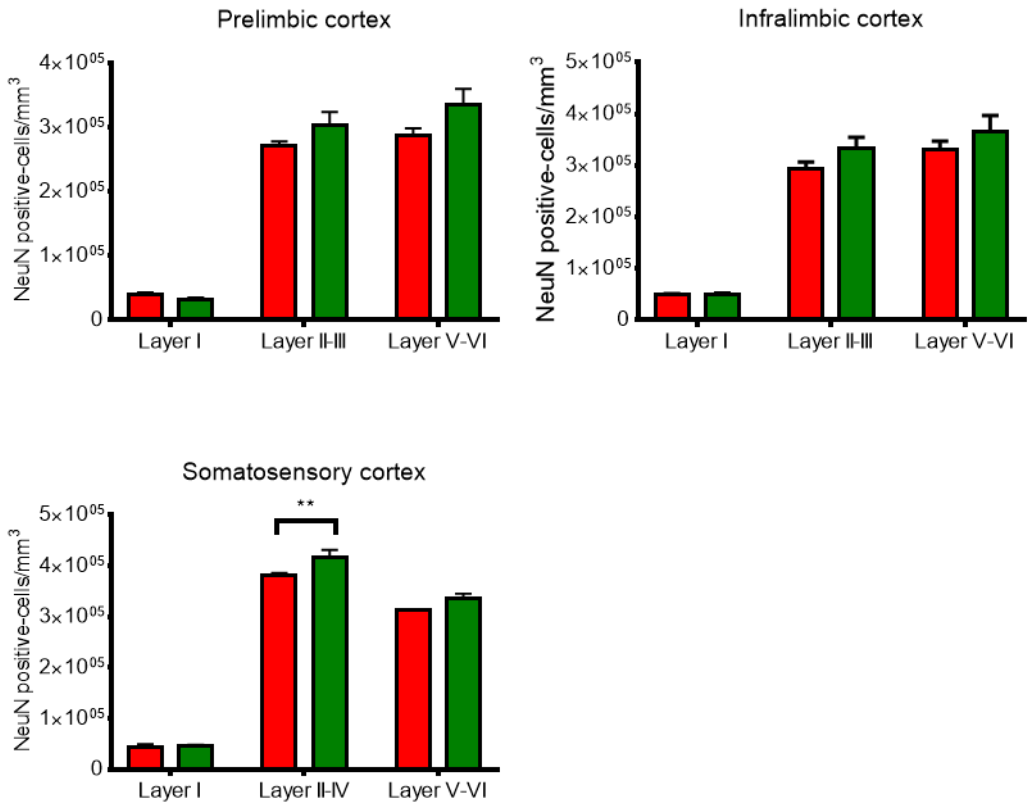


Figure 6-16: Neuronal densities in the prefrontal and somatosensory cortices

No statistically significant differences in the neuronal cell counts in the prelimbic and infralimbic cortices (n=3 brains /group), but significant difference in layers II-IV in the somatosensory cortex, between CASPR2 and HC IgG-exposed animals . * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Data presented as mean±SEM. Staining and confocal imaging by Dr Menassa. Quantification analysis by Dr West.

of the brain that include cortical dysplasia and neuronal disorganisation (Strauss, Puffenberger et al. 2006), while the *Cntnap2* knockout mice displays ectopic neurons in the corpus callosum, abnormalities of the cytoarchitecture of the somatosensory cortex and reduction of inhibitory gabaergic interneurons (Penagarikano, Abrahams et al. 2011). In our model, we also detected an increase in CUX1 positive-neurons in the lower layers which, given the data on patients with the *CNTNAP2* mutations and on the knockout model, likely represents an abnormality in the normal migration of cortical projection neurons and not a change in cell fate identity. However, we have not performed the BrdU neuron birthdating experiments that would allow us to firmly reach this conclusion. CUX1 neurons have previously been shown to be glutamatergic (Ferrere, 2006) and so were the majority of neurons found to be in the deeper cortical layers in the CASPR2 IgG-exposed mice. However, some of the Cux-1-positive neurons didn't express glutaminase or GAD65/67, and their identity is unknown. Interestinlgy, a significant reduction in neuronal counts was detected in layers II-IV of the somatosensory cortex. It is possible that this reduction relates to a reduced number of neurons that migrated to their final position, given the increase in CUX-1 positive-cells in the deeper cortical layers of CASPR2 IgG-exposed mice. However, CUX-1 positive-cells were not quantified in the upper layers.

We next explored the effects of CASPR2 IgG exposure *in utero* on microglia activation. Microglia activation has been shown to occur as a consequence of early immune activation in animal models of neurodevelopmental disorders (Hornig, Weissenbock et al. 1999) (Juckel, Manitz et al. 2011) and post-mortem human tissue studies demonstrated abnormally reactive microglia in brain regions relevant to the physiopathology of several neuropsychiatric disorders, many of which are now considered to be neurodevelopmental, such as autism or schizophrenia (reviewed in (Frick, Williams et al. 2013)). Microglia regulate neuronal progenitor cell numbers by actively initiating cell death and by engulfing dead or dying cells (Cunningham, Martinez-

Cerdeno et al. 2013) and it is involved in both initial wiring of the embryonic brain and maturation of synapses in the postnatal brain. (Roumier, Pascual et al. 2008) (Paolicelli, Bolasco et al. 2011). It is also interesting to note that several genetic human diseases occur due to mutations in microglial surface receptors (CSF1R, DAP12, TREM2) that manifest in adulthood as mood, social, cognitive deficits or psychosis and early-onset progressive dementia (Rademakers, Baker et al. 2012) (Paloneva, Kestila et al. 2000) (Colonna and Wang 2016). All these findings highlight the importance of microglial cells during neurodevelopment and the potential for causing long-term behavioural abnormalities if defective, making them interesting to study in animal models of neurodeveopmental diseases. An increase in reactive microglia densities was shown in CASPR2 IgG exposed animals in the prefrontal and somatosensory cortices using the CD68 marker (marcrosialin / ED1). Although CD68 has been used extensively in the literature as a marker of activated microglia (Mosher, Andres et al. 2012) (Supramaniam, Vontell et al. 2013), one must note that other defining characteristics of microglia activation, either other histological markers (for instance, HLA-DR) or morphological phenotype, were not analysed, which may constitute a limitation to our finding.

Of relevance, a decrease in the number of glutamatergic synapses was shown in the same brain areas, and this was shown not to relate to loss of neurons. An improved method of staining using pepsin-retrieval, and previously shown to specifically stain PSD-95 puncta (Fukaya and Watanabe 2000) (Polgar, Watanabe et al. 2008), was used, which together with an automated counting method allowed accurate quantification of thousands of puncta per layer. Naturally, there is no direct evidence that these two findings are related, but based on the accepted role of microglia in synaptic pruning (Paolicelli, Bolasco et al. 2011), one can postulate that these findings reflect an early disruption in this physiological mechanism, but further experiments would be necessary to establish causality.

It is unknown the mechanism by which CASPR2 antibodies might cause these changes. It is possible that these antibodies cause cell death or destabilization of synapses and then activation of microglia that is perpetuated beyond the original insult. On the other hand, these antibodies might have a direct effect on activating the microglia, eventually by complement activation, and this in turn would lead to excessive synaptic pruning. Ideally, this would be further explored by histological analysis of the embryonic brains, which was impossible to perform during the time of my research project. These findings, although preliminary given the low number of brains analysed (3 brains/ group), raise new questions regarding the role of maternal antibodies and their effects on microglia-dependent synaptic development, which will hopefully be pursued in the future.

In conclusion, the work described in this chapter showed that CASPR2 IgG-exposed mice *in utero* have persistent histological abnormalities in late adult life, and that are characterized by abnormalities in the distribution of neurons in cortical layers, increased reactive microglial densities and decreased number of glutamatergic synapses in the prefrontal and somatosensory cortices.

Chapter 7: General discussion

The recent history of autoimmune neurology is notable for the discovery of many antibody-mediated disorders that cause diseases of the CNS (Crisp, Kullmann et al. 2016). This led to the realization that many patients who, until recently had not been identified, have treatable autoimmune conditions. Following these discoveries, the diagnostic algorithms changed in many neurology subspecialties and there has been increasing interest as to whether these antibodies are also relevant in patients with psychiatric disorders (Coutinho, Harrison et al. 2014). However, the consequences of exposure to these antibodies during neurodevelopment has hardly been considered.

The work in this thesis aimed at filling this gap in our current knowledge by studying the relationship between maternal antibodies towards neuronal surface proteins and the neurodevelopment of the foetus. This work showed, through the study of maternal samples from a Danish historic cohort, that the presence of antibodies to neuronal surface proteins in the maternal circulation, particularly CASPR2 antibodies, can be associated with an increased risk of mental retardation and disorders of psychological development in the progeny. A mouse model of maternal-to-foetal transfer was established to explore experimentally the relationship between maternal CASPR2 antibodies and neurodevelopmental disorders in the offspring. This animal model showed that mice exposed *in utero* to CASPR2 antibodies have long term behavioural sequelae, manifested as deficits in social interaction and social activities, and increased repetitive, non-social behaviours. The brain of these mice provided evidence of an abnormal migration of cortical projection neurons, increased microglial activation and reduction in the number of glutamatergic synapses in adult life, confirming that there had been long-term changes in neurodevelopment.

These novel data should stimulate further discussion into the role of maternal antibodies to neuronal proteins in altered brain development, as well as the interaction between immune regulation / glial cell function and neural circuitry development. Major areas of debate and future directions are highlighted below.

Autoantibodies in mothers of children with and without neurodevelopmental disorders

Our study suggests that a subgroup of children with neurodevelopmental disorders will have been exposed to antibodies towards a neuronal surface protein. This subgroup of children represents a small proportion of the affected children, approximately 5%, as expected given the highly heterogeneous nature of these disorders. Nevertheless, the identification of this subgroup is of crucial importance. Not only could these antibodies serve as biomarkers, allowing the identification of at-risk children, and facilitating diagnosis and early intervention, but could also lead to treatment or preventive measures. The best example of the potential benefits from identifying mothers with pathogenic antibodies during gestation comes from another antibody-mediated disorder, myasthenia gravis. Women with AChR antibodies are usually advised to achieve clinical remission / stabilisation before pregnancy (Norwood, Dhanjal et al. 2014). When this is not possible, the offspring can benefit from treatment during pregnancy, particularly in the cases of AMC (Hacohen, Jacobson et al. 2015). However, even before treatments were available, not all babies developed neonatal myasthenia gravis (Namba, Brown et al. 1970), which suggests that the final outcome is influenced by many other factors, such as genetic background or other immune effectors. The interpretation of antibodies targeting CNS proteins is even more complex, as factors such as permeability of the blood-brain barrier or the relationship of timing of exposure with period of brain development are likely to influence the outcome. In fact, in our study, we found antibodies towards CNS proteins in both mothers of children with neurodevelopmental disorders

and in mothers of healthy progeny, highlighting the importance of further research into associated predisposing factors. Maternal infections are of particular interest in this context, given that they can lead to a pro-inflammatory maternal immune activation state (Blomstrom, Gardner et al. 2015) and are known to be risk factors for neurodevelopmental disorders (Abdallah, Hougaard et al. 2012). Unfortunately, we did not have access to this information. Likewise, the efficacy of immunotherapy in this scenario requires further research. It is possible that more selective therapeutics, eventually targeting the nFcR and thus preventing the antibody transfer, could be beneficial and should also be pursued. An anti-rat nFcR monoclonal antibody was shown to ameliorate disease symptoms and reduce the levels of pathogenic antibodies in an animal model of myasthenia gravis (Liu, Garcia et al. 2007). This was achieved due to increased degradation of IgG as a consequence of the blockade of the nFcR present on the endothelial cells, which usually re-circulates IgG, prolonging its half-life. A similar action in the placental nFcR would be expected.

The foetal blood brain barrier

It is well established that IgG antibodies are efficiently transferred from the maternal to the foetal circulation (Garty, Ludomirsky et al. 1994, Malek, Sager et al. 1994, Palmeira, Quinello et al. 2012). It is also well established that the transfer of human antibodies occurs from dam to foetus in murine models (Jacobson, Polizzi et al. 1999). Thus, it was expected that CASPR2 antibodies would transfer from the dam into the foetal circulation in our model, as indeed was demonstrated. However, whether these antibodies would cross the foetal blood-brain barrier was uncertain, particularly in the absence of other factors that are known to cause permeabilisation, such as infections or inflammatory cytokines (reviewed in (Almutairi, Gong et al. 2016)). The common notion that the foetal blood-brain barrier is “leaky” is unfounded, since the foetal brain has tight junctions at several barrier interfaces from a very early stage in development, such as the blood brain

barrier (composed of tight junctions between endothelial cells) and the blood CSF barrier (composed of tight junctions between choroid plexus epithelial cells) (reviewed in (Saunders, Liddelow et al. 2012)). In addition, a CSF-brain barrier is also present during foetal development (disappearing later in life), which is formed by strap junctions between neuroependymal cells. In the mouse, this barrier disappears postnatally and molecules diffuse freely from CSF to brain parenchyma from P20 onwards (Whish, Dziegielewska et al. 2015). Also, there is very limited evidence on the crossing of human IgG into the foetal mouse parenchyma after peripheral injection, with only one study demonstrating histologically that human IgG could be found in the brain parenchyma in foetal mice up until E17.5 (Braniste, Al-Asmakh et al. 2014).

In our model, staining for human IgG in the embryonic brain, exposed to both patient and control IgG, also suggested its presence beyond the foetal vasculature. This poses questions on the pathway of passage for IgG. It is known that some proteins, like albumin, are transferred transcellularly through the choroid plexus epithelial cells; a transport mechanism dependent on the presence of specific receptors / protein-binding molecules in these cells, namely the SPARC (secreted protein acidic and rich in cysteine) and GYP A and B (Glycophorin A and B) (Liddelow, Temple et al. 2012). A specific transport mechanism for IgG, was described in the guinea pig (Zlokovic, Skundric et al. 1990). Interestingly, this study showed that this transport mechanism is saturated at physiological levels, thus providing evidence for a yet unknown carrier-mediated type of transport. The study of the mechanism by which these antibodies reach the foetal parenchyma, identification of the carrier protein and whether this is influenced by a maternal inflammatory response occurring at the periphery, is of relevance for the future, since it could potentially provide therapeutic targets.

Phenotypic and genetic overlap of neurodevelopmental disorders and implications for animal modelling

Traditional diagnostic categories for neurodevelopmental disorders represent umbrella terms for collections of heterogeneous syndromes characterized by considerable phenotypic and genetic overlap. Core symptoms can be shared among different disorders, for instance social deficits are a core symptom in schizophrenia, autism and in some cases of mental retardation; and also different neurodevelopmental disorders have a common genetic cause (reviewed in (Zhu, Need et al. 2014)). A perfect example of this heterogeneity is the multitude of neuropsychiatric and neurodevelopmental disorders associated with disruptions in the *CNTNAP2* gene (reviewed in (Rodenas-Cuadrado, Ho et al. 2014). It is possible that dose effects of gene disruption or extrinsic factors serve as modulators on the same genetic background. Conversely, disruption of different genes can lead to the same clinical phenotype. For instance, both mutations in the *FMR1* (*Fragile X mental retardation 1*) gene or the *CNTNAP2* gene can cause an autistic or intellectual disability phenotype. This can be explained by convergence towards a core set of neurodevelopmental pathways involved in synapse and circuit formation. It is perhaps logical, therefore, to suppose that different maternal antibodies could lead to a specific neurodevelopmental disorder and that a specific maternal antibody could cause different neurodevelopmental disorders in the progeny. This highlights the need for future studies aiming at a full clinical characterization of the progeny of mothers with the antibodies identified here and other studies using novel antigen discovery methods in these and other cohorts.

The phenotypic overlap of neurodevelopmental disorders will also have implications for the interpretation of results from animal models. A classical approach to modelling of neurodevelopmental disorders has been to study similarities between behavioural assays in rodents and domains of psychopathology in humans (Mitchell, Huang et al. 2011). Given the clinical overlap of these disorders, it is easy to understand that an

interpretation of behavioural deficits as a specific disease trait is difficult and even misleading. In our model, CASPR2 IgG-exposed animals had deficits in social interaction and repetitive non-social behaviours, which can be interpreted as correlates of symptomatic features seen in autism, schizophrenia and mental retardation (Cervantes and Matson 2015). It is thus more accurate to interpret them in the context of long-term behavioural deficits caused by neurodevelopmental abnormalities rather than a specific disease manifestation. This is highlighted by the fact that in the human cohorts, maternal CASPR2 antibodies were associated with a range of neurodevelopmental disorders, such as mental retardation, speech and language impairments and, in fact, undefined neurodevelopmental disorders.

CASPR2 and neuronal migration defects

The normal functioning of the neocortex is highly dependent on two crucial processes that occur during neurodevelopment: the correct migration of neurons into their final position in one of the six cortical layers and the acquisition of distinct neuronal identities and formation of precise synaptic connections (reviewed in (Kwan, Sestan et al. 2012)). Two main types of migration have been identified. The main type, known as radial migration, occurs when cells migrate from the dorsal germinal zones, the ventricular and subventricular zones, towards the most superficial cortical layers, the marginal zone. This is the route for excitatory glutamatergic projection neurons. The inhibitory interneurons are generated in the ventral forebrain, in the medial and caudal ganglionic eminences, and then undergo a tangential migration, descending towards the subventricular zone before finally migrating radially to their specific laminar position (Anderson, Eisenstat et al. 1997) (Tamamaki, Fujimori et al. 1997). This neuronal migratory activity is a highly orchestrated set of events. Dysfunction in any of the intervening molecules can lead to neurodevelopmental disorders, as it was shown in

cases of cortical malformations, schizophrenia, epilepsy or autism (reviewed in (Valiente and Marin 2010)).

Despite the evidence, from both patients and animal models, that CASPR2 is essential for normal neuronal migration during development, the mechanism by which CASPR2 is involved in this process is still unknown. An *in vitro* study using induced pluripotent stem cells from two individuals with a heterozygous deletion in the *CNTNAP2* gene showed aberrantly reduced migration in one proband, a patient with schizophrenia, using a neurosphere migration assay (Lee, Carvalho et al. 2015). However, the molecular mechanism was not explored or even postulated in this study. CASPR2 is a cell-adhesion molecule, so we can hypothesise that it may be involved in neuron-glia interactions, which are known to be essential for radial migration (Rakic 1972) (Nadarajah, Alifragis et al. 2003). Another possibility relates to the second mechanism of neuronal movement across the neocortex, somal translocation (Nadarajah, Alifragis et al. 2003). The intracellular portion of CASPR2 is anchored to actin via protein 4.1B (Denisenko-Nehrbass, Oguievetskaia et al. 2003). Disruption of actin can have profound influences on neuronal migration, as documented experimentally (Rivas and Hatten 1995), and as known to occur in patients with mutations that lead to destabilization of actin, such as the X-linked dominant periventricular heterotopia (Fox, Lamperti et al. 1998). Our CASPR2 IgG-exposed mice showed abnormalities in cortical patterning, which could result from an interference by the antibody with the participation of the protein in one of these mechanisms of neuronal movement during development. A better understanding of CASPR2 function in cortical migration is necessary.

CASPR2 and synaptic development

The role of CASPR2 in synapse formation is still largely unknown, despite being the subject of recent studies (Varea, Martin-de-Saavedra et al. 2015) (Anderson, Eisenstat

et al. 1997). *Cntnap2* knockout or knockdown neurons *in vitro* were shown to have reduced density of spines, particularly glutamatergic synapses, and an abnormality of AMPA receptor morphology characterised by a deficit in AMPA trafficking to the spine membrane and accumulation as cytoplasmic aggregates (Varea, Martin-de-Saavedra et al. 2015). Another study showed that loss of CASPR2, in an *in vitro* knockdown model, causes a decrease in the total number of both excitatory and inhibitory synapses in a neuron, resulting in an abnormal neural circuit assembly that manifests as a change in network activity (Anderson, Galfin et al. 2012). In our model, we detected a decrease in excitatory synapses in mice exposed to CASPR2 IgG *in utero*, which could suggest an interference with CASPR2 in synaptic formation.

In the early stages of development, the nervous system begins with excess of neuronal connections. As the nervous system develops and the brain wiring is established, a process of synapse elimination ensues. Recent evidence has shown that this process is regulated by complement-mediated synaptic pruning (reviewed in (Stephan, Barres et al. 2012)). Using a classical model for activity-dependent developmental synapse elimination, the mouse retinogeniculate system, complement activation products were shown to modulate synapse formation during brain development. C1q and C3 (important molecules of the classical complement cascade) knockout mice exhibited sustained defects in synaptic refinement and elimination in the developing visual system (Stevens, Allen et al. 2007). A further study in C1q knockout mice showed enhanced excitatory synaptic connectivity manifested as increased epileptiform activity *in vivo* and *in vitro*, thus indicating the same role of complement in synapse elimination in the neocortex (Chu, Jin et al. 2010). Microglia have recently been implicated as the cellular key players of this complement-mediated synaptic pruning. It is now known that microglia engulf synaptic material during brain development and that this process is partly mediated by the microglial C3 receptor and its ligand the complement component C3 (Paolicelli, Bolasco et al. 2011, Schafer, Lehrman et al. 2012).

In our animal model, CASPR2 IgG-exposed mice showed both increased number of reactive microglia and decreased number of glutamatergic synapses, suggesting that excessive microglia-dependent pruning could have occurred. The role of CASPR2 antibodies in the onset of this disturbance is unclear. Knowing the role of CASPR2 in the development of glutamatergic synapses (discussed above), it is possible that mice exposed to these antibodies had an increased number of aberrant or weaker synapses that were then tagged for elimination. Another possibility is that the antibodies themselves have activated the complement cascade. Excessive complement activation during development could target synapses for elimination. Complement-mediated destruction of neurons is thought to be an important immune mechanism in patients with limbic encephalitis, as suggested by the neuropathological study of a biopsy from a patient with CASPR2 encephalitis (Kortvelyessy, Bauer et al. 2015). However, mice have a low serum complement activity that has, in fact, limited the use of mouse models in other complement- antibody-mediated disorders (Ratelade and Verkman 2014), which might make activation of complement a less likely mechanism in our animal model. Even though the exact mechanism of microglia activation was not fully explored, excessive synaptic pruning can alter neural connectivity and behaviour (Zhan, Paolicelli et al. 2014), which would explain the long-term behavioural sequelae observed in these mice. This supports the notion that neurodevelopmental disorders arise from disruptions in both neural circuitry and immune regulation/glial cell function pathways; two apparently distinct mechanisms that recent evidence on the function of glial cells in network pruning and circuitry assembly brought together.

Another interesting question relates to the perpetuation of the microglia activation after an initial insult during neurodevelopment. There has been no convincing evidence suggesting a mechanism by which microglia remains activated, even though excess of reactive microglia is seen in neuropathology studies from patients with different neuropsychiatric disorders (Vargas, Nascimbene et al. 2005, Wierzba-Bobrowicz T

2005, Morgan, Chana et al. 2010, Xue and Streit 2011). There seems to be a period in neurodevelopment when an immune challenge can induce long-term changes in microglia. In rodents, this period goes from E14 to the first postnatal week, corresponding in humans to the late second and third trimesters. One theory states that after an initial activation during this period, the microglia remain in the brain in a "primed" state (morphologically similar to activated cells but without chronically producing cytokines and other pro-inflammatory mediators) and that these "primed" cells are more likely to become overactivated later in life upon a second challenge (reviewed in (Bilbo and Schwarz 2012)).

In conclusion, the work in this thesis shows, for the first time, how an extrinsic factor, in this case a pathogenic maternal antibody, can alter fundamental neurodevelopmental events in a manner similar to that described in intrinsic genetic abnormalities. Importantly, these findings support a model in which maternal antibodies towards foetal neuronal proteins can cause long-term behavioural deficits and permanent abnormalities at the cellular and synaptic level in a subset of children with neurodevelopmental disorders (Figure 7-1).

As discussed above, many questions remain unanswered and require future work. The data collected in this thesis should stimulate further research, both epidemiological and experimental, into the role of these and other potentially pathogenic antibodies in disorders of brain development. The exact physiopathological mechanisms by which CASPR2 antibodies affect brain development during the foetal period are still largely unknown. An assessment of the intervening molecules (complement proteins C1q and C3, C3 receptor) and cell effectors in the neuron-glia axis in the context of CASPR2 antibody-exposure during development is needed. The extent to which those findings apply to other maternal neuronal-surface antibodies also needs assessment.

This is a novel field in autoimmune neurology, with the potential to change clinical assessment of many patients with neurodevelopmental disorders, and hopefully improve treatment and quality of life.

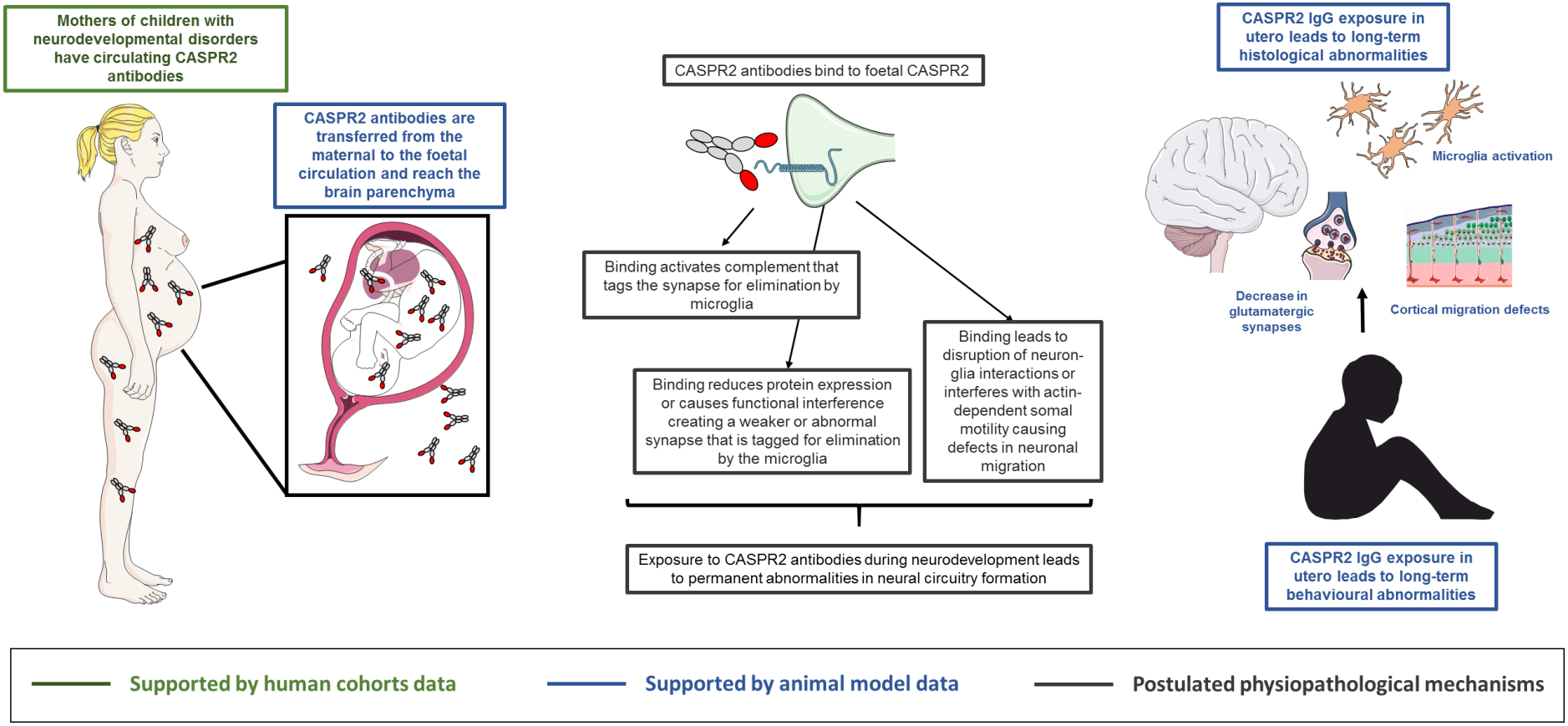


Figure 7-1: Proposed model

References

Abdallah, M. W., D. M. Hougaard, B. Norgaard-Pedersen, J. Grove, E. C. Bonefeld-Jorgensen and E. L. Mortensen (2012). "Infections during pregnancy and after birth, and the risk of autism spectrum disorders: a register-based study utilizing a Danish historic birth cohort." Turk Psikiyatri Derg **23**(4): 229-235.

Almutairi, M. M., C. Gong, Y. G. Xu, Y. Chang and H. Shi (2016). "Factors controlling permeability of the blood-brain barrier." Cell Mol Life Sci **73**(1): 57-77.

Anderson, G. R., T. Galfin, W. Xu, J. Aoto, R. C. Malenka and T. C. Sudhof (2012). "Candidate autism gene screen identifies critical role for cell-adhesion molecule CASPR2 in dendritic arborization and spine development." Proc Natl Acad Sci U S A **109**(44): 18120-18125.

Anderson, S. A., D. D. Eisenstat, L. Shi and J. L. Rubenstein (1997). "Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes." Science **278**(5337): 474-476.

Ashwood, P., P. Krakowiak, I. Hertz-Picciotto, R. Hansen, I. Pessah and J. Van de Water (2011). "Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome." Brain Behav Immun **25**(1): 40-45.

Atladottir, H. O., M. G. Pedersen, P. Thorsen, P. B. Mortensen, B. Deleuran, W. W. Eaton and E. T. Parner (2009). "Association of family history of autoimmune diseases and autism spectrum disorders." Pediatrics **124**(2): 687-694.

Baird, G., E. Simonoff, A. Pickles, S. Chandler, T. Loucas, D. Meldrum and T. Charman (2006). "Prevalence of disorders of the autism spectrum in a population cohort of children in South Thames: the Special Needs and Autism Project (SNAP)." Lancet **368**(9531): 210-215.

Barkus, C., M. Feyder, C. Graybeal, T. Wright, L. Wiedholz, A. Izquierdo, C. Kiselycznyk, W. Schmitt, D. J. Sanderson, J. N. Rawlins, L. M. Saksida, T. J. Bussey, R. Sprengel, D. Bannerman and A. Holmes (2012). "Do GluA1 knockout mice exhibit behavioral abnormalities relevant to the negative or cognitive symptoms of schizophrenia and schizoaffective disorder?" Neuropharmacology **62**(3): 1263-1272.

Baron-Cohen, S., F. J. Scott, C. Allison, J. Williams, P. Bolton, F. E. Matthews and C. Brayne (2009). "Prevalence of autism-spectrum conditions: UK school-based population study." Br J Psychiatry **194**(6): 500-509.

Bauman, M. D., A. M. Iosif, P. Ashwood, D. Braunschweig, A. Lee, C. M. Schumann, J. Van de Water and D. G. Amaral (2013). "Maternal antibodies from mothers of children with autism alter brain growth and social behavior development in the rhesus monkey." Transl Psychiatry **3**: e278.

Baumann, N. and D. Pham-Dinh (2001). "Biology of oligodendrocyte and myelin in the mammalian central nervous system." Physiol Rev **81**(2): 871-927.

Becker, E. B., L. Zuliani, R. Pettingill, B. Lang, P. Waters, A. Dulneva, F. Sobott, M. Wardle, F. Graus, L. Bataller, N. P. Robertson and A. Vincent (2012). "Contactin-associated protein-2 antibodies in non-paraneoplastic cerebellar ataxia." J Neurol Neurosurg Psychiatry **83**(4): 437-440.

Bender, A. N., S. P. Ringel, W. K. Engel, M. P. Daniels and Z. Vogel (1975). "Myasthenia gravis: a serum factor blocking acetylcholine receptors of the human neuromuscular junction." Lancet **1**(7907): 607-609.

Bergink, V., T. Armangue, M. J. Titulaer, S. Markx, J. Dalmau and S. A. Kushner (2015). "Autoimmune Encephalitis in Postpartum Psychosis." Am J Psychiatry **172**(9): 901-908.

Bergink, V., K. M. Burgerhout, K. Weigelt, V. J. Pop, H. de Wit, R. C. Drexhage, S. A. Kushner and H. A. Drexhage (2013). "Immune system dysregulation in first-onset postpartum psychosis." Biol Psychiatry **73**(10): 1000-1007.
Bergink, V., S. A. Kushner, V. Pop, H. Kuijpens, M. P. Lambregtse-van den Berg, R. C. Drexhage, W. Wiersinga, W. A. Nolen and H. A. Drexhage (2011). "Prevalence of autoimmune thyroid dysfunction in postpartum psychosis." Br J Psychiatry **198**(4): 264-268.
Bergink, V., T. M. Laursen, B. M. Johannsen, S. A. Kushner, S. Meltzer-Brody and T. Munk-Olsen (2015). "Pre-eclampsia and first-onset postpartum psychiatric episodes: a Danish population-based cohort study." Psychol Med **45**(16): 3481-3489.
Bien, C. G., A. Vincent, M. H. Barnett, A. J. Becker, I. Blumcke, F. Graus, K. A. Jellinger, D. E. Reuss, T. Ribalta, J. Schlegel, I. Sutton, H. Lassmann and J. Bauer (2012). "Immunopathology of autoantibody-associated encephalitides: clues for pathogenesis." Brain **135**(Pt 5): 1622-1638.
Bilbo, S. D. and J. M. Schwarz (2012). "The immune system and developmental programming of brain and behavior." Front Neuroendocrinol **33**(3): 267-286.
Bilimoria, P. M. and A. Bonni (2008). "Cultures of cerebellar granule neurons." CSH Protoc **2008**: pdb prot5107.
Blomstrom, A., R. M. Gardner, C. Dalman, R. H. Yolken and H. Karlsson (2015). "Influence of maternal infections on neonatal acute phase proteins and their interaction in the development of non-affective psychosis." Transl Psychiatry **5**: e502.
Boronat, A., J. M. Gelfand, N. Gresa-Arribas, H. Y. Jeong, M. Walsh, K. Roberts, E. Martinez-Hernandez, M. R. Rosenfeld, R. Balice-Gordon, F. Graus, B. Rudy and J. Dalmau (2013). "Encephalitis and antibodies to dipeptidyl-peptidase-like protein-6, a subunit of Kv4.2 potassium channels." Ann Neurol **73**(1): 120-128.
Boulanger, L. M. (2004). "MHC class I in activity-dependent structural and functional plasticity." Neuron Glia Biol **1**(3): 283-289.
Boyce, P. and E. Barriball (2010). "Puerperal psychosis." Arch Womens Ment Health **13**(1): 45-47.
Brambell, F. W. (1966). "The transmission of immunity from mother to young and the catabolism of immunoglobulins." Lancet **2**(7473): 1087-1093.
Brambell, F. W. and R. Halliday (1956). "The route by which passive immunity is transmitted from mother to foetus in the rat." Proc R Soc Lond B Biol Sci **145**(919): 170-178.
Brambell, F. W. and W. A. Hemmings (1949). "The passage into the embryonic yolk-sac cavity of maternal plasma proteins in rabbits." J Physiol **108**(2): 177-185.
Branchi, I. (2009). "The mouse communal nest: investigating the epigenetic influences of the early social environment on brain and behavior development." Neurosci Biobehav Rev **33**(4): 551-559.
Braniste, V., M. Al-Asmakh, C. Kowal, F. Anuar, A. Abbaspour, M. Toth, A. Korecka, N. Bakocevic, L. G. Ng, P. Kundu, B. Gulyas, C. Halldin, K. Hultenby, H. Nilsson, H. Hebert, B. T. Volpe, B. Diamond and S. Pettersson (2014). "The gut microbiota influences blood-brain barrier permeability in mice." Sci Transl Med **6**(263): 263ra158.
Braunschweig, D., P. Ashwood, P. Krakowiak, I. Hertz-Picciotto, R. Hansen, L. A. Croen, I. N. Pessah and J. Van de Water (2008). "Autism: maternally derived antibodies specific for fetal brain proteins." Neurotoxicology **29**(2): 226-231.
Braunschweig, D., P. Duncanson, R. Boyce, R. Hansen, P. Ashwood, I. N. Pessah, I. Hertz-Picciotto and J. Van de Water (2012). "Behavioral correlates of maternal antibody status among children with autism." J Autism Dev Disord **42**(7): 1435-1445.
Braunschweig, D., M. S. Golub, C. M. Koenig, L. Qi, I. N. Pessah, J. Van de Water and R. F. Berman (2012). "Maternal autism-associated IgG antibodies delay development and produce anxiety in a mouse gestational transfer model." J Neuroimmunol **252**(1-2): 56-65.

Braunschweig, D., P. Krakowiak, P. Duncanson, R. Boyce, R. L. Hansen, P. Ashwood, I. Hertz-Picciotto, I. N. Pessah and J. Van de Water (2013). "Autism-specific maternal autoantibodies recognize critical proteins in developing brain." Transl Psychiatry **3**: e277.
Brimberg, L., S. Mader, Y. Fujieda, Y. Arinuma, C. Kowal, B. T. Volpe and B. Diamond (2015). "Antibodies as Mediators of Brain Pathology." Trends Immunol.
Brimberg, L., A. Sadiq, P. K. Gregersen and B. Diamond (2013). "Brain-reactive IgG correlates with autoimmunity in mothers of a child with an autism spectrum disorder." Mol Psychiatry **18**(11): 1171-1177.
Brucato, A., R. Cimaz, R. Caporali, V. Ramoni and J. Buyon (2011). "Pregnancy outcomes in patients with autoimmune diseases and anti-Ro/SSA antibodies." Clin Rev Allergy Immunol **40**(1): 27-41.
Buckley, C., J. Oger, L. Clover, E. Tuzun, K. Carpenter, M. Jackson and A. Vincent (2001). "Potassium channel antibodies in two patients with reversible limbic encephalitis." Ann Neurol **50**(1): 73-78.
Burnashev, N. and P. Szepetowski (2015). "NMDA receptor subunit mutations in neurodevelopmental disorders." Curr Opin Pharmacol **20**: 73-82.
Buyon, J. P., R. J. Winchester, S. G. Slade, F. Arnett, J. Copel, D. Friedman and M. D. Lockshin (1993). "Identification of mothers at risk for congenital heart block and other neonatal lupus syndromes in their children. Comparison of enzyme-linked immunosorbent assay and immunoblot for measurement of anti-SS-A/Ro and anti-SS-B/La antibodies." Arthritis Rheum **36**(9): 1263-1273.
Camacho, J., K. Jones, E. Miller, J. Ariza, S. Noctor, J. Van de Water and V. Martinez-Cerdeno (2014). "Embryonic intraventricular exposure to autism-specific maternal autoantibodies produces alterations in autistic-like stereotypical behaviors in offspring mice." Behav Brain Res **266**: 46-51.
Cardenas-Roldan, J., A. Rojas-Villarraga and J. M. Anaya (2013). "How do autoimmune diseases cluster in families? A systematic review and meta-analysis." BMC Med **11**: 73.
Carvajal-Gonzalez, A., M. I. Leite, P. Waters, M. Woodhall, E. Coutinho, B. Balint, B. Lang, P. Pettingill, A. Carr, U. M. Sheerin, R. Press, M. P. Lunn, M. Lim, P. Maddison, H. M. Meinck, W. Vandenberghe and A. Vincent (2014). "Glycine receptor antibodies in PERM and related syndromes: characteristics, clinical features and outcomes." Brain **137**(Pt 8): 2178-2192.
Castillo-Gomez, E., A. Kastner, J. Steiner, A. Schneider, B. Hettling, G. Poggi, K. Ostehr, M. Uhr, A. R. Asif, M. Matzke, U. Schmidt, V. Pfander, C. Hammer, T. F. Schulz, L. Binder, W. Stocker, F. Weber and H. Ehrenreich (2015). "The brain as 'immunoprecipitator' of serum autoantibodies against NMDAR1." Ann Neurol.
Caviness, V. S., Jr. and T. Takahashi (1995). "Proliferative events in the cerebral ventricular zone." Brain Dev **17**(3): 159-163.
Cervantes, P. E. and J. L. Matson (2015). "Comorbid Symptomology in Adults with Autism Spectrum Disorder and Intellectual Disability." J Autism Dev Disord **45**(12): 3961-3970.
Chang, C. (2012). "Neonatal autoimmune diseases." Lupus **21**(14): 1487-1491.
Chen, J., S. Yu, Y. Fu and X. Li (2014). "Synaptic proteins and receptors defects in autism spectrum disorders." Front Cell Neurosci **8**: 276.
Chen, M. H., T. P. Su, Y. S. Chen, J. W. Hsu, K. L. Huang, W. H. Chang, T. J. Chen and Y. M. Bai (2013). "Comorbidity of Allergic and Autoimmune Diseases Among Patients With ADHD: A Nationwide Population-Based Study." J Atten Disord.
Chez, M. G., T. Dowling, P. B. Patel, P. Khanna and M. Kominsky (2007). "Elevation of tumor necrosis factor-alpha in cerebrospinal fluid of autistic children." Pediatr Neurol **36**(6): 361-365.
Christadoss, P., M. Poussin and C. Deng (2000). "Animal models of myasthenia gravis." Clin Immunol **94**(2): 75-87.
Chu, Y., X. Jin, I. Parada, A. Pesic, B. Stevens, B. Barres and D. A. Prince (2010). "Enhanced synaptic connectivity and epilepsy in C1q knockout mice." Proc Natl Acad Sci U S A **107**(17): 7975-7980.

Cohen, I. R. and D. B. Young (1991). "Autoimmunity, microbial immunity and the immunological homunculus." Immunol Today **12**(4): 105-110.
Colonna, M. and Y. Wang (2016). "TREM2 variants: new keys to decipher Alzheimer disease pathogenesis." Nat Rev Neurosci **17**(4): 201-207.
Coutinho, A., M. D. Kazatchkine and S. Avrameas (1995). "Natural autoantibodies." Curr Opin Immunol **7**(6): 812-818.
Coutinho, E., P. Harrison and A. Vincent (2014). "Do neuronal autoantibodies cause psychosis? A neuroimmunological perspective." Biol Psychiatry **75**(4): 269-275.
Creten, C., S. van der Zwaan, R. J. Blankespoor, A. Maatkamp, J. Nicolai, J. van Os and J. N. Schieveld (2011). "Late onset autism and anti-NMDA-receptor encephalitis." Lancet **378**(9785): 98.
Crisp, S. J., D. M. Kullmann and A. Vincent (2016). "Autoimmune synaptopathies." Nat Rev Neurosci **17**(2): 103-117.
Croen, L. A., D. Braunschweig, L. Haapanen, C. K. Yoshida, B. Fireman, J. K. Grether, M. Kharrazi, R. L. Hansen, P. Ashwood and J. Van de Water (2008). "Maternal mid-pregnancy autoantibodies to fetal brain protein: the early markers for autism study." Biol Psychiatry **64**(7): 583-588.
Cunningham, C. L., V. Martinez-Cerdeno and S. C. Noctor (2013). "Microglia regulate the number of neural precursor cells in the developing cerebral cortex." J Neurosci **33**(10): 4216-4233.
Curtis, B. R. (2015). "Recent progress in understanding the pathogenesis of fetal and neonatal alloimmune thrombocytopenia." Br J Haematol **171**(5): 671-682.
Dahm, L., C. Ott, J. Steiner, B. Stepniak, B. Teegen, S. Saschenbrecker, C. Hammer, K. Borowski, M. Begemann, S. Lemke, K. Rentzsch, C. Probst, H. Martens, J. Wienands, G. Spalletta, K. Weissenborn, W. Stocker and H. Ehrenreich (2014). "Seroprevalence of autoantibodies against brain antigens in health and disease." Ann Neurol **76**(1): 82-94.
Dale, R. C., V. Merheb, S. Pillai, D. Wang, L. Cantrill, T. K. Murphy, H. Ben-Pazi, S. Varadkar, T. D. Aumann, M. K. Horne, A. J. Church, T. Fath and F. Brilot (2012). "Antibodies to surface dopamine-2 receptor in autoimmune movement and psychiatric disorders." Brain **135**(Pt 11): 3453-3468.
Dalmau, J., A. J. Gleichman, E. G. Hughes, J. E. Rossi, X. Peng, M. Lai, S. K. Dessain, M. R. Rosenfeld, R. Balice-Gordon and D. R. Lynch (2008). "Anti-NMDA-receptor encephalitis: case series and analysis of the effects of antibodies." Lancet Neurol **7**(12): 1091-1098.
Dalmau, J. and M. R. Rosenfeld (2008). "Paraneoplastic syndromes of the CNS." Lancet Neurol **7**(4): 327-340.
Dalmau, J., E. Tuzun, H. Y. Wu, J. Masjuan, J. E. Rossi, A. Voloschin, J. M. Baehring, H. Shimazaki, R. Koide, D. King, W. Mason, L. H. Sansing, M. A. Dichter, M. R. Rosenfeld and D. R. Lynch (2007). "Paraneoplastic anti-N-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma." Ann Neurol **61**(1): 25-36.
Dalton, P., L. Clover, R. Wallerstein, H. Stewart, O. Genzel-Boroviczeny, A. Dean and A. Vincent (2006). "Fetal arthrogryposis and maternal serum antibodies." Neuromuscul Disord **16**(8): 481-491.
Dalton, P., R. Deacon, A. Blamire, M. Pike, I. McKinlay, J. Stein, P. Styles and A. Vincent (2003). "Maternal neuronal antibodies associated with autism and a language disorder." Ann Neurol **53**(4): 533-537.
de Witte, L. D., C. Hoffmann, H. C. van Mierlo, M. J. Titulaer, R. S. Kahn, P. Martinez-Martinez and D. European Consortium of Autoimmune Mental (2015). "Absence of N-Methyl-D-Aspartate Receptor IgG Autoantibodies in Schizophrenia: The Importance of Cross-Validation Studies." JAMA Psychiatry.
Deacon, R. M. (2006). "Assessing nest building in mice." Nat Protoc **1**(3): 1117-1119.
Deacon, R. M. (2011). "Hyponeophagia: a measure of anxiety in the mouse." J Vis Exp(51).
Deacon, R. M. (2013). "Measuring motor coordination in mice." J Vis Exp(75): e2609.

Deacon, R. M. and J. N. Rawlins (2006). "T-maze alternation in the rodent." Nat Protoc **1**(1): 7-12.
DeGiorgio, L. A., K. N. Konstantinov, S. C. Lee, J. A. Hardin, B. T. Volpe and B. Diamond (2001). "A subset of lupus anti-DNA antibodies cross-reacts with the NR2 glutamate receptor in systemic lupus erythematosus." Nat Med **7**(11): 1189-1193.
Denisenko-Nehrbass, N., K. Oguievetskaia, L. Goutebroze, T. Galvez, H. Yamakawa, O. Ohara, M. Carnaud and J. A. Girault (2003). "Protein 4.1B associates with both Caspr/paranodin and Caspr2 at paranodes and juxtaparanodes of myelinated fibres." Eur J Neurosci **17**(2): 411-416.
Drachman, D. B. (1990). "How to recognize an antibody-mediated autoimmune disease: criteria." Res Publ Assoc Res Nerv Ment Dis **68**: 183-186.
Emerson, E. (2012). "Deprivation, ethnicity and the prevalence of intellectual and developmental disabilities." J Epidemiol Community Health **66**(3): 218-224.
Estes, M. L. and A. K. McAllister (2015). "Immune mediators in the brain and peripheral tissues in autism spectrum disorder." Nat Rev Neurosci **16**(8): 469-486.
Ewald, R. C. and H. T. Cline (2009). NMDA Receptors and Brain Development. Biology of the NMDA Receptor. A. M. Van Dongen. Boca Raton (FL).
Fallier-Becker, P., J. P. Vollmer, H. C. Bauer, S. Noell, H. Wolburg and A. F. Mack (2014). "Onset of aquaporin-4 expression in the developing mouse brain." International Journal of Developmental Neuroscience **36**: 81-89.
Festing, M. F. (2006). "Design and statistical methods in studies using animal models of development." ILAR J **47**(1): 5-14.
Forrest, D., M. Yuzaki, H. D. Soares, L. Ng, D. C. Luk, M. Sheng, C. L. Stewart, J. I. Morgan, J. A. Connor and T. Curran (1994). "Targeted disruption of NMDA receptor 1 gene abolishes NMDA response and results in neonatal death." Neuron **13**(2): 325-338.
Fox, J. W., E. D. Lamperti, Y. Z. Eksioglu, S. E. Hong, Y. Feng, D. A. Graham, I. E. Scheffer, W. B. Dobyns, B. A. Hirsch, R. A. Radtke, S. F. Berkovic, P. R. Huttenlocher and C. A. Walsh (1998). "Mutations in filamin 1 prevent migration of cerebral cortical neurons in human periventricular heterotopia." Neuron **21**(6): 1315-1325.
Frick, L. R., K. Williams and C. Pittenger (2013). "Microglial dysregulation in psychiatric disease." Clin Dev Immunol **2013**: 608654.
Frost, J. L. and D. P. Schafer (2016). "Microglia: Architects of the Developing Nervous System." Trends Cell Biol.
Fujita, K., T. Yuasa, Y. Takahashi, K. Tanaka, W. Sako, H. Koizumi, Y. Iwasaki, M. Yoshida, Y. Izumi and R. Kaji (2012). "Antibodies to N-methyl-D-aspartate glutamate receptors in Creutzfeldt-Jakob disease patients." J Neuroimmunol **251**(1-2): 90-93.
Fukata, Y., K. L. Lovero, T. Iwanaga, A. Watanabe, N. Yokoi, K. Tabuchi, R. Shigemoto, R. A. Nicoll and M. Fukata (2010). "Disruption of LGI1-linked synaptic complex causes abnormal synaptic transmission and epilepsy." Proc Natl Acad Sci U S A **107**(8): 3799-3804.
Fukaya, M. and M. Watanabe (2000). "Improved immunohistochemical detection of postsynaptically located PSD-95/SAP90 protein family by protease section pretreatment: a study in the adult mouse brain." J Comp Neurol **426**(4): 572-586.
Garty, B. Z., A. Ludomirsky, Y. L. Danon, J. B. Peter and S. D. Douglas (1994). "Placental transfer of immunoglobulin G subclasses." Clin Diagn Lab Immunol **1**(6): 667-669.
Gilhus, N. E. and J. J. Verschuuren (2015). "Myasthenia gravis: subgroup classification and therapeutic strategies." Lancet Neurol **14**(10): 1023-1036.
Gleichman, A. J., L. A. Spruce, J. Dalmau, S. H. Seeholzer and D. R. Lynch (2012). "Anti-NMDA receptor encephalitis antibody binding is dependent on amino acid identity of a small region within the GluN1 amino terminal domain." J Neurosci **32**(32): 11082-11094.
Gotkine, M., T. Ben-Hur, A. Vincent and A. Vaknin-Dembinsky (2011). "Limbic encephalitis presenting as a post-partum psychiatric condition." J Neurol Sci **308**(1-2): 152-154.

Gottfried, C., V. Bambini-Junior, F. Francis, R. Riesgo and W. Savino (2015). "The Impact of Neuroimmune Alterations in Autism Spectrum Disorder." Front Psychiatry **6**: 121.
Graus, F., A. Boronat, X. Xifro, M. Boix, V. Svigelj, A. Garcia, A. Palomino, L. Sabater, J. Alberch and A. Saiz (2010). "The expanding clinical profile of anti-AMPA receptor encephalitis." Neurology **74**(10): 857-859.
Graus, F., I. Illa, M. Agusti, T. Ribalta, F. Cruz-Sanchez and C. Juarez (1991). "Effect of intraventricular injection of an anti-Purkinje cell antibody (anti-Yo) in a guinea pig model." J Neurol Sci **106**(1): 82-87.
Graus, F., A. Saiz and J. Dalmau (2010). "Antibodies and neuronal autoimmune disorders of the CNS." J Neurol **257**(4): 509-517.
Greenlee, J. E. and H. R. Brashear (1983). "Antibodies to cerebellar Purkinje cells in patients with paraneoplastic cerebellar degeneration and ovarian carcinoma." Ann Neurol **14**(6): 609-613.
Grunwald, G. B., R. Klein, M. A. Simmonds and S. E. Kornguth (1985). "Autoimmune basis for visual paraneoplastic syndrome in patients with small-cell lung carcinoma." Lancet **1**(8430): 658-661.
Hacohen, Y., K. Deiva, P. Pettingill, P. Waters, A. Siddiqui, P. Chretien, E. Menson, J. P. Lin, M. Tardieu, A. Vincent and M. J. Lim (2013). "N-methyl-D-aspartate receptor antibodies in post-herpes simplex virus encephalitis neurological relapse." Mov Disord.
Hacohen, Y., L. W. Jacobson, S. Byrne, F. Norwood, A. Lall, S. Robb, R. Dilena, M. Fumagalli, A. P. Born, D. Clarke, M. Lim, A. Vincent and H. Jungbluth (2015). "Fetal acetylcholine receptor inactivation syndrome: A myopathy due to maternal antibodies." Neurol Neuroimmunol Neuroinflamm **2**(1): e57.
Hacohen, Y., S. Wright, A. Siddiqui, N. Pandya, J. P. Lin, A. Vincent and M. Lim (2012). "A clinico-radiological phenotype of voltage-gated potassium channel complex antibody-mediated disorder presenting with seizures and basal ganglia changes." Dev Med Child Neurol **54**(12): 1157-1159.
Hahn, M. E. and M. J. Lavooy (2005). "A review of the methods of studies on infant ultrasound production and maternal retrieval in small rodents." Behav Genet **35**(1): 31-52.
Halliday, R. (1955). "Prenatal and postnatal transmission of passive immunity to young rats." Proc R Soc Lond B Biol Sci **144**(916): 427-430.
Hammer, C., B. Stepniak, A. Schneider, S. Papiol, M. Tantra, M. Begemann, A. L. Siren, L. A. Pardo, S. Sperling, S. Mohd Jofrry, A. Gurvich, N. Jensen, K. Ostmeier, F. Luhder, C. Probst, H. Martens, M. Gillis, G. Saher, F. Assogna, G. Spalletta, W. Stocker, T. F. Schulz, K. A. Nave and H. Ehrenreich (2013). "Neuropsychiatric disease relevance of circulating anti-NMDA receptor autoantibodies depends on blood-brain barrier integrity." Mol Psychiatry.
Hammer, C., M. Zerche, A. Schneider, M. Begemann, K. A. Nave and H. Ehrenreich (2014). "Apolipoprotein E4 carrier status plus circulating anti-NMDAR1 autoantibodies: association with schizoaffective disorder." Mol Psychiatry **19**(10): 1054-1056.
Harrison, P. J. and V. A. West (2006). "Six degrees of separation: on the prior probability that schizophrenia susceptibility genes converge on synapses, glutamate and NMDA receptors." Mol Psychiatry **11**(11): 981-983.
Hart, I. K., C. Waters, A. Vincent, C. Newland, D. Beeson, O. Pongs, C. Morris and J. Newsom-Davis (1997). "Autoantibodies detected to expressed K+ channels are implicated in neuromyotonia." Ann Neurol **41**(2): 238-246.
Hartter, H. K., O. I. Oyedele, K. Dietz, S. Kreis, J. P. Hoffman and C. P. Muller (2000). "Placental transfer and decay of maternally acquired antimeasles antibodies in Nigerian children." Pediatr Infect Dis J **19**(7): 635-641.
Haussleiter, I. S., B. Emons, M. Schaub, K. Borowski, M. Brune, K. P. Wandinger and G. Juckel (2012). "Investigation of antibodies against synaptic proteins in a cross-sectional cohort of psychotic patients." Schizophr Res **140**(1-3): 258-259.

Hoch, W., J. McConville, S. Helms, J. Newsom-Davis, A. Melms and A. Vincent (2001). "Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies." Nat Med **7**(3): 365-368.
Hoerder-Suabedissen, A. and Z. Molnar (2015). "Development, evolution and pathology of neocortical subplate neurons." Nat Rev Neurosci **16**(3): 133-146.
Hofer, M. A., H. N. Shair and S. A. Brunelli (2002). "Ultrasonic vocalizations in rat and mouse pups." Curr Protoc Neurosci **Chapter 8**: Unit 8 14.
Hoftberger, R., M. J. Titulaer, L. Sabater, B. Dome, A. Rozsas, B. Hegedus, M. A. Hoda, V. Laszlo, H. J. Ankersmit, L. Harms, S. Boyero, A. de Felipe, A. Saiz, J. Dalmau and F. Graus (2013). "Encephalitis and GABAB receptor antibodies: novel findings in a new case series of 20 patients." Neurology **81**(17): 1500-1506.
Hoftberger, R., A. van Sonderen, F. Leypoldt, D. Houghton, M. Geschwind, J. Gelfand, M. Paredes, L. Sabater, A. Saiz, M. J. Titulaer, F. Graus and J. Dalmau (2015). "Encephalitis and AMPA receptor antibodies: Novel findings in a case series of 22 patients." Neurology.
Hornig, M., H. Weissenbock, N. Horscroft and W. I. Lipkin (1999). "An infection-based model of neurodevelopmental damage." Proc Natl Acad Sci U S A **96**(21): 12102-12107.
Horresh, I., S. Poliak, S. Grant, D. Bredt, M. N. Rasband and E. Peles (2008). "Multiple molecular interactions determine the clustering of Caspr2 and Kv1 channels in myelinated axons." J Neurosci **28**(52): 14213-14222.
Hughes, E. G., X. Peng, A. J. Gleichman, M. Lai, L. Zhou, R. Tsou, T. D. Parsons, D. R. Lynch, J. Dalmau and R. J. Balice-Gordon (2010). "Cellular and synaptic mechanisms of anti-NMDA receptor encephalitis." J Neurosci **30**(17): 5866-5875.
Huttenlocher, P. R. (1979). "Synaptic density in human frontal cortex - developmental changes and effects of aging." Brain Res **163**(2): 195-205.
Hytten, F. (1985). "Blood volume changes in normal pregnancy." Clin Haematol **14**(3): 601-612.
Irani, S. R., S. Alexander, P. Waters, K. A. Kleopa, P. Pettingill, L. Zuliani, E. Peles, C. Buckley, B. Lang and A. Vincent (2010). "Antibodies to Kv1 potassium channel-complex proteins leucine-rich, glioma inactivated 1 protein and contactin-associated protein-2 in limbic encephalitis, Morvan's syndrome and acquired neuromyotonia." Brain **133**(9): 2734-2748.
Irani, S. R., K. Bera, P. Waters, L. Zuliani, S. Maxwell, M. S. Zandi, M. A. Friese, I. Galea, D. M. Kullmann, D. Beeson, B. Lang, C. G. Bien and A. Vincent (2010). "N-methyl-D-aspartate antibody encephalitis: temporal progression of clinical and paraclinical observations in a predominantly non-paraneoplastic disorder of both sexes." Brain **133**(Pt 6): 1655-1667.
Irani, S. R., A. W. Michell, B. Lang, P. Pettingill, P. Waters, M. R. Johnson, J. M. Schott, R. J. Armstrong, S. Z. A, A. Bleasel, E. R. Somerville, S. M. Smith and A. Vincent (2011). "Faciobrachial dystonic seizures precede Lgi1 antibody limbic encephalitis." Ann Neurol **69**(5): 892-900.
Irani, S. R., C. J. Stagg, J. M. Schott, C. R. Rosenthal, S. A. Schneider, P. Pettingill, R. Pettingill, P. Waters, A. Thomas, N. L. Voets, M. J. Cardoso, D. M. Cash, E. N. Manning, B. Lang, S. J. Smith, A. Vincent and M. R. Johnson (2013). "Faciobrachial dystonic seizures: the influence of immunotherapy on seizure control and prevention of cognitive impairment in a broadening phenotype." Brain **136**(Pt 10): 3151-3162.
Ito, Y., T. Abe, R. Tomioka, T. Komori and N. Araki (2010). "[Anti-NMDA receptor encephalitis during pregnancy]." Rinsho Shinkeigaku **50**(2): 103-107.
J. Pittock, S. e. and A. e. Vincent (2016). Autoimmune neurology.
Jacobson, L., A. Polizzi, G. Morriss-Kay and A. Vincent (1999). "Plasma from human mothers of fetuses with severe arthrogryposis multiplex congenita causes deformities in mice." J Clin Invest **103**(7): 1031-1038.
Jacobson, L., A. Polizzi and A. Vincent (1998). "An animal model of maternal antibody-mediated arthrogryposis multiplex congenita (AMC)." Ann N Y Acad Sci **841**: 565-567.

Jaeckle, K. A., F. Graus, A. Houghton, C. Cardon-Cardo, S. L. Nielsen and J. B. Posner (1985). "Autoimmune response of patients with paraneoplastic cerebellar degeneration to a Purkinje cell cytoplasmic protein antigen." Ann Neurol **18**(5): 592-600.
Jagota, P., A. Vincent and R. Bhidayasiri (2014). "Transplacental transfer of NMDA receptor antibodies in an infant with cortical dysplasia." Neurology **82**(18): 1662-1663.
Jantzie, L. L., D. M. Talos, M. C. Jackson, H. K. Park, D. A. Graham, M. Lechpammer, R. D. Folkerth, J. J. Volpe and F. E. Jensen (2015). "Developmental expression of N-methyl-D-aspartate (NMDA) receptor subunits in human white and gray matter: potential mechanism of increased vulnerability in the immature brain." Cereb Cortex **25**(2): 482-495.
Javitt, D. C., S. R. Zukin, U. Heresco-Levy and D. Umbricht (2012). "Has an angel shown the way? Etiological and therapeutic implications of the PCP/NMDA model of schizophrenia." Schizophr Bull **38**(5): 958-966.
Jeffery, O. J., V. A. Lennon, S. J. Pittock, J. K. Gregory, J. W. Britton and A. McKeon (2013). "GABAB receptor autoantibody frequency in service serologic evaluation." Neurology **81**(10): 882-887.
Johnson, B. (2014). "Overview of neonatal lupus." J Pediatr Health Care **28**(4): 331-341.
Jones, I., P. S. Chandra, P. Dazzan and L. M. Howard (2014). "Bipolar disorder, affective psychosis, and schizophrenia in pregnancy and the post-partum period." The Lancet **384**(9956): 1789-1799.
Joubert, B., P. Kerschen, A. Zekeridou, V. Desestret, V. Rogemond, M. O. Chaffois, F. Ducray, V. Larrue, B. Daubail, A. Idbaih, D. Psimaras, J. C. Antoine, J. Y. Delattre and J. Honnorat (2015). "Clinical Spectrum of Encephalitis Associated With Antibodies Against the alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid Receptor: Case Series and Review of the Literature." JAMA Neurol **72**(10): 1163-1169.
Juckel, G., M. P. Manitz, M. Brune, A. Friebe, M. T. Heneka and R. J. Wolf (2011). "Microglial activation in a neuroinflammational animal model of schizophrenia--a pilot study." Schizophr Res **131**(1-3): 96-100.
Kadam, S. D., B. M. French, S. T. Kim, C. M. Morris-Berry, A. W. Zimmerman, M. E. Blue and H. S. Singer (2013). "Altered postnatal cell proliferation in brains of mouse pups prenatally exposed to IgG from mothers of children with autistic disorder." J Exp Neurosci **7**: 93-99.
Kadotani, H., T. Hirano, M. Masugi, K. Nakamura, K. Nakao, M. Katsuki and S. Nakanishi (1996). "Motor discoordination results from combined gene disruption of the NMDA receptor NR2A and NR2C subunits, but not from single disruption of the NR2A or NR2C subunit." J Neurosci **16**(24): 7859-7867.
Kaech, S. and G. Banker (2006). "Culturing hippocampal neurons." Nat Protoc **1**(5): 2406-2415.
Kayser, M. S. and J. Dalmau (2011). "The emerging link between autoimmune disorders and neuropsychiatric disease." J Neuropsychiatry Clin Neurosci **23**(1): 90-97.
Kayser, M. S., M. J. Titulaer, N. Gresa-Arribas and J. Dalmau (2013). "Frequency and Characteristics of Isolated Psychiatric Episodes in Anti-N-Methyl-d-Aspartate Receptor Encephalitis." JAMA Neurol.
Keith, D. and A. El-Husseini (2008). "Excitation Control: Balancing PSD-95 Function at the Synapse." Front Mol Neurosci **1**: 4.
Kim, W., S. H. Kim, I. Nakashima, Y. Takai, K. Fujihara, M. I. Leite, J. Kitley, J. Palace, E. Santos, E. Coutinho, A. M. Silva, B. J. Kim, B. J. Kim, S. W. Ahn and H. J. Kim (2012). "Influence of pregnancy on neuromyelitis optica spectrum disorder." Neurology **78**(16): 1264-1267.
Kitten, S., N. Gupta, R. M. Bloch and C. K. Dunham (2011). "Voltage-gated potassium channel antibody associated mood disorder without paraneoplastic disease." Biol Psychiatry **70**(4): e15-17.
Klang, A., P. Schmidt, S. Kneissl, Z. Bago, A. Vincent, B. Lang, T. Moloney, C. G. Bien, P. Halasz, J. Bauer and A. Pakozdy (2014). "IgG and complement deposition and

neuronal loss in cats and humans with epilepsy and voltage-gated potassium channel complex antibodies." J Neuropathol Exp Neurol **73**(5): 403-413.
Knuesel, I., L. Chicha, M. Britschgi, S. A. Schobel, M. Bodmer, J. A. Hellings, S. Toovey and E. P. Prinssen (2014). "Maternal immune activation and abnormal brain development across CNS disorders." Nat Rev Neurol **10**(11): 643-660.
Kohane, I. S., A. McMurry, G. Weber, D. MacFadden, L. Rappaport, L. Kunkel, J. Bickel, N. Wattanasin, S. Spence, S. Murphy and S. Churchill (2012). "The co-morbidity burden of children and young adults with autism spectrum disorders." PLoS One **7**(4): e33224.
Koksal, A., S. Baybas, B. Mutluay, Y. Altunkaynak and A. Keskek (2015). "A case of NMDAR encephalitis misdiagnosed as postpartum psychosis and neuroleptic malignant syndrome." Neurol Sci **36**(7): 1257-1258.
Kondo, S., H. Al-Hasani, A. Hoerder-Suabedissen, W. Z. Wang and Z. Molnar (2015). "Secretory function in subplate neurons during cortical development." Front Neurosci **9**: 100.
Kortvelyessy, P., J. Bauer, C. M. Stoppel, W. Bruck, I. Gerth, S. Vielhaber, F. R. Wiedemann, H. J. Heinze, C. Bartels and C. G. Bien (2015). "Complement-associated neuronal loss in a patient with CASPR2 antibody-associated encephalitis." Neurol Neuroimmunol Neuroinflamm **2**(2): e75.
Kowal, C., L. A. Degiorgio, J. Y. Lee, M. A. Edgar, P. T. Huerta, B. T. Volpe and B. Diamond (2006). "Human lupus autoantibodies against NMDA receptors mediate cognitive impairment." Proc Natl Acad Sci U S A **103**(52): 19854-19859.
Kruse, J. L., M. I. Lapid, V. A. Lennon, C. J. Klein, O. O. Toole, S. J. Pittock, E. A. Strand, M. A. Frye and A. McKeon (2015). "Psychiatric Autoimmunity: N-Methyl-D-Aspartate Receptor IgG and Beyond." Psychosomatics **56**(3): 227-241.
Kumar, M. A., A. Jain, V. E. Dechant, T. Saito, T. Rafael, H. Aizawa, K. C. Dysart, T. Katayama, Y. Ito, N. Araki, T. Abe, R. Balice-Gordon and J. Dalmau (2010). "Anti-N-methyl-D-aspartate receptor encephalitis during pregnancy." Arch Neurol **67**(7): 884-887.
Kutsuwada, T., K. Sakimura, T. Manabe, C. Takayama, N. Katakura, E. Kushiya, R. Natsume, M. Watanabe, Y. Inoue, T. Yagi, S. Aizawa, M. Arakawa, T. Takahashi, Y. Nakamura, H. Mori and M. Mishina (1996). "Impairment of suckling response, trigeminal neuronal pattern formation, and hippocampal LTD in NMDA receptor epsilon 2 subunit mutant mice." Neuron **16**(2): 333-344.
Kwan, K. Y., N. Sestan and E. S. Anton (2012). "Transcriptional co-regulation of neuronal migration and laminar identity in the neocortex." Development **139**(9): 1535-1546.
Lai, M.-C., M. V. Lombardo and S. Baron-Cohen (2014). "Autism." The Lancet **383**(9920): 896-910.
Lai, M., M. G. Huijbers, E. Lancaster, F. Graus, L. Bataller, R. Balice-Gordon, J. K. Cowell and J. Dalmau (2010). "Investigation of LGI1 as the antigen in limbic encephalitis previously attributed to potassium channels: a case series." Lancet Neurol **9**(8): 776-785.
Lalic, T., P. Pettingill, A. Vincent and M. Capogna (2011). "Human limbic encephalitis serum enhances hippocampal mossy fiber-CA3 pyramidal cell synaptic transmission." Epilepsia **52**(1): 121-131.
Lamale-Smith, L. M., G. S. Moore, S. R. Guntupalli and J. B. Scott (2015). "Maternal-fetal transfer of anti-N-methyl-D-aspartate receptor antibodies." Obstet Gynecol **125**(5): 1056-1058.
Lancaster, E. and J. Dalmau (2012). "Neuronal autoantigens--pathogenesis, associated disorders and antibody testing." Nat Rev Neurol **8**(7): 380-390.
Lancaster, E., M. G. Huijbers, V. Bar, A. Boronat, A. Wong, E. Martinez-Hernandez, C. Wilson, D. Jacobs, M. Lai, R. W. Walker, F. Graus, L. Bataller, I. Illa, S. Markx, K. A. Strauss, E. Peles, S. S. Scherer and J. Dalmau (2011). "Investigations of caspr2, an autoantigen of encephalitis and neuromyotonia." Ann Neurol **69**(2): 303-311.
Lancaster, E., E. Martinez-Hernandez, M. J. Titulaer, M. Boulos, S. Weaver, J. C. Antoine, E. Liebers, C. Kornblum, C. G. Bien, J. Honnorat, S. Wong, J. Xu, A. Contractor,

R. Balice-Gordon and J. Dalmau (2011). "Antibodies to metabotropic glutamate receptor 5 in the Ophelia syndrome." Neurology **77**(18): 1698-1701.
Lauvsnes, M. B. and R. Omdal (2012). "Systemic lupus erythematosus, the brain, and anti-NR2 antibodies." J Neurol **259**(4): 622-629.
Lee, E. J., S. Y. Choi and E. Kim (2015). "NMDA receptor dysfunction in autism spectrum disorders." Curr Opin Pharmacol **20**: 8-13.
Lee, I. S., C. M. B. Carvalho, P. Douvaras, S.-M. Ho, B. J. Hartley, L. W. Zuccherato, I. G. Ladran, A. J. Siegel, S. McCarthy, D. Malhotra, J. Sebat, J. Rapoport, V. Fossati, J. R. Lupski, D. L. Levy and K. J. Brennand (2015). "Characterization of molecular and cellular phenotypes associated with a heterozygous CNTNAP2 deletion using patient-derived hiPSC neural cells." npj Schizophrenia **1**: 15019.
Lee, J. Y., P. T. Huerta, J. Zhang, C. Kowal, E. Bertini, B. T. Volpe and B. Diamond (2009). "Neurotoxic autoantibodies mediate congenital cortical impairment of offspring in maternal lupus." Nat Med **15**(1): 91-96.
Leite, M. I., E. Coutinho, M. Lana-Peixoto, S. Apostolos, P. Waters, D. Sato, L. Melamud, M. Marta, A. Graham, J. Spillane, A. M. Villa, D. Callegaro, E. Santos, A. M. da Silva, S. Jarius, R. Howard, I. Nakashima, G. Giovannoni, C. Buckley, D. Hilton-Jones, A. Vincent and J. Palace (2012). "Myasthenia gravis and neuromyelitis optica spectrum disorder: a multicenter study of 16 patients." Neurology **78**(20): 1601-1607.
Lemke, J. R., K. Geider, K. L. Helbig, H. O. Heyne, H. Schutz, J. Hentschel, C. Courage, C. Depienne, C. Nava, D. Heron, R. S. Moller, H. Hjalgrim, D. Lal, B. A. Neubauer, P. Nurnberg, H. Thiele, G. Kurlemann, G. L. Arnold, V. Bhambhani, D. Bartholdi, C. R. Pedurupillay, D. Misceo, E. Frengen, P. Stromme, D. J. Dlugos, E. S. Doherty, E. K. Bijlsma, C. A. Ruivenkamp, M. J. Hoffer, A. Goldstein, D. S. Rajan, V. Narayanan, K. Ramsey, N. Belnap, I. Schrauwen, R. Richholt, B. P. Koeleman, J. Sa, C. Mendonca, C. G. de Kovel, S. Weckhuysen, K. Hardies, P. De Jonghe, L. De Meirleir, M. Milh, C. Badens, M. Lebrun, T. Busa, C. Francannet, A. Piton, E. Riesch, S. Biskup, H. Vogt, T. Dorn, I. Helbig, J. L. Michaud, B. Laube and S. Syrbe (2016). "Delineating the GRIN1 phenotypic spectrum: A distinct genetic NMDA receptor encephalopathy." Neurology **86**(23): 2171-2178.
Lencer, W. I. and R. S. Blumberg (2005). "A passionate kiss, then run: exocytosis and recycling of IgG by FcRn." Trends Cell Biol **15**(1): 5-9.
Levy, R. A., F. C. Dos Santos, G. R. de Jesus and N. R. de Jesus (2015). "Antiphospholipid Antibodies and Antiphospholipid Syndrome during Pregnancy: Diagnostic Concepts." Front Immunol **6**: 205.
Liddelow, S. A., S. Temple, K. Mollgard, R. Gehwolf, A. Wagner, H. Bauer, H. C. Bauer, T. N. Phoenix, K. M. Dziegielewska and N. R. Saunders (2012). "Molecular characterisation of transport mechanisms at the developing mouse blood-CSF interface: a transcriptome approach." PLoS One **7**(3): e33554.
Liederman, J. and K. A. Flannery (1994). "Fall conception increases the risk of neurodevelopmental disorder in offspring." J Clin Exp Neuropsychol **16**(5): 754-768.
Liguori, R., A. Vincent, L. Clover, P. Avoni, G. Plazzi, P. Cortelli, A. Baruzzi, T. Carey, P. Gambetti, E. Lugaresi and P. Montagna (2001). "Morvan's syndrome: peripheral and central nervous system and cardiac involvement with antibodies to voltage-gated potassium channels." Brain **124**(Pt 12): 2417-2426.
Liu, L., A. M. Garcia, H. Santoro, Y. Zhang, K. McDonnell, J. Dumont and A. Bitonti (2007). "Amelioration of experimental autoimmune myasthenia gravis in rats by neonatal FcR blockade." J Immunol **178**(8): 5390-5398.
Love, S. e., H. e. Budka, J. W. e. Ironside, A. e. Perry and J. G. N. Greenfield (2015). Greenfield's neuropathology.
Lu, J., S. Samson, J. Kass and N. Ram (2015). "Acute psychosis in a pregnant patient with Graves' hyperthyroidism and anti-NMDA receptor encephalitis." BMJ Case Rep **2015**.
M. Page, R. T. (1996). Purification of IgG by precipitation with sodium sulfate or ammonium sulfate. The Protein Protocols Handbook, Springer. New York.

Mackay, G., K. Ahmad, J. Stone, C. Sudlow, D. Summers, R. Knight, R. Will, S. R. Irani, A. Vincent and P. Maddison (2012). "NMDA receptor autoantibodies in sporadic Creutzfeldt-Jakob disease." J Neurol **259**(9): 1979-1981.
Mackay, I. R. (2010). "Travels and travails of autoimmunity: a historical journey from discovery to rediscovery." Autoimmun Rev **9**(5): A251-258.
Maddison, P. (2006). "Neuromyotonia." Clinical Neurophysiology **117**(10): 2118-2127.
Magley, J., D. Towner, V. Tache and M. L. Apperson (2012). "Pregnancy outcome in anti-N-methyl-D-aspartate receptor encephalitis." Obstet Gynecol **120**(2 Pt 2): 480-483.
Malek, A., R. Sager and H. Schneider (1994). "Maternal-fetal transport of immunoglobulin G and its subclasses during the third trimester of human pregnancy." Am J Reprod Immunol **32**(1): 8-14.
Mandarim-de-Lacerda, C. A. (2003). "Stereological tools in biomedical research." An Acad Bras Cienc **75**(4): 469-486.
Manto, M., J. Dalmau, A. Didelot, V. Rogemond and J. Honnorat (2010). "In vivo effects of antibodies from patients with anti-NMDA receptor encephalitis: further evidence of synaptic glutamatergic dysfunction." Orphanet J Rare Dis **5**: 31.
Martin, L. A., P. Ashwood, D. Braunschweig, M. Cabanlit, J. Van de Water and D. G. Amaral (2008). "Stereotypies and hyperactivity in rhesus monkeys exposed to IgG from mothers of children with autism." Brain Behav Immun **22**(6): 806-816.
Martinez-Cerdeno, V., J. Camacho, E. Fox, E. Miller, J. Ariza, D. Kienzle, K. Plank, S. C. Noctor and J. Van de Water (2014). "Prenatal Exposure to Autism-Specific Maternal Autoantibodies Alters Proliferation of Cortical Neural Precursor Cells, Enlarges Brain, and Increases Neuronal Size in Adult Animals." Cereb Cortex.
Martinez-Cerdeno, V. and S. C. Noctor (2014). "Cajal, Retzius, and Cajal-Retzius cells." Front Neuroanat **8**: 48.
Masdeu, J. C., A. Gonzalez-Pinto, C. Matute, S. Ruiz De Azua, A. Palomino, J. De Leon, K. F. Berman and J. Dalmau (2012). "Serum IgG antibodies against the NR1 subunit of the NMDA receptor not detected in schizophrenia." Am J Psychiatry **169**(10): 1120-1121.
Masopust, J., C. Andrys, J. Bazant, O. Vysata, K. Kuca and M. Valis (2015). "Anti-NMDA receptor antibodies in patients with a first episode of schizophrenia." Neuropsychiatr Dis Treat **11**: 619-623.
Mat, A., H. Adler, A. Merwick, G. Chadwick, G. Gullo, J. O. Dalmau and N. Tubridy (2013). "Ophelia syndrome with metabotropic glutamate receptor 5 antibodies in CSF." Neurology **80**(14): 1349-1350.
McAllister, D. L., B. J. Kaplan, S. M. Edworthy, L. Martin, S. G. Crawford, R. Ramsey-Goldman, S. Manzi, J. F. Fries and J. Sibley (1997). "The influence of systemic lupus erythematosus on fetal development: cognitive, behavioral, and health trends." J Int Neuropsychol Soc **3**(4): 370-376.
McCarthy, A., J. Dineen, P. McKenna, M. Keogan, J. Sheehan, T. Lynch and K. O'Rourke (2012). "Anti-NMDA receptor encephalitis with associated catatonia during pregnancy." J Neurol **259**(12): 2632-2635.
Meyer, U., J. Feldon and S. H. Fatemi (2009). "In-vivo rodent models for the experimental investigation of prenatal immune activation effects in neurodevelopmental brain disorders." Neurosci Biobehav Rev **33**(7): 1061-1079.
Mikasova, L., P. De Rossi, D. Bouchet, F. Georges, V. Rogemond, A. Didelot, C. Meissirel, J. Honnorat and L. Groc (2012). "Disrupted surface cross-talk between NMDA and Ephrin-B2 receptors in anti-NMDA encephalitis." Brain **135**(Pt 5): 1606-1621.
Mitchell, K. J., Z. J. Huang, B. Moghaddam and A. Sawa (2011). "Following the genes: a framework for animal modeling of psychiatric disorders." BMC Biol **9**: 76.
Moffett, A. and Y. W. Loke (2004). "The immunological paradox of pregnancy: a reappraisal." Placenta **25**(1): 1-8.
Moghaddam, B. and J. H. Krystal (2012). "Capturing the angel in "angel dust": twenty years of translational neuroscience studies of NMDA receptor antagonists in animals and humans." Schizophr Bull **38**(5): 942-949.

Mohn, A. R., R. R. Gainetdinov, M. G. Caron and B. H. Koller (1999). "Mice with reduced NMDA receptor expression display behaviors related to schizophrenia." Cell **98**(4): 427-436.
Molyneaux, B. J., P. Arlotta, J. R. Menezes and J. D. Macklis (2007). "Neuronal subtype specification in the cerebral cortex." Nat Rev Neurosci **8**(6): 427-437.
Morgan, J. T., G. Chana, C. A. Pardo, C. Achim, K. Semendeferi, J. Buckwalter, E. Courchesne and I. P. Everall (2010). "Microglial activation and increased microglial density observed in the dorsolateral prefrontal cortex in autism." Biol Psychiatry **68**(4): 368-376.
Morphis, L. G. and D. Gitlin (1970). "Maturation of the maternofoetal transport system for human gamma-globulin in the mouse." Nature **228**(5271): 573.
Morris, C. M., A. W. Zimmerman and H. S. Singer (2009). "Childhood serum anti-fetal brain antibodies do not predict autism." Pediatr Neurol **41**(4): 288-290.
Mors, O., G. P. Perto and P. B. Mortensen (2011). "The Danish Psychiatric Central Research Register." Scand J Public Health **39**(7 Suppl): 54-57.
Mosher, K. I., R. H. Andres, T. Fukuhara, G. Bieri, M. Hasegawa-Moriyama, Y. He, R. Guzman and T. Wyss-Coray (2012). "Neural progenitor cells regulate microglia functions and activity." Nat Neurosci **15**(11): 1485-1487.
Mostafa, G. A. and A. A. Shehab (2010). "The link of C4B null allele to autism and to a family history of autoimmunity in Egyptian autistic children." J Neuroimmunol **223**(1-2): 115-119.
Motomura, M., I. Johnston, B. Lang, A. Vincent and J. Newsom-Davis (1995). "An improved diagnostic assay for Lambert-Eaton myasthenic syndrome." J Neurol Neurosurg Psychiatry **58**(1): 85-87.
Muller, U. J., B. Teegen, C. Probst, H. G. Bernstein, S. Busse, B. Bogerts, K. Schiltz, W. Stoecker and J. Steiner (2014). "Absence of dopamine receptor serum autoantibodies in schizophrenia patients with an acute disease episode." Schizophr Res **158**(1-3): 272-274.
Murray, E. L., S. Kedar and V. V. Vedanarayanan (2010). "Transmission of maternal muscle-specific tyrosine kinase (MuSK) to offspring: report of two cases." J Clin Neuromuscul Dis **12**(2): 76-79.
Murray, N. A. and I. A. Roberts (2007). "Haemolytic disease of the newborn." Arch Dis Child Fetal Neonatal Ed **92**(2): F83-88.
Nadarajah, B., P. Alifragis, R. O. Wong and J. G. Parnavelas (2003). "Neuronal migration in the developing cerebral cortex: observations based on real-time imaging." Cereb Cortex **13**(6): 607-611.
Nadarajah, B., J. E. Brunstrom, J. Grutzendler, R. O. Wong and A. L. Pearlman (2001). "Two modes of radial migration in early development of the cerebral cortex." Nat Neurosci **4**(2): 143-150.
Nadler, J. J., S. S. Moy, G. Dold, D. Trang, N. Simmons, A. Perez, N. B. Young, R. P. Barbaro, J. Piven, T. R. Magnuson and J. N. Crawley (2004). "Automated apparatus for quantitation of social approach behaviors in mice." Genes Brain Behav **3**(5): 303-314.
Nagado, T., K. Arimura, Y. Sonoda, A. Kurono, Y. Horikiri, A. Kameyama, M. Kameyama, O. Pongs and M. Osame (1999). "Potassium current suppression in patients with peripheral nerve hyperexcitability." Brain **122 (Pt 11)**: 2057-2066.
Namba, T., S. B. Brown and D. Grob (1970). "Neonatal myasthenia gravis: report of two cases and review of the literature." Pediatrics **45**(3): 488-504.
Nasse, M. J. and J. C. Woehl (2010). "Realistic modeling of the illumination point spread function in confocal scanning optical microscopy." Journal of the Optical Society of America A **27**(2): 295-302.
Needleman, L. A. and A. K. McAllister (2012). "The major histocompatibility complex and autism spectrum disorder." Dev Neurobiol **72**(10): 1288-1301.
Neri, F., L. Chimini, F. Bonomi, E. Filippini, M. Motta, D. Faden, A. Lojacono, C. B. Rebaioli, M. Frassi, E. Danieli and A. Tincani (2004). "Neuropsychological development of children born to patients with systemic lupus erythematosus." Lupus **13**(10): 805-811.

Neuber, T., K. Frese, J. Jaehrling, S. Jager, D. Daubert, K. Felderer, M. Linnemann, A. Hohne, S. Kaden, J. Kolln, T. Tiller, B. Brocks, R. Ostendorp and S. Pabst (2014). "Characterization and screening of IgG binding to the neonatal Fc receptor." MAbs **6**(4): 928-942.
Niks, E. H., A. Verrips, B. A. Semmekrot, M. J. Prick, A. Vincent, M. J. van Tol, C. M. Jol-van der Zijde and J. J. Verschuuren (2008). "A transient neonatal myasthenic syndrome with anti-musk antibodies." Neurology **70**(14): 1215-1216.
Nobile, C., R. Michelucci, S. Andreazza, E. Pasini, S. C. Tosatto and P. Striano (2009). "LGI1 mutations in autosomal dominant and sporadic lateral temporal epilepsy." Hum Mutat **30**(4): 530-536.
Noctor, S. C., A. C. Flint, T. A. Weissman, R. S. Dammerman and A. R. Kriegstein (2001). "Neurons derived from radial glial cells establish radial units in neocortex." Nature **409**(6821): 714-720.
Nordahl, C. W., D. Braunschweig, A. M. Iosif, A. Lee, S. Rogers, P. Ashwood, D. G. Amaral and J. Van de Water (2013). "Maternal autoantibodies are associated with abnormal brain enlargement in a subgroup of children with autism spectrum disorder." Brain Behav Immun **30**: 61-65.
Norwood, F., M. Dhanjal, M. Hill, N. James, H. Jungbluth, P. Kyle, G. O'Sullivan, J. Palace, S. Robb, C. Williamson, D. Hilton-Jones and C. Nelson-Piercy (2014). "Myasthenia in pregnancy: best practice guidelines from a U.K. multispecialty working group." J Neurol Neurosurg Psychiatry **85**(5): 538-543.
Nour, M. M., I. Nakashima, E. Coutinho, M. Woodhall, F. Sousa, J. Revis, Y. Takai, J. George, J. Kitley, M. E. Santos, J. M. Nour, F. Cheng, H. Kuroda, T. Misu, A. Martins-da-Silva, G. C. DeLuca, A. Vincent, J. Palace, P. Waters, K. Fujihara and M. I. Leite (2016). "Pregnancy outcomes in aquaporin-4-positive neuromyelitis optica spectrum disorder." Neurology **86**(1): 79-87.
O'Carroll, P., T. E. Bertorini, G. Jacob, C. W. Mitchell and J. Graff (2009). "Transient neonatal myasthenia gravis in a baby born to a mother with new-onset anti-MuSK-mediated myasthenia gravis." J Clin Neuromuscul Dis **11**(2): 69-71.
Odell, D., A. Maciulis, A. Cutler, L. Warren, W. M. McMahon, H. Coon, G. Stubbs, K. Henley and A. Torres (2005). "Confirmation of the association of the C4B null allelle in autism." Hum Immunol **66**(2): 140-145.
Ohkawa, T., Y. Fukata, M. Yamasaki, T. Miyazaki, N. Yokoi, H. Takashima, M. Watanabe, O. Watanabe and M. Fukata (2013). "Autoantibodies to epilepsy-related LGI1 in limbic encephalitis neutralize LGI1-ADAM22 interaction and reduce synaptic AMPA receptors." J Neurosci **33**(46): 18161-18174.
Ohkawa, T., S. Satake, N. Yokoi, Y. Miyazaki, T. Ohshita, G. Sobue, H. Takashima, O. Watanabe, Y. Fukata and M. Fukata (2014). "Identification and characterization of GABA(A) receptor autoantibodies in autoimmune encephalitis." J Neurosci **34**(24): 8151-8163.
Pakozdy, A., U. Glantschnigg, M. Leschnik, H. Hechinger, T. Moloney, B. Lang, P. Halasz and A. Vincent (2014). "EEG-confirmed epileptic activity in a cat with VGKC-complex/LGI1 antibody-associated limbic encephalitis." Epileptic Disord **16**(1): 116-120.
Pakozdy, A., P. Halasz, A. Klang, J. Bauer, M. Leschnik, A. Tichy, J. G. Thalhammer, B. Lang and A. Vincent (2013). "Suspected limbic encephalitis and seizure in cats associated with voltage-gated potassium channel (VGKC) complex antibody." J Vet Intern Med **27**(1): 212-214.
Palmeira, P., C. Quinello, A. L. Silveira-Lessa, C. A. Zago and M. Carneiro-Sampaio (2012). "IgG placental transfer in healthy and pathological pregnancies." Clin Dev Immunol **2012**: 985646.
Paloneva, J., M. Kestila, J. Wu, A. Salminen, T. Bohling, V. Ruotsalainen, P. Hakola, A. B. Bakker, J. H. Phillips, P. Pekkarinen, L. L. Lanier, T. Timonen and L. Peltonen (2000). "Loss-of-function mutations in TYROBP (DAP12) result in a presenile dementia with bone cysts." Nat Genet **25**(3): 357-361.

Paoletti, P., C. Bellone and Q. Zhou (2013). "NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease." Nat Rev Neurosci **14**(6): 383-400.
Paolicelli, R. C., G. Bolasco, F. Pagani, L. Maggi, M. Scianni, P. Panzanelli, M. Giustetto, T. A. Ferreira, E. Guiducci, L. Dumas, D. Ragozzino and C. T. Gross (2011). "Synaptic pruning by microglia is necessary for normal brain development." Science **333**(6048): 1456-1458.
Parthasarathi, U. D., T. Harrower, M. Tempest, J. R. Hodges, C. Walsh, P. J. McKenna and P. C. Fletcher (2006). "Psychiatric presentation of voltage-gated potassium channel antibody-associated encephalopathy. Case report." Br J Psychiatry **189**: 182-183.
Pathmanandavel, K., J. Starling, V. Merheb, S. Ramanathan, N. Sinmaz, R. C. Dale and F. Brilot (2015). "Antibodies to surface dopamine-2 receptor and N-methyl-D-aspartate receptor in the first episode of acute psychosis in children." Biol Psychiatry **77**(6): 537-547.
Patrick, J. and J. Lindstrom (1973). "Autoimmune response to acetylcholine receptor." Science **180**(4088): 871-872.
Patterson, P. H. (2002). "Maternal infection: window on neuroimmune interactions in fetal brain development and mental illness." Curr Opin Neurobiol **12**(1): 115-118.
Pedersen, C. B. (2011). "The Danish Civil Registration System." Scand J Public Health **39**(7 Suppl): 22-25.
Pedersen, C. B., H. Gotzsche, J. O. Moller and P. B. Mortensen (2006). "The Danish Civil Registration System. A cohort of eight million persons." Dan Med Bull **53**(4): 441-449.
Penagarikano, O., B. S. Abrahams, E. I. Herman, K. D. Winden, A. Gdalyahu, H. Dong, L. I. Sonnenblick, R. Gruver, J. Almajano, A. Bragin, P. Golshani, J. T. Trachtenberg, E. Peles and D. H. Geschwind (2011). "Absence of CNTNAP2 leads to epilepsy, neuronal migration abnormalities, and core autism-related deficits." Cell **147**(1): 235-246.
Petanjek, Z., M. Judas, G. Simic, M. R. Rasin, H. B. Uylings, P. Rakic and I. Kostovic (2011). "Extraordinary neoteny of synaptic spines in the human prefrontal cortex." Proc Natl Acad Sci U S A **108**(32): 13281-13286.
Petit-Pedrol, M., T. Armangue, X. Peng, L. Bataller, T. Cellucci, R. Davis, L. McCracken, E. Martinez-Hernandez, W. P. Mason, M. C. Kruer, D. G. Ritacco, W. Grisold, B. F. Meaney, C. Alcala, P. Sillevis-Smitt, M. J. Titulaer, R. Balice-Gordon, F. Graus and J. Dalmau (2014). "Encephalitis with refractory seizures, status epilepticus, and antibodies to the GABAA receptor: a case series, characterisation of the antigen, and analysis of the effects of antibodies." Lancet Neurol **13**(3): 276-286.
Pettingill, P., H. B. Kramer, J. A. Coebergh, R. Pettingill, S. Maxwell, A. Nibber, A. Malaspina, A. Jacob, S. R. Irani, C. Buckley, D. Beeson, B. Lang, P. Waters and A. Vincent (2015). "Antibodies to GABAA receptor alpha1 and gamma2 subunits: clinical and serologic characterization." Neurology **84**(12): 1233-1241.
Piepgras, J., M. Holtje, K. Michel, Q. Li, C. Otto, C. Drenckhahn, C. Probst, M. Schemann, S. Jarius, W. Stocker, B. Balint, H. M. Meinck, R. Buchert, J. Dalmau, G. Ahnert-Hilger and K. Ruprecht (2015). "Anti-DPPX encephalitis: pathogenic effects of antibodies on gut and brain neurons." Neurology **85**(10): 890-897.
Piras, I. S., L. Haapanen, V. Napolioni, R. Sacco, J. Van de Water and A. M. Persico (2014). "Anti-brain antibodies are associated with more severe cognitive and behavioral profiles in Italian children with Autism Spectrum Disorder." Brain Behav Immun **38**: 91-99.
Planaguma, J., F. Leypoldt, F. Mannara, J. Gutierrez-Cuesta, E. Martin-Garcia, E. Aguilar, M. J. Titulaer, M. Petit-Pedrol, A. Jain, R. Balice-Gordon, M. Lakadamyali, F. Graus, R. Maldonado and J. Dalmau (2015). "Human N-methyl D-aspartate receptor antibodies alter memory and behaviour in mice." Brain **138**(Pt 1): 94-109.
Polgar, E., M. Watanabe, B. Hartmann, S. G. Grant and A. J. Todd (2008). "Expression of AMPA receptor subunits at synapses in laminae I-III of the rodent spinal dorsal horn." Mol Pain **4**: 5.

Poliak, S., D. Salomon, H. Elhanany, H. Sabanay, B. Kiernan, L. Pevny, C. L. Stewart, X. Xu, S. Y. Chiu, P. Shrager, A. J. Furley and E. Peles (2003). "Juxtaparanodal clustering of Shaker-like K+ channels in myelinated axons depends on Caspr2 and TAG-1." J Cell Biol **162**(6): 1149-1160.
Price, B. H., R. D. Adams and J. T. Coyle (2000). "Neurology and psychiatry: closing the great divide." Neurology **54**(1): 8-14.
Pruss, H., J. Leubner, N. K. Wenke, G. A. Czirjak, C. A. Szentiks and A. D. Greenwood (2015). "Anti-NMDA Receptor Encephalitis in the Polar Bear (Ursus maritimus) Knut." Sci Rep **5**: 12805.
Pruss, H., M. Rothkirch, U. Kopp, H. M. Hamer, M. Hagge, P. Sterzer, S. Saschenbrecker, W. Stocker, L. Harms and M. Endres (2014). "Limbic encephalitis with mGluR5 antibodies and immunotherapy-responsive prosopagnosia." Neurology **83**(15): 1384-1386.
Rademakers, R., M. Baker, A. M. Nicholson, N. J. Rutherford, N. Finch, A. Soto-Ortolaza, J. Lash, C. Wider, A. Wojtas, M. DeJesus-Hernandez, J. Adamson, N. Kouri, C. Sundal, E. A. Shuster, J. Aasly, J. MacKenzie, S. Roeber, H. A. Kretzschmar, B. F. Boeve, D. S. Knopman, R. C. Petersen, N. J. Cairns, B. Ghetti, S. Spina, J. Garbern, A. C. Tselis, R. Uitti, P. Das, J. A. Van Gerpen, J. F. Meschia, S. Levy, D. F. Broderick, N. Graff-Radford, O. A. Ross, B. B. Miller, R. H. Swerdlow, D. W. Dickson and Z. K. Wszolek (2012). "Mutations in the colony stimulating factor 1 receptor (CSF1R) gene cause hereditary diffuse leukoencephalopathy with spheroids." Nat Genet **44**(2): 200-205.
Rakic, P. (1972). "Mode of cell migration to the superficial layers of fetal monkey neocortex." J Comp Neurol **145**(1): 61-83.
Rasband, M. N. (2010). "Clustered K+ channel complexes in axons." Neurosci Lett **486**(2): 101-106.
Ratelade, J. and A. S. Verkman (2014). "Inhibitor(s) of the classical complement pathway in mouse serum limit the utility of mice as experimental models of neuromyelitis optica." Mol Immunol **62**(1): 104-113.
Rhoads, J., H. Guirgis, C. McKnight and A. M. Duchemin (2011). "Lack of anti-NMDA receptor autoantibodies in the serum of subjects with schizophrenia." Schizophr Res **129**(2-3): 213-214.
Riemersma, S., A. Vincent, D. Beeson, C. Newland, S. Hawke, B. Vernet-der Garabedian, B. Eymard and J. Newsom-Davis (1996). "Association of arthrogryposis multiplex congenita with maternal antibodies inhibiting fetal acetylcholine receptor function." J Clin Invest **98**(10): 2358-2363.
Rivas, R. J. and M. E. Hatten (1995). "Motility and cytoskeletal organization of migrating cerebellar granule neurons." J Neurosci **15**(2): 981-989.
Robert, D. (2005). Extensions of DAMAS and Benefits and Limitations of Deconvolution in Beamforming. 11th AIAA/CEAS Aeroacoustics Conference, American Institute of Aeronautics and Astronautics.
Roberts, W. K. and R. B. Darnell (2004). "Neuroimmunology of the paraneoplastic neurological degenerations." Curr Opin Immunol **16**(5): 616-622.
Rodenas-Cuadrado, P., J. Ho and S. C. Vernes (2014). "Shining a light on CNTNAP2: complex functions to complex disorders." Eur J Hum Genet **22**(2): 171-178.
Rodriguez, J. I. and J. K. Kern (2011). "Evidence of microglial activation in autism and its possible role in brain underconnectivity." Neuron Glia Biol **7**(2-4): 205-213.
Roitt, I. M., D. Doniach, P. N. Campbell and R. V. Hudson (1956). "Auto-antibodies in Hashimoto's disease (lymphadenoid goitre)." Lancet **271**(6947): 820-821.
Roopenian, D. C. and S. Akilesh (2007). "FcRn: the neonatal Fc receptor comes of age." Nat Rev Immunol **7**(9): 715-725.
Rose, N. R. and C. Bona (1993). "Defining criteria for autoimmune diseases (Witebsky's postulates revisited)." Immunol Today **14**(9): 426-430.
Rossi, C. C., J. Fuentes, J. Van de Water and D. G. Amaral (2013). "Brief Report: Antibodies Reacting to Brain Tissue in Basque Spanish Children with Autism Spectrum Disorder and Their Mothers." J Autism Dev Disord.

Rossi, C. C., J. Van de Water, S. J. Rogers and D. G. Amaral (2011). "Detection of plasma autoantibodies to brain tissue in young children with and without autism spectrum disorders." Brain Behav Immun **25**(6): 1123-1135.
Rossi, M., S. Mead, J. Collinge, P. Rudge and A. Vincent (2015). "Neuronal antibodies in patients with suspected or confirmed sporadic Creutzfeldt-Jakob disease." J Neurol Neurosurg Psychiatry **86**(6): 692-694.
Roumier, A., O. Pascual, C. Bechade, S. Wakselman, J. C. Poncer, E. Real, A. Triller and A. Bessis (2008). "Prenatal activation of microglia induces delayed impairment of glutamatergic synaptic function." PLoS One **3**(7): e2595.
Saadoun, S., P. Waters, M. I. Leite, J. L. Bennett, A. Vincent and M. C. Papadopoulos (2013). "Neuromyelitis Optica IgG Causes Placental Inflammation and Fetal Death." J Immunol.
Saunders, N. R., S. A. Liddelow and K. M. Dziegielewska (2012). "Barrier mechanisms in the developing brain." Front Pharmacol **3**: 46.
Schafer, D. P., E. K. Lehrman, A. G. Kautzman, R. Koyama, A. R. Mardinly, R. Yamasaki, R. M. Ransohoff, M. E. Greenberg, B. A. Barres and B. Stevens (2012). "Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner." Neuron **74**(4): 691-705.
Schuler, T., I. Mesic, C. Madry, I. Bartholomaus and B. Laube (2008). "Formation of NR1/NR2 and NR1/NR3 heterodimers constitutes the initial step in N-methyl-D-aspartate receptor assembly." J Biol Chem **283**(1): 37-46.
Scott, O., L. Richer, K. Forbes, L. Sonnenberg, A. Currie, M. Eliyashevska and H. R. Goez (2014). "Anti-N-methyl-D-aspartate (NMDA) receptor encephalitis: an unusual cause of autistic regression in a toddler." J Child Neurol **29**(5): 691-694.
Sekar, A., A. R. Bialas, H. de Rivera, A. Davis, T. R. Hammond, N. Kamitaki, K. Tooley, J. Presumey, M. Baum, V. Van Doren, G. Genovese, S. A. Rose, R. E. Handsaker, C. Schizophrenia Working Group of the Psychiatric Genomics, M. J. Daly, M. C. Carroll, B. Stevens and S. A. McCarroll (2016). "Schizophrenia risk from complex variation of complement component 4." Nature.
Senechal, K. R., C. Thaller and J. L. Noebels (2005). "ADPEAF mutations reduce levels of secreted LGI1, a putative tumor suppressor protein linked to epilepsy." Hum Mol Genet **14**(12): 1613-1620.
Shaaban, H. S., H. F. Choo and J. W. Sensakovic (2012). "Anti-NMDA-receptor encephalitis presenting as postpartum psychosis in a young woman, treated with rituximab." Ann Saudi Med **32**(4): 421-423.
Shillito, P., P. C. Molenaar, A. Vincent, K. Leys, W. Zheng, R. J. van den Berg, J. J. Plomp, G. T. van Kempen, G. Chauplannaz, A. R. Wintzen and et al. (1995). "Acquired neuromyotonia: evidence for autoantibodies directed against K+ channels of peripheral nerves." Ann Neurol **38**(5): 714-722.
Sillevis Smitt, P., A. Kinoshita, B. De Leeuw, W. Moll, M. Coesmans, D. Jaarsma, S. Henzen-Logmans, C. Vecht, C. De Zeeuw, N. Sekiyama, S. Nakanishi and R. Shigemoto (2000). "Paraneoplastic cerebellar ataxia due to autoantibodies against a glutamate receptor." N Engl J Med **342**(1): 21-27.
Silva, J., G. Wang and J. K. Cowell (2011). "The temporal and spatial expression pattern of the LGI1 epilepsy predisposition gene during mouse embryonic cranial development." BMC Neurosci **12**: 43.
Silverstein, A. M. (1996). "Paul Ehrlich: the founding of pediatric immunology." Cell Immunol **174**(1): 1-6.
Simister, N. E. and K. E. Mostov (1989). "An Fc receptor structurally related to MHC class I antigens." Nature **337**(6203): 184-187.
Singer, H. S., C. Morris, C. Gause, M. Pollard, A. W. Zimmerman and M. Pletnikov (2009). "Prenatal exposure to antibodies from mothers of children with autism produces neurobehavioral alterations: A pregnant dam mouse model." J Neuroimmunol **211**(1-2): 39-48.

Singer, H. S., C. M. Morris, C. D. Gause, P. K. Gillin, S. Crawford and A. W. Zimmerman (2008). "Antibodies against fetal brain in sera of mothers with autistic children." J Neuroimmunol **194**(1-2): 165-172.
Sinha, S., J. Newsom-Davis, K. Mills, N. Byrne, B. Lang and A. Vincent (1991). "Autoimmune aetiology for acquired neuromyotonia (Isaacs' syndrome)." Lancet **338**(8759): 75-77.
Somers, K. J., V. A. Lennon, J. R. Rundell, S. J. Pittock, D. A. Drubach, M. R. Trenerry, D. H. Lachance, C. J. Klein, P. A. Aston and A. McKeon (2011). "Psychiatric manifestations of voltage-gated potassium-channel complex autoimmunity." J Neuropsychiatry Clin Neurosci **23**(4): 425-433.
Sonoda, Y., K. Arimura, A. Kurono, M. Suehara, M. Kameyama, S. Minato, A. Hayashi and M. Osame (1996). "Serum of Isaacs' syndrome suppresses potassium channels in PC-12 cell lines." Muscle Nerve **19**(11): 1439-1446.
Spinazzi, M., V. Argentiero, L. Zuliani, A. Palmieri, B. Tavolato and A. Vincent (2008). "Immunotherapy-reversed compulsive, monoaminergic, circadian rhythm disorder in Morvan syndrome." Neurology **71**(24): 2008-2010.
Steiner, J., M. Walter, W. Glanz, Z. Sarnyai, H. G. Bernstein, S. Vielhaber, A. Kastner, M. Skalej, W. Jordan, K. Schiltz, C. Klingbeil, K. P. Wandinger, B. Bogerts and W. Stoecker (2013). "Increased prevalence of diverse N-methyl-D-aspartate glutamate receptor antibodies in patients with an initial diagnosis of schizophrenia: specific relevance of IgG NR1a antibodies for distinction from N-methyl-D-aspartate glutamate receptor encephalitis." JAMA Psychiatry **70**(3): 271-278.
Stephan, A. H., B. A. Barres and B. Stevens (2012). "The complement system: an unexpected role in synaptic pruning during development and disease." Annu Rev Neurosci **35**: 369-389.
Stevens, B., N. J. Allen, L. E. Vazquez, G. R. Howell, K. S. Christopherson, N. Nouri, K. D. Micheva, A. K. Mehalow, A. D. Huberman, B. Stafford, A. Sher, A. M. Litke, J. D. Lambris, S. J. Smith, S. W. John and B. A. Barres (2007). "The classical complement cascade mediates CNS synapse elimination." Cell **131**(6): 1164-1178.
Stiles, J. and T. L. Jernigan (2010). "The basics of brain development." Neuropsychol Rev **20**(4): 327-348.
Strauss, K. A., E. G. Puffenberger, M. J. Huentelman, S. Gottlieb, S. E. Dobrin, J. M. Parod, D. A. Stephan and D. H. Morton (2006). "Recessive symptomatic focal epilepsy and mutant contactin-associated protein-like 2." N Engl J Med **354**(13): 1370-1377.
Supramaniam, V., R. Vontell, L. Srinivasan, J. Wyatt-Ashmead, H. Hagberg and M. Rutherford (2013). "Microglia activation in the extremely preterm human brain." Pediatr Res **73**(3): 301-309.
Tamamaki, N., K. E. Fujimori and R. Takauji (1997). "Origin and route of tangentially migrating neurons in the developing neocortical intermediate zone." J Neurosci **17**(21): 8313-8323.
Tanaka, D. H. and K. Nakajima (2012). "Migratory pathways of GABAergic interneurons when they enter the neocortex." Eur J Neurosci **35**(11): 1655-1660.
Tanaka, K., M. Tanaka, S. Igarashi, O. Onodera, T. Miyatake and S. Tsuji (1995). "Trial to establish an animal model of paraneoplastic cerebellar degeneration with anti-Yo antibody. 2. Passive transfer of murine mononuclear cells activated with recombinant Yo protein to paraneoplastic cerebellar degeneration lymphocytes in severe combined immunodeficiency mice." Clin Neurol Neurosurg **97**(1): 101-105.
Titulaer, M. J., B. Lang and J. J. Verschuuren (2011). "Lambert-Eaton myasthenic syndrome: from clinical characteristics to therapeutic strategies." Lancet Neurol **10**(12): 1098-1107.
Titulaer, M. J., L. McCracken, I. Gabilondo, T. Armangué, C. Glaser, T. Iizuka, L. S. Honig, S. M. Benseler, I. Kawachi, E. Martinez-Hernandez, E. Aguilar, N. Gresa-Arribas, N. Ryan-Florance, A. Torrents, A. Saiz, M. R. Rosenfeld, R. Balice-Gordon, F. Graus and J. Dalmau (2013). "Treatment and prognostic factors for long-term outcome in

patients with anti-NMDA receptor encephalitis: an observational cohort study." The Lancet Neurology **12**(2): 157-165.
Tomimitsu, H., K. Arimura, T. Nagado, O. Watanabe, R. Otsuka, A. Kurono, Y. Sonoda, M. Osame and M. Kameyama (2004). "Mechanism of action of voltage-gated K+ channel antibodies in acquired neuromyotonia." Ann Neurol **56**(3): 440-444.
Traynelis, S. F., L. P. Wollmuth, C. J. McBain, F. S. Menniti, K. M. Vance, K. K. Ogden, K. B. Hansen, H. Yuan, S. J. Myers and R. Dingledine (2010). "Glutamate receptor ion channels: structure, regulation, and function." Pharmacol Rev **62**(3): 405-496.
Trowsdale, J. and A. G. Betz (2006). "Mother's little helpers: mechanisms of maternal-fetal tolerance." Nat Immunol **7**(3): 241-246.
Tsutsui, K., T. Kanbayashi, K. Tanaka, S. Boku, W. Ito, J. Tokunaga, A. Mori, Y. Hishikawa, T. Shimizu and S. Nishino (2012). "Anti-NMDA-receptor antibody detected in encephalitis, schizophrenia, and narcolepsy with psychotic features." BMC Psychiatry **12**: 37.
Urowitz, M. B., D. D. Gladman, A. MacKinnon, D. Ibanez, V. Bruto, J. Rovet and E. Silverman (2008). "Neurocognitive abnormalities in offspring of mothers with systemic lupus erythematosus." Lupus **17**(6): 555-560.
Valiente, M. and O. Marin (2010). "Neuronal migration mechanisms in development and disease." Curr Opin Neurobiol **20**(1): 68-78.
van Loo, K. M. and G. J. Martens (2007). "Genetic and environmental factors in complex neurodevelopmental disorders." Curr Genomics **8**(7): 429-444.
Varea, O., M. D. Martin-de-Saavedra, K. J. Kopeikina, B. Schurmann, H. J. Fleming, J. M. Fawcett-Patel, A. Bach, S. Jang, E. Peles, E. Kim and P. Penzes (2015). "Synaptic abnormalities and cytoplasmic glutamate receptor aggregates in contactin associated protein-like 2/Caspr2 knockout neurons." Proc Natl Acad Sci U S A **112**(19): 6176-6181.
Vargas, D. L., C. Nascimbene, C. Krishnan, A. W. Zimmerman and C. A. Pardo (2005). "Neuroglial activation and neuroinflammation in the brain of patients with autism." Ann Neurol **57**(1): 67-81.
Vedeler, C. A., J. C. Antoine, B. Giometto, F. Graus, W. Grisold, I. K. Hart, J. Honnorat, P. A. Sillevis Smitt, J. J. Verschuuren, R. Voltz and E. Paraneoplastic Neurological Syndrome (2006). "Management of paraneoplastic neurological syndromes: report of an EFNS Task Force." Eur J Neurol **13**(7): 682-690.
Vernet-der Garabedian, B., M. Lacokova, B. Eymard, E. Morel, M. Faltin, J. Zajac, O. Sadovsky, M. Dommergues, P. Tripon and J. F. Bach (1994). "Association of neonatal myasthenia gravis with antibodies against the fetal acetylcholine receptor." J Clin Invest **94**(2): 555-559.
Vincent, A., P. Dalton, L. Clover, J. Palace and B. Lang (2003). "Antibodies to neuronal targets in neurological and psychiatric diseases." Ann N Y Acad Sci **992**: 48-55.
Vincent, A., R. Deacon, P. Dalton, C. Salmond, A. M. Blamire, S. Pendlebury, H. Johansen-Berg, B. Rajogopalan, P. Styles and J. Stein (2002). "Maternal antibody-mediated dyslexia? Evidence for a pathogenic serum factor in a mother of two dyslexic children shown by transfer to mice using behavioural studies and magnetic resonance spectroscopy." J Neuroimmunol **130**(1-2): 243-247.
Vitaliani, R., W. Mason, B. Ances, T. Zwerdling, Z. Jiang and J. Dalmau (2005). "Paraneoplastic encephalitis, psychiatric symptoms, and hypoventilation in ovarian teratoma." Ann Neurol **58**(4): 594-604.
Warren, R. P., P. Cole, J. D. Odell, C. B. Pingree, W. L. Warren, E. White, J. Yonk and V. K. Singh (1990). "Detection of maternal antibodies in infantile autism." J Am Acad Child Adolesc Psychiatry **29**(6): 873-877.
Warren, R. P., V. K. Singh, P. Cole, J. D. Odell, C. B. Pingree, W. L. Warren and E. White (1991). "Increased frequency of the null allele at the complement C4b locus in autism." Clin Exp Immunol **83**(3): 438-440.
Waters, P. J., A. McKeon, M. I. Leite, S. Rajasekharan, V. A. Lennon, A. Villalobos, J. Palace, J. N. Mandrekar, A. Vincent, A. Bar-Or and S. J. Pittock (2012). "Serologic

diagnosis of NMO: a multicenter comparison of aquaporin-4-IgG assays." Neurology **78**(9): 665-671; discussion 669.
Wendykier, P. and J. G. Nagy (2010). "Parallel Colt: A High-Performance Java Library for Scientific Computing and Image Processing." ACM Trans. Math. Softw. **37**(3): 1-22.
Whish, S., K. M. Dziegielewska, K. Mollgard, N. M. Noor, S. A. Liddelow, M. D. Habgood, S. J. Richardson and N. R. Saunders (2015). "The inner CSF-brain barrier: developmentally controlled access to the brain via intercellular junctions." Front Neurosci **9**: 16.
Wierzba-Bobrowicz T, L. E., Lechowicz W, Stepień T, Pasennik E. (2005). "Quantitative analysis of activated microglia, ramified and damage of processes in the frontal and temporal lobes of chronic schizophrenics." Folia Neuropathol **43**(2): 81.
Wills, S., M. Cabanlit, J. Bennett, P. Ashwood, D. Amaral and J. Van de Water (2007). "Autoantibodies in autism spectrum disorders (ASD)." Ann N Y Acad Sci **1107**: 79-91.
Wright, S., K. Hashemi, L. Stasiak, J. Bartram, B. Lang, A. Vincent and A. L. Upton (2015). "Epileptogenic effects of NMDAR antibodies in a passive transfer mouse model." Brain **138**(Pt 11): 3159-3167.
Xu, G., J. Jing, K. Bowers, B. Liu and W. Bao (2014). "Maternal diabetes and the risk of autism spectrum disorders in the offspring: a systematic review and meta-analysis." J Autism Dev Disord **44**(4): 766-775.
Xue, Q. S. and W. J. Streit (2011). "Microglial pathology in Down syndrome." Acta Neuropathol **122**(4): 455-466.
Yu, A. Y. and F. G. Moore (2011). "Paraneoplastic encephalitis presenting as postpartum psychosis." Psychosomatics **52**(6): 568-570.
Zandi, M. S., S. R. Irani, B. Lang, P. Waters, P. B. Jones, P. McKenna, A. J. Coles, A. Vincent and B. R. Lennox (2011). "Disease-relevant autoantibodies in first episode schizophrenia." J Neurol **258**(4): 686-688.
Zerbo, O., A. M. Iosif, L. Delwiche, C. Walker and I. Hertz-Picciotto (2011). "Month of conception and risk of autism." Epidemiology **22**(4): 469-475.
Zerbo, O., A. Leong, L. Barcellos, P. Bernal, B. Fireman and L. A. Croen (2015). "Immune mediated conditions in autism spectrum disorders." Brain Behav Immun **46**: 232-236.
Zerbo, O., C. Yoshida, J. K. Grether, J. Van de Water, P. Ashwood, G. N. Delorenze, R. L. Hansen, M. Kharrazi and L. A. Croen (2014). "Neonatal cytokines and chemokines and risk of Autism Spectrum Disorder: the Early Markers for Autism (EMA) study: a case-control study." J Neuroinflammation **11**: 113.
Zhan, Y., R. C. Paolicelli, F. Sforazzini, L. Weinhard, G. Bolasco, F. Pagani, A. L. Vyssotski, A. Bifone, A. Gozzi, D. Ragozzino and C. T. Gross (2014). "Deficient neuron-microglia signaling results in impaired functional brain connectivity and social behavior." Nat Neurosci **17**(3): 400-406.
Zhang, Q., K. Tanaka, P. Sun, M. Nakata, R. Yamamoto, K. Sakimura, M. Matsui and N. Kato (2012). "Suppression of synaptic plasticity by cerebrospinal fluid from anti-NMDA receptor encephalitis patients." Neurobiol Dis **45**(1): 610-615.
Zhou, Y. D., S. Lee, Z. Jin, M. Wright, S. E. Smith and M. P. Anderson (2009). "Arrested maturation of excitatory synapses in autosomal dominant lateral temporal lobe epilepsy." Nat Med **15**(10): 1208-1214.
Zhu, X., A. C. Need, S. Petrovski and D. B. Goldstein (2014). "One gene, many neuropsychiatric disorders: lessons from Mendelian diseases." Nat Neurosci **17**(6): 773-781.
Zimmerman, A. W., S. L. Connors, K. J. Matteson, L. C. Lee, H. S. Singer, J. A. Castaneda and D. A. Pearce (2007). "Maternal antibrain antibodies in autism." Brain Behav Immun **21**(3): 351-357.
Zlokovic, B. V., D. S. Skundric, M. B. Segal, M. N. Lipovac, J. B. Mackic and H. Davson (1990). "A saturable mechanism for transport of immunoglobulin G across the blood-brain barrier of the guinea pig." Exp Neurol **107**(3): 263-270.

Appendix

ICD-10 codes

Full description of the ICD-10 diagnostic codes used in this thesis is given in the next pages. Information was copied from http://apps.who.int/classifications/icd10