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*Pathological implications of
anti-GluA3 antibodies in
Frontotemporal Dementia:
from animal model to rescue strategy*

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Abstract

Over the last two decades, growing attention has been paid to the role of autoantibodies that target brain antigens in disorders of the central nervous system. Within this field, several studies detected anti-GluA3 antibodies in the serum of patients with autoimmune encephalitis and epilepsy. More recently, our research group identified anti-GluA3 antibodies in 20%–25% of patients with frontotemporal dementia (FTD), introducing novel implications for a pathogenic role of glutamate receptor autoantibodies in neurodegenerative diseases. Although previous *in vitro* studies showed that anti-GluA3 autoantibodies affect glutamatergic neurotransmission through different mechanisms, the impact of these autoantibodies on cognitive and social functions *in vivo* is still unknown, as well as the biological substrates of these putative modifications.

To address this gap of knowledge and better dissect the contribution of anti-GluA3 autoantibodies to the above-mentioned neurological conditions, in the present study, we employed two models of *in vivo* administration of anti-GluA3 antibodies isolated from patients (anti-GluA3 hIgGs) to mice. First, we demonstrated that a single injection of anti-GluA3 hIgGs into the lateral ventricles induced profound molecular and morphological alterations into the prefrontal cortex of mice. Accordingly, mice presented significant changes in recognition memory and impairments in social behavior and in social cognitive functions. Given the negative effects of acute GluA3 hIgGs administration on synapses, behavior, and cognition, whether and how the chronic presence of autoantibodies triggers a neurodegenerative process and the association between this process and the appearance of FTD-related neuropathological and behavioral signature should be further investigated. Thus, we developed a chronic model by infusing mice with anti-GluA3 hIgGs for one month through an intracerebroventricular cannula. Data showed that chronic anti-GluA3 hIgGs administration in mice mediated the internalization of GluA3-containing AMPARs and led to selective accumulation of the phosphorylated form of tau in the post-synaptic fraction and dendritic spine loss in the prefrontal cortex of mice. Remarkably, we detected an increase in p-tau levels in cerebrospinal fluid of FTD patients positive for anti-GluA3 hIgGs (anti-GluA3-Ab+) compared with negative ones. In addition, mice chronically treated with anti-GluA3 hIgGs exhibited behavioral disturbances that mostly reflected the deficits detected in anti-GluA3-Ab+ patients, such as apathy, binge-like eating, and impulsivity. Finally, anti-GluA3 hIgG-mediated alterations were rescued in the chronic animal model by enhancing glutamatergic neurotransmission by a positive allosteric modulator of AMPAR.

Overall, our study clarified the contribution of anti-GluA3 antibodies to central nervous system symptoms and pathology and identified a new subgroup of FTD patients. Our findings will be instrumental in the development of a therapeutic personalized medicine strategy for anti-GluA3-Ab+ patients.

Sommario

Nel corso degli ultimi decenni, numerosi gruppi di ricerca hanno focalizzato la loro attenzione sullo studio del ruolo svolto da autoanticorpi diretti contro antigeni neuronali nelle malattie del sistema nervoso centrale. Tra questi, autoanticorpi diretti contro la sub-unità GluA3 (anti-GluA3) del recettore per il glutammato AMPA sono stati inizialmente individuati nel siero di pazienti affetti da encefalite autoimmune ed epilessia. Più di recente, tuttavia, il gruppo di ricerca coordinato dal professor Fabrizio Gardoni ha identificato questi stessi anticorpi in circa il 20%-25% di pazienti affetti da Demenza Frontotemporale (FTD). Questa scoperta è di particolare rilevanza in quanto, per la prima volta, rende possibile ipotizzare un ruolo patologico di autoanticorpi diretti contro recettori del glutammato anche in malattie neurodegenerative. Precedenti studi condotti su modelli in vitro hanno dimostrato come gli autoanticorpi anti-GluA3 vadano ad influenzare negativamente la neurotrasmissione glutammatergica attraverso diversi meccanismi. Tuttavia, l'impatto di questi autoanticorpi sulle funzioni cognitive e sociali in vivo è ancora sconosciuto, così come i substrati biologici di queste potenziali alterazioni.

Per colmare questa lacuna e comprendere il contributo degli anticorpi anti-GluA3 nelle condizioni neurologiche sopra descritte, in questo studio, abbiamo sviluppato ed utilizzato due modelli di somministrazione in vivo di anticorpi anti-GluA3 isolati dai pazienti (anti-GluA3 hIgGs) in modelli murini. In primo luogo, abbiamo dimostrato come una singola iniezione di anti-GluA3 hIgGs nei ventricoli laterali sia sufficiente per indurre profonde alterazioni molecolari e morfologiche a livello della corteccia prefrontale del modello animale. Coerentemente con questi risultati, gli animali trattati in acuto con anti-GluA3 hIgGs mostrano alterazioni nella memoria e nel comportamento sociale. Avendo dimostrato come la somministrazione acuta di GluA3 hIgGs porti ai difetti sopra riportati, abbiamo successivamente voluto indagare se e come la presenza cronica di anti-GluA3 hIgGs potesse innescare un processo neurodegenerativo e l'associazione tra questo processo e la comparsa di caratteristiche neuropatologiche e comportamentali legate allo spettro FTD. Con questa finalità, abbiamo sviluppato un modello cronico di autoimmunità in FTD trattando topi con anti-GluA3 hIgGs per un mese attraverso una cannula intracerebroventricolare. I dati ottenuti da questo modello hanno mostrato come la somministrazione cronica di anti-GluA3 hIgGs nei topi porti all'internalizzazione degli AMPAR contenenti GluA3 così come ad un accumulo selettivo della forma fosforilata di tau nella frazione post-sinaptica unitamente alla perdita di spine dendritiche nella corteccia prefrontale. È importante sottolineare come un aumento dei livelli di p-tau sia stato anche identificato nel liquido cerebrospinale dei pazienti FTD positivi agli anticorpi contro GluA3 (anti-GluA3-Ab+) quando paragonati ai pazienti negativi. Inoltre, l'amministrazione cronica di anti-GluA3 hIgGs nel

topo ha portato alla comparsa di disturbi comportamentali che, almeno in parte, riflettono i deficit propri dei pazienti FTD GluA3-Ab+, come apatia, binge-eating ed impulsività. Infine, abbiamo dimostrato come quasi tutte le alterazioni mediate da anti-GluA3 hIgGs possano essere migliorate, nel modello murino, andando a potenziare la neurotrasmissione glutammatergica con un modulatore allosterico positivo (PAM) degli AMPAR.

Nel complesso, questo studio ha permesso di chiarificare il contributo degli autoanticorpi anti-GluA3 ai sintomi e alla patologia della FTD. Inoltre, ha permesso di identificare un sottogruppo specifico di pazienti FTD con tratti clinici e neuropatologici distinti. Alla luce di questo, i risultati qui riportati saranno potenzialmente utili per lo sviluppo di una strategia terapeutica di medicina personalizzata per i pazienti FTD anti-GluA3-Ab+.

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1. Introduction

My PhD project stems from a fundamental clinical observation: the presence of autoantibodies directed against the GluA3 sub-unit of AMPA-type glutamate receptors (anti-GluA3 antibodies) in the serum and in the cerebrospinal fluid (CSF) of approximately 20% to 25% of patients with Frontotemporal dementia (FTD) (Borroni et al. 2017). During my PhD, I employed *in vivo* models to address the following question: *do anti-GluA3 autoantibodies play a role in the pathogenesis of this neurodegenerative disease?*

This question highlights three principal elements at the core of my project: autoimmunity and autoantibodies, the GluA3 sub-unit of AMPAR, and the neurodegenerative disease FTD. Within this introduction, I will delve into these three elements with a primary focus on aspects relevant to the project itself. In section 1.1, I will discuss some data related to autoantibodies in neurological disorders, particularly emphasizing those that target ionotropic glutamate receptors. Subsequently, in section 1.2, I will present the primary findings concerning the physiological functions of the GluA3 sub-unit, as well as its role in various pathological conditions. The concluding part of this introduction (section 1.3) is dedicated to FTD.

1.1 Autoantibodies in neurological disorders

There is a growing consensus emphasizing the importance of autoimmunity in the etiopathogenesis of certain central nervous system (CNS) disorders. In last twenty years, more than 25 new types of autoantibodies targeting proteins relevant for synapse functionality have been identified in a great variety of patients. The realization that these autoantibodies are not solely a consequence of concurrent diseases but actively contribute to defining specific pathological phenotypes, has led to a significant shift in the understanding of some neurological disorders. Thanks to this new knowledge, a better comprehension of these diseases is now possible, thereby paving the way for improved diagnostic accuracy and the development of more effective therapeutic strategies.

In the following paragraphs, I will provide a summary of the current knowledge on the mechanisms underlying the production of anti-neuronal au-

toantibodies (subsection 1.1.1), along with their shared pathogenic processes (subsection 1.1.2). Subsequently, I will delve into the details of autoantibodies targeting the sub-units of ionotropic glutamate receptors and their relevance in various CNS disorders (subsection 1.1.4). Given that my PhD project is based on the employment of a pre-clinical model of chronic administration of autoantibodies, I have included in this section a concise overview of the models that have been designed for investigating the contribution of autoantibodies to CNS disorders (subsection 1.1.3).

1.1.1 Reasons for autoantibodies production

VDJ recombination is a process of somatic recombination by which developing lymphocytes randomly assemble different gene segments known as variable (V), diversity (D), and joining (J) genes. This process ultimately results in novel amino acid sequences in the antigen-binding regions of immunoglobulins and T-cell receptors (TCRs) of B and T cells respectively. In this process, there is no prior knowledge of the antigens toward which the immune system is supposed to react: as a result, the adaptive immune system generates lymphocytes that can virtually recognize any antigens. The inevitable consequence of this powerful process that guarantees receptors' diversity is the creation of receptors reactive to self antigens.

The lymphocytes that react against self-antigens are called auto-reactive or self-reactive lymphocytes. Even though self-reactive lymphocytes are continuously generated in our body, the immune system reacts with foreign antigens while it is tolerant to the self-antigens. The state of unresponsiveness of the immune system to self-antigens is called self-tolerance. If self-tolerance processes fail, the innate-immune system reacts toward self antigens, attacking cells and tissues of the body. This process is called autoimmunity and could lead to autoimmune diseases.

Self-tolerance is achieved by exposing lymphocytes to numerous checkpoints and fail-safe mechanisms. Tolerance checkpoints are classified into central and peripheral mechanisms based on anatomical location and the lymphocytes' stage of differentiation at which they act. The central tolerance is the tolerance induced during early development of lymphocytes at the level of primary or central lymphoid organs (bone marrow and thymus); as a result of this tolerance, most auto-reactive T and B cells are eliminated. Since this process is not foolproof, peripheral tolerance exists. The peripheral tolerance prevents auto-reactive mature lymphocytes from attacking self-antigens and it is induced in mature lymphocytes. Focusing on B cells, self-tolerance is established through 3 main mechanisms: clonal deletion, receptor editing and anergy. At the stage of immature B cell development, central tolerance acts mainly by clonal deletion (i.e., the induction of apoptosis of those B cells expressing B-cell-receptors (BCRs) that strongly bind the presented self-antigens), and by receptor editing. This last process consists in modification

of BCR through secondary recombination of antibody genes. The editing of the BCR that can happen in this phase is different from the process of somatic hyper-mutation that conversely takes place into secondary lymphoid organs and it is aimed at achieving higher affinity antibodies during a normal immune response. The residual auto-reactivity is controlled by peripheral tolerance that is achieved mostly through anergy, a state where B cells exist in the periphery but they are unresponsive and eventually die by apoptosis.

Many pieces of evidence suggests that the production of anti-neuronal antibodies could result from deregulated central and peripheral negative selection of B cells and, thus, a failure into the mechanisms of tolerance, including clonal deletion, BCR editing and anergy. For example, in patients with neuromyelitis optica spectrum disorder (NMOSD) with circulating autoantibodies targeting the aquaporin-4 water channel (AQP4) the presence of elevated frequencies of autoreactive B cells recently emigrated from the bone marrow suggests a failure into central B cell tolerance (Cotzomi et al. 2019). There are other (external) factors that can lead to a failure in self-tolerance mechanisms. For example, it has been demonstrated that viral infections could contribute to the breakdown of peripheral B cell tolerance with the subsequent emergence of autoantibodies against synaptic antigens. In this case, auto-antibodies are produced as a result of the co-engagement of the BCR together with co-stimulatory signals from pathogen-associated molecular patterns. A plausible scenario that can lead to this event is the release of brain specific “neo-antigens” (like NMDAR proteins) as a result of viral-induced tissue damage and cell apoptosis. In this context, viral components or debris antigens from necrotic tissue (such as nucleic acids) are co-presented to the “neo-antigen” antibody-specific B cells in local lymph nodes allowing escape from tolerance (Rawlings et al. 2017; Kuraoka et al. 2017; Kolhatkar et al. 2015). Alternatively, B cells may simultaneously capture the self-brain protein and the virus. Presenting viral proteins to virus-specific T cells could trigger T cell help, leading to subsequent B cell proliferation, class switch, and the generation of autoantibodies. Coherently, viral infections are a common cause of antibody-triggered encephalitis. Clinical studies correlate herpes simplex infection and encephalitis (HSE) with the development of NMDAR autoantibodies (Prüss et al. 2012; Armangue et al. 2014; Armangue et al. 2018) and (as a proof of concept of the casual relation between HSE and anti-NMDAR antibodies) experimental mice inoculated intranasally with HSV-1 developed serum NMDAR antibodies (Linnoila et al. 2018). Along with HSV, a large group of virus, including Epstein–Barr virus, varicella-zoster virus, human herpes-virus 6, HIV, hepatitis viruses, and Japanese encephalitis B virus, can trigger similar events leading to the generation of a great variety of autoantibodies targeting not only NMDA but also GABAA, AMPA, and dopamine D2 receptors (Linnoila et al. 2016).

Tumor represents another pathological event whose occurrence has been

correlated to auto-antibodies production. Indeed, the aberrant presentation of neuronal antigens within malignant cell can trigger an immune reaction against the tumor; however, this same reaction can also inadvertently target the corresponding central nervous system (CNS) proteins. This frequently results in severe para-neoplastic neurological disorders (Graus et al. 2019; Darnell et al. 2003). Some examples are subacute sensory neuronopathy, paraneoplastic cerebellar degeneration, and limbic encephalitis, conditions that are commonly associated with underlying malignancies such as small cell lung cancer, breast cancer, and ovarian cancer. Even though these syndromes are generally the result of tissue destruction mediated by cytotoxic T cells, antibody-mediated neurological diseases can also arise from underlying tumors. In this regard, it is worth to mention the case of ovarian teratomas: it has been found that in 20%-40% of women with NMDAR encephalitis there is a concurrence of ovarian teratomas (Titulaer et al. 2013; Dalmau et al. 2019). Indeed, the teratomas contain dense B cell and T cell infiltrates and dysplastic neurons expressing NMDARs (Makuch et al. 2018; Day et al. 2014). Overall, these data indicate autoantibodies-mediated encephalitis could result from an autoimmune process that is initiated in the periphery.

1.1.2 Pathogenic mechanisms of action

Several autoantibody-mediated pathogenic mechanisms of action have been proposed to explain the clinical phenotype of the patients positive for anti-neuronal antibodies. In this paragraph, I will discuss these mechanisms. It is important to underline that the variable clinical presentation of patients with anti-neuronal autoantibodies (in some cases, even in patients carrying the same ones) suggests that, in addition to some common mechanisms of action, multiple other factors come in action and contribute to the disease's manifestation. Examples are different biophysical antibody properties, such as affinity or glycosylation patterns as well as the different local anatomical enrichment of B cells in the brain.

Autoantibodies directed against ion channels often induce the cross-linking and the subsequent internalization of the targeted sub-units (Fig. 1.1a). This phenomenon is quite common and it has been observed in presence of anti-NMDAR (Jézéquel et al. 2017; Moscato et al. 2014; Ladépêche et al. 2018), anti-AMPA (Lai et al. 2009; Peng et al. 2015; Borroni et al. 2017), anti-GABAA (Pettingill et al. 2015) and anti-glycine receptors (GlyR) (Crisp et al. 2019; Carvajal-González et al. 2014). The reduction of functional receptors on the neuronal surface is problematic since it can mediate a reduction of the corresponding mediated-currents. A similar surface-expression reduction has also been described in presence of anti-neuroxin3 α antibodies which in turn leads to a reduction in the number of synapses (at least in hippocampal neurons) (Gresa-Arribas et al. 2016).

In contrast, complement activation is apparently restricted to a few

autoantibodies-mediated encephalitis (Bien et al. 2012), such as the one mediated by AQP4 antibodies (Fig. 1.1b).

In some cases, autoantibodies mediate stimulatory or inhibitory effects on receptors (fig. 1.1c). For example, anti-GABAB receptor antibodies do not change the surface expression of synaptic receptors but block their function (Lancaster et al. 2010). Similarly, direct antagonistic actions on glycine receptors also contribute to the pathogenic mechanism of anti-GlyR antibodies (Crisp et al. 2019).

Another mechanism through which autoantibodies interfere with their targets' activity, is the disruption of protein–protein interaction (Fig. 1.1d). For example, Leucine-rich glioma-inactivated 1 (LGI1) autoantibodies induce neuronal dysfunction by interrupting the trans-synaptic binding of LGI1 to its postsynaptic receptor ADAM22 (and likewise ADAM23 at the presynaptic site) (Kornau et al. 2020; Petit-Pedrol et al. 2018).

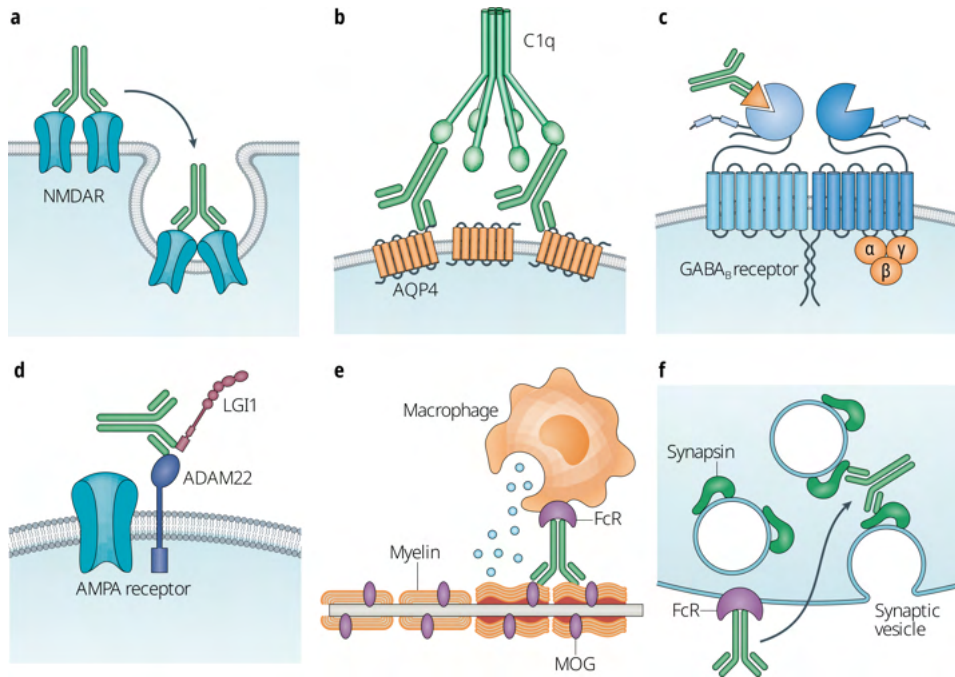


Figure 1.1: *Anti-neuronal antibodies' pathogenetic mechanisms of action.* (a) Cross-linking and target receptor internalization; (b) complement-dependent cytotoxicity; (c) inhibition of receptors' signalling; (d) disruption of protein–protein interaction; (e) antibody-dependent cellular cytotoxicity; (f) inactivation of the target intracellularly. The figure is adapted from Prüss 2021.

A few pieces of evidence indicate that the uptake of antibodies by neurons could induce neuronal dysfunction. More in detail, selected autoantibodies, such as to synapsin, are internalized via neuronal FcRs and lead to target

inactivation intracellularly (Fig. 1.1f) (Rocchi et al. 2019).

As it happens for non-self immunity, autoantibodies can also mediate cellular-dependent cytotoxicity which results from IgG-Fc binding to Fc γ Rs on phagocytes and natural killer cells (Fig. 1.1e). This event leads to degranulation and destruction of the antibody-bound target structure. This is the case of Myelin oligodendrocyte glycoprotein (MOG) autoantibodies that target the myelin sheath of axons and induce FcR-mediated antibody-dependent cellular cytotoxicity (Brilot et al. 2009).

1.1.3 Pre-clinical animal models

The continuous discovery of autoantibodies directed against synaptic proteins in a great variety of patients affected by neurological diseases clearly indicates the need of developing both *in vitro* and *in vivo* pre-clinical models. Different animal models of antibody-mediated neurological diseases have been developed, exploiting passive or active immunization depending on the biologic question they were supposed to answer. Passive immunization consists into the direct administration of the auto-antibodies under investigation to the animal—as a consequence, the immune process that mediates the auto-antibody production is not recapitulated. However, these models are ideal for analyzing the specific contribution of a given auto-antibody to a certain pathological condition, including its role in mediating molecular synaptic changes and behavioral abnormalities. By contrast, active immunization models are essential to study the early steps in the immunological cascade that lead to auto-antibody production as well as the role of other immune cells. In this second case, the immune-system of the animal is stimulated to produce the auto-antibody of interest.

The majority of pre-clinical models available exploits passive-immunization. In these models, the administered immunoglobulins are often of human origin since they are isolated from patients. In addition, immunoglobulins (or CSF or serum positive for the autoantibody of interest) are usually administered directly into the brain of animals to guarantee their presence into the CNS. Table 1.1 reports some examples of studies in which passive immunization pre-clinical models were used. Conversely, active immunization models are quite rare and few examples are now-a-day available. For example, active immunization with conformationally stabilized NMDAR tetramers in mice, induce encephalitis mimicking behavioral and pathological aspects of the disease (Jones et al. 2019). Similarly, active immunization with AQP4 mimotopes supports AQP4 antibody production in rats (Tsymala et al. 2020). Finally, anti-GluA3 antibodies were produced in mice immunized with GluA3-peptide; the presence of these autoantibodies induced lower seizure threshold and abnormal behavior (Ganor et al. 2014).

Table 1.1: Examples of pre-clinical models achieved through passive immunization to study anti-neuronal antibodies

Target	IgG		Mimicked disease	Site of injection	Effects
Amphiphysin ^a	Patient-derived IgG fraction containing anti-amphiphysin antibody		Stiff person syndrome	Intrathecal injection	Stiff person syndrome-like symptoms, including stiffness and muscle spasms; decreased presynaptic inhibition due to disturbed GABAergic inhibition
Aquaporin-4 ^b	Monoclonal recombinant antibodies (rAbs)		Neuromyelitis optica (NMO)	Retrobulbar venous plexus	Perivascular astrocyte depletion, myelinolysis, complement and Ig deposition (NMO immunopathology)
mGluR1 ^c	Patient-derived serum or CSF		Cerebellar ataxia	Cisterna magna	Transient severe ataxia
GluN1 ^d	Patient-derived CSF		NMDAR encephalitis	Lateral ventricles	Memory deficits, anhedonic and depressive-like behaviours
GluN1 ^e	Recombinant GluN1-specific clonal antibody	human monoclonal	NMDAR encephalitis	Lateral ventricles	Decreased synaptic NMDAR density in hippocampus; severe reversible memory impairment
NMDAR ^f	Patient-derived (from plasma) IgG fraction containing anti-NMDAR antibody		NMDAR encephalitis	Lateral ventricles	Low threshold for seizures

Continued on next page

Table 1.1: Examples of pre-clinical models achieved through passive immunization to study anti-neuronal antibodies (Continued)

Target	IgG	Mimicked disease	Site of injection	Effects
GluA2 ^g	Patient-derived (from serum or CSF) IgG fraction containing anti-GluA2 antibody	Autoimmune encephalitis	Hippocampus / lateral ventricles	Impairment in long-term synaptic plasticity, learning and memory
GABAA ^h	Anti-GABAA monoclonal antibody isolated from patients' CSF	Autoimmune encephalitis	Lateral ventricles	Severe catatonia and epileptic seizures

^a Geis et al. 2010^b Bennett et al. 2009^c Smitt et al. 2000^d Planagumà et al. 2016^e Malviya et al. 2017^f Wright et al. 2015^g Haselmann et al. 2018^h Kreye et al. 2021

1.1.4 Autoantibodies against ionotropic glutamate receptors

Autoantibodies directed against subunits of both N-methyl-D-aspartate receptor (NMDAR) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) seem to be among the most diffuse and clinically relevant autoantibodies identified in the last decades.

In the following paragraphs, I will present findings from both clinical and pre-clinical studies that link the presence of these autoantibodies to specific CNS diseases. Additionally, I will describe, when available, the proposed molecular mechanism(s) through which these autoantibodies can influence the synaptic localization and function of the target receptor—and consequently, how this interference may disrupt excitatory signaling within affected brain circuits.

1.1.4.1 NMDAR autoantibodies: anti-GluN1

NMDARs are ligand- and voltage-gated ionotropic glutamate receptors that regulate Ca^{2+} influx. They are tetramers composed of two obligatory GluN1 sub-units and two regulatory sub-units of alternatively GluN2-type (GluN2A-D) or GluN3-type (GluN3A-B); the expression of the regulatory sub-units varies depending on the brain region and the developmental period (Franchini et al. 2020).

Autoantibodies against the GluN1 sub-unit of the NMDAR and, specifically, its extracellular N368/G369 region domain, were originally found in patients affected by encephalitis (Dalmau et al. 2007; Dalmau et al. 2008). This discovery (and the subsequent clinical characterization of these patients) allowed the definition of a new pathological condition known as anti-NMDAR encephalitis (NMDARE) (Dalmau et al. 2007). NMDARE is the most common form of autoimmune encephalitis and it accounts for about 4% of patients with any form of encephalitis (Granerod et al. 2010). It is a synaptic brain disorder characterized by complex neuropsychiatric features: adult patients present with psychosis, behavioral disorders, or confusion, as well as other symptoms such as seizures, movement disorders, sleep disorders, and irritability (Dalmau et al. 2019, Muñoz-Lopetegi et al. 2020). The presence of both psychiatric or behavioral symptoms makes difficult to differentiate this condition, at least at its onset, from a primary psychiatric disease (Kayser et al. 2013).

Two confirmed triggers of anti-NMDAR encephalitis are tumors (mainly teratomas, Dalmau et al. 2007) and herpes simplex encephalitis (Nosadini et al. 2017; Armangue et al. 2018). Of note, between 4% and 7.5% of patients with NMDARE present concurrent glial or neuronal-surface antibodies. The most frequent association is with myelin oligodendrocyte glycoprotein (MOG) or aquaporin 4 (AQP4) antibodies, indicating a possible overlap between NMDARE and demyelinating disorders (Martinez-Hernandez et al. 2020,

Titulaer et al. 2014).

Given the clinical relevance of anti-GluN1 antibodies, great effort has been placed in trying to dissect the molecular mechanisms by which their presence could support the appearance of observed behavioral phenotypes. I will now report some studies aiming at understating the impact of these autoantibodies on glutamatergic neurotransmission and the expression of specific behaviors.

In vitro studies showed that the incubation of primary hippocampal neurons with anti-GluN1 antibodies isolated from patients mediates a decrease in GluN1 or NMDAR surface levels and synaptic clusters in a titer-dependent fashion (Hughes et al. 2010). Specifically, the antibodies bind and cross-link the NMDARs, altering their surface dynamics and disrupting the normal interaction with other synaptic proteins, such as ephrin-B2 receptors (EPHB2R). These events ultimately culminate in the receptor internalization. Focusing on EPHB2R, it has been demonstrated that its stimulation prevents anti-GluN1 antibody-dependent reduction of NMDAR clusters (Mikasova et al. 2012). In this light, it is possible to speculate that the decreased interaction with EPHB2R leads to an increased lateral diffusion toward extrasynaptic sites where the receptor is internalized (Moscato et al. 2014). However, other studies also indicate a direct action of the anti-GluN1 antibodies on the extrasynaptic pool of NMDARs which in turn leads to fast receptor internalization and/or a decrease in receptor diffusion and synaptic incorporation (Hunter et al. 2021). Whatever the mechanism, anti-GluN1 antibody-mediated internalization of NMDAR and interacting proteins, is most likely at the root of anti-GluN1 antibodies-mediated alterations in glutamatergic neurotransmission—consistently with the decrease in NMDARs surface levels, anti-GluN1 antibodies reduce NMDAR-mediated currents without affecting AMPAR-mediated ones (Hughes et al. 2010) and electrophysiological analysis supports the idea that this event is associated with an antibody-mediated internalization of the target receptor rather than a direct antibody blockade (Hughes et al. 2010, Moscato et al. 2014). Of relevance, anti-GluN1 antibody-mediated decrease in surface expression of NMDAR as well as in their currents also leads to reduced synaptic plasticity, as indicated by profound modifications of molecular signatures of long-term potentiation (LTP) (Mikasova et al. 2012). In this context, the fact that treatment with anti-GluN1 antibodies does not affect miniature excitatory postsynaptic currents (mEPSC) frequency or amplitude of hippocampal neurons indicates the absence of any effect on presynaptic release probability (Hughes et al. 2010). Despite of these main effects in term of glutamatergic neurotransmission, anti-GluN1 antibodies administration to neuronal cultures is not associated with alterations of AMPAR and PSD-95 clusters neither with morphological events such as reduced branching or spine loss and with modifications of neuronal viability (Hughes et al. 2010). It must be highlighted that the above-described effects on NMDARs are reversible

after anti-GluN1 antibody removal from cultures. In *in vivo* model of passive immunization, anti-GluN1 antibodies elicit similar synaptic alterations as those described in cultured primary neurons with a significant decrease in NMDARs cluster density and severe impairment of long-term plasticity. The antibodies do not affect the number of synapses, the density of other synaptic components and do not cause neuronal death (Hughes et al. 2010). The observed changes in synaptic function and plasticity are associated with memory deficits, anhedonia, and depression-like behavior but no effect on other social or locomotor skills have been detected (Malviya et al. 2017; Kreye et al. 2021; Planagumà et al. 2014; Planagumà et al. 2016)—of note, some of the detected behavioral defects reflect symptoms proper of the human pathology. Interestingly, as for the *in vitro* model, the above-mentioned molecular and behavioral deficits were progressive and fully reversible one week after the end of the infusion (Planagumà et al. 2014): this observation further supports the existence of a direct link between behavioral deficits and antibody-induced effects on NMDARs. Notably, *in vivo* treatment of ephrin-B2 prevented memory impairments and depressive-like behavior, as well as molecular alterations of synaptic NMDAR and impairments of synaptic plasticity (Planagumà et al. 2016).

The described models of passive immunization against GluN1 allowed to have insights into the role of anti-GluN1 antibodies in mediating molecular synaptic changes and behavioral abnormalities; however, they did not provide any clues in term of immune and inflammatory events' contribution to the pathology—which is quite relevant if we consider the autoimmune nature of NMDARE. To address this issue, other studies employing an active immunization model are available. In the work of Wagnon and colleagues, after two week from the immunization, mice developed clinical symptoms reminiscent of encephalitis, including anxiety- and depressive-like behaviors, a deficit in spatial memory, and increased sensitivity to seizures. This response was associated with B-cell infiltration toward the ventricles, where they differentiated into plasmacytes (Wagnon et al. 2020). Notably, the use of depleting antibodies to block the B-cell response induced a reduction of the severity of symptoms (Wagnon et al. 2020). Similar results, in term of developed symptoms and immune cell infiltration in the mouse brain, were achieved by Jones and colleagues (Jones et al. 2019).

Even though anti-GluN1 antibodies have been traditionally associated to NMDARE, recent studies pointed out a role for these autoantibodies in other disorders of the CNS. For example, anti-NMDAR antibodies were found in about 20% of patients diagnosed with schizophrenia (Jézéquel et al. 2017). It is also worth to mention that anti-GluN1 antibodies of IgM- and IgA-type have been identified in patients affected by schizophrenia, dementia, stroke, and Parkinson's disease (Busse et al. 2014, Doss et al. 2014, Zerche et al. 2015). However, conversely to what demonstrated for anti-GluN1 IgG found in NMDARE, these IgM and IgA do not alter NMDAR synaptic function

(Hara et al. 2018).

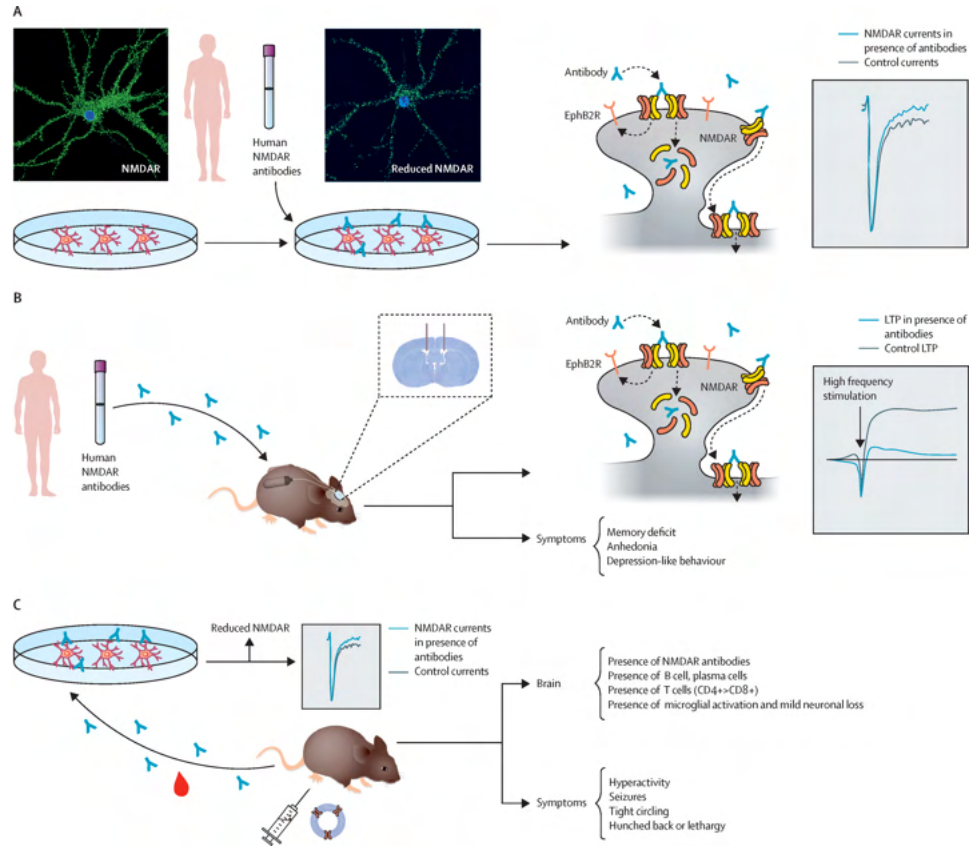


Figure 1.2: *Models and mechanisms of NMDARE*. (A) Effects of anti-GluN1 antibodies on primary cultures of rat hippocampal neurons; molecular and behavioral characterization of a mouse model of passive (B) or active (C) immunization with GluN1. The figure is adapted from Dalmau et al. 2019.

1.1.4.2 NMDAR autoantibodies: anti-GluN2B

Given the prominent role played by anti-GluN1 autoantibodies in autoimmune encephalitis, much attention has been paid also to autoantibodies targeting other NMDAR sub-units. In this regard, some studies identified the presence of anti-GluN2B antibodies mainly in patients with epilepsy.

In an initial study, anti-GluN2B antibodies were detected in the serum of patients with chronic epilepsia partialis continua, a rare condition involving recurrent and sometimes intractable focal onset seizures. More in detail, anti-GluN2B antibodies were found in patients with histologically proven Rasmussen's encephalitis, but not in patients with West syndrome or Lennox-Gastaut syndrome (Takahashi et al. 2003). In this study, the iden-

tified antibody recognized predominantly GluN2B C-terminal epitopes and rarely the N-terminal epitope. However, in a more recent study, anti-GluN2B antibodies against both N-terminal and C-terminal epitopes have been identified in patients with Rasmussen's syndrome. In these patients, the presence of anti-GluN2B antibodies (which increased to the highest levels from 12 to 23 months after epilepsy onset) was associated with seizure aggravation (Fukuyama et al. 2015). In contrast to Takahashi's findings, anti-GluN2B antibodies were also found in patients with West syndrome; in this more recent study, GluN2B antibodies were detected after the onset of epileptic spasms, and probably were associated with neural damage (Mori et al. 2016). Of note, anti-GluN2B antibodies have been identified also in patients with systemic lupus erythematosus (SLE); in this study, those patients also affected by fibromyalgia (FM) had higher anti-GluN2B antibody titers (Park et al. 2017).

1.1.4.3 AMPAR autoantibodies: anti-GluA1 and anti-GluA2

AMPA receptors are tetrameric ligand-gated ion channels composed of GluA1-4 sub-units which play a key role in regulating fast excitatory glutamatergic neurotransmission.

As for NMDARE, the identification of autoantibodies directed against AMPAR sub-units GluA1 and GluA2 in patients diagnosed with autoimmune encephalitis has allowed the definition of a new pathological condition called anti-AMPA encephalitis. Anti-AMPA encephalitis is a severe but treatment-responsive disorder; the patients manifest limbic encephalitis with short-term memory impairment, confusion, mood fluctuations, seizures, and disturbances in sleep patterns. In some cases, patients can also manifest additional symptoms or even psychosis. Neoplastic syndromes seem to have a role in triggering anti-GluA1/2 antibodies production since in 64% of cases, anti-AMPA encephalitis is associated with them (Peng et al. 2015; Laurido-Soto et al. 2018; Gleichman et al. 2014; Lai et al. 2009; Hoftberger et al. 2015; Muñoz-Lopetegi et al. 2020).

In vitro models were used to investigate the pathogenic mechanism of action of anti-GluA1/2 antibodies. As already described for anti-NMDAR antibodies, the treatment of hippocampal neuronal cultures with anti-GluA1/2 antibodies negatively influences glutamatergic synapses by mediating a significant decrease in the synaptic levels of AMPAR sub-units; coherently, electrophysiological analysis showed that the patients' antibodies reduced AMPAR- but not NMDAR-mediated currents (Lai et al. 2009; Peng et al. 2015). No differences in NMDAR clusters, dendritic spine density, or cell survival were detected in this same model (Peng et al. 2015). Importantly, the above-described detrimental effects strictly depend on the presence of the autoantibodies since, upon their removal, all of them disappear (Lai et al. 2009; Peng et al. 2015).

In a recent study, Haselmann and colleagues specifically addressed the role played by anti-GluA2 antibodies in both *in vitro* and *in vivo* models (Haselmann et al. 2018). By combining electrophysiology and high-resolution imaging with neuronal culture preparations and a murine model of passive immunization, they analyzed how anti-GluA2 antibodies affect receptor function and composition, synaptic transmission, plasticity and behavior. In their study, they confirmed that anti-GluA2 antibodies induce receptor internalization; of interest, they also demonstrated that this antibody-mediated reduction of synaptic GluA2-containing AMPARs is followed by compensatory ryanodine receptor-dependent incorporation of synaptic AMPARs not containing GluA2 sub-unit. They also showed that the injection of anti-GluA2 antibodies in the hippocampus of mice leads to defects in long-term synaptic plasticity *ex vivo* and negatively affects learning and memory *in vivo*.

1.1.4.4 AMPAR autoantibodies: anti-GluA3

The interest in anti-GluA3 autoantibodies stems from the observation of their presence in the serum of patients affected by Rasmussen’s encephalitis and in up to 20% to 30% of patients affected by different types of epilepsy (Levite 2014; Ganor et al. 2005a; Goldberg-Stern et al. 2014; Rogers et al. 1994). Relevantly, in patients with epilepsy, the presence of anti-GluA3 antibodies correlated with cognitive, psychiatric and behavioral abnormalities (Goldberg-Stern et al. 2014). Starting from these findings, several pre-clinical studies have sought to clarify both the effects and the underlying pathogenic mechanisms of anti-GluA3 antibodies.

In animal models of active immunization against GluA3-specific peptides, anti-GluA3 antibodies were found to enhance seizures and correlate with behavioral abnormalities (Rogers et al. 1994; Ganor et al. 2014). However, it’s worth noting that this outcome wasn’t consistently replicated in other studies (Levite et al. 1999a; Levite et al. 1999b) and, for instance, it was demonstrated that anti-GluA3 antibodies could also provide partial protection against seizures in rats (Ganor et al. 2005b).

Various research groups have employed *in vitro* models to gain insights into the molecular events mediated by anti-GluA3 antibodies. From these studies, it emerges that anti-GluA3 antibodies bind to GluA3-containing AMPARs and cause an agonist-like excitotoxic effect with subsequent neuronal death (Levite et al. 1999a; Twyman et al. 1995; Malina et al. 2006). Within this context, Malina and colleague demonstrated that anti-GluA3 antibodies trigger receptor activation: indeed, the acute application of anti-GluA3 antibodies prompted a rapid inward current in GluA3-expressing oocytes (Malina et al. 2006). In line with these *in vitro* studies, histopathological analysis revealed an increase in neuronal death accompanied by reactive gliosis subsequent to GluA3 peptide immunization in rats (Ganor et al. 2005b).

However, a more recent study (Day et al. 2023) showed that anti-GluA3

autoantibodies mediate inhibitory effects on neuronal excitability at the synaptic level. More in detail, acute (10 or 30 min) or longer-term (24 h) application of anti-GluA3 antibodies to hippocampal neurons caused a significant reduction in the mean frequency of spontaneous and miniature EPSCs (mEPSCs) in hippocampal neurons.

It is possible that these contrasting effects stemmed from variations in methodology, dosage, affinity, and/or specificity of the anti-GluA3 antibodies. Alternatively, they might represent two distinct phases of the same phenomenon. Initially, anti-GluA3 autoantibodies could exert an agonistic action on the receptor. This initial event could then be followed by the receptor's internalization (see subsection 1.1.2), culminating in the observed reduction in neuronal excitability. Another plausible explanation for these apparently contradictory findings could involve a temporal-dependent rearrangement in AMPAR composition, specifically involving the Ca^{2+} -permeable sub-unit GluA2.

The antibody-mediated internalization of GluA3 sub-unit has been confirmed in both rat hippocampal neuronal primary cultures and human induced pluripotent stem cell (hiPSCs)-derived neurons: here, anti-GluA3 antibodies administration caused a reduction in GluA3-containing AMPARs via increase in endocytosis and/or increased trafficking from cell surface, together with a reduction in dendritic spine density and, ultimately, decreased glutamate release (Borroni et al. 2017; Palese et al. 2020).

1.2 GluA3-containing AMPARs

Note. This paragraph partially reflects the content of my first-name review titled “*GluA3-containing AMPA receptors: From physiology to synaptic dysfunction in brain disorders*” published in 2021 on the open-access journal *Neurobiology of Disease* (Italia et al. 2021).

In this section, an overview of the physiological roles (both in term of behavior and synaptic plasticity) of GluA3 sub-unit is provided (subsection 1.2.1). Following that, its involvement in different pathological conditions is discussed (subsection 1.2.2).

1.2.1 Physiological roles

In the mammalian brain, AMPARs are cation-permeable, tetrameric receptors composed of various combinations of the pore-forming subunits GluA1–GluA4. Although homotetrameric AMPARs have been described, most AMPARs are heteromeric (Wenthold et al. 1996; Lu et al. 2009). The biophysical properties of the assembled AMPAR depend on the specific combination of the pore-forming sub-units (Isaac et al. 2007; Jonas 2000). Indeed, each sub-unit has its own distinct features that influence the activity of assembled channels. For example, the presence of GluA2 makes AMPAR impermeable to Ca_2^+ thanks to the presence of a positively charged arginine lining in GluA2 channel pore which ensures the impermeability to Ca_2^+ . Conversely, GluA1, 3, and 4 feature neutral glutamine residues on the pore face rather than arginine, allowing Ca_2^+ passage upon activation (Burnashev et al. 1992; Geiger et al. 1995; Jonas 1994).

AMPARs are widely distributed throughout the CNS but the relative abundance and sub-unit composition of AMPARs varies across brain areas (Petrallia et al. 1992; Schwenk et al. 2014). In hippocampal neurons, GluA1 and GluA2 are the most abundant pore-forming sub-units, comprising 80% of the entire pool of AMPARs (Lu et al. 2009; Schwenk et al. 2014). In other regions, such as the cortex and the striatum, GluA2 is the predominant subunit, whereas GluA1 and GluA3 are expressed at similar levels (Gold et al. 1996; Kessels et al. 2009; Schwenk et al. 2014; Jacob et al. 2015). Along with this difference in term of regional expression, AMPAR-sub-units show distinct sub-cellular localization. GluA3-containing AMPARs are uniformly enriched at synaptic sites and are only rarely distributed perisynaptically (Jacob et al. 2015). A proteomics study observed that the GluA3 subunit is the most enriched subunit within the excitatory postsynaptic density (PSD) under basal conditions (Pandya et al. 2017). Conversely, although GluA1/GluA2 AMPARs are enriched at synapses, they can also be located at extrasynaptic domains (He et al. 2009; Lu et al. 2009). A large number of studies also demonstrated the existence of specific trafficking mechanisms that regulate the localization of different AMPAR pools. GluA1/GluA2 AMPARs

are primarily inserted in the postsynaptic membrane following induction of synaptic plasticity (Shi et al. 2001), whereas GluA2/GluA3-containing AMPARs undergo constitutive recycling and replace GluA1/GluA2 AMPARs in synapses at rest (Shi et al. 2001).

	Temporal expression	Regional predominance	Ca ₂ ⁺ permeability	Synaptic targeting
GluA1	development, mature brain	hippocampus	yes	upon neuronal activity
GluA2	late development, mature brain	hippocampus	no	constitutively, upon neuronal activity
GluA3	development, mature brain	cortex	yes	constitutively
GluA4	early development	cerebellum	yes	upon neuronal activity

Table 1.2: *Summary of the main properties of each AMPAR subunit.*

The majority of AMPAR research over the last two decades has focused on understanding the physiopathological roles played by GluA1/GluA2 AMPARs. Conversely, the number of publications that examine the physiological role of GluA3-containing AMPARs currently available is quite modest (Diering et al. 2018). The limited interest in GluA3-containing AMPAR possibly come from the observation that global genetic deletion of the gene encoding for GluA3 (GRIA3) produces no clear effects in term of synaptic plasticity (Adamczyk et al. 2012, Meng et al. 2003) as well as no gross behavioral abnormalities. Is it then possible to assume that this sub-unit play little or no role in mammalian central nervous system? Evidence seems to be against this conclusion: mutations in GRIA3 has been linked to different brain disorders (Wu et al. 2007), but most importantly GluA3 is present at many central synapses (as described above) where it contributes to the formation of functional AMPARs—why should we (as mammals) employ energies to express a protein whose presence is not essential?

On the base of these considerations, a growing consensus is now pointing towards the importance of GluA3 in specific forms of synaptic plasticity, as well as in the expression of certain behaviors. In the next paragraphs, I will discuss recent research articles in which a key role for GluA3 in brain functions is proposed.

It must be said that many of these articles are based on traditional KO approaches of GRIA3 gene. However, the absence of a protein throughout

neurodevelopment could lead to compensatory changes: as a consequence, the resulting effect may represent a false readout of the true contribution of the protein in the native condition (Elias et al. 2006). Thus, in the evaluation of data coming from GRIA3 KO model, this big limitation must be considered.

1.2.1.1 Involvement of GluA3-containing AMPARs in synaptic transmission and synaptic plasticity

Synaptic plasticity is the ability of synapses to strengthen or weaken over time. Synaptic plasticity events are traditionally classified in two main groups according to their duration. Plasticity events that last from milliseconds to a few minutes are classified as short-term; long-term ones conversely last from minutes to hours. The most studied forms of long-term synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD) at glutamatergic synapses. LTP and LTD are key mechanisms for learning and memory, and alterations in their correct establishment and induction are associated to many neurological and mental disorders (Henley and Wilkinson, 2016).

Changes in properties or number of AMPAR are functional to the expression of long-term synaptic plasticity at many central synapses in the mammalian brain (Malinow et al. 2002; Anggono et al. 2012). In this regard, much research have been carried out, mainly at the level of the excitatory synapses between CA3 and CA1 pyramidal neurons in the hippocampus; results demonstrated that different AMPAR sub-units play distinct roles in the expression of these forms of plasticity. For example, GluA1-containing AMPARs have been mainly associated to LTP induction whereas GluA2-containing AMPARs seem to play a role in LTD (Diering et al. 2018).

Focusing on GluA3, GRIA2/GRIA3 double-knockout (KO) mice are characterized by a dramatic decrease in the amplitude of basal synaptic transmission in the hippocampus (Meng et al. 2003): this indicates that that GluA2/GluA3 AMPARs are essential for *in vivo* physiological synaptic transmission. However, these animals do not show any impairments in the establishment of several forms of long-lasting hippocampal synaptic plasticity, including LTP and LTD (Meng et al. 2003), suggesting that GluA1 alone is capable of various forms of synaptic plasticity. Conversely to what happens in the double-KO model, GRIA3 single-KO mice exhibit physiological hippocampal synaptic transmission, showing only a small reduction in synaptic currents and little changes in extra-synaptic currents (Meng et al. 2003; Humeau et al. 2007; Lu et al. 2009). However, the AMPA/NMDA ratio and the frequency of mEPSC in CA1 hippocampal neurons in GluA3-deficient organotypic slices show significant reductions compared with wild-type (WT) neurons (Reinders et al. 2016). As for the GRIA2/GRIA3 double-KO model, no alterations in LTD and enhanced or unchanged LTP can be observed in GRIA3 KO mice (Meng et al. 2003; Humeau et al. 2007). Coherently with

this last result, the use of a theta-burst stimulation at CA3–CA1 synapses results in an unchanged physiological LTP magnitude in GluA3-deficient slices (Reinders et al. 2016).

Overall, these data suggest that GluA3 sub-unit is not involved in the expression of these primary forms of hippocampal synaptic plasticity. However, Renner and colleagues recently demonstrated that GluA3 sub-unit contributes to a novel type of hippocampal synaptic potentiation. In their study, they showed that under basal conditions GluA3 channels at CA1 hippocampal synapses are in a low conductance and probability opening state and, as a consequence, they transmit no or low currents upon glutamate binding. However, when postsynaptic cyclic adenosine monophosphate (cAMP) concentrations increase, GluA3-containing AMPAR become functional. Indeed, they shift to a high-conductance state leading to augmented glutamate-gated channel opening and synaptic potentiation. The increase in cAMP levels that supports the activation of “dormant” GluA3-containing receptors requires the activation of protein kinase A (PKA) and GTPase Ras, which in turn could result from the activation of β -adrenergic receptors (Renner et al. 2017). In other words, this study indicates that GluA3 has a role in synaptic transmission which depends on cAMP levels in hippocampal pyramidal neurons. The same research group demonstrated that a similar mechanism of plasticity exists also in cerebellar Purkinje cells. In these cells, LTP is strictly dependent on the presence of GluA3- but not GluA1-containing AMPARs and, as in the hippocampus, GluA3-dependent LTP at cerebellar synapses does not involve GluA3 trafficking but correlates to a cAMP-dependent increase in the GluA3-containing AMPAR channel open-probability (Gutierrez-Castellanos et al. 2017). Together, these results suggest that this peculiar GluA3-dependent plasticity may be operative throughout the brain.

Indeed, besides the hippocampus and cerebellum, GluA3-containing AMPARs are present in other brain regions, including the cortex, amygdala, striatum, thalamus, brain stem, nucleus accumbens and the olfactory bulb (Breese et al. 1996; Reimers et al. 2011; Schwenk et al. 2014). Coherently, other studies have addressed the role of GluA3-containing AMPARs in brain circuits different from hippocampus. At thalamo- and cortico-lateral amygdala synapses, GluA3 deletion does not modify AMPAR-mediated synaptic transmission, although significant reductions in the amplitude and frequency of mEPSCs were observed (Humeau et al. 2007). However, if the amplitude of LTP at thalamo-amygdala synapses is not altered in GRIA3-KO mice (Humeau et al. 2007), LTP is absent in the cortico-amygdala pathway. This data suggests the specific involvement of GluA3 at cortico-amygdala synapses (Humeau et al. 2007). Analysis of GRIA3-KO mice also showed a fundamental role for this sub-unit in synaptic transmission and activity-dependent plasticity in endbulb–bushy cell synapses in the anteroventral cochlear nucleus of the auditory system (Antunes et al. 2020). At these synapses, the GluA3 sub-unit plays a relevant role in ultrastructural modifications (Rubio et al.

2017), activity-dependent plasticity, and normal auditory processing (García-Hernández et al. 2017; Antunes et al. 2020). In particular, GluA3 drives the ultrafast kinetics of endbulb synapse glutamatergic currents and is necessary for the function and maturation of the presynaptic terminal, modulating short-term plasticity (Antunes et al. 2020). In addition, Rutherford et al., showed that GluA3 subunit is required for appropriate assembly of AMPAR GluA2 and GluA4 subunits on cochlear afferent synapses and for presynaptic ribbon modiolar-pillar morphology (Rutherford et al. 2023)

Recently, Peng and colleagues also demonstrated that GRIA3-KO mice exhibit a deficiency in excitatory transmission at mPFC. They showed that both amplitude and frequency of mEPSCs is reduced in pyramidal neurons from layer II-III of the mPFC from GRIA3-KO mice compared to WT. Consistent with this observation, they observed a reduced AMPA/NMDA ratio by recording the evoked EPSCs in layer II-III pyramidal neurons while electrically stimulating the layer IV. This result further supports the hypothesis that synaptic AMPAR function is impaired when GluA3 is deleted. Of relevance, the paired pulse ratio (PPR) does not differ between the WT and GRIA3-KO mice, excluding the possibility of a difference in presynaptic glutamate release. Finally, they also demonstrated that GRIA3-KO reduces firing activity of pyramidal neurons in mPFC by measuring the number of action potentials evoked following a train stimulus of pulses applied to layer IV (Peng et al. 2022).

Overall, these studies provide an indication that GluA3 has a role in synaptic transmission and plasticity in different brain circuits that are relevant for learning and memory, motor, and social behavior—having established this physiological role for GluA3 at glutamatergic synapses, it is of interest to investigate which could be the behaviors whose expression strictly depends on GluA3 activity.

1.2.1.2 Role of GluA3-containing AMPARs in mouse behavior

The behavioral characterization of GRIA3-KO mice provides the *in vivo* correlation of the role exerted by GluA3 sub-unit in synaptic transmission and synaptic plasticity in different brain areas. In this paragraph, I will summarize the main findings concerning GluA3 contribution to the expression of mouse behaviors.

The global deletion of GluA3 does not induce gross behavioral abnormalities: GRIA3-KO mice are similar to WT mice in term of growth, general appearance, and olfaction (Sanchis-Segura et al. 2006; Adamczyk et al. 2012; Li et al. 2023). However, GluA3 sub-unit is involved in regulating neural networks necessary for the orchestration of electroencephalogram and breathing: in a free behavior setting, GRIA3-KO mice show an almost total absence of electroencephalographic signatures of non-rapid eye movement (NREM) sleep as well as a selective reduction in breathing rate during behavioral inactivity.

What is more, GluA3 deficiency may predispose mice to seizures (Steenland et al. 2008).

Mild or null impairments in motor coordination and locomotor activity are present in GRIA3-KO mice compared with control mice (Sanchis-Segura et al. 2006; Adamczyk et al. 2012; Peng et al. 2022). However, the reduced performance of GluA3-deficient mice in motor tests, such as the accelerated rotarod assay, is not associated with any significant impairments in grip strength performance (Adamczyk et al. 2012). Coherently with the role for GluA3 in cerebellar Purkinje neurons, GluA3-deficient mice show significant impairments in cerebellum-dependent learning involving the adaptation of compensatory eye movements (Gutierrez-Castellanos et al. 2017).

GluA3-deficient mice do not display deficits relative to WT mice in the cognitive functions evaluated by the Morris water maze or Y-maze tests (Adamczyk et al. 2012; Sanchis-Segura et al. 2006), indicating that GluA3-containing AMPARs do not play key roles in plasticity mechanisms associated with spatial memory and learning.

GRIA3-KO mice have a delayed acquisition of conditioned freezing behavior. However, they show physiological freezing behavior after the third conditioned stimulus, during an aversive unconditioned stimulus pairing, and during conditioned stimulus and context memory tests (Humeau et al. 2007).

The data concerning GluA3 contribution to anxiety behavior are controversial. In the recent article of Peng and colleagues, GRIA3-KO mice display a clear reduction in anxiety behavior. Specifically, they found that GRIA3-KO mice exhibit increased time and entries into the open arms of elevated plus maze (EPM) apparatus (Peng et al. 2022). However, this data is not confirmed by Adamczyk et al. and Sanchis-Segura et al. which did not identify any differences between GRIA3-KO mice and control ones in EPM or o-maze and light/dark box tasks (Adamczyk et al. 2012; Sanchis-Segura et al. 2006).

Adamczyk and colleagues made a great effort in trying to define the role played by GluA3 sub-unit in the modulation of social behavior. They showed that GRIA3-KO mice display a moderate increase in sociability but not in preference for social novelty when they are free to interact with con-specifics in a neutral arena (Adamczyk et al. 2012). What is more, GluA3-deficient mice show increased aggressive behavior in the resident-intruder test (Adamczyk et al. 2012; Peng et al. 2022); in this task, an increased number of aggressive interactions is measured when a neutral con-specific is inserted into the actor mouse's cage. Of note, the expression of GluA3 back in the mPFC reduces the aggressive phenotype of GRIA3-KO mice (Peng et al. 2022). In addition, the increase in sociability as well as in aggressive behavior proper of GRIA3 KO mice, correlates with increase levels of dopamine in the striatum (Adamczyk et al. 2012). In agreement with these pre-clinical findings, GRIA3 gene has been identified as a quantitative trait locus for aggression in humans (Brodtkin et al. 2002; Veroude et al. 2015; Odintsova et al. 2019; Zhang-James et al.

2018).

Alcohol-seeking and relapse behavior are known to be associated with AMPAR function. Thus, Li and colleagues evaluated the role played by GluA3 sub-unit in this specific behavior. They showed that GRIA3-KO mice show no alterations in ethanol self-administration behavior under operant or home cage drinking conditions. However, these animals show a blunted cue-induced reinstatement response and an alcohol deprivation effect compared with WT animals. Overall, these data suggest a role for GluA3 in alcohol seeking and relapse (Li et al. 2017).

On the base of clinical research data, Li and colleagues claimed that the concurrence of obesity and mutations in GRIA3 can support the appearance of symptoms of intellectual disability (ID) (Li et al. 2023). To validate their hypothesis, they tested GRIA3-KO mice under high fat diet (HFD) in a series of behavioral paradigms associated with symptoms of ID. They found that GRIA3-KO mice under HFD show working spatial memory behavior impairment in Y-maze test and deficits in reversal learning in T-maze task, abnormal anxiety in open-field test as well as decrease sociability and preference for social novelty (Li et al. 2023)—a series of deficits proper of ID. These data are different (and in some cases opposite) if compared to the behavioral characterization of GRIA3-KO mice reported above; however, that difference can be attributed to the oxidative stress to which GRIA3-KO mice were subjected in the study of Li et al.

Even though it does not employ a KO model for GRIA3, it is worth to mention the recent study of Mao and colleagues (Mao et al. 2022). In their article, they showed that social isolation supports the appearance of depressive and anxiety behaviors as well as a decrease of both GluA3 and GluA1 sub-units in the limbic striatum. Of note, the chronic treatment with the metabotropic glutamate (mGlu) receptor 5 antagonist MTEP reverses the reduction of AMPA receptors in the NAc and CPu but also prevents depression- and anxiety-like behavior induced by social isolation—Could we thus infer that striatal levels of GluA3 could be involved in the expression of depressive phenotype?

From the studies reported above, it is difficult to identify an univocal role of GluA3 sub-unit. What is for sure evident is that GluA3's correct activity supports the expression of a wide range of behaviors. However, further studies, possibly employing temporal or spatial GRIA3-KO models, will be needed to better dissect a specific role for GluA3.

Table 1.3: Summary of GluA3 KO effects in term of synaptic transmission and plasticity. **Effect correlated to a cAMP increase.*

Synaptic transmission and plasticity	GluA3 KO effects
Hippocampal synaptic and extra-synaptic currents	Mildly reduced ^{abc}
AMPA/NMDA ratio at CA1 synapses	Reduced ^d
Frequency of mEPSC at CA1 synapses	Reduced ^d
LTD at hippocampal synapses	Not affected ^{ab}
LTP at hippocampal synapses	Not affected ^{ab}
LTP at hippocampal synapses	Reduced ^{*e}
LTP at cerebellar Purkinje neurons	Reduced ^{*e}
LTD at cerebellar Purkinje neurons	Not affected ^d
AMPA-mediated synaptic transmission at thalamo- and cortico-lateral amygdala synapses	Not affected ^b
Amplitude and frequency of mEPSCs at thalamo- and cortico-lateral amygdala synapses	Reduced ^b
LTP at thalamo-amygdala synapses	Not affected ^b
LTP at cortico-amygdala synapses	Reduced ^b
Ultrafast kinetics of endbulb-bushy cell synapses glutamatergic currents	Reduced ^g
Short term depression at endbulb-bushy cell synapses	Enhanced ^g

^a Meng et al. 2003^b Humeau et al. 2007^c Lu et al. 2009^d Reinders et al. 2016^e Renner et al. 2017^f Gutierrez-Castellanos et al. 2017^g Antunes et al. 2020

Table 1.4: Summary of GluA3 KO effects in term of behavior.

Animal behavior	GluA3 KO effects
Growth, general appearance, and olfaction	Not affected ^{abc}
Breathing rate during inactivity	Reduced ^d
Non-rapid eye movement sleep	Reduced ^d
Seizure activity	Enhanced ^d
Motor coordination and locomotor activity	Mildly reduced ^{abe}
Grip strength performance	Not affected ^b
Cerebellum-dependent learning	Reduced ^f
Spatial learning and memory	Not affected ^{ab}
Conditioned freezing behavior	Delayed acquisition ^g
Anxiety behavior	Reduced ^e or not affected ^{ab}
Sociability	Enhanced ^b
Preference for social novelty	Not affected ^b
Aggressive behavior	Enhanced ^{be}
Alcohol-seeking and relapse behavior	Reduced ^h

^a Sanchis-Segura et al. 2006^b Adamczyk et al. 2012^c Li et al. 2023^d Steenland et al. 2008^e Peng et al. 2022^f Gutierrez-Castellanos et al. 2017^g Humeau et al. 2007^h Li et al. 2017

1.2.2 Role in synaptic dysfunction associated with brain disorders

1.2.2.1 Neurodevelopmental disorders and intellectual disability

The recent advances in DNA sequencing technology have enabled whole-genome studies on large cohorts of patients which in turn provide a better characterization of the roles and prevalence of various genes in the complex genetic landscapes of neurodevelopmental disorders (NDD) and intellectual disability (ID) (Koboldt et al. 2013). From these studies, it emerges a role for chromosomal rearrangements and gene mutations involving glutamate receptors and related synaptic scaffolding in the etiopathogenesis of these pathological conditions (Moretto et al. 2018; Soto et al. 2014).

In the next paragraph, I will focus on the clinical findings that correlate

GRIA3 gene with both NDD and ID.

In 1999 the first evidence of GRIA3 as a causative gene for ID was identified in a female patient carrying a balanced translocation involving the X-chromosome (Gécz et al. 1999). The patient presented with bipolar affective disorder, mental retardation, and epilepsy. Data collected on this clinical case did not allow for a detailed description of the specific pathogenic mechanism. However, the clinical presentation strongly supports the contribution of GluA3 alterations to at least some of the observed clinical manifestations.

Subsequently, several other studies reported the involvement of GRIA3 rearrangements in the etiology of different forms of ID. The whole-genome array comparative genomic hybridization studies performed in 2006 and 2013 correlated a genomic duplication containing GRIA3 to syndromic autism spectrum disorder (Jacquemont et al. 2006) and syndromic ID (Philippe et al. 2013) in two different families. In this regard, also partial duplications of GRIA3 gene have been associated with X-linked mental retardation. Bonnet et al., in 2009 (Bonnet et al. 2009), discovered a family bearing a partial tandem duplication of GRIA3, extending from exon 1 to exon 12, in association with X-linked mental retardation. This work not only described the clinical and genomic alterations identified in affected individuals but also provided mechanistic insights on the underlying pathogenic process. Through expression analyses, they identified three aberrant transcripts originating from the partial duplication which resulted in premature termination codons. They hypothesized that the resulting aberrant truncated transcripts generated truncated proteins that are either non-functional or undergo premature degradation: as a consequence, the partial duplication in GRIA3 gene, could lead to the loss of AMPAR function due to an imbalance in sub-unit composition. A similar pathogenic mechanism of action was hypothesized by Chiyonobu and colleagues who identified a partial tandem duplication of GRIA3 in a male with mental retardation (Chiyonobu et al. 2007).

Wu and colleagues demonstrated that specific mutations in GRIA3 gene alter GluA3 channel properties and lead to moderate cognitive impairment in humans (Wu et al. 2007). More in detail, by sequencing a large cohort of patients with X-linked mental retardation, they identified a complete gene deletion and four different GRIA3 variants (G833R, M706T, R631S, and R450Q), all of which fall within important functional domains of GluA3 AMPAR subunit. They used *in vitro* functional studies to assess the impact of these missense variants on GluA3 mRNA stability, protein levels, channel assembly, and AMPAR electrophysiological properties. Among them, the G833R mutation resulted in a dramatic 78% reduction in the receptor protein level, and no or minimal channel currents were recorded in the presence of the M706T or R631S mutation. These results demonstrated, from both genetic and functional perspectives, that mutations in the GluA3 sub-unit have consequences on AMPAR-complex kinetics and functionality. Interestingly,

these alterations were not only observed for GluA3 homomeric receptors but were also observed in GluA2-GluA3 heteromers. Despite being limited to an *in vitro* approach, this study suggested that specific neurological signs and mental retardation in human patients can be ascribed to decreased GluA3 channel activity.

Several lines of evidence have reported a role for glutamate in the regulation of sleep patterns and circadian rhythms (Ebling 1996; Vyazovskiy et al. 2008). A large sequencing project in 2017 associated a GluA3 mutation with a presentation of ID accompanied by severe sleep disturbances (Davies et al. 2017). The identified single nucleotide variant (A653T) falls within the transmembrane domain of GluA3, which is highly conserved among species and among ionotropic glutamate receptors. This amino acid substitution affects the structure of the ion conduction pore, resulting in stabilization of the channel closed state, as demonstrated by *in vitro* functional and electrophysiological experiments. Moreover, gene editing using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9)-mediated technique allowed for the study of this mutation in an *in vivo* mice model. Similar to the findings reported for the GluA3 KO mouse model, the results of A653T variant expression in mice were cryptic and did not phenocopy the human clinical presentation (Davies et al. 2017). However, mice exhibit more subtle alterations in activity and sleep than humans, in addition to an aberrant sensitivity to constant light. Therefore, the results of this study indicated that GluA3 activity might serve as a determinant of the sleep-wake cycle.

In addition to these studies, other more recent articles correlate variants in GRIA3 to NDD (Allen et al. 2015; Aspromonte et al. 2020; Chérot et al. 2017; Fernández-Marmiesse et al. 2019).

In the majority of the studies reported above, the authors hypothesized (or demonstrated) that the detected variants in GRIA3 gene have loss-of-function (LOF) effects on GluA3. In these cases, the observed pathological phenotype could result from a reduction of AMPAR activities. In contrast, Hamanaka and colleagues (Hamanaka et al. 2022) recently reported a gain-of-function (GOF) variant at GRIA3 in a male patient with NDD. Indeed they showed, through electrophysiological and fly model experiments, that this variant allows more ion current due to slow desensitization and deactivation. Of course, the correct identification of the effect (LOF or GOF) of a specific variant on the gene functionality is crucial since it permits to adopt a personalized therapy approach to treat the disorder.

1.2.2.2 Neurodegenerative disorders

Alterations in the glutamatergic system have been described in many neurodegenerative diseases, and a substantial body of evidence has identified excitotoxicity as a mechanism underlying cell death in several pathological

conditions. Coherently, compounds that interfere with NMDAR and AMPAR activities have been proposed to counteract neurodegenerative disease onset and progression (Stone et al. 2002; Jayakar et al. 2004).

In this section, I will describe the role played by GluA3-containing AMPARs in two different neurodegenerative disorders, Alzheimer’s disease (AD) and amyotrophic lateral sclerosis (ALS). In these two pathological conditions, the GluA3 subunit contributes to neurodegeneration through different and – at least in part – opposite mechanisms. In AD, the increased endocytosis of the GluA3 subunit is required for beta-amyloid ($A\beta$)-mediated synaptic and cognitive deficits (Reinders et al., 2016), whereas, in ALS, the increased expression of GluA3-containing AMPARs in motor neurons promotes excitotoxicity and neuronal death (Rembach et al. 2004; Spalloni et al. 2004). Regardless of the mechanism, GluA3 plays a role in these pathological conditions and might represent a potential target for therapeutic strategies.

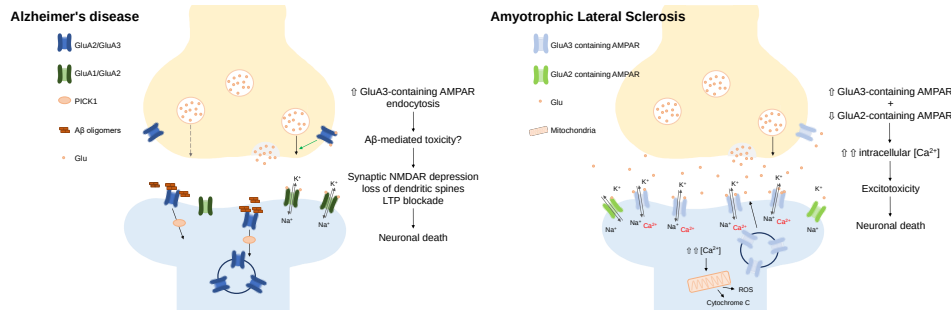


Figure 1.3: *Schematic representation of GluA3 subunit involvement in AD and ALS.* The figure is adapted from Italia et al. 2021.

Alzheimer’s disease

AD is the most common form of senile dementia, accounting for approximately 60%–80% of all dementia cases. The initial stages of AD are characterized by deficits in the process of encoding and storing new memories, followed by changes in cognition and behavior, which appear in later stages. These dramatic symptoms result from a diffuse neurodegenerative process throughout the brain (Lopez et al. 2019).

Two primary pathological events are thought to be responsible for AD: alterations in the cleavage of amyloid precursor protein, followed by the subsequent production and oligomerization of the $A\beta$ peptide, and the aggregation of hyperphosphorylated tau protein (Selkoe et al. 2016). The production and oligomerization of $A\beta$ result in the formation of extracellular senile plaques, whereas tau hyperphosphorylation results in the accumulation of intracellular neurofibrillary tangles. The accumulation of $A\beta$ appears to play a prevalent role in neurodegeneration. According to the “amyloid cascade hypothesis”, the pathological extracellular secretion of $A\beta$ triggers multiple, detrimental,

downstream effects, such as the formation of phospho-tau-based neurofibrillary tangles, excitotoxicity, inflammation, and oxidative stress (Selkoe 2004; Querfurth et al. 2010). Senile plaques and neurofibrillary tangles, combined with metabolic, vascular, and inflammatory changes, reduce synaptic strength, leading to synaptic loss and neurodegeneration.

Many pieces of evidence indicate that AD can be categorized primarily as a synaptopathy, in which the progressive loss of synaptic plasticity and synapses parallels the observed cognitive decline and other AD symptoms (Shankar et al. 2008; Talantova et al. 2013). Importantly, the loss of synapses and dendritic spines, which characterize the early phases of AD, are associated with the active $A\beta$ -mediated removal of postsynaptic receptors. Specifically, $A\beta$ generates structural and synaptic abnormalities via the endocytosis of AMPARs, suggesting that AMPARs down-regulation underlies the observed $A\beta$ -induced synaptic depression and dendritic spine loss (Baglietto-Vargas et al. 2018; Miyamoto et al. 2016; Hsieh et al. 2006; Miñano-Molina et al. 2011; Alfonso et al. 2014; Henley et al. 2016; Zhang et al. 2017). These observations are coherent with the general hypothesis that perturbations in glutamatergic signaling, specifically in the glutamate tripartite synapse, contribute to the pathogenic mechanisms of AD (Rudy et al. 2015; Findley et al. 2019).

Intriguingly, Reinders et al. (Reinders et al. 2016) demonstrated that the GluA3 AMPAR subunit plays a prominent role in $A\beta$ -dependent toxicity. Hippocampal neurons that did not express GluA3 were protected against $A\beta$ -mediated synaptic depression and spine loss, and only disrupted long-term synaptic potentiation in neurons that expressed GluA3. $A\beta$ -overproducing mice with GluA3 deficiency showed increased resistance against $A\beta$ -induced memory impairments compared with animals expressing normal levels of GluA3. These experiments demonstrated that the presence of GluA3-containing AMPARs is critical for $A\beta$ -mediated synaptic and cognitive deficits, and the active removal of GluA3-containing AMPARs is necessary to trigger the subsequent synaptic pathogenic events. Importantly, the GluA3 subunit has been described to play a role in the homeostatic scaling of synaptic strength (Makino et al. 2011); we can, thus, speculate that the $A\beta$ -induced removal of synaptic GluA3 may also impact homeostatic plasticity, which has been shown to be altered in AD (Jang et al. 2016). Coherently, protein interacting with C kinase 1 (PICK1), which is known to be involved in GluA3 endocytosis (Diering et al. 2018), is also required for the synaptic-depressive effects of $A\beta$ (Alfonso et al. 2014).

Another mechanism has been identified that might link GluA3 to AD development: GluA3 is modified by site-specific limited proteolysis, including cleavage by γ -secretase (Meyer et al. 2003). Therefore, alterations in the γ -secretase proteolytic system are associated with AD pathogenesis through the generation of the toxic $A\beta$ peptide and may also directly affect AMPAR function by modifying its physiological cleavage.

These preclinical studies are further supported by a recent study showing altered GluA3 levels in the biological fluids of AD patients. Enache et al. (Enache et al. 2020) reported increased GluA3 levels in the cerebrospinal fluid (CSF) of AD patients compared with those from cognitively normal subjective cognitive decline (SCD) patients. Although the aim of this study was to identify synaptic proteins capable of marking differences between SCD and clinical dementia, the reported increase in GluA3 concentrations in the CSF from AD patients further indicates the involvement of this receptor subunit in AD.

Overall, these data demonstrate the increasing attention being paid to glutamate receptors, including AMPARs, in the AD field. Recently, treatment with an AMPAR positive allosteric modulator (PAM) was shown to ameliorate cognitive and memory deficits in a non-transgenic murine model of A β -oligomer injection, reducing the accumulation of soluble tau in the hippocampus and resulting in decreased excitotoxic synaptic signaling (Monteiro-Fernandes et al. 2020). These findings suggest that the specific potentiation of AMPAR signaling can inhibit the effects of A β during the early disease stage. Interference with the effects of A β on the GluA3 AMPAR subunit might, therefore, represents a potential new strategy for counteracting early-stage AD progression. If A β requires GluA3 to exert its detrimental effects on synapses, as suggested by Reinders et al. (Reinders et al. 2016), preventing this interaction may represent an opportunity to prevent the negative effects of A β .

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by muscle atrophy and weakness, fasciculations, and spasticity, which result from the selective death of lower motor neurons in the brainstem and spinal cord and upper motor neurons in the motor cortex. In some cases, neurodegeneration also involves neurons in the prefrontal and temporal cortices, possibly leading to frontal executive dysfunctions and the concomitant appearance of frontotemporal dementia (FTD) symptoms (Robberecht et al. 2013).

A variety of mechanisms have been proposed to serve as triggers or enhancing factors that contribute to ALS pathogenesis, such as interference with normal proteasomal or autophagic protein degradation and the disturbance of normal RNA processing. Most of the genes associated with familial forms of ALS (FALS) are involved in these pathways, and the resulting alterations progressively lead to cellular failure. Other factors might contribute to the vulnerability of motor neurons, including the increased susceptibility to glutamate excitotoxicity, which appears to play a prevalent role (Robberecht et al. 2013). Only a few drugs have been approved for ALS treatment, including riluzole, a drug that exerts neuroprotective effects by attenuating glutamatergic neurotransmission (Bryson et al. 1996; Doble 1996; Geevasinga

et al. 2016).

Glutamate excitotoxicity results from glutamate-mediated neuronal overstimulation, which induces increased Ca_2^+ influx and triggers the subsequent aberrant activation of downstream pathways (Heath et al. 2002). Multiple factors can contribute to excessive Ca_2^+ ion influx, such as pathologically high glutamate levels and changes in the AMPAR composition. AMPAR subunits have been shown to differ in Ca_2^+ permeability: the presence of a positively charged arginine lining in the channel pore makes AMPARs containing GluA2 impermeable to Ca_2^+ , whereas GluA1, -3, and -4 feature neutral glutamine residues on the pore face rather than arginine, allowing Ca_2^+ passage upon activation (Burnashev et al. 1992; Geiger et al. 1995; Jonas 1994). Therefore, GluA2 plays a crucial role in counteracting glutamate excitotoxicity. Many pieces of evidence show that AMPAR-mediated Ca_2^+ influx contributes to selective motor neuron death (Shaw 1994; Takuma et al. 1999). Reports show that motor neurons are physiologically GluA2-deficient, increasing their susceptibility to Ca_2^+ -mediated excitotoxicity (Petrulia et al. 1997; Williams et al. 1997; Shaw et al. 1997; Bar-Peled et al. 1996; Heath et al. 2002; Damme et al. 2002; Kawahara et al. 2003).

A role for AMPARs, especially the GluA3 and GluA2 subunits, in ALS pathogenesis has been speculated. Multiple groups identified alterations in GluA3 expression in a well-validated transgenic mouse model of FALS, which overexpresses the mutant human superoxide dismutase (SOD1) gene, containing a glycine $\rightarrow$ alanine (G93A) substitution (SOD1G93A mice; Gold et al. 1996). Increased levels of GluA3 protein and mRNA expression were identified in spinal cords of SOD1G93A mice (Rembach et al. 2004) and cultured motor neurons derived from SOD1G93A mice (Spalloni et al. 2004). In the motor neurons of SOD1G93A mice, GluA3 mRNA and protein overexpression are accompanied by GluA2 protein downregulation (Tortarolo et al. 2006). Consistent with these alterations in AMPAR composition, SOD1G93A cultured motor neurons showed an increased vulnerability to AMPAR-mediated excitotoxicity (Spalloni et al. 2004).

Together, these independent results support the idea that the altered expression of Ca_2^+ permeable (GluA2-lacking) and impermeable (GluA2-containing) AMPARs contributes to disease onset and progression. Compared with WT motor neurons, the presence of higher numbers of Ca_2^+ -permeable AMPARs in motor neurons with SOD1 mutations predisposes them to injury due to AMPAR-mediated glutamate stimulation. Two different therapeutic strategies for ALS have been tested, targeting the AMPAR system. Rembach et al. (Rembach et al. 2004) proposed a rescue strategy using an antisense peptide nucleic acid that directly targets GluA3 and that demonstrated substantial reductions in *in vitro* GluA3 expression. Surprisingly, the administration of this compound to SOD1G96A mice extended the animal survival and delayed disease onset and progression, possibly due to the prevention of AMPAR-mediated excitotoxicity (as was demonstrated in the *in vitro* model).

Similarly, the treatment of SOD1G96A mice with a non-competitive AMPAR antagonist (ZK 187638) partially protected motor neurons, improved motor function, and prolonged animal survival (Tortarolo et al. 2006).

These data further support the involvement of GluA3 in ALS pathogenesis. Although the timing of GluA3 dysregulation relative to disease progression and the interactions between AMPARs and ALS-causing mutations require further investigation, these findings suggest that interference with GluA3 may represent a novel strategy for counteracting glutamate-mediated excitotoxicity in the motor neurons of ALS patients, which might ameliorate disease progression.

1.2.2.3 Autoantibodies targeting the GluA3 AMPAR subunit

As already described in the sub-section 1.1.4.4, autoantibodies directed against GluA3 sub-unit have been identified in patients affected by Rasmussen's encephalitis, different types of epilepsy and FTD. These data indicate that also auto-antibody-mediated interference with the physiological activity of GluA3 can elicit a wide spectrum of pathological conditions. A detailed discussion of the role played by anti-GluA3 antibodies in FTD is provided in the section 1.3.6.1 of the next chapter.

Alterations in the function or expression levels of GluA3 appear to contribute to a vast array of pathological brain conditions, ranging from neurodevelopmental to neurodegenerative disorders. Based on these observations, the identification and characterization of novel tools and pharmacological approaches able to selectively target and modulate the GluA3 subunit represent currently unmet needs that might counteract brain disorders characterized by AMPAR alterations at the glutamatergic synapse.

1.3 Frontotemporal Dementia (FTD)

Frontotemporal Dementia (FTD) is an heterogeneous neurodegenerative disorder enclosing a wide range of different neuropathological entities and clinical presentations. It presents with behavioral abnormalities, personality change, deficits of executive functions and language impairment due the progressive atrophy of frontal and temporal lobes.

From an epidemiological point of view, is the second most prevalent form of dementia with early onset (under 65 years old) (Vieira 2013), while it is the third most common form of dementia considering all age groups (Bang et al. 2015).

Clinically, FTD encompasses different syndromes: behavioral variant FTD (bvFTD); non-fluent variant of Primary Progressive Aphasia (nfvPPA); semantic variant of PPA (svPPA); FTD with motor neuron disease (which includes amyotrophic lateral sclerosis (ALS), corticobasal syndrome and progressive supranuclear palsy syndrome) (Hodges et al. 2018).

FTD neuropathological substrate is heterogeneous; hyperphosphorylated tau and transactive response DNA-binding protein 43 (TDP-43) are the most frequently identified underlying proteinopathies, responsible for frontotemporal lobar degeneration (FTLD)-tau and FTLD-TDP43, respectively (Mackenzie et al. 2009; Mackenzie et al. 2010).

In recent years, there has been marked progress in defining FTD syndromes, which requires much effort considering FTD great heterogeneity in terms of clinical diagnostic criteria (Gorno-Tempini et al. 2011; Rascovsky et al. 2011; Armstrong et al. 2013; Höglinger et al. 2017), genetic association (Seelaar et al. 2010; Baizabal-Carvallo et al. 2016), pathology (Mackenzie et al. 2010), and clinical and imaging biomarkers (Whitwell et al. 2005; Hughes et al. 2013; Skillback et al. 2014; Rohrer et al. 2010; Ranasinghe et al. 2016).

In this paragraph, I will discuss some aspects related to FTD: I will initially provide an overview of the main clinical sub-types of FTD (subsection 1.3.1) to then move to its neuropathological (subsection 1.3.2) and genetic substrate (subsection 1.3.3). Since many pieces of evidence suggest that both an impairment of specific neurotransmitter systems as well as deficits in immune system could have a role in FTD pathogenesis, two paragraphs are dedicated to these topics (sections: 1.3.4; 1.3.6). I will also discuss the therapeutic strategies now-a-days available (section 1.3.5).

1.3.1 Clinical features

According to the latest criteria, three core clinical variants have been recognized: the behavioral variant of FTD (bvFTD), which is associated with early behavioral and executive deficits, the agrammatic/non-fluent variant of primary progressive aphasia (avPPA/nfvPPA), with progressive deficits in speech, grammar, and word output, and the semantic variant of PPA

(svPPA), which is a progressive disorder of semantic knowledge and naming (Rascovsky et al. 2011; Gorno-Tempini et al. 2011). As the disease progresses, this sub-classification in bv-FTD, nfv-PPA and sv-PPA can become harder: the seminal features that define the three clinical variants can indeed converge as result of the spreading of the initial focal degeneration to larger regions in frontal and temporal lobes. FTD also frequently overlaps clinically with three additional neurodegenerative diseases with profound motor deficits: corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), and amyotrophic lateral sclerosis (ALS) (Bang et al. 2015). The occurrence of global cognitive impairment and the above-stated motor diseases, enriches the spectrum of FTD related disorders (Burrell et al. 2011).

At the end-stage of the disease, patients have difficulty in eating, moving, and swallowing. Death usually happens about 8 years after symptom onset and it is typically caused by pneumonia or other secondary infections (Bang et al. 2015).

In the following paragraphs, I will delve into details of FTD main variants (Bang et al. 2015) and I will discuss why the differential diagnosis of FTD can be challenging.

1.3.1.1 Behavioural-variant frontotemporal dementia

The hallmarks of bvFTD are personality changes, apathy, and disinhibition. Other common symptoms are loss of sympathy or empathy, stereotypical, compulsive, or perseverative behavior, hyper-orality or dietary changes and executive deficits with relative sparing of visuospatial skills.

Disinhibition can be defined as a loss or lack of restraint based on social norms thus potentially leading to tactless or socially inappropriate behaviors (such making uncharacteristic rude or offensive comments, ignoring other people's personal space) and impulsivity (such as shoplifting or reckless spending). Sometimes, it could also result in aggressive behaviors. *Apathy* is the indifference or lack of interest in previously meaningful activities. It manifests as a reduced interest in work, hobbies, social relationships and personal hygiene as well as in loss of initiative. Patients often manifest *loss of sympathy or empathy* for others; as a result, they can be indifferent to important events (i.e., death of a family member or friend) or can not be capable of recognizing that loved ones are upset or unhappy. *Compulsive behaviors* consist in single behavior or routines that are performed over and over, including simple repetitive movements, compulsive ritualistic behaviors, and repetitive use of verbal phrases. Patients often show excessive, compulsive or inappropriate *eating* and *drinking*, or other pronounced changes in dietary preferences. Finally, *deficits* in various *executive tasks* can be identified; indeed, patients can present poor decision-making, judgment, problem-solving, and organizational skills.

1.3.1.2 Primary progressive aphasia

Patients with primary progressive aphasia (PPA) have a progressive, decline in linguistic skills during the initial phase of the disease with no behavioral disturbances. Deficits include language production, object naming, syntax, or word comprehension, and are apparent during conversation or through speech and language assessment. PPA is further subdivided into two clinical sub-types based on the specific language skills that are most affected: non-fluent variant (nfv-PPA) and semantic-variant (sv-PPA) primary progressive aphasia. PPAs patients show from the beginning an important language dysfunction: given that these symptoms can be mistaken for Alzheimer's disease, a careful diagnosis is necessary.

Patients affected by nfv-PPA find increasingly difficult to speak even though they can still recall the meanings of individual words. Common symptoms are: *apraxia* (i.e., difficulty in producing movements of lips and tongue needed for speech), *agrammatism* (i.e., omitting words in sentences, especially short connecting words), *impaired comprehension of complex sentences* (but not of single-word), *mutism* and *difficulty swallowing* (later in the disease). As a result of these symptoms, the speech is slow, laboured, and halting and it is characterized by omission or misuse of grammar (agrammatism) and by inconsistent sound errors, including insertions, deletions, substitutions, transpositions, and distortions. Some patients maintain intact writing despite the presence of marked deficits in spoken language.

In contrast, patients with svPPA progressively lose the meanings of words—they lose the ability to understand or formulate words in a spoken sentence. If there are additional major problems in identifying objects or faces, the condition is also called semantic dementia. Patients' language skills, including the ability to produce speech and to repeat phrases and sentences spoken by others, are unaffected. However, although the affected person may continue to speak fluently with correct grammar, their speech becomes vague and difficult to understand because many words are omitted or substituted. More in details, common symptoms are: *anomia* (i.e., inability to recall the names of objects), *reduced single-word comprehension* (i.e., the person affected is unable to recall what words mean), *associative agnosia* and *impaired object knowledge* (i.e., being unable to remember what a familiar object is or how it is used) and *surface dyslexia/dysgraphia* (i.e., difficulty reading and writing words that do not follow pronunciation or spelling rules) (Neary et al. 1998). Sv-PPA symptoms result from early asymmetrical degeneration of anterior temporal lobes and amygdala. Left temporal lobe variant presents with mainly linguistic semantic loss, whereas the right temporal lobe variant presents with behavior changes (Seeley et al. 2005). As the disease spreads from the temporal lobes into the orbitofrontal cortex, also behavioral deficits appear, such as irritability, emotional withdrawal, insomnia and strict or selective eating; sometimes also depression emerges.

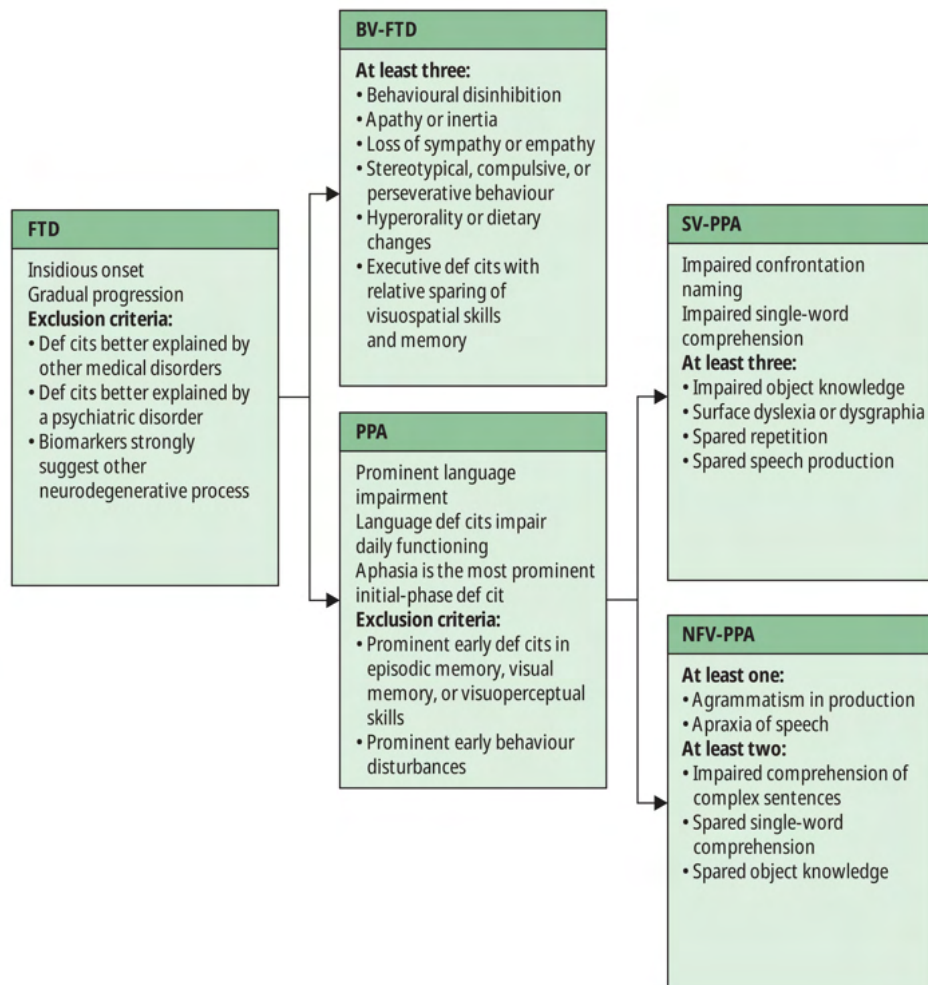


Figure 1.4: *Diagnostic criteria for clinical diagnosis of FTD.* The figure is adapted from Bang et al. 2015.

1.3.1.3 Motor syndromes

About 12.5% of patients with behavioral-variant frontotemporal dementia develop motor neuron disease and mild features can occur in up to 40% of patients with FTD (Burrell et al. 2011). Motor system dysfunction in frontotemporal dementia may result from pathological involvement of the primary motor cortex, with secondary degeneration of lower motor neurons in the brainstem and anterior horn of the spinal cord. The patients typically develop upper motor neuron signs (hyper-reflexia, extensor plantar response, spasticity), lower motor neuron signs (weakness, muscle atrophy, fasciculations), dysarthria, dysphagia, and pseudobulbar affect. In addition, early parkinsonism occurs in up to 20% of patient, more often in patients with bvFTD, followed by those with nvPPA (Ber et al. 2006). Disturbances of motor (movement or muscle) function include three disorders that are part of the frontotemporal degeneration spectrum that produce changes in muscle or motor functions with or without behavior (bvFTD) or language (PPA) problems: amyotrophic lateral sclerosis (ALS), corticobasal syndrome and progressive supranuclear palsy syndrome. Corticobasal syndrome causes symmetrical parkinsonism, sensory–motor cortical dysfunction, alien-limb syndrome and dystonia; progressive supranuclear palsy syndrome, instead, is characterized by vertical supranuclear palsy, decreased saccade velocity and early postural instability with falls. As described in 1.2.2.2, Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by muscle atrophy, weakness, fasciculations, and spasticity, which result from the selective death of lower motor neurons in the brainstem and spinal cord and upper motor neurons in the motor cortex. Even though ALS is primarily characterized by motor symptoms, half of people diagnosed with ALS exhibit also behavioral changes or a decline in language skills similar to those observed in bvFTD or PPA—and, similarly, up to 30% of people diagnosed with FTD develop motor symptoms consistent with ALS. This observation (together with the discovery that the C9orf72 gene mutation can cause both ALS and FTD) has progressively transformed the belief that ALS is ‘purely’ a movement disorder and that FTD is ‘purely’ a cognitive or behavioral form of dementia: indeed, at present, it is possible to talk about *ALS-Frontotemporal spectrum disorder*. The patients within this spectrum present both changes in behavior, personality, and language skills that characterize FTD, but have also difficulties in walking, standing, using their hands, speaking, swallowing, or breathing. These symptoms result from a neurodegenerative process that involves both motor neurons and neurons in frontal and temporal lobes.

1.3.1.4 Differential diagnosis

One of the current challenges in FTD clinical practice is the establishment of a correct and rapid diagnosis of FTD. The differential diagnosis of frontotem-

poral dementia actually needs a careful history that examines the progression of behavioral changes, family history, behavior in face-to-face interviews, performance on neuropsychological testing but also laboratory studies (blood analysis with a comprehensive metabolic panel, blood count, vitamin B12 concentration, thyroid studies; β -amyloid and tau CSF concentration) and neuroimaging.

There are two main groups of disorders that, for their symptoms, can be misdiagnosed for FTD: adult-onset psychiatric disorders and Alzheimer's disease (AD) and other neurodegenerative diseases. Of the FTD variants, the one that is most likely misdiagnosed as a psychiatric disorder is bvFTD. For example, the repetitive and compulsive behaviors proper of bvFTD can support a misdiagnosis of obsessive-compulsive disorder; apathy and emotional withdrawal can be interpreted as signs of depression; delusion and euphoria are also features of bipolar disorder and schizophrenia.

There is a substantial overlap of symptoms between Alzheimer's disease (AD) and FTD; however, there are some key differences between these two conditions. The age at diagnosis, for example, is an important clue: most people with FTD are diagnosed earlier than the ones with AD, in their 40s and early 60s. In terms of symptoms, memory loss tends to be a more prominent symptom in early AD than in early FTD as well as problems with spatial orientation. Behavior changes are often the first noticeable symptoms in bvFTD; contrary, behavioral changes can occur in AD, but they tend to occur later in the disease. Finally, although people with AD may have trouble thinking of the right word or remembering names, they tend to have less difficulty making sense when they speak, understanding the speech of others, or reading than those with FTD.

An important tool that can help to correctly diagnose FTD is imaging. For example, it is possible to take advantage structural MRI and CT to identify typical patterns of atrophy—FTD is characterized by predominant frontal or temporal atrophy and atrophy in the frontoinsular region is especially indicative of FTD (Rosen et al. 2002). Fluorodeoxyglucose PET, functional MRI, and singlephoton-emission CT can also help since they can allow to show disproportionate hypoperfusion and hypometabolism in these regions (Ber et al. 2006). In addition, thanks to PET imaging, it is possible to differentiate FTD from AD (for example exploiting amyloid tracers or tau ones, even though the latter are still under investigation (Maruyama et al. 2013)) but also to distinguish the different frontotemporal lobar degeneration syndromes. Finally, functional connectivity network mapping achieved with MRI can help into the differential diagnosis with AD (Raichle et al. 2001, Buckner et al. 2005).

Fluid biomarkers in both CSF and blood (plasma or serum) can be useful for FTD diagnosis. For example, phosphorylated tau (p-Tau), total tau (T-tau), amyloid- β peptide 1–42 ($A\beta_{42}$) can be used to differentiate FTD from AD. Neurofilament light chain protein (NfL) has been measured in

many studies as a CSF bio-marker of FTD cases; of relevance, it has been correlated to disease severity and brain atrophy as well as with specific clinical sub-types of FTD. TDP-43 has also been suggested as a potential biomarker for the discrimination of patients with FTD or AD with TDP-43 pathology from those without TDP-43 pathology and GFAP seems to be a potential biomarker of FTD patients, especially for GRN mutation carriers (Ntymenou et al. 2021).

1.3.2 Neuropathology of FTD

Common to all FTD clinical syndromes is the underlying *frontotemporal lobar degeneration* (FTLD). FTLD is comprehensive term referring to distinct pathological processes which include neuronal loss, gliosis, and microvacuolar changes. These processes ultimately lead to degeneration and atrophy of frontal lobes, anterior temporal lobes, anterior cingulate cortex, and insular cortex. Of note, the initial patterns of atrophy represent another criteria to classify the sub-types of FTD: the degeneration of the right lobe with simultaneous behavioral disorders are typical of semantic aphasia whereas the degeneration of the left lobe is often associated with language deficits (Gorno-Tempini et al. 2011; Rabinovici et al. 2010).

Neuropathologically, FTD is considered a proteinopathy: as it happens in many other neurodegenerative disorders, FTD pathology often includes abnormal and toxic intracellular aggregation and accumulation of some proteins. According to the predominant constituent proteins of intracellular inclusions, three main sub-types of FTD have been identified: FTLD-Tau, FTLD-TDP and FTLD-FUS with respect to the deposition of tau protein, TAR DNA Binding protein 43 (TDP-43) and fused-in-sarcoma protein (FUS) (Mackenzie et al. 2010; Neumann et al. 2006; Mackenzie et al. 2009).

In the next paragraphs, I will focus on FTLD-Tau and FTLD-TDP since they are the most common proteinopathies underlying FTD. In addition, I will provide some notions regarding the physiological functions of tau and TDP-43 proteins and how, in turn, they can become pathological.

1.3.2.1 FTLD-Tau

FTLD-Tau is part of a group of neurodegenerative diseases collectively referred to as tauopathies because of the presence of tau inclusions (Lee et al. 2001). Specicially, FTLD-Tau is defined by toxic intracellular aggregates of hyperphosphorylated tau.

Tau protein is encoded by the MAPT gene on chromosome 17; it is a member of microtubule(MT)-associated protein family, mainly enriched in the axonal compartment of neurons. In this compartment, it is primary involved in the regulation of cytoskeletal turnover by promoting MT stabilization and axonal elongation (Kovacs 2015). However, recent studies demonstrated that

tau can exert physiological functions also in synaptic compartment (Italia et al. 2022).

Tau exists as six major alternative splicing variants; indeed, the human MAPT gene includes 11 exons of which 2, 3 and 10 are alternative spliced (Goedert et al. 1989). Of notice, the inclusion of exon 10 determines whether the protein contains either 3 or 4 microtubule-binding repeat regions and, on the base of this, we distinguish between tau three-repeat (3R) and four-repeat (4R) isoforms—the relative amount of these two isoforms varies in the course of human brain development and it concurs to characterize the pathological profile of the different tauopathies.

Once synthesized, tau undergoes different post-translational modifications such as phosphorylation, acetylation, glycation, nitration, O-GlcNAcylation, oxidation, polyamination, SUMOylation, and ubiquitination (Morris et al. 2015). The presence of hyper-phosphorylated tau is a hallmark of tauopathies (Spillantini et al. 1997): indeed, once hyper-phosphorylated tau tends to aggregate and form insoluble toxic filaments. In addition, also an alteration of the relative amount of tau 3R and 4R isoforms could be pathological (Fernández-Nogales et al. 2020).

FTLD-tau accounts for about 40% of all FTLD cases and 20% of those with high heritability; these latter are related to mutations in MAPT gene, with more than sixty pathogenic variations reported (Greaves et al. 2019; Moore et al. 2020; Baborie et al. 2010; Sieben et al. 2012). According to the relative amount of tau-3R and tau-4R in the inclusions, FTLD-tau accomplishes four main histological sub-types: Pick’s disease (PiD), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), and globular glial tauopathy (GGT). These sub-types differ in term of patterns of atrophy as well as in term of aspects of the intracellular depositions of tau (Josephs et al. 2011; Barker et al. 2002; Rebeiz et al. 1968).

1.3.2.2 FTLD-TDP

TDP-43, encoded by the gene TARDBP, is a member of the large heterogeneous family of nuclear ribonucleoproteins(hnRNPs), a group of proteins able to bind the RNA with high specificity (Sephton et al. 2010; Sephton et al. 2012; Geuens et al. 2016). Specifically, TDP-43 activity is critical for RNA-related metabolism.

Inclusions of pathogenic deposits containing TAR DNA-binding protein 43 (TDP-43) are evident in the brain and spinal cord of patients with different neurodegenerative diseases such as sporadic and familiar ALS as well as FTLD-FTD, which is characterized by ubiquitin and TDP-43 positive, tau negative, FUS negative inclusion bodies (Boer et al. 2020). In this regard, over-abundant TDP-43 inclusions are detrimental to neuronal cells on account of a contemporary loss-of-function and gain-of-toxic-function (Cannon et al. 2012; Moussa et al. 2016; Lu et al. 2016). The exact mechanism of inclusions’

formation is still unclear. However, it is accepted that before the formation of toxic TDP43 aggregates, nuclear TDP-43 undergoes depletion (Lee et al. 2011; Xu 2012): the presence of TDP-43 inclusions in the cytoplasm reduces the amount of TDP-43 required for mRNA transport producing a detrimental effect in neuronal cells (Igaz et al. 2009; Pesiridis et al. 2011; Yang et al. 2010; Wang et al. 2013). This first loss of function event is then followed by the toxic gain of function which supports TDP-43 toxic aggregates formation (Broeck et al. 2015; Ederle et al. 2017). The main post-translational modifications of TDP-43 are phosphorylation and ubiquitination (Neumann et al. 2006; Arai et al. 2006; Neumann et al. 2009; Hasegawa et al. 2008; Inukai et al. 2008).

FTLD-TDP accounts for about 50% of all FTLD cases including both sporadic and familial forms (Neumann et al. 2019). Despite the common molecular substrate, FTLD-TDP covers a wide range of anatomopathological changes and different sub-types of FTLD-TDP are recognized on the basis of the patterns of cytoplasmic or intra-nuclear pathology, and cortical association.

1.3.3 Genetics of FTD

Up to 40% of FTD cases have a family history of dementia, with an autosomal dominant inheritance in around a third of patients (Stevens et al. 1998; Benussi et al. 2015; Rohrer et al. 2015; Ferrari et al. 2014). In 60% of these cases (Ber 2013) (and in 10-20% of all FTD cases, Rohrer et al. 2009), the mutation is in microtubule-associated protein tau (MAPT) (Hutton et al. 1998), chromosome 9 open reading frame 72 (C9orf72) (DeJesus-Hernandez et al. 2011; Renton et al. 2011) or Granulin precursor (GRN) genes (Baker et al. 2006; Cruts et al. 2006). In the last twenty years, other less common mutations associated with FTD have been found.

	Prevalence among familial FTD cases	Geographic prevalence of mutation carriers	Atrophy patterns	Common clinical presentations	FTLD proteinopathy	Mechanisms of neurodegeneration
<i>C9orf72</i> (9p21.2)	13-50%	Scandinavia, west Europe, USA, Australia, rare in Asia	Symmetrical, orbitofrontal, medial and dorsolateral frontal, followed by temporal lobes, parietal and occipital lobes, cerebellum, posterior thalamus	BV-FTD FTD-MND ALS Parkinsonism Late-onset psychosis	TDP type B (less commonly type A) dipeptide repeat proteins in neocortex, thalamus, cerebellum, and hippocampus	Endosomal trafficking dysregulation; RNA-foci formation and impaired transcription processing; dipeptide repeat protein toxicity
<i>MAPT</i> (17q21.1)	5-20%	Northwest Europe, USA	Symmetric frontal, anterior cingulate cortex, insular, anterior, and medial temporal lobe	BV-FTD Parkinsonism	Tau (often unclassifiable, occasionally resembling Pick's disease), corticobasal degeneration or progressive supranuclear palsy	Altered microtubule stabilisation and/or increased propensity of tau self-aggregation
<i>GRN</i> (17q21.32)	5-20%	England, central and southern Europe, USA	Asymmetrical, anterior temporal, temporo-parietal, frontal (left > PPA; right > BV-FTD), anterior cingulate cortex, insular	BV-FTD NFV-PPA Parkinsonism CBS	TDP type A	Impaired neurotrophic function, promotion of inflammation, impaired lysosomal-mediated protein degradation

Figure 1.5: *Genetic and pathological correlation in FTLD*. The figure is adapted from Bang et al. 2015.

In term of neuropathology, in FTD cases associated with pathogenic mutations in MAPT, the tau accumulation in neurons and glia has been explained in terms of abnormal phosphorylation of the protein or in an altered proportion in the production ratio of the 4R and 3R Tau isoforms; conversely,

GRN mutations and C9orf72 expansion lead to TDP43 aggregation, with a less defined mechanism. Within this context, it must be said that for the majority of FTD patients without known pathogenic mutations, the molecular pathways related to either Tau or TDP43 protein aggregations are still uncovered. In the next part of the paragraph, I will provide more details on the mutations associated with MAPT, C9orf72 and GRN.

MAPT. Researchers described many different mutations in MAPT gene. About half of the known mutations mediate their primary effect at the protein level: they reduce the ability of tau protein to interact with MT (thus altering MT stabilization, loss of function mechanism) and increase its propensity to self-aggregate and assemble into abnormal filaments (gain of function mechanism). For example, one of the most common mutations, P301L, is known to increase the propensity for β -sheets aggregation (Bergen et al. 2001). Other mutations mediate their primary effect at the RNA level, for example perturbing the normal ratio of 3R to 4R tau isoforms (Goedert 2005). In term of FTLT proteinopathy, FTD patients with MAPT mutation present aggregates of insoluble tau (Kirola et al. 2022). Relevantly, thanks to the discovery of MAPT as one of the major genetic causes of FTD, transgenic mice models with most common MAPT mutations are now used as *in vivo* models to study FTD pathology (Götz 2001; Crews et al. 2007).

C9orf72. The expansion of a non-coding GGGGCC hexanucleotide repeat in the chromosome 9 open reading frame 72 (C9orf72) gene is the most common cause of inherited frontotemporal dementia and ALS worldwide (DeJesus-Hernandez et al. 2011; Renton et al. 2011; Majounie et al. 2012). Even if the role of C9orf72 has not been completely clarified yet, TDP-43 inclusions are expressed in most of patients with C9orf72 mutation (Majounie et al. 2012). Endosomal trafficking dysregulation, RNA-foci formation, impaired transcription processing and dipeptide repeat protein toxicity represent the mechanisms of neurodegeneration linked with C9orf72 hexanucleotide expansion.

GRN. A huge number of mutations in granulin precursor (GRN) gene were found in FTD patients (Kirola et al. 2022). If the heterozygous mutations in GRN causes FTD, the homozygous mutations cause lysosomal storage disorder (Ferrari et al. 2014). GRN gene encodes progranulin (PGRN), a secreted protein involved into cell-cycle regulation, wound repair, axonal growth, and modulation of inflammation (Toh et al. 2011). Increased lipofuscinosis and lysosomal storage materials are considered as toxic effects of heterozygous GRN mutations (Valdez et al. 2017). Moreover, GRN mutations correlate with inflammation (altered TNF- α (Tumor Necrosis Factor) levels) (Krabbe et al. 2017) as well as TDP-43 dysfunction (with both nuclear depletion and pathogenic accumulation) (Chang et al. 2017).

The table in figure 1.5 summarizes the patterns of brain atrophy and proposed mechanisms of neurodegeneration associated with C9orf72, MAPT

and GRN gene mutations.

1.3.4 Neurotransmission impairment

As reported by Murley and Rowe (Murley et al. 2018), several studies showed that FTDL is associated with changes in many neurotransmitter systems—these changes possibly contribute to symptomatology of FTLD.

I will briefly discuss the regional changes in neurotransmitter synthesis, release, reuptake, catabolism, and synaptic binding associated with FTD focusing on the major neurotransmitter systems. A summary of the main neurotransmitter deficits in FTD is also reported in table 1.5.

Table 1.5: *Summary of neurotransmitter deficits in FTD*

Neurotransmitter pathway	FTD
Dopamine	
Dopaminergic neurons	severe-moderate deficits
Dopaminergic receptors	mild deficits
Noradrenaline	
Noradrenergic neurons	no significant change
Noradrenergic receptors	no available evidence
Serotonin	
Serotonergic neurons	severe-moderate deficits
Serotonergic receptors	severe-moderate deficits
Acetylcholine	
Cholinergic neurons	no significant change
Cholinergic receptors	inconsistent results
Glutamate	
Glutamatergic neurons	severe-moderate deficits
Glutamatergic receptors	severe-moderate deficits
GABA	
GABAergic neurons	mild deficits
GABAergic receptors	no available evidence

Glutamate is the principle excitatory neurotransmitter in the CNS. While glutamatergic transmission is essential for cognition, an excess can lead to toxicity and subsequent neuronal death; of note, this phenomenon is known to be detrimental for the neuronal survival in many neurodegenerative diseases (like AD) (Danysz et al. 2000; Kalia et al. 2008). Several pre-clinical and clinical

evidence indicate that glutamate plays a crucial role in the pathogenesis of FTD (Borroni et al. 2017; Benussi et al. 2019). For what concerns pre-clinical models, the transgenic animal model of FTD expressing mutations in FTD-associated gene CHMP2B presented altered AMPAR composition together with alterations in sociability and this behavioral phenotype was reversed when normal AMPAR composition is restored. In murine cortical neurons, silencing the FTD-associated gene granulin (GRN) decreased the expression of extra synaptic NR2B-containing NMDAR (Longhena et al. 2017). In addition, mouse models expressing pathological human tau presented accelerated neuronal loss mediated by glutamate transmission (Decker et al. 2016). In frontal and temporal lobes of patients, a reduction in both NMDARs and AMPARs (Francis et al. 1993; Procter et al. 1999; Bowen et al. 2007), as well as in glutamate levels (Ernst et al. 1997; Sarac et al. 2008), was detected. These results came from, respectively, post-mortem analysis and magnetic resonance spectroscopy. What is more, AMPARs appeared to be abnormal also in their composition (Gascon et al. 2014). Finally, a reduction of the overall levels of glutamatergic pyramidal neurons was detected in FTD patients (Ferrer 1999).

GABA (Gamma-aminobutyric acid) is the main inhibitory neurotransmitter in the CNS. A particular subgroup of GABAergic neurons, those that bind calbindin-D28k, are reduced in the frontal and temporal cortex of FTD patients (Ferrer 1999). This aspect might explain the cognitive decline and the loss of cortical feedforward information processing proper of FTD patients (Mann et al. 2007).

Serotonin regulates many higher brain functions related to cognitive control and learning (Harvey 2003; Ciranna 2006; Canli et al. 2007; Artigas 2013). Studies on FTD patients revealed that dysfunctions in serotonin system contribute to behavioral and cognitive symptoms proper of bvFTD (Huey et al. 2006; Hughes et al. 2015). More in detail, a reduction in both serotonin transmission and post-synaptic receptor densities have been detected in association with several symptoms seen in FTD including aggression, impulsivity, increased appetite and depression (Huey et al. 2006). Post-mortem analysis revealed that both 5HT1A and 2A serotonin receptors are reduced in frontal and temporal lobes and hypothalamus (Sparks et al. 1991; Francis et al. 1993; Procter et al. 1999; Bowen et al. 2007). *In vivo* PET studies confirmed these data showing that the 5-HT2A receptor binding potential is reduced in the midbrain and medial frontal cortex (Franceschi et al. 2005) and the 5-HT1A binding potential is reduced across all cortical areas (Lanctôt et al. 2007). Of notice, a length polymorphism in the promoter of the serotonin transporter gene (SLC6A4) has been associated to FTD. Indeed, the short allele (5-HTTLPR-s) was associated with a greater susceptibility to FTD in one study (Albani et al. 2008) although this was not replicated (Yokoyama et al. 2015). 5-HTTLPR variants also affect brain atrophy in FTD with different degree and spatial pattern of atrophy depending on its

length (Premi et al. 2015; Yokoyama et al. 2015).

Dopaminergic deficits are widely associated with PD; however, they are also a common feature of FTLD. In many cases of FTD, a nigrostriatal deficit, with loss of pre-synaptic dopaminergic neurons, reduced dopamine levels, reduced dopamine transporter binding, and abnormal dopamine receptor binding, has been detected with associated occurrence of Parkinsonism and motor symptoms (Rinne et al. 2002; Sedaghat et al. 2007; Kanazawa et al. 1988; Pal et al. 2001; Boeve et al. 1999; O'Dowd et al. 2012; Gil-Navarro et al. 2013). In contrast, degeneration of dopaminergic tract in mesocortical pathway (with a reduction of dopamine receptors, Frisoni et al. 2009) can contribute to behavioral symptoms of FTD.

It seems that also noradrenalin and acetylcholine deficits might play a role in the definition of FTD pathology, even though minor—indeed, noradrenergic and cholinergic pathways are relatively preserved in FTD patients (Murley et al. 2018).

Until that point of the discussion, I have considered the neurotransmitter systems as separate units. However, a complex cross-talk between them exists: as a consequence, dysfunctions in each of the above described neurotransmitter system could have some consequences on the correct functionality of all the others. In this regard, Benussi and colleagues proposed a model in which an initial dysfunction in glutamatergic system negatively influences both GABAergic and dopaminergic systems, finally supporting the appearance of both the positive and the negative symptoms proper of FTD (Benussi et al. 2019). In this glutamate-driven hypothesis, the deficits observed in FTD patients in terms of glutamate, GABA, serotonin and dopamine are brought together in a unified and interconnected framework.

In conclusion, it seems that the major clinical syndromes associated with FTLD present different neurotransmitter deficits (see table 1.5)—Restoring these deficits, individually or in combination, has thus the potential to improve cognitive, behavioral and motor symptoms.

1.3.5 Therapeutic strategies

Currently, there are no approved therapies for FTD or treatments that can stop or alter the course of disease progression. To date, pharmacological treatment has mostly involved off-label use of medications for symptomatic management. However, the advancements in the understanding of FTLD pathophysiology, genetics and neuropathology as well as the development of cellular and animal models, have allowed both the development and the testing of new molecules. These molecules target the pathological events underlying FTLD pathology and thus aim at achieving a disease-modifying effect. In the next paragraphs, I will briefly discuss both symptomatic and the potential disease-modifying treatments now-a-days available.

1.3.5.1 Symptomatic treatments

The medications commonly used in clinical practice with FTD patients aim at treating symptoms. Specifically, they are commonly employed to improve behavioral, cognitive, or motor symptoms of FTD. It must be highlighted that they were originally developed for use in psychiatric disorders or Alzheimer’s disease (AD), reason why it is incorrect to consider them as “approved” therapies for FTD (Tsai et al. 2016): they are symptomatic therapies used off-label. The majority of these agents work by modulating the levels or downstream effects of various neurotransmitters. The rationale of their employment is that, as described above (1.3.4), abnormalities in the cholinergic, serotonergic, dopaminergic, noradrenergic, and glutamatergic systems have been described also in FTD. In the table 1.6, a summary of the available symptomatic treatments for FTD are reported (Tsai et al. 2016).

1.3.5.2 Potential disease-modifying treatments

The recent advancements in the understanding of the genetics, pathophysiology, and neuropathology of FTD and its related disorders have led to the generation of new therapeutics which aim at a disease-modifying effect. In table 1.7, the available therapeutics and currently ongoing or recently completed clinical trials with potentially disease-modifying agents in FTD and its related disorders are reported. As clearly indicated in the table, these new therapies aim at interfering with those pathogenic mechanisms that are known to underlie FTLTD. For examples, some of them decrease tau levels (as in the case of the vaccines anti-tau (Boutajangout et al. 2011; Chai et al. 2011)) or interfere with both the hyper-phosphorylation of tau by glycogen synthase kinase (GSK-3) (Tolosa et al. 2014; Höglinger et al. 2014) and the formation of tau aggregates (d’Abramo et al. 2013; Castillo-Carranza et al. 2014). Conversely, others approaches contrast both tau and GRN loss of function/haplo-insufficiency (Boxer et al. 2014; Alberici et al. 2014). Some strategies also interfere with inflammation which apparently exert a negative role at least in some patients with GRN mutations (Tsai et al. 2016). In addition, anti-sense oligonucleotide (ASO) against C9orf72 expansion are in pre-clinical development (Donnelly et al. 2013; Lagier-Tourenne et al. 2013)—ASO are synthetic nucleic acids that can inactivate the mRNA of a target gene by direct binding or inducing RNase H mediated cleavage via a DNA/RNA hetero-duplex: within the FTD context, they can consequently counteract both the loss of function and the toxic gain of function proper of expanded C9orf72 transcripts.

Table 1.6: Clinical therapeutics for frontotemporal dementia (FTD) spectrum disorders

Drug class	Examples	Effect	Types of evidence
Behavioral symptoms			
Anti-depressants	Citalopram, paroxetine, sertraline, trazodone	May improve behavioral symptoms	Case reports and series, open label trials, randomized double-blind placebo-controlled trial
Anti-psychotics	Quetiapine, risperidone, aripiprazole, olanzapine	May improve behavioral symptoms	Case reports and series
Anti-epileptics	Carbamazepine, topiramate, valproic acid	May improve behavioral symptoms	Case reports and series
Cognitive symptoms			
Acetylcholinesterase inhibitors	Acetylcholinesterase inhibitors	No improvements, may worsen behavioral symptoms	Open label trials, randomized, double-blind placebo-controlled trials
NMDA-antagonist	Memantine	No improvements, may worsen cognition	Case series, open label trials, randomized double-blind placebo-controlled trials
Movement symptoms			
Dopamine replacement	Carbidopa-levodopa	Modest to no benefits	Case series

Table 1.7: Clinical trials with potentially disease-modifying agents

Compound	Mechanism	Indication	Clinical trial reference
C2N-8E12	Anti-tau antibody	PSP	NCT02494024
BMS-986168	Anti-tau antibody	PSP	NCT02460094
TRx0237 (LMTx)	Tau aggregation inhibitor	bvFTD	NCT01626378
Tideglusib	Glycogen synthase kinase-3 inhibitor	PSP	NCT01049399
Salsalate	Tau acetylation inhibitor	PSP	NCT02422485
TPI-287	Microtubule stabilizer	CBS, PSP	NCT02133846
Davunetide	Microtubule stabilizer	PSP	NCT01110720
FRM-0334	Increase progranulin expression	GRN carrier	NCT02149160
Nimodipine	Increase progranulin release	GRN carrier	NCT01835665

1.3.6 Toward an autoimmune hypothesis of FTD

Many pieces of evidence suggested that immune system dysregulation (both the inflammatory and the autoimmune component) plays a role in FTD pathogenesis. Genetic research (and, specifically, genome-wide association analysis) argued for a significant correlation between immune-associated loci (particularly within the HLA region) and increased FTD risk (Ferrari et al. 2014; Broce et al. 2018). In addition, epidemiological data and clinical studies reported a significantly increased risk of autoimmune disorders, represented by inflammatory arthritis, cutaneous disorders, and gastrointestinal conditions (Miller et al. 2013), and autoimmune system dysregulation in FTD patients. Concerning this last point, FTD patients presented a significant higher frequency of anti-neuronal antibody (ANA) if compared to healthy controls (Cavazzana et al. 2018)—of note, the occurrence of ANA at significant titre could represent a first sign of the immune system aberrant activation and consequently of the occurrence of autoimmune disorders. Our group also reported a high frequency (from 20% to 25%) of autoantibodies recognizing the GluA3 sub-unit of AMPA-type glutamate receptors (anti-GluA3 hIgGs) in patients with FTD (Borroni et al. 2017). In the next paragraph, I will focus my discussion on this finding.

It must be highlighted that, in the complex scenario of FTD, the identification of a dysregulation of the immune system in FTD might open new routes for therapeutic perspectives in autoimmune related neurodegeneration to reduce or revert the disease progression.

1.3.6.1 Anti-GluA3 autoantibodies

As stated above, our group identified autoantibodies directed against the GluA3 sub-unit of AMPARs in approximately 20% to 25% of FTD patients (Borroni et al. 2017) (GluA3-Ab+ patient). In this first study, one hundred seventy-five FTD patients were recruited and their serum and CSF were tested for the presence of anti-GluA3 antibodies and compared to those of healthy controls. Having detected a significant higher proportion of anti-GluA3 antibodies in FTD patients, Borroni and colleagues started to investigate the contribution of these autoantibodies through both clinical and pre-clinical evaluations.

Anti-GluA3-Ab+ FTD patients did not differ from anti-GluA3-Ab- ones in term of genetic substrate as well as for major clinical characteristics (like age of onset, sex, age of diagnosis or % of bvFTD cases). However, an inverse correlation between the disease age of onset and the serum dosage of the anti-GluA3 antibodies was found with the higher the autoantibodies dosage, the lower the age of onset. Of note, none of anti-GluA3-Ab+ patients presented a positive family history for autoimmune disorders. Neuopathologically, GluA3-Ab+ FTD patients presented bitemporal atrophy at MRI scan; however, no

neuroimaging differences between FTD patients with and without anti-GluA3 antibodies were found (Borroni et al. 2017).

Post-mortem analysis revealed that GluA3 sub-unit levels were reduced in the post-synaptic fraction but not in the total homogenate of temporal cortex specimens from GluA3-Ab+ patients (Palese et al. 2020). This finding suggested that anti-GluA3 hIgGs could exert their primary toxic effect by inducing the internalization of target sub-unit, as already described for other autoantibodies directed against receptors' sub-units (see section 1.1.2). To further investigate this hypothesis, the levels of PICK1 and GRIP1, scaffolding proteins involved in receptor insertion/internalization at the postsynaptic membrane, were measured. More in detail, PICK1 regulates the regulated removal of AMPA receptors from the synaptic plasma membrane (Hanley 2008) while GRIP1 is necessary for the anchorage of the receptor at the postsynaptic density (Anggono et al. 2012). In accordance with the reduced expression of GluA3 at the synapse, PICK1 and GRIP1 were respectively increased and reduced in both post-synaptic fraction and total homogenate (although GRIP1 decrease did not reach a statistical significance). Coherently, quantification of PICK1/GRIP1 ratio highlighted a 4-fold increase GluA3-Ab+ patients both in the postsynaptic compartment and in the total homogenate (Palese et al. 2020). Since AMPARs mainly exist as heterotrimeric structures, Borroni and colleagues investigated the levels of the other AMPAR sub-units. No differences in GluA1 and GluA2 or pGluA1 were detected in post-synaptic fraction; however, GluA2 levels were slightly higher in the total homogenate of GluA3-Ab+ patients. In addition, no significant alteration was observed in term of synaptic and total levels of NMDA-type receptor sub-units in the temporal cortex (Palese et al. 2020). The occipital cortex from the same patients was used as internal control area not affected by the disease; the analysis of postsynaptic fractions and total homogenate obtained from this area did not show any significant difference in AMPAR-sub-units or PICK-GRIP1 levels between GluA3-Ab+ or GluA3-Ab- patients (Palese et al. 2020).

Overall, these data suggest that in FTD patients, GluA3 antibodies target specifically GluA3-containing AMPAR in temporal cortex without interfering with other glutamate receptor sub-types. To investigate which could be the consequences, in term of glutamatergic neurotransmission, of anti-GluA3 antibodies interference with GluA3-containing AMPAR, intracortical facilitation (ICF) was investigated by Transcranial Magnetic Stimulation (TMS). Indeed, ICF is a paired-pulse protocol which induces facilitation of the motor evoked potential which is likely mediated by excitatory glutamatergic receptors; thus, ICF allows the indirect and *in vivo* assessment of excitatory glutamatergic circuits (Benussi et al. 2017). Of note, ICF has been previously shown to be deficient both in sporadic and genetic FTD patients compared to healthy controls (Benussi et al. 2017), and to correlate with disease progression (Benussi et al. 2019). A significant difference in ICF not only between healthy

controls and FTD patients but also within FTD patients between GluA3-Ab+ and GluA3-Ab- patients was observed (Palese et al. 2020), with a more pronounced glutamatergic deficit in anti-GluA3-Ab+ patients.

These results are in agreement with several recent preclinical (Udagawa et al. 2015; Decker et al. 2016; Longhena et al. 2017) and clinical studies (Leuzy et al. 2015; Benussi et al. 2017; Benussi et al. 2019) indicating a key role of glutamate receptors and glutamate neurotransmission in the pathogenesis of FTD and seems to assign a specific role for anti-GluA3 antibodies in a specific subgroup of FTD patients.

Having detected the above-described abnormalities in anti-GluA3-Ab+ patients, *in vitro* models were used to further dissect the molecular effects mediated by anti-GluA3 antibodies. The antibody-mediated internalization of GluA3 sub-unit was confirmed in both rat hippocampal neuronal primary cultures and human induced pluripotent stem cell (hiPSCs)-derived neurons acutely treated with patients' CSF containing anti-GluA3 hIgGs (Borroni et al. 2017). In rat primary hippocampal neurons, the decrease in GluA3 post-synaptic levels was also accompanied by a reduction in dendritic spine density, indicating that anti-GluA3 autoantibodies negatively impact also on this aspect (Borroni et al. 2017). Of note, anti-GluA3 autoantibodies also affect the pre-synaptic compartment, mediating a decrease of glutamate release, assessed thanks to a synaptosome-based assay (Palese et al. 2020). Finally, in the cell homogenate of differentiated neurons obtained from hiPSCs treated with anti-GluA3 hIgGs+ CSF, an increase of intracellular tau levels was detected with no differences in TDP-43 levels.

Overall, these clinical and pre-clinical findings opened new indications for a pathogenic role of glutamate receptors' autoantibodies in neurodegenerative diseases (Alberici et al. 2018; Benussi et al. 2019). However, even though it appears clear, from both clinical and pre-clinical data, that anti-GluA3 hIgGs antibodies negatively affect glutamatergic neurotransmission (see figure 1.6), further studies are needed to clarify whether these defects can in turn result into the expression of specific abnormal phenotypes which contribute to the definition of a distinct population of FTD patients.

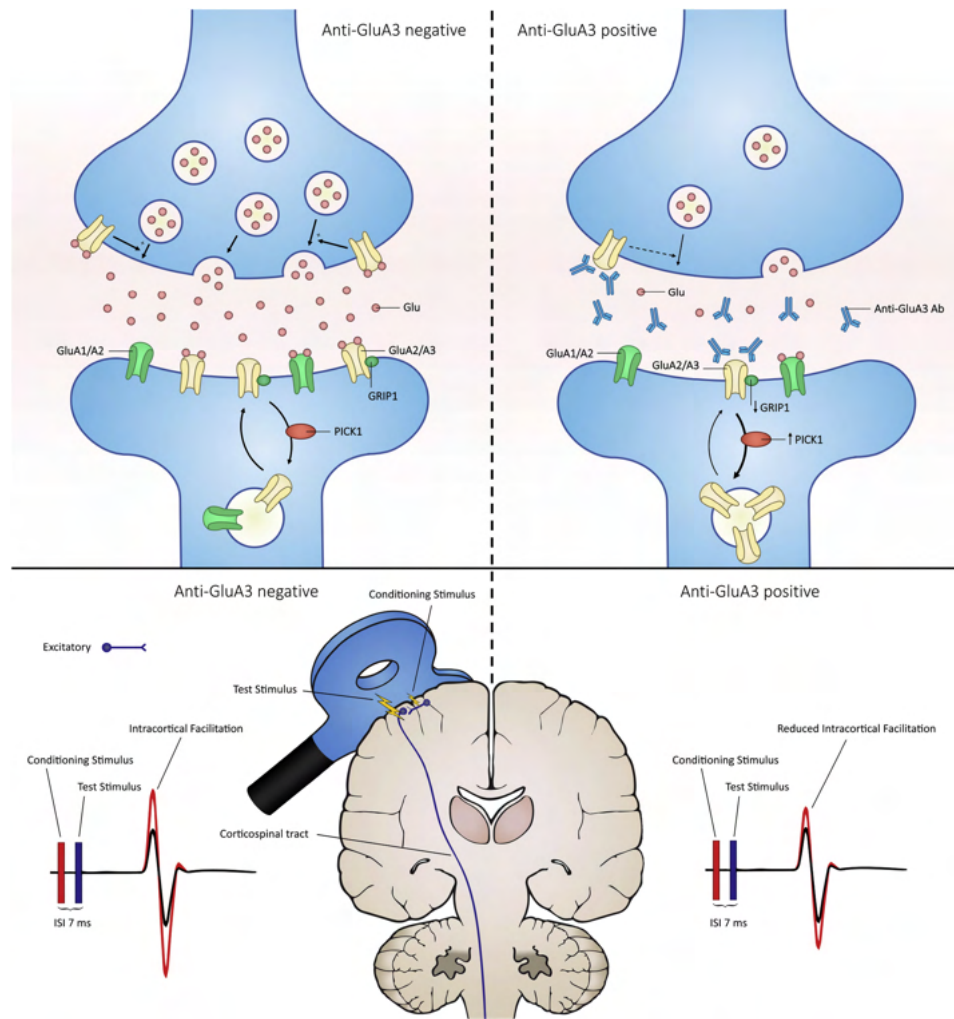


Figure 1.6: *Schematic representation of the molecular and functional effects induced by the presence of anti-GluA3 antibodies.* (Upper panels) Effect of GluA3 antibody on synaptic AMPAR sub-unit composition and glutamate release at excitatory glutamatergic synapses. (Lower panels) Reduced ICF as measured by TMS in anti-GluA3-Ab+ patients vs anti-GluA3-Ab- ones. The figure is adapted from Palese et al. 2020.

2. Aim

Autoantibodies directed against the GluA3 sub-unit of AMPARs have been identified in approximately 20% to 25% of FTD patients. Previous *in vitro* studies from professor Fabrizio Gardoni's group indicate that the presence of anti-GluA3 antibodies negatively affects glutamatergic neurotransmission at both pre- and postsynaptic sites, decreasing glutamate release and altering GluA3-containing AMPA receptor levels, ultimately leading to a significant reduction in dendritic spine density. Of notice, the detrimental effects of anti-GluA3 antibodies on glutamatergic neurotransmission have been verified in FTD GluA3-Ab+ patients.

Altogether, these findings suggest that anti-GluA3 antibodies not only affect GluA3-containing AMPARs but can trigger more profound morphological alterations in neuronal function that, in turn, might affect cognition and behavior. However, these aspects and, consequently, whether the presence of anti-GluA3 antibodies defined a specific sub-population of patients with distinct features, have not been investigated yet.

Within this context, my PhD project primarily aims at further clarifying the role of anti-GluA3 antibodies in FTD exploiting a mouse model injected with anti-GluA3 hIgGs purified from FTD patients. First of all, I will evaluate the biochemical, morphological, cognitive, and behavioral effects of either acute or chronic administration of GluA3 hIgGs in mice. These evaluations will allow me to study whether and how the presence of anti-GluA3 hIgGs triggers synaptic dysfunction and neurodegenerative process in mice and the association between these events and the appearance of FTD-related neuropathological and behavioral signature. As a secondary yet no less important point, I will use the chronic model to identify a possible rescue strategy to counteract the detrimental effects induced by anti-GluA3 antibodies administration.

Overall, this research will better define the contribution of anti-GluA3 antibodies to FTD neuropathology and will possibly allow to identify a therapeutic strategy of personalized medicine for anti-GluA3-Ab+ patients.

3. Materials and methods

3.1 Serum dosage and purification of anti-GluA3 antibody from FTD patients

Serum samples were frozen immediately after centrifugation and stored at -80°C before enzyme-linked immunosorbent assay. The detection of anti-GluA3 antibodies was performed by enzyme-linked immunosorbent assay according to a previously published protocol (Borroni et al. 2017; Mantegazza et al. 2002). Affinity purification protocol of the anti-GluA3 IgG from FTD patients' serum was performed by sulfo-link affinity chromatography. We purified anti-GluA3 antibodies from a pool of patients positive for the autoantibodies. Both the detection and the purification were performed using a peptide with following aminoacidic sequence: NEYERFVPFSDQQISNDSASSENRT (GluA3 peptide B, amino acids 399–424, in GluA3 extracellular domain), as previously reported (Borroni et al. 2017).

3.2 Mice

All procedures were approved by the Italian Ministry of Health and the local Animal Use Committee (projects n° 1161-2020, 182-2022) and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the European Community Council Directives. Routine veterinary care and animal maintenance were provided by dedicated and trained personnel. Two- to three-month-old male C57BL/6J animals were used. Animals were housed two per cage in a climate-controlled facility ($22 \pm 2^{\circ}\text{C}$, humidity 45-65%), with ad libitum access to food and water throughout, and with a 12 h light-dark cycle (19:00 07:00 schedule). Experiments were run during the light phase (between 10:00-17:00). All mice were handled on alternate days during the week preceding the first behavioural testing.

In both acute and chronic animal models, we employed male mice. This decision was driven by the fact that, in anti-GluA3-Ab+ FTD patients, clinicians did not observe any differences between sexes. Similarly, the percentage of patients positive for anti-GluA3 hIgGs was similar between

females and males. Thus, given that male animals represent a simpler model (at least for behavioral analyses, where conversely females' menstrual cycle must be monitored and could influence animals' performance), we excluded females from our evaluations. In addition, the choice of using only male animals is in accordance with the ethical principle of *reduction* since it allows to reduce the variability among animals and consequently the number of mice employed into the study.

3.2.1 Surgical procedures

3.2.1.1 Acute mouse model

C57BL/6J male mice were anesthetized with a mix of isoflurane (2%) and oxygen (1.5%) by inhalation and mounted into a stereotaxic frame (Kopf Instruments) linked to a digital micromanipulator. Brain coordinates of unilateral intracerebroventricular injection were chosen in accordance with the mouse brain atlas: anterior posterior (AP), -0,34 mm; medial lateral (ML): 1 mm; and dorsal ventral (DV): -2.5 mm. The volume of anti-GluA3 IgG and control human IgG (RandD Systems, cat. 1-001-A) was 4 μ l (0.12 mg/ml). Anti-GluA3 IgG or control IgG, were infused through a 10 μ l Hamilton syringe using a microinjection pump at flow rate 1.0 μ l/min. Surgical procedure lasted about 20 min for each animal. Mice received carprofen (5 mg/kg) in drinking water for three consecutive days.

3.2.1.2 Chronic mouse model

To chronically administer hIgGs to male mice, we used an intracerebroventricular cannula (Bilaney, Mouse guide cut 1.5mm below pedestal, C315GS-4/Spc). To implant the cannula, C57BL/6J male mice were anesthetized with a mix of isoflurane (2%) and oxygen (1.5%) by inhalation and mounted onto a stereotaxic frame (Kopf Instruments) linked to a digital micromanipulator. Brain coordinates of intracerebroventricular cannula positioning were chosen in accordance with the mouse brain atlas: anterior-posterior (AP): -0.34 mm; medial-lateral (ML): $\pm$ 1 mm; and dorsal-ventral (DV): -2.5 mm. Once positioned, the cannula was fixed on mice skull through dental cement (Super bond universal sun medical kit dx, 410446782). Surgical procedure lasted about 30 min for each animal. Mice received carprofen (5 mg/kg) in drinking water for three consecutive days after the surgery. Through the cannula, intracerebroventricular administration of control (CTRL) hIgGs or anti-GluA3 hIgGs was performed once a week for four consecutive weeks. Specifically, each animals received 2 μ l of anti-GluA3 hIgGs or CTRL hIgGs at a concentration of 0.12 μ g/ μ l. The hIgGs were infused through an injector (Bilaney, Mouse internal fits 1.5 mm guide with 1 mm projection, C315IS-4/Spc) connected to a 50 μ l Hamilton syringe.

3.2.1.3 Fiber implant

To perform fiber photometry experiments, during the surgery for intracerebroventricular cannula positioning, male mice were implanted with a optic fiber into the medial PFC (AP +2.1; ML + 0.25; DV -2.5) and injected with a virus with retrograde activity encoding for a calcium sensor (ssAAV-retro/2-mCaMKII α -jGCaMP7s-WPRE-bGHp(A)) into the Nucleus Accumbens (NAc) core (AP +1.2; ML + 1; DV -4.6). After 4 weeks (time required for the expression of the sensor), the activity of neurons projecting from mPFC to NAc was monitored. At the end of the tasks, we sacrificed mice to verify the position of the fiber and the infection with the GCaMP virus through fluorescent microscopy. With this aim, we perfused mice with PFA 4% in PBS1X; after 1 hour of post-fix with PFA 4% in PBS1X, we cut 50 μ m brain slices that, after DAPI staining, were observed at confocal microscope.

3.2.2 S 47445 administration

S 47445 (also known as CX-1632 and Tulrampator, MedChemExpress, HY-109046), a positive allosteric modulator (PAM) of AMPAR, was administrated daily through intraperitoneal injections from day 15 to day 29. The side of the injection was changed every day. According to its datasheet, S 47445 was dissolved in 10% Dimethyl sulfoxide (DMSO, Sigma-Aldrich, 276855), 40% poly(ethylene glycol)300 (PEG 300, Sigma-Aldrich, 81162), 5% Tween 80 (Sigma-Aldrich, P4780), 45% saline. On the basis of previous pre-clinical studies, we administered S 47445 at a dose of 3 mg/kg for a final volume of 100 μ l. As negative control, mice were injected with the emulsion used to solve S 47445 (10% DMSO, 40% PEG 300, 5% Tween 80, 45% saline).

3.2.3 *Ex-vivo* analyses

3.2.3.1 Subcellular fractionation

Triton insoluble postsynaptic fractions (TIF), a fraction highly enriched in PSD proteins but absent of presynaptic markers, was isolated from adult mouse PFC and hippocampus. Mice were sacrificed, PFCs and hippocampi were dissected within 2 min and pooled. A representative schema of the portion of PFC dissected for biochemical assays is reported in figure 3.1 (pink area). Tissues homogenization was carried out by 10 strokes in a glass-Teflon homogenizer in 700 μ L of cold 0.32 M sucrose containing 1 mM HEPES, 1 mM NaF, 1 mM MgCl₂, 1 mM NaHCO₂ and 0.1 mM phenylmethylsulfonyl fluoride (PMSF, pH 7.4) in the presence of protease inhibitors (Complete, GE Healthcare) and phosphatase inhibitors (PhosSTOP, Roche Diagnostics GmbH). Aliquots of homogenates were kept for total homogenate analysis. Homogenates were centrifuged at 13,000*g for 15 min

at 4°C. Triton X-100 extraction of the resulting pellet was carried out at 4°C for 15 min in 500 μ L of extraction buffer (0.5% Triton X-100, 150 mM KCl and Complete). After extraction, samples were centrifuged at 100,000*g for 1 h at 4°C and the TIFs obtained were resuspended by 10 strokes in a glass-glass homogenizer in an adequate volume of 20 mM HEPES with Complete. Protein content of the samples was quantified by using Bio-Rad protein assay. After measuring protein concentration, all samples were standardized at 1 mg/mL concentration and the same protein amount loaded in each lane.

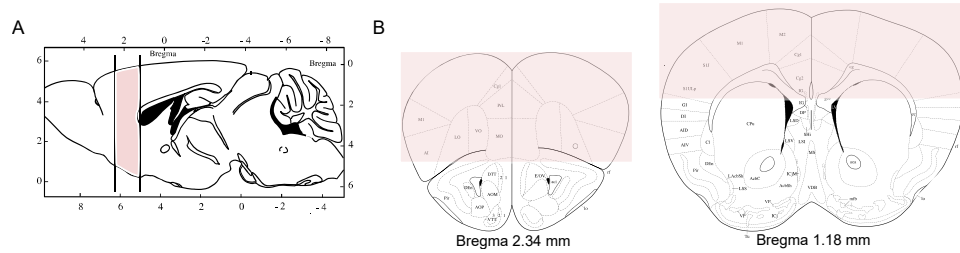


Figure 3.1: *Representative schema of the portion of PFC dissected for biochemical assays.* (A) Sagittal diagram of a mouse brain; the region delimited by black lines and highlighted in pink is the portion of the brain that was dissected to perform biochemical assays; (B) Coronal diagrams corresponding to the antero-posterior coordinates of the two black lines in (A). The figures are adapted from "*The mouse brain atlas*" by G. Paxinos and K.B.J. Franklin.

3.2.3.2 Western blot (WB) analysis

WB analysis was performed in total homogenate and in TIF purified from mouse PFC and hippocampus and in BS3 samples. Protein samples were separated on an acrylamide/bisacrylamide gel at the appropriate concentration depending on their molecular weight. Nitrocellulose membranes (Biorad) were developed with electrochemiluminescence reagents (Biorad) and scanned with a Chemidoc (Biorad Universal Hood III). Images were quantified with computer-assisted imaging (Image Lab, Biorad). Protein levels were expressed as relative optical density (OD) measurements normalized to a housekeeping protein. pCREB, pERK and pGluA1S845 bands were normalized against CREB, ERK and GluA1 bands, respectively.

3.2.3.3 Immunohistochemistry (IHC) and relative analyses

Mice were perfused with cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS). The brain was removed from the skull and post-fixed in 4% PFA in PBS for 1 h at 4 °C. The brain was sliced into 50 μ m coronal sections using the Vibratome 1000 Plus Sectioning System (3 M). Brain slices were permeabilized in 0.3% Triton X-100 in PBS (0.3% T-PBS) for 1 h at RT,

shaking. After permeabilization, brain slices were blocked with 0.1% Triton X-100 in PBS (0.1% T-PBS) supplemented with 10% normal goat serum (NGS) for 2 hours at RT, shaking. After permeabilization and blocking, slices were incubated with primary antibodies in 0.1% T-PBS supplemented with 3% NGS for 3 overnights (o/n) at 4 °C, shaking. The appropriate Alexa Fluor-conjugated secondary antibodies in 0.1% T-PBS with 3% NGS were applied for 2 hours at RT, shaking, followed by nuclei staining with the fluorescent dye 4,6-diamidino-2-phenylindole (DAPI; 1:50,000 in PBS; Thermo Fisher Scientific). Slices were mounted on glass slides with Fluoromount mounting medium (Sigma-Aldrich) for confocal imaging.

To count Iba1 and CD68 positive cells, Z-stacks of 3 μm step (for a final Z distance of 12 μm) were taken with LSM900 confocal microscope (Zeiss) with a 20X objective at 0.2 μm pixel size and analysed automatically using Fiji (ImageJ) software.

To perform Sholl analysis on Iba1+ cells, Z-stacks of 0.45 μm step were taken using LSM900 (Zeiss) confocal microscopes with 63X objective at 0.09 μm pixel size and analysed using ImageJ software. Specifically, we employed the Simple Neurite Tracer plugin indicating 5 μm as radius step size. The number of intersections was plotted as a function of the radial distance from the cell soma and the total length expressed the sum of the length of the cell's ramification.

3.2.3.4 Cross-linking assay

Mice were sacrificed by cervical dislocation and the brains were immediately transferred into artificial cerebrospinal fluid (aCSF) (NaCl 120 mM, KCl 2.5 mM, NaHCO₃ 26 mM, NaH₂PO₄ 1.24 mM, CaCl₂ 2 mM, MgSO₄ 2 mM, D-glucose 10 mM, water; pH = 7.4/7.5) previously treated with carboxigen and maintained cold in ice. Coronal slices of 200 μm of thickness containing PFC were obtained with the Vibratome 1000 Plus sectioning system (3M). Slices were treated with 1 mg/ml (Bis (Sulfosuccinimidyl) suberate (BS3, 21580, Thermo scientific) solved in aCSF for 30 minutes at 4°C in presence of carboxigen. BS3-treated slices underwent quenching in glycine (HelloBio, HB0299) 100 mM for 10 minutes at 4°C. Control slices were maintained in cold aCSF in presence of carboxigen. BS3 treated and control slices were separately centrifugated at 13000 rpm for 2 minutes at 4°C. The pellets were frozen and finally resuspended with Lysis buffer (NaCl 500 mM, HEPES pH 7.4 25 mM, DTT 1 mM, EDTA 2 mM, PMSF 1 mM, NaF 20 mM, NONIDET P-40 0.10%, water; pH = 7.4/7.5) with protease inhibitor 1X (Complete, GE Healthcare) and phosphatase inhibitor 1X (PhosSTOP, Roche Diagnostics GmbH) in a glass-glass potter. After measuring protein concentration, all samples were standardized at 1 mg/mL concentration in and then analysed through western blot analysis. BS3 does not cross cell membranes so it covalently crosslinks cell surface expressed receptors while intracellular receptors are not modified

enabling surface and intracellular receptor pools to be distinguished and measured. Quantitative analysis was performed as a comparison between the portion of slices treated with BS3 (BS3+-treated slices) and the portion of the slices from the same animal not treated with BS3 (CTRL slices).

3.2.3.5 Spine morphology

Carbocyanine dye DiI (D282, Invitrogen) was used to label neurons as previously reported (Kim et al. 2007; Stanic et al. 2015). Z-stacks of 0.45 μm steps were taken with a confocal microscope (Zeiss) and analysed using Fiji (ImageJ) software. Specifically, for each dendritic spine, spine length, width and neck were manually measured at selected regions of interest. These measurements were used to classify dendritic spines into three categories (thin, stubby, and mushroom) (see also Gardoni et al. 2012; Harris et al. 1992). For each neuron, an average of 3 dendrites that are distant from the neuron soma no more than 200 μm , for a total dendritic length of about 200–300 μm , was considered. For spine morphology studies, we analysed brain samples only from mice that did not undergo behavioural and cognitive tests. To avoid issues related to a possible different effect of the Abs on the two sides, confocal imaging was performed on a balanced number of stained neurons of the two hemispheres.

3.2.4 Behavioral tasks

3.2.4.1 Open Field (OF)

To assess general locomotor activity, mice were individually placed in the centre of the open field arena (Ugo Basile, 44 $\times$ 44 cm) and video-tracking software (ANY-maze 6.2, Stoelting) was used to record and analyse their movements for one hour. The distance travelled and the time spent at the centre and the corners of the arena were automatically calculated by the ANY-maze software.

3.2.4.2 Recognition memory task

The procedures for novel object recognition, object-in-place and object location tasks were adapted from a previous study (Barker et al. 2011). All the tasks involved an acquisition phase (5 min) and a recognition test (5 min), separated by a 5-minute delay. During the delay period, all the objects were cleaned with alcohol to remove olfactory cues and any sawdust that had stuck to the object. Mice were tested in a standard open field arena (UgoBasile, 44 $\times$ 44 cm) with black PVC walls. The stimuli were objects constructed from Duplo blocks (Lego) and varied in shape, color, and size, and were too heavy to be displaced. A digital camera (Imaging Source, DMK 22AUC03 monochrome) was placed above the apparatus to record the test using a

behavioral tracking system (Anymaze 6.0, Stoelting). These videos were used offline by experimenters blind to the manipulations for a posteriori scoring of the time spent in the different zones of the apparatus and exploratory behavior, which was defined as the animal directing its nose toward an object at a distance of at least 2 cm. To express the discrimination between the objects we calculated a discrimination ratio as the absolute difference in the time spent exploring the novel, displaced or exchanged objects and the familiar objects divided by the total time spent exploring the objects. All mice were habituated to the open field arena for 15 min the day before testing. Animals that failed to complete a minimum of 10 s of exploration in the test phase were excluded from the analysis.

Novel object recognition task. Mice were tested in a standard open field arena (UgoBasile, 44 × 44 cm) with black PVC walls. The stimuli were objects constructed from Duplo blocks (Lego) and varied in shape, color, and size, and were too heavy to be displaced. In the acquisition phase, two identical objects were placed near the corners on one wall in the arena (10 cm from the walls). The animals were placed into the arena and allowed to explore for 5 min. During the test, one of the two objects was replaced with a novel object. The positions of the objects in the test and the objects used as novel or familiar were counterbalanced between the animals.

Object location task. We measured the ability of mice to recognize an object that had changed location compared to the acquisition phase. In the acquisition phase, two identical objects were placed near the corners on one wall in the arena. In the test, one object was left in the same position, the other one was displaced to the corner adjacent to the original position, such that the two objects were diagonal from each other.

Object-in-place task. In this task four objects were located in the four corners of the arena. In the test phase, two of the objects, both on the left or right of the arena, exchanged positions. If object-in-place memory is intact, the subject should spend more time exploring the two objects that are in different locations compared with the two objects that are in the same locations.

3.2.4.3 Fear conditioning

This task was performed as in a previous study (Scheggia et al. 2017). Fear conditioning took place in a standard conditioning chamber (Ugo Basile). The conditioned stimulus (CS) was a tone (4 kHz, 80 dB sound pressure level, 30 s) and the unconditioned stimulus (US) was a scrambled shock (0.7 mA) delivered through the grid floor that terminated simultaneously with the tone (2 s). On experimental day 1, mice were placed in the training chamber and after a 2 min habituation period (baseline) three conditioning trials were presented (tones paired with shock) with an intertrial interval of 90 s. Then, the animals were returned to their home cages 2 min after the last CS–US pairing. After 72 h, mice were retested for long-term memory in the same chamber for 5 min without tone or footshock (contextual memory). At 1 h

after the contextual memory recall, mice were placed in a new chamber and after 2 min of habituation (baseline) exposed to the conditioning tone for 2 min (cue) to test cued memory. Then, the animals were returned to their home cages after 2 min (post cue).

3.2.4.4 Emotion-affective discrimination task

This task was adapted from previous studies (Ferretti et al. 2019, Scheggia et al. 2019 2020).

Testing mice (observers) were tested in a standard three-chamber sociability cage (Ugo Basile, 60x40x22cm) equipped with transparent PVC walls and two grid enclosure that hosted the demonstrators (15 cm in height, diameter 7 cm). After each test, the apparatus was wiped down with 70% ethanol and allowed to air dry.

Both habituation and behavioral testing, were carried under dimly lit (6 ± 1 lx). Digital cameras (Imaging Source, DMK 22AUC03 monochrome) were placed above the apparatus to record the test using a behavioral tracking system (Anymaze 6.0, Stoelting). These videos were used offline by experimenters blind to the manipulations of both the observers and demonstrators for a posteriori scoring of the time spent in the different zones of the apparatus. Animals that failed to complete a minimum of 10s of exploration were excluded from the analysis.

Habituation. Habituation (10 min) to the testing setting occurred on the day before the first experiment. Demonstrator mice (both neutral, fear and stress), matched by age, sex, and strain to the observers, were habituated, without the observer, inside the grid enclosure. The neutral demonstrators supposed to be matched, during the test, with the fear demonstrators, were also habituated to the tone cue (4 kHz, once for 120 s) without any conditioning. Similarly, also observers mice were habituated to the experimental setting; the observers mice that were supposed to discriminate between neutral and fear demonstrators, were also habituated to the tone cue (4 kHz, once for 120 s) without any conditioning.

Demonstrators. For both fear and stress conditions, *neutral demonstrators* did not receive any manipulation and were left undisturbed, with ad libitum water access, in their home cage. All the neutral demonstrators were group-housed, separately from the cages of the emotionally altered demonstrators. *Fear demonstrators* were fear conditioned one day before the test using the parameters and context previously described (Scheggia et al. 2017), and using the same tone delivered to the observers and neutral demonstrators during their habituation process. In particular, the conditioned stimulus was a tone (4 kHz, 80 dB sound pressure level, 30s) and the unconditioned stimulus were three scrambled shocks (0.7 mA, 2s duration, 90 s intershock interval) delivered through the grid floor that terminated simultaneously with the tone (2s). Fear mice were conditioned only once, in a separate room and

using distinct apparatus (Ugo Basile, Italy) from the one where the emotion discrimination task would be performed. *Stress demonstrators* were subjected to a mild stress consisting of the restraint tube test, a standard procedure to induce physiological stress in rodents for 15 min before the beginning of the test. These mice were then immediately moved to the testing arena. Demonstrators were test-naïve and used a maximum of two times (with the exception of fear demonstrators that were used only once).

Test. On the day of testing, neutral demonstrators and observers were brought, inside their home cages, into the experimental room 1 h before the experiment began. 10 min before the experiment, observer mice were gently moved into the dimly lit testing apparatus for habituation. Then, one neutral demonstrator and one emotionally altered demonstrator (fear or stress) mouse were placed under the grid enclosure, and the 6 min experiment started. In the test with fear animals, from minute 3 to 4 of the test, the tone cue was played. We counterbalanced the presentation of the neutral versus affectively altered demonstrators in the two sides of the testing arena to avoid position biases.

3.2.4.5 Observational fear conditioning

This task was performed as in a previous study (Jeon et al. 2010). The apparatus consisted of two identical and adjacent fear conditioning chambers (Ugo Basile, $24 \times 20 \times 30$ cm) separated by a transparent Plexiglas partition. Olfactory and auditory cues could be transmitted between the chambers. An observer mouse (IgG control or anti-GluA3 IgG) and a sex and age-matched demonstrators were individually placed in the two chamber and allowed to explore the chambers for 5 min (baseline). Then, a 2-s foot shock (0.7 mA) was delivered every 10 s for 4 min to the demonstrator mouse using the behavior tracking software. Demonstrators were used only once. Based on previous studies (Jeon et al. 2010), we used 10-s intervals for foot shocks and a 4-min training. At the end of the procedure demonstrator and observer mice returned to their respective home-cage.

3.2.4.6 Sociability and social memory tests

We used the same three-chamber sociability cage employed for the emotion discrimination task. Habituation (10 min) to the testing setting occurred on the day before the experiment. On the day of testing, after habituation in the apparatus without stimuli (10 min), an adult conspecific mouse (novel mouse 1) that had no previous contact with the observer, was placed in the grid enclosure in one side of the apparatus, whereas the other grid enclosure in the opposite chamber was empty. To measure sociability (the tendency of the subject mouse to spend time with a conspecific, compared with time spent with an object), a discrimination ratio was calculated (time spent with

novel mouse 1 – time spent with novel object / total time spent with novel mouse 1 and novel object). Following the sociability test, a novel mouse (novel mouse 2) was placed in the empty grid enclosure and the observer was tested for another 5 min to assess the p for social novelty. This is defined as more time spent in the chamber with novel mouse 2 than time in the chamber with novel mouse 1. Most mice prefer to spend more time near the unfamiliar novel mouse (novel mouse 2). To assess social novelty, we calculated a discrimination ratio for each mouse (time spent with novel mouse 2 – time spent with novel mouse 1 / total time spent with novel mouse 1 and novel mouse 2). The procedures and analyses were performed as in previous study (Scheggia et al. 2019).

3.2.4.7 Rotarod task

To assess motor learning, coordination and balance, mice were tested on a rotarod apparatus (Ugo Basile) as described by Ferrari and colleagues (Ferrari et al. 2022). To familiarize them with the instrument, each mouse was given training sessions (three trials/day for a maximum of 300 s/trial) for 3 days before the testing days. Each test session was composed of three consecutive trials of the duration of maximum 300 s with 2 min as inter-trial interval; the trial was stopped as the mouse fell off the rotarod bar. For the accelerating rotarod test, each mouse was placed on the rotarod with the speed increasing from 4 rpm to 40 rpm. The latency to fall off the rotarod bar was recorded for each mouse in each test and the mean of the duration of three consecutive trials was used for the analysis.

3.2.4.8 Elevated Plus Maze

Mice were tested in the elevated plus maze to assess anxiety-like behaviour (Walf and Frye, 2007). Mice were placed in the maze at the junction of the four arms (i.e., two open and two enclosed arms, Ugo Basile), and entries and duration in each arm were recorded by a video-tracking system (Anymaze 6.2, Stoelting) for 5 min.

3.2.4.9 Free Reward Consumption task

As habituation to the experimental setting, the day before the task, mice were placed individually in empty standard mouse cages for one hour. On the day of the task (performed in the same room and in the same cages of the habituation), mice were left individually in the cages with 50 pellets of palatable food (14 mg, Test Diet, 5-TUL) for 2 hours. The number of pellets eaten was monitored over time. The result of the task was expressed as number of pellets eaten (50 - pellet/s left in the cage) at the end of the two hours. We tested mice both after a period of food restriction, to stimulate their interest in food consumption, and in satiety.

3.2.4.10 Food Seeking Task

Food seeking task was performed in a standard operant chamber (length of 24cm, width of 20cm, height of 18.5cm; ENV-307W-CT, Med Associates). A food magazine connected to an external dispenser of food pellets was available in the centre of the operant chamber and two circular holes (i.e., nose-poke holes) were located on the food magazine's right and left respectively. Mice were mildly food restricted to 85-90% of their free-feeding body weights to promote food-seeking behaviour. Each session lasted 40 minutes. Before the beginning of the test, every operant box was cleaned with ethanol 50%. During the task, mice were individually positioned in a single operant chamber where their movements were monitored by custom scripts written in MED-PC V (Med Associates). First, mice had to associate the insertion of the nose in one of the two nose-poke holes with the delivery of the food reward pellet(s) in the magazine. The number of nose-pokes required to obtain one food reward pellet represents the fixed ratio. The nose-poke hole that allowed the delivery of the food reward pellet(s) in the food magazine, represented the active nose-poke hole. The other nose-poke hole did not produce any effect and represented the inactive one. We named the choice of the active nose-poke hole as "Food-related response" and the choice of the inactive nose-poke hole as "Non-food related response". The location of the Food-related response was counterbalanced between left and right nose-pokes across mice to avoid position bias. Once assigned in the habituation phase, however, the position of the Food-related response never changed across test sessions of the same mouse. The habituation continued until all the mice understood the task. This was indicated by a positive preference index, calculated as the number of food-related responses – the number of non-food related responses / the total number of responses. Generally, mice took two days to understand the task. Once habituated, we measured mice task performance by measuring the preference index, the total number of food-related responses, the total number of responses and the time interval between a food-related response and the subsequent one (i.e., average food latency). We tested mice both after a period of food restriction, to stimulate their interest in food seeking, and in satiety. Mice were tested both with a fixed ratio = 1 and a fixed ratio = 4 or 8.

3.2.4.11 Sucrose Preference task

Sucrose Preference Task was partially adapted from the protocol of Amodeo and colleagues (Amodeo et al. 2021). As habituation to the experimental setting, mice were placed individually in cages with two drinking bottles both containing water for 12 hours. Then, after a night in their original cages, mice were re-placed individually in the same cages of the habituation with a bottle containing water and another with 1% sucrose solution (Sigma-Aldrich,

84097). During the test, mice had free access to the two bottles for 24 hours. The two bottles were not visibly distinguishable and were both weighed before, after 12 hours and at the end of the test. The two bottles were also switched in their position after 12 hours to avoid position bias in drinking behaviour. Sucrose preference was calculated as the percentage of the volume of sucrose intake over the total volume of fluid intake for each tested animal.

3.2.4.12 Free interaction

In sessions of 20 minutes, actor mice were let free to interact into an open field arena with stimuli that entail, respectively, a neutral valence (an object), an increasing positive valence (a palatable food or social stimuli such as juvenile males or females in oestrus) or a negative valence (CD1 aggressive mice). More in detail, after 5 minutes of habituation to the arena, the stimulus (an object, a palatable food, a juvenile male, a female in oestrus or an aggressive CD1 mouse) was introduced into the OF, and the actor mouse interacted with it for the remaining 15 minutes of the session. During the entire task, the activity of the glutamatergic neurons projecting from mPFC to NAc was monitored through fiber photometry. Actor mice were tested with the above-mentioned stimuli on different days with at least one day of rest between one session and the subsequent one.

3.3 Antibodies

The following primary antibodies were used:

- mouse anti-GluA3 (MAB5416, Millipore; dilution: 1:1000 WB)
- mouse anti-GluA2 (75-002, Neuromab; dilution: 1:2000 WB)
- rabbit anti-GluA1 (13185S, Cell Signalling; dilution: 1:1,000 WB)
- rabbit anti-pGluA1S845 (3420, Abcam, dilution: 1:1000 WB)
- rabbit anti-GluN2A (M264, Sigma; dilution: 1:1000 WB)
- rabbit anti-GluN2B (14544S, Cell Signalling; dilution: 1:1000 WB)
- mouse anti-PSD95 (192757, Abcam, dilution: 1:2000 WB)
- rabbit anti-PICK1 (3420, Abcam, dilution: 1:1000 WB)
- rabbit anti-pERK (9101, Cell Signalling, dilution: 1:1000 WB)
- rabbit anti-ERK (9102, Cell Signalling, dilution: 1:1000 WB)
- rabbit anti-pCREBS133 (06-519, Millipore, dilution: 1:1000 WB)

- rabbit anti-CREB (AB3006, Millipore, dilution: 1:1000 WB)
- mouse anti-phospho tau (MN1020, Invitrogen; dilution: 1:1000 WB)
- rabbit anti-phosphoTDP43 (22309-1-AP, Proteintech; dilution: 1:5000 WB)
- rabbit anti-human-tau (A0024, Dako; dilution: 1:1000 WB)
- rabbit anti-CD11b,c (bs-1014R, BIOSS; dilution: 1:1000 WB)
- rat anti-CD68 (1957, Biorad; dilution: 1:400 IHC)
- rabbit anti-Iba1 (019-19741, Fujifilm; dilution 1:700 IHC)
- mouse anti-actin (A5441, Sigma; dilution: 1:5000 WB)
- mouse anti-tubulin (T9026, Sigma-Aldrich; dilution: 1:5000 WB)

The following secondary antibodies were used:

- goat anti-mouse-HRP (172–1011, Bio-Rad)
- goat anti-rabbit-HRP (170–6515, Bio-Rad)
- goat anti-rabbit-Alexa488 (A-11034, Invitrogen)
- goat anti-rat-Alexa488 (A11006, Invitrogen)
- goat anti-rabbit-Alexa555 (A21428, Invitrogen)

3.4 Statistical analyses

All the group values are expressed as mean $\pm$ s.e.m. Comparisons between groups were performed using the following tests as appropriate: two-tailed unpaired Student's t test, Mann–Whitney test, ordinary one-way ANOVA, Kruskal-Wallis test and 2-way ANOVA. Statistical analyses were performed using the GraphPad Prism statistical package (GraphPad software). Number of neurons and mice used as well as all the statistical details of experiments are reported in the figure legends. Normal distribution was checked using D'Agostino and Pearson normality test or by using Shapiro-Wilk normality test when n was insufficient.

No statistical methods were used to predetermine sample size for single experiments. The animal numbers were based on estimation from previous studies, including our own published studies (Ferretti et al. 2019; Haselmann et al. 2018, Scheggia et al. 2019). For all behavioural tests, littermates were randomly assigned to the different groups. Experimenters were not blinded during data acquisition, but all analyses were performed with blinding of the experimental conditions. Outliers were identified using ROUT ($Q = 1\%$) method.

4. Results

4.1 Acute animal model

Note. This chapter reflects the “*Results*” section of the article titled “*GluA3 autoantibodies induce alterations in dendritic spine and behavior in mice*” published in 2021 on the journal *Brain Behaviour and Immunity* (Scheggia et al. 2021) and to which I contributed for the *ex-vivo* analyses. All the *in-vivo* behavioral tests were performed in collaboration with Diego Scheggia.

4.1.1 Acute injection of anti-GluA3 hIgGs induced molecular and morphological alterations of dendritic spine in the PFC

To evaluate the *in vivo* effects induced by anti-GluA3 IgG purified from FTD patients, male C57BL/6J mice received unilateral stereotaxic cerebroventricular injection of the purified antibody or control IgG (Fig. 4.1).

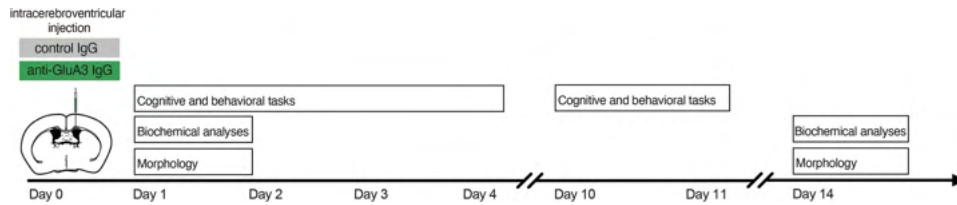


Figure 4.1: *Scheme representing the timeline of the experimental procedures.* Mice were injected with anti-GluA3 IgG or control IgG at 3 months of age. After injection the mice were enrolled in behavioral tests or used for biochemical and morphological experiments.

One day after injection, mice were sacrificed and biochemical analyses by WB assay (Fig. 4.1) were performed both in a purified triton-insoluble postsynaptic fraction (Mellone et al. 2019) (TIF) and in a total homogenate. As shown in Fig. 4.2A, B, single injection of anti-GluA3 IgG induced a significant reduction of the GluA3 subunit of AMPARs at synapses of the prefrontal cortex (PFC) without any modification of the level of this subunit in the total homogenate of the same brain area (Fig. 4.2C, D).

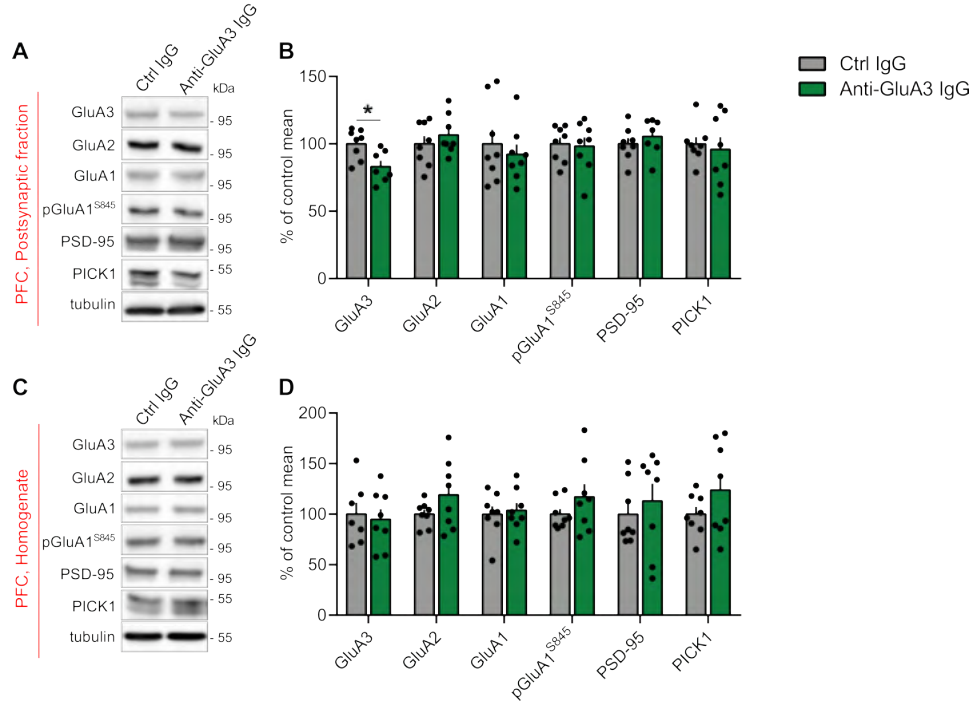


Figure 4.2: Molecular effects induced by acute administration of *GluA3* IgG in mice's PFC. (A-D): Western blot representative images (left panels) and bar graph of densitometric quantification (right panels) of GluA3, GluA2, GluA1 and phosphorylated ser845 GluA1 subunits, PSD-95 and PICK in Triton-insoluble postsynaptic fractions (TIF) (A,B) (two-tailed unpaired t-test; GluA3: $t = 2.861$, $df = 13$, $P = 0.0134$; GluA2: $t = 0.8624$, $df = 14$, $P = 0.4030$; GluA1: $t = 0.6059$, $df = 14$, $P = 0.554$; PSD95: $t = 0.8223$, $df = 13$, $P = 0.4258$; pGluA1: $t = 0.2165$, $df = 14$, $P = 0.8317$; PICK1: $t = 0.3945$, $df = 14$, $P = 0.6992$) or in total homogenate (C,D) (two-tailed unpaired t-test; GluA3: $t = 0.3365$, $df = 13$, $P = 0.7419$; GluA2: $t = 1.476$, $df = 14$, $P = 0.1620$; GluA1: $t = 0.3512$, $df = 14$, $P = 0.7307$; pGluA1: $t = 1.248$, $df = 14$, $P = 0.2327$; PICK1: $t = 1.345$, $df = 14$, $P = 0.2001$ - Mann-Whitney test; PSD95: $P = 0.4418$) obtained from mice PFC 24 h after injection of either GluA3 IgG or control IgG. Tubulin band was used for normalization. $n = 7-8$ /group. * $P < 0.05$.

No modifications of the AMPAR GluA1 and GluA2 subunits, of the AMPAR-binding protein PICK1 and of the postsynaptic marker PSD-95 were detected at PFC synapses (Fig. 4.2A, B) and in PFC homogenate (Fig. 4.2C, D). Moreover, the phosphorylated form of GluA1 (pGluA1-Ser845; Fig. 4.2A-D) and the phosphorylated forms of ERK and CREB (Fig. 4.3A, B) were not altered by the treatment with anti-GluA3 IgG thus suggesting no alteration of the main signaling pathways associated to the activation of synaptic glutamate receptors (Hardingham et al. 2010).

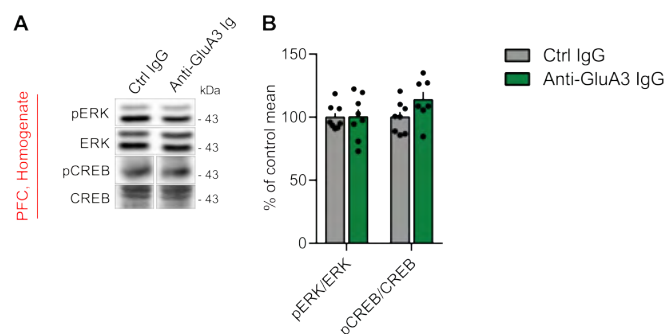


Figure 4.3: *Acute administration of GluA3 IgG does not affect pCREB and pERK in mice's PFC.* Western blot representative images (A) and bar graph of densitometric quantification (B) of pERK and pCREB in total homogenate (two-tailed unpaired t-test; pERK: $t = 0.03026$, $df = 14$, $P = 0.9763$; pCREB: $t = 1.803$, $df = 13$, $P = 0.0947$) obtained from mice PFC 24 h after injection of either GluA3 IgG or control IgG. pERK and pCREB proteins were normalized against ERK and CREB bands respectively. $n = 7-8$ /group.

These same molecular targets were analyzed in TIF and total homogenate extracted from mice hippocampus. In this area, single injection of anti-GluA3 IgG did not alter any of the proteins under analysis (data not shown).

AMPA activation is a key event in spine enlargement/maturation and formation of new spines (Diering et al. 2018, Hanley 2008). Previous *in vitro* studies in neuronal cultures demonstrated that treatment with anti-GluA3 IgG induces activation of AMPAR channel (Malina et al. 2006, Levite et al. 1999a) and this event is followed by receptor endocytosis (Bretin et al. 2017). However, the role of anti-GluA3 IgG in dendritic spine remodeling *in vivo* was unexplored. Morphological analysis of PFC neurons twenty-four hours after intracerebroventricular injection of anti-GluA3 IgG showed a significant increase in dendritic protrusion density compared with control IgG (Fig. 4.4A, B). In addition, the anti-GluA3 IgG modified the percentage of spine versus filopodia-like protrusions, leading to a significant increase of filopodia suggesting the ongoing formation of new spines (Fig. 4.4C, D). For a more detailed morphological analysis, the dendritic spine length, head and neck width were measured allowing for spines categorization according

to their shape (mushroom, stubby and thin). Interestingly, anti-GluA3 IgG induced a statistically significant increase in spine head width (Fig. 4.4E) and spine length (Fig. 4.4F) thus indicating an overall increase in spine size. Accordingly, anti-GluA3 IgG induced a significant modification of spine type, namely an increase of the percentage of mature mushroom spines and thin spines and a concomitant decrease of stubby spines (Fig. 4.4G).

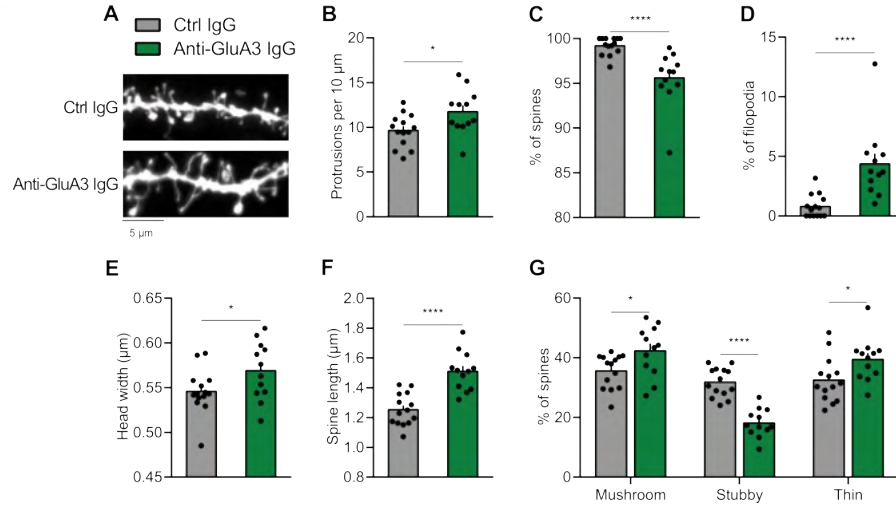


Figure 4.4: *Effect of acute administration of GluA3 IgG in mice on spine morphology.* (A) Representative images showing dendrites of adult mice PFC 24 h after injection of either anti-GluA3 IgG or control IgG (scale bar = 5 μm). (B-G) Bar graphs representing in both conditions (B) protrusion densities (two-tailed unpaired t-test, $t = 2.523$, $df = 24$, $P = 0.0187$), (C) percentages of spines (Mann-Whitney test, $P < 0.0001$), (D) percentages of filopodia (Mann-Whitney test, $P < 0.0001$), (E) spine head width (two-tailed unpaired t-test, $t = 2.067$, $df = 24$, $P = 0.0497$), (F) spine length (two-tailed unpaired t-test, $t = 5.503$, $df = 24$, $P < 0.0001$), (G) proportions of different spine types in percentage of total spines (mushroom, stubby and thin) (two-tailed unpaired t-test; mushroom: $t = 2.42$, $df = 24$, $P = 0.0235$; stubby: $t = 7.181$, $df = 24$, $P < 0.0001$; thin: $t = 2.368$, $df = 24$, $P = 0.0263$). $n = 12-14$ neurons/group. * $P < 0.05$, **** $P < 0.0001$.

4.1.2 Acute injection of anti-GluA3 hIgGs induced impairments of cortical-dependent cognitive functions

To detect whether antibodies against GluA3 produced *in vivo* behavioral and cognitive changes we first tested cortical-dependent cognitive functions. A new cohort of mice have been injected with anti-GluA3 IgG or control IgG through stereotaxic unilateral intracerebroventricular infusion (Fig. 4.1, 4.5).

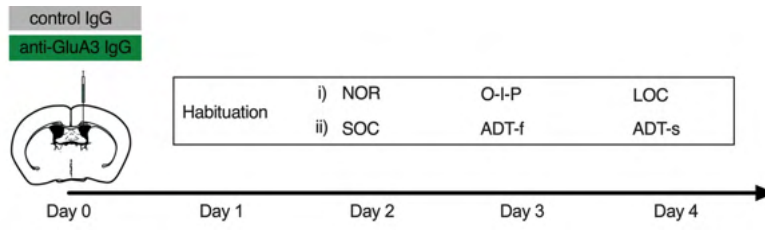


Figure 4.5: *Experimental design.* Naïve mice were injected unilaterally in the cerebral ventricle with anti-GluA3 IgG or control IgG and after 24 h tested for behavioral procedures. Mice were assigned to two different groups: i) mice were tested in two of three tasks among novel object recognition (NOR), object-in-place (O-I-P) and object location tasks (LOC). ii) Mice were tested in two of three tasks among social approach (SOC), and emotion discrimination with fear (ADT-f) or stress demonstrators (ADT-s). The day before testing mice were habituated in the open field arena (i) or the three-chamber arena (ii).

Twenty-four hours after the injection we did not detect any difference between groups in locomotor activity (Fig. 4.6A), exploration of the center of the arena, which did not suggest anxiety-like behaviors (Fig. 4.6B) and grooming behavior (Fig. 4.6C).

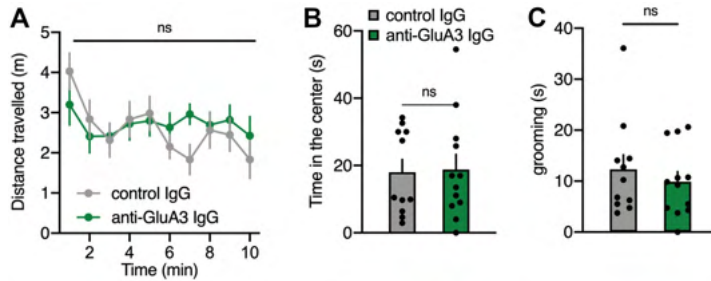


Figure 4.6: *Injection of antibodies against GluA3 does not impair locomotor activity and does not trigger anxiety-like behaviour.* (A) Locomotor activity measured as total distance travelled in the open field arena (two-way ANOVA, treatment, $F_{1,21} = 0.11$, $P = 0.74$). (B) Time spent in the center zone of the open field arena (two-tailed unpaired t-test, $t = 0.13$, $df = 21$, $p = 0.89$, $n = 11-12$ /group). (C) Time spent in grooming behavior (two-tailed unpaired t-test, $t = 0.62$, $df = 10$, $P = 0.54$, $n = 6$ /group). n.s., not significant.

On the following day we tested the mice on recognition memory abilities, which require mice to judge about whether a stimulus has been previously encountered. Importantly, recognition memory judgements require the functioning of different cortical regions, such as the perirhinal cortex and the PFC (Barker et al. 2011).

We tested anti-GluA3 IgG- or control IgG-injected mice in the novel object recognition task, where the relative familiarity of an object is used to judge prior encounter. Both groups of mice were presented with a pair of identical objects (Fig. 4.7A) and after a 5-minutes delay, one of the two was replaced with a novel object. Whereas control mice spent more time in the zone related to the novel object, compared to the familiar, mice injected with anti-GluA3 IgG did not show any difference in such behavior (Fig. 4.7B). We also calculated a discrimination ratio, where positive values indicated increased exploration of the novel over the familiar object and we found that mice injected with anti-GluA3 IgG showed a significantly worse performance compared to control group (Fig. 4.7C). This effect did not depend on total exploration of the objects, which did not change between the two groups of mice (Fig. 4.7D).

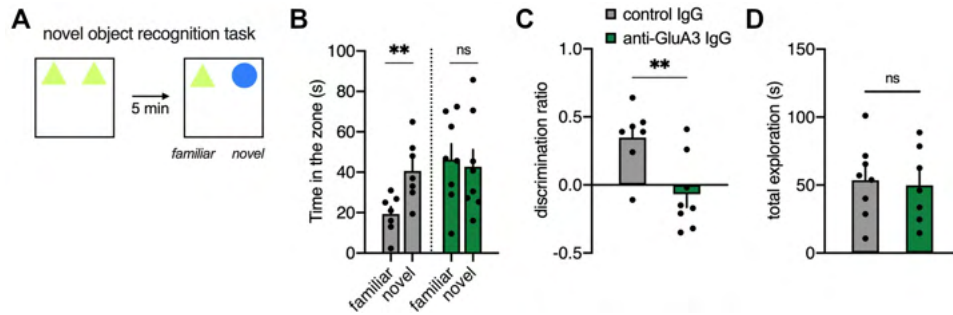


Figure 4.7: *Injection of antibodies against GluA3 induce impairments of cortical-dependent cognitive functions assessed with NORT.* (A) Schematic of the novel object recognition test (NORT). (B) Time spent in the zones related to the familiar or the novel objects (two-tailed multiple t-test, Bonferroni correction, control IgG $t = 5.58$, $df = 6$, $P = 0.002$; anti-GluA3 IgG, $t = 0.29$, $df = 7$, $P = 0.77$). (C) Performance of mice injected with anti-GluA3 IgG or control IgG in the novel object recognition task expressed as discrimination ratio (two-tailed unpaired t-test, $t = 3.15$, $df = 13$, $P = 0.0076$, $n = 7-8/\text{group}$). (D) Total exploration time (two-tailed unpaired t-test, $t = 0.04$, $df = 12$, $P = 0.38$, $n = 7/\text{group}$). ** $P < 0.01$, not significant.

We next tested a different type of recognition memory involving information about where an object has been previously encountered. To do this we used the object-in-place task (Fig. 4.8A), in which mice were presented with four objects that differed in shape and color, and following a 5-min delay, two of the objects (both in the left or right side of the arena) exchanged position. Also, in this task anti-GluA3 IgG-injected mice failed to show a significant discrimination between the displaced objects and the two that remained in the same position (Fig. 4.8B, C).

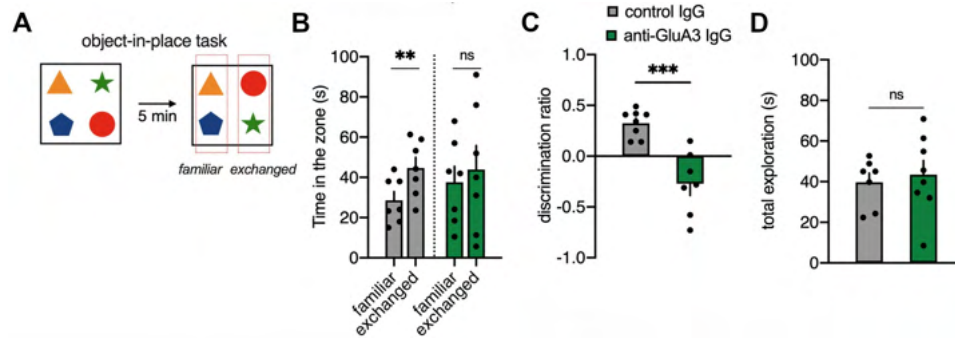


Figure 4.8: *Injection of antibodies against GluA3 induce impairments of cortical-dependent cognitive functions assessed with O-I-P task.* (A) Schematic of the object-in-place task. (B) Time spent in the zones related to the familiar or the exchanged objects (two-tailed multiple t-test, Bonferroni correction, control IgG $t = 0.56$, $df = 6$, $P = 0.001$; anti-GluA3 IgG, $t = 0.69$, $df = 6$, $P = 0.51$). (C) Performance of mice injected with anti-GluA3 IgG or control IgG in the object-in-place task expressed as discrimination ratio (two-tailed unpaired t-test, $t = 4.92$, $df = 13$, $P = 0.0003$). (D) Total exploration time (two-tailed multiple t-test, Bonferroni correction, control IgG $t = 5.58$, $df = 6$, $P = 0.002$; anti-GluA3 IgG, $t = 0.29$, $df = 7$, $P = 0.77$, $n = 7-8/\text{group}$). ** $P < 0.01$, *** $P < 0.0005$, n.s., not significant.

We then tested the object location recognition memory (Fig. 4.9A).

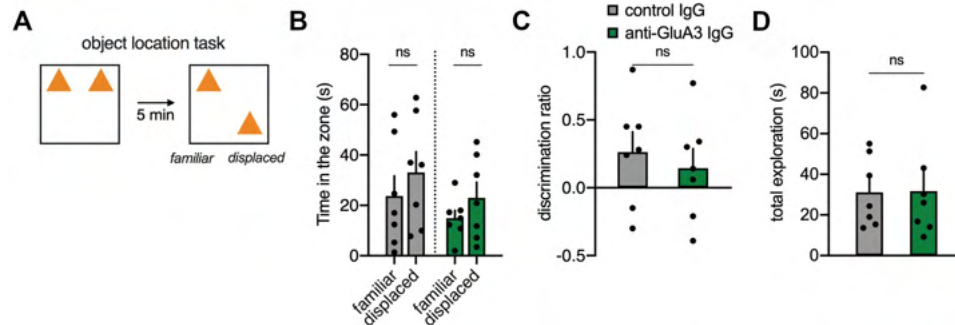


Figure 4.9: *Injection of antibodies against GluA3 does not impair hippocampal-dependent cognitive functions.* (A) Schematic of the object location task. (B) Time spent in the zones related to the familiar or the displaced objects (two-tailed multiple t-test, Bonferroni correction, control IgG $t = 1.04$, $df = 6$, $P = 0.33$; anti-GluA3 IgG, $t = 1.89$, $df = 6$, $P = 0.10$). (C) Performance of mice injected with anti-GluA3 IgG or control IgG in the object location task expressed as discrimination ratio (two-tailed unpaired t-test, $t = 0.57$, $df = 12$, $P = 0.52$). (D) Total exploration time (two-tailed unpaired t-test, $t = 0.04$, $df = 14$, $P = 0.96$, $n = 7/\text{group}$). n.s., not significant.

Indeed, object location memory is known to depend more on the functioning of the hippocampus (Barker et al. 2011). In this case we did not observe any difference between anti-GluA3 IgG- and control IgG-injected mice (Fig. 4.9B, C). These results suggest that injection of the purified anti-GluA3 IgG in mice has an impact on cognitive functions.

In the recognition memory tasks mice were tested after a short delay (5 min) thus to assess whether purified anti-GluA3 IgG could have an impact on long-term memory we used a fear conditioning paradigm that allows the formation of long-lasting memories (Scheggia et al. 2017). Mice were injected with anti-GluA3 IgG or control IgG and then tested in the fear conditioning three days later (Fig. 4.10A). Then, 72 h following the conditioning, mice were re-exposed to the same conditioned context (Fig. 4.10B) and to a novel context (Fig. 4.10C) in which were presented with the conditioned tone (Fig. 4.10D), however, we did not observe any difference as both group of mice displayed similar level of freezing in all these conditions.

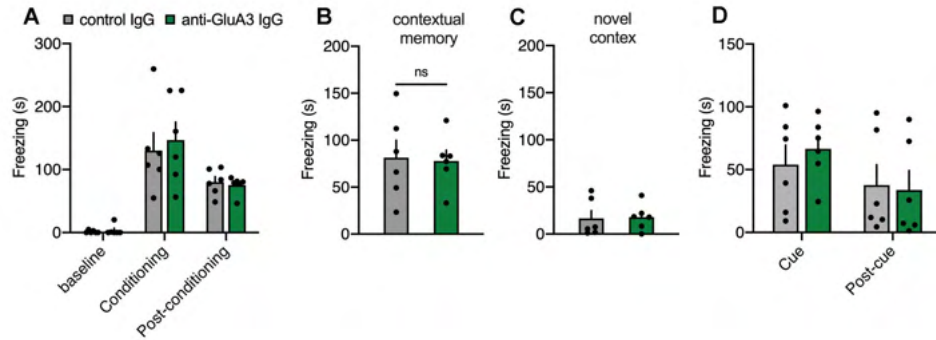


Figure 4.10: *Anti-GluA3 IgG injection did not produce long-term memory alterations.* (A) Freezing behavior measured during baseline, conditioning and post-conditioning phases of the test (two-way ANOVA, group (anti-GluA3 IgG, control IgG) $F_{1,5}=0.13$, $P=0.73$). (B) Contextual memory recall 72 h after conditioning (two-tailed unpaired t-test, $t=0.15$, $df=10$, $P=0.87$, $n=6$ /group). (C) 1 h after contextual memory recall mice were tested in a different context to assess fear generalization (two-tailed unpaired t-test, $t=0.13$, $df=10$, $P=0.89$). (D) Mice were presented in a novel context with the conditioned tone for 2 m to test cued auditory memory (two-way ANOVA, group (anti-GluA3 IgG, control IgG), $F_{1,10}=0.04$, $P=0.82$). n.s., not significant.

4.1.3 Injection of antibodies against GluA3 induced alteration of social behavior and social cognition

We tested whether injection with antibodies against GluA3 might affect social behavior. Thus, we assessed mice injected with anti-GluA3 IgG or control IgG in a well-validated social approach task (Fig. 4.11A).

Sociability, defined as the tendency of the subject mouse to spend more time with a conspecific, compared with time spent with an inanimate object, was evident in both groups (Fig. 4.11B). However, this effect was much larger in control IgG-injected mice, thus we calculated a preference score which showed that injection with anti-GluA3 IgG diminished mice social preference versus a conspecific compared to an inanimate object (Fig. 4.11C). As control we also tested mice social memory, which is their ability to discriminate a familiar conspecific compared to a novel one (Fig. 4.11D). We did not detect any differences as both groups of mice spent more time in the chamber with the novel mouse than time in the chamber with the familiar mouse (Fig. 4.11E, F). We then set out to understand the cause of the different social preference in anti-GluA3 IgG-injected mice. Social cognition and empathy are both regarded as central in social interaction, thus we checked whether these abilities were affected following injection with anti-GluA3 IgG. To test empathy-like behavior in mice we used the observational fear conditioning (Fig. 4.11G) where empathy occurs by vicariously sensing the emotions of a conspecific (emotional contagion). In this task, an unfamiliar conspecific (demonstrator) and a mouse injected with anti-GluA3 IgG or control IgG (observer) were placed in an adjacent compartment, and then the demonstrator received foot shocks (Fig. 4.11G). We measured freezing behavior, which reflected the observational fear induced by social transmission and we found that mice injected with anti-GluA3 IgG displayed reduced freezing reaction, compared to control IgG-injected mice (Fig. 4.11H). Both groups of mice spent similar amount of time in exploration of their conspecific demonstrator in the proximity of the divider (Fig. 4.11I). These results suggested that social transmission and emotional contagion was impaired in anti-GluA3 IgG-injected mice.

Further, because this process might depend on social perception and social cognitive processes, we assessed mice abilities to discriminate emotional states in their conspecifics (Scheggia et al. 2019). To test this, mice injected with anti-GluA3 IgG or control IgG were tested in the emotion discrimination task where mice were presented with two demonstrator mice, one that underwent fear conditioning (“demonstrator fear”), and the other one that did not receive any manipulation (“demonstrator neutral”, Fig. 4.12A). During the test, mice were presented with the tone cue used for conditioning of the demonstrator fear mouse, and we measured mice preference to spend time with the demonstrators. Whereas control mice spent more time with the emotionally altered demonstrator, mice injected with anti-GluA3 IgG

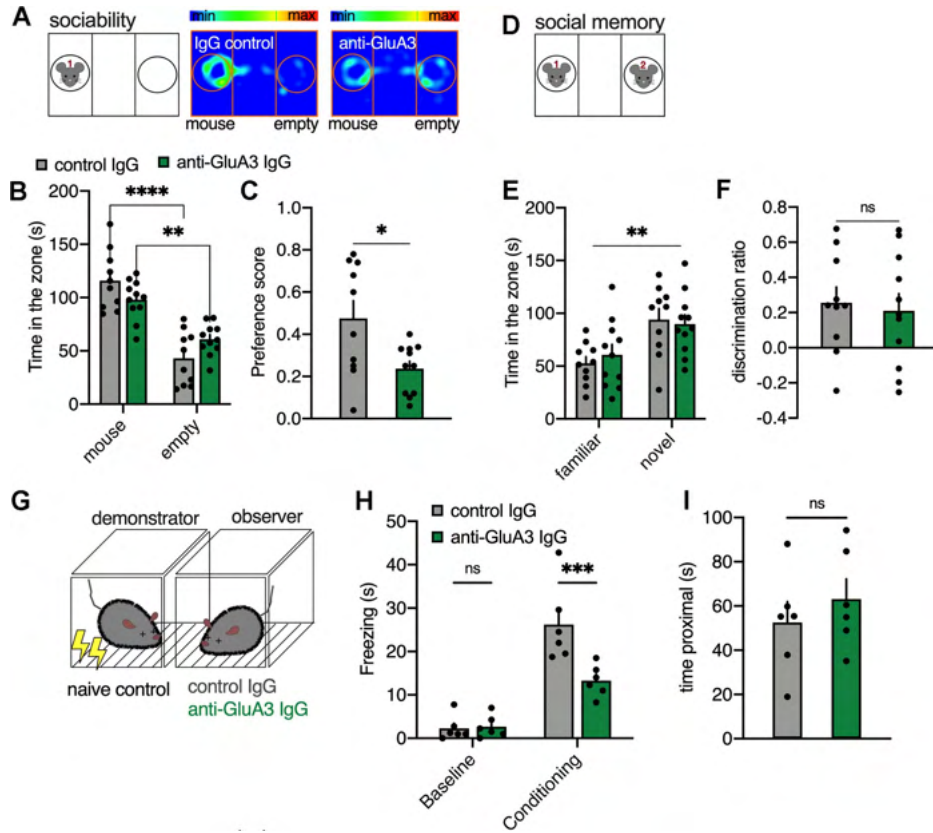


Figure 4.11: *Alteration of social behavior and social cognition after anti-GluA3 IgG injection.* (A) Schematic of the social approach task to test sociability in mice injected with anti-GluA3 IgG or control IgG and graphical representation of the amount of time mice spent in different parts of the apparatus (with blue as the shortest and red as the longest time). (B) Time spent by the mice in the chamber with a mouse (mouse 1) or in the empty chamber during the sociability test (two-way ANOVA, group (anti-GluA3 IgG, control IgG) $\times$ stimulus (empty, mouse), $F_{1,19} = 7.0$, $P = 0.015$, $n = 10-11/\text{group}$). (C) Preference to spend time exploring a conspecific compared to an inanimate object measured as preference score (two-tailed unpaired t-test, $t = 2.68$, $df = 19$, $P = 0.01$). (D) After sociability, mice were tested for social memory where a novel mouse (mouse 2) was presented in the previously empty chamber. (E) Time spent by the mice in the chamber with the familiar mouse or in the chamber with the novel mouse during the social memory test (two-way ANOVA, stimulus (familiar, novel mouse), $F_{1,19} = 12.84$, $P = 0.002$, $n = 10-11/\text{group}$). (F) Preference to explore a novel conspecific compared to a familiar one measured as discrimination ratio (two-tailed unpaired t-test, $t = 0.34$, $df = 19$, $P = 0.73$). (G) Schematic representation of the observational fear learning. (H) Freezing behavior measured during baseline and conditioning phases of the task in mice injected with anti-GluA3 IgG or control IgG (two-way ANOVA, group (anti-GluA3 IgG, control IgG) $\times$ phase (baseline, conditioning), $F_{1,10} = 18.48$, $P = 0.0016$, $n = 6/\text{group}$). (I) Time spent by the observer in the zone proximal to the demonstrator mouse (two-tailed unpaired t-test, $t = 0.80$, $df = 10$, $P = 0.43$). $P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.0005$. n.s., not significant.

spent similar amount of time with both demonstrators (Fig. 4.12A, data not showed). The total number of visits did not differ between the two demonstrators (data not showed). Furthermore, while the latency to make the first visit towards the demonstrator fear mouse was shorter than towards the neutral demonstrator in the control group (Fig. 4.12A), anti-GluA3 IgG-injected mice took longer time to explore demonstrator fear mouse.

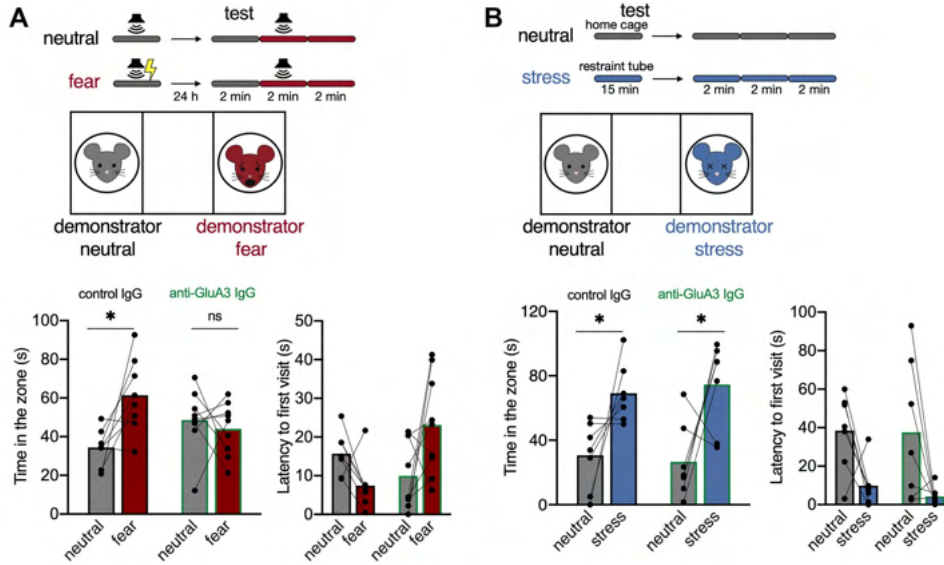


Figure 4.12: *Anti-GluA3 IgG injections affect mice capabilities to discriminate fear but not stress conspecifics.* (A,B) Top, Schematic representation of the emotion discrimination task with ‘neutral’ and ‘fear’ (A) or ‘stress’ (B) demonstrators. (A) Bottom, time spent by the mice during the presentation (2-min) of the conditioned tone (two-tailed multiple t-test, Bonferroni correction, control IgG $t = 3.14$, $df = 7$, $P = 0.03$; anti-GluA3 IgG, $t = 0.55$, $df = 8$, $P = 0.59$; $n = 8-9$ /group) and latency to make the first visit (two-tailed multiple t-test, Bonferroni correction, control IgG $t = 2.61$, $df = 6$, $P = 0.03$; anti-GluA3 IgG, $t = 2.63$, $df = 6$, $P = 0.03$) in the chamber with a neutral demonstrator and in the chamber with fear demonstrator. (B) time spent by the mice during the first 2 min of the task (two-tailed multiple t-test, Bonferroni correction, control IgG $t = 3.14$, $df = 7$, $P = 0.03$; anti-GluA3 IgG, $t = 0.55$, $df = 8$, $P = 0.59$; $n = 6-7$ /group) and latency to make the first visit (two-tailed multiple t-test, Bonferroni correction, control IgG $t = 2.64$, $df = 6$, $P = 0.03$; anti-GluA3 IgG, $t = 2.24$, $df = 6$, $P = 0.06$) in the chamber with a neutral demonstrator and in the chamber with stress demonstrator. * $P < 0.05$, n.s., not significant.

These results suggest that they were not able to make the distinction between the two based on their emotional state. We also tested these mice in a different condition involving a demonstrator mouse subjected to stress

(“demonstrator stress”, Fig. 4.12B). However, in this condition, we did not observe any difference between mice injected with anti-GluA3 IgG and control IgG. Also, both group of mice first visited the stressed demonstrator (Fig. 4.12B).

Thus, our results suggested that injection with anti-GluA3 IgG led to significant changes in social transmission and discrimination of emotional states both in relation with affective states of fear.

4.1.4 Effects mediated by acute injection of anti-GluA3 hIgGs were transient

To assess whether the observed molecular and behavioral alterations induced by acute intracerebroventricular injection of anti-GluA3 IgG were transient and correlated with anti-GluA3 IgG presence, we repeated the same experiments presented in the previous paragraphs 14 days after anti-GluA3 IgG administration (see Fig. 4.1). Indeed, 14 days represent a spell of time long enough to ensure the complete wash out of injected IgG from mice brain (Planagumà et al. 2014, Planagumà et al. 2016).

As shown in Fig. 4.13A-G, the profound and significant alterations of spine morphology that were present 24 h after anti-GluA3 IgG intracerebroventricular injection, were no longer detectable at day 14. Similarly, WB analysis indicated that anti-GluA3 IgG-injected mice did not differ significantly from control mice in GluA3-containing AMPARs level at synapses of the prefrontal cortex (Fig. 4.13H, I). These data are in line with our hypothesis of a time-dependent loss of anti-GluA3 IgG effects that accompanies the progressive dilution of this autoantibody and suggests a kind of reversibility of anti-GluA3 IgG-induced alterations.

Similarly, to determine whether a single injection of anti-GluA3 IgG led to transient or permanent effects, mice received anti-GluA3 IgG or control IgG and then were tested 10 days following the injection (see Fig. 4.1 and Fig. 4.14A). We first tested mice in the object-in-place, which did not show any difference between the groups as anti-GluA3 IgG-injected mice performed similarly to control IgG mice (Fig. 4.14B, C). Moreover, we tested mice social behavior, using the three-chamber social approach task that also did not reveal differences between mice injected with anti-GluA3 IgG and control IgG, as both groups of mice similarly spent more time with their conspecific compared to an inanimate object and showed normal social preference (Fig. 4.14D, E).

Thus, these results suggest that behavioral and cognitive effects induced by anti-GluA3 IgG injection were transient and reverted within two weeks after the injection.

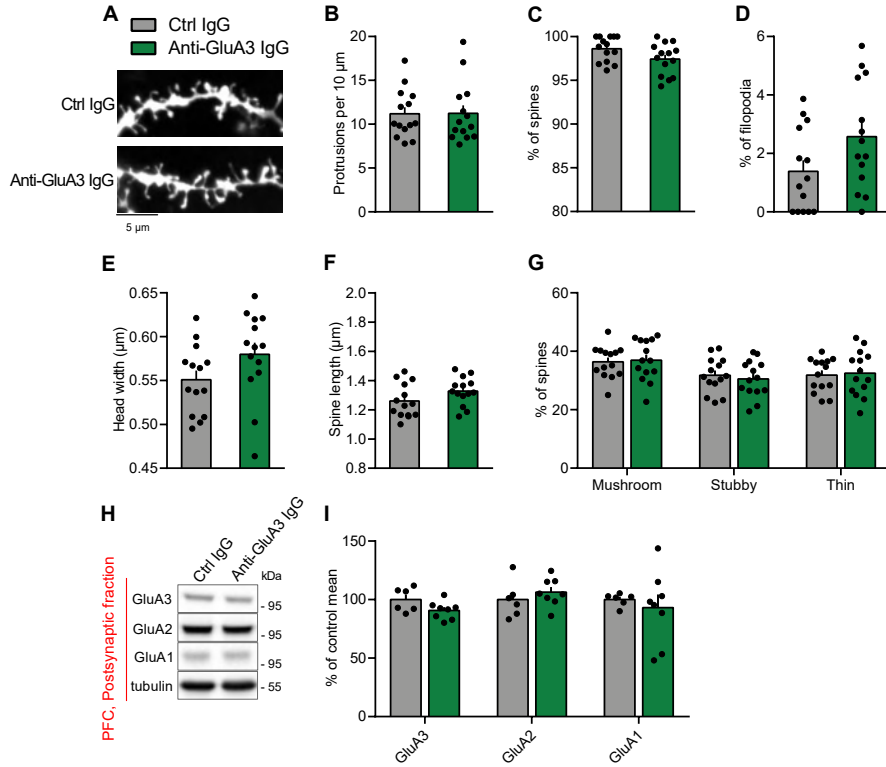


Figure 4.13: *Molecular and morphological effects in mice of anti-GluA3 IgG are transient.* (A) Representative images showing dendrites of adult mice PFC 2 weeks after injection of either anti-GluA3 IgG or control IgG (scale bar = 5 μ m). (B-G) Bar graphs representing, in both conditions, (B) the protrusion densities (Mann-Whitney test, $P = 0.7345$), (C) percentages of spines (Mann-Whitney test, $P = 0.0695$), (D) percentages of filopodia (Mann-Whitney test, $P = 0.0695$), (E) spine head width (two-tailed unpaired t-test, $t = 1.725$, $df = 26$, $P = 0.0965$), (F) spine length (two-tailed unpaired t-test, $t = 1.685$, $df = 26$, $P = 0.1040$), (G) proportions of different spine types in percentage of total spines (mushroom, stubby and thin) (two-tailed unpaired t-test; mushroom: $t = 0.2511$, $df = 26$, $P = 0.8037$; stubby: $t = 0.5266$, $df = 26$, $P = 0.6029$; thin: $t = 0.245$, $df = 26$, $P = 0.8083$). (H) Western blot representative image of GluA3, GluA2 and GluA1 subunits in Triton-insoluble postsynaptic fractions (TIF) obtained from mice PFC 2 weeks after injection of either anti-GluA3 IgG or control IgG and (I) corresponding bar graph of densitometric quantification (two-tailed unpaired t-test; GluA3: $t = 1.985$, $df = 12$, $P = 0.0705$; GluA2: $t = 0.8632$, $df = 12$, $P = 0.4049$; GluA1: $t = 0.5265$, $df = 12$, $P = 0.6081$; $n = 6-8$ /group). In B-G: $n = 14$ neurons from 3 animals per condition, in H,I: $n = 6-8$ animals.

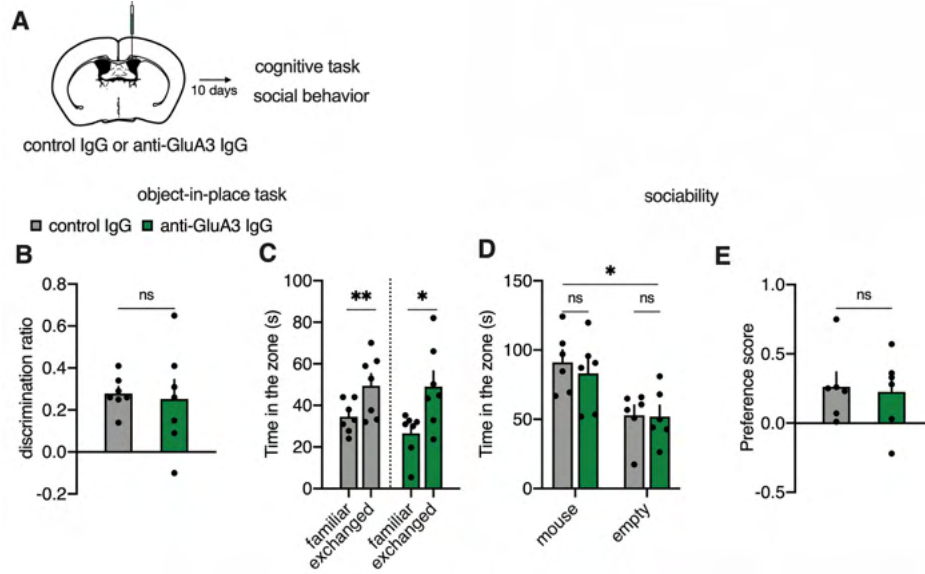


Figure 4.14: *Effects of anti-GluA3 IgG injection are transient.* (A) Experimental design: naïve mice were injected unilaterally in the cerebral ventricle with anti-GluA3 IgG or control IgG and after 10 days cognitive functions and social behavior were assessed. (B) Performance of mice in the object-in-place task expressed as discrimination ratio (two-tailed unpaired t-test, $t = 0.26$, $df = 12$, $P = 0.079$, $n = 7$ /group) (C) Time spent in the zones related to the familiar or the exchanged objects (two-tailed multiple t-test, Bonferroni correction, control IgG $t = 4.68$, $df = 6$, $P = 0.006$; anti-GluA3 IgG, $t = 2.52$, $df = 6$, $P = 0.04$). (D) Time spent by the mice in the chamber with the mouse or in the empty chamber with the novel mouse during the sociability test (two-way ANOVA, stimulus (familiar, novel mouse), $F_{1,10} = 10.64$, $P = 0.008$, $n = 6$ /group). (E) Preference to explore a conspecific compared to an inanimate object measured as preference score (two-tailed unpaired t-test, $t = 0.23$, $df = 10$, $P = 0.81$). * $P < 0.05$. n.s., not significant.

4.2 Chronic animal model

Note. This section reflects the “Results” section of my first name article titled “*Anti-GluA3 autoantibodies define a new sub-population of Frontotemporal dementia patients with distinct neuropathological features*” that is currently under revision to *Brain Behavior and Immunity*.

4.2.1 Chronic anti-GluA3 hIgGs administration induced a reduction in GluA3-containing AMPAR surface retention and the appearance of early signs of synapse loss and tau phosphorylation

To evaluate the effects of chronic anti-GluA3 hIgG administration, we injected male mice in the lateral ventricles once a week for 1 month (on days 1, 8, 15, 22) with anti-GluA3 hIgGs that were isolated from FTD patients (Fig. 4.15). As a control, mice were injected with a pool of human IgG (CTRL hIgGs).

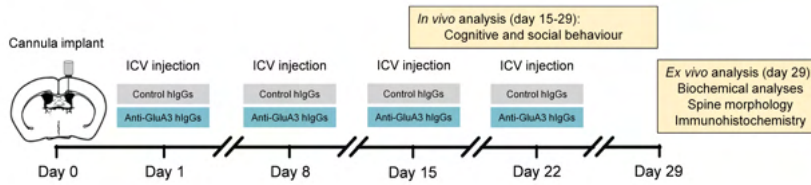


Figure 4.15: *Schematic representation of the experimental timeline.* At day 0, mice are implanted with an intracerebroventricular cannula and then injected once a week (at days 1,8,15,22) with purified anti-GluA3 hIgGs or CTRL hIgGs. From day 15 to day 29, mice are tested in different behavioural tasks. At day 29, mice are sacrificed for *ex vivo* analyses on PFC.

Numerous studies (Haselmann et al. 2018, Hughes et al. 2010, Lai et al. 2009, Maudes et al. 2022, Mikasova et al. 2012, Peng et al. 2015) reported that pathogenic IgG antibodies targeting extracellular epitopes of membrane receptors often promote the endocytosis of the receptor itself. By employing a crosslinking assay of surface proteins, we found that chronic anti-GluA3 hIgG injection mediated an increase in the intracellular levels of GluA3 and GluA2 subunits (Fig. 4.16A, B) and consequently decreased their surface retention. Interestingly, we did not detect any differences in the levels of the subunits in a purified triton-insoluble post-synaptic fraction (TIF) (Fig. 4.16A, C). This suggests that anti-GluA3 hIgGs induce an endocytic process of GluA3-containing AMPARs within the dendritic spine compartment. The surface insertion of other subunits of ionotropic glutamate receptors (i.e., GluA1, GluN2A, and GluN2B) was unaffected by chronic anti-GluA3 hIgG injection (Fig. 4.17A, B).

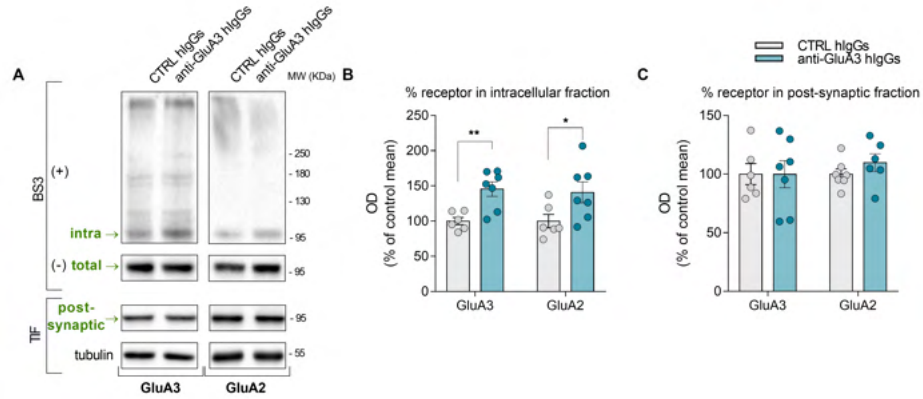


Figure 4.16: *Effects of chronic injection of anti-GluA3 hIgGs on GluA3 and GluA2 surface expression.* (A) Western blot representative images and bar graph of densitometric quantification of GluA3 and GluA2 in (B) intracellular fraction (from BS3 experiment, the blotted values represent the intracellular fraction band normalized on the total amount band) (two-tailed unpaired t-test; GluA3: $t(11)=3.722$, $p=0.0034$; GluA2: $t(11)=2.215$, $p=0.0488$) or in (D) Triton-insoluble postsynaptic fractions (TIF) obtained from mice PFC at the end of chronic treatment (two-tailed unpaired t-test; GluA3: $t(11)=0.008576$, $p=0.9933$; GluA2: $t(11)=1.131$, $p=0.2823$). $n=7-6/\text{group}$. * $P < 0.05$; ** $P < 0.01$.

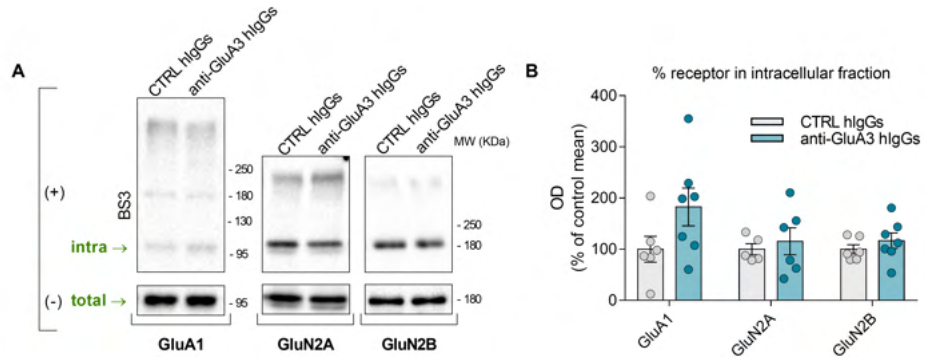


Figure 4.17: *Anti-GluA3 hIgG administration did not affect surface GluA1, GluN2A, or GluN2B levels.* (A) Western blot representative images and (B) bar graph of densitometric quantification of GluA1, GluN2A and GluN2B in intracellular fraction (from BS3 experiment, the blotted values represent the intracellular fraction band normalized on the total amount band) (two-tailed unpaired t-test; GluA1: $t(11)=1.792$, $p=0.1007$, $n=6-7/\text{group}$; GluN2A: $t(9)=0.5023$, $p=0.6275$, $n=5-6/\text{group}$; GluN2B: $t(11)=0.9124$, $p=0.3811$, $n=6-7/\text{group}$).

Overall, these data indicate that anti-GluA3 hIgGs selectively target GluA2/GluA3 AMPAR heteromers, affecting their correct retention into the synaptic membrane.

Tau protein and TDP43 are the most frequently identified proteinopathies in FTD (Mackenzie et al. 2009). Recent studies have indicated that tau hyperphosphorylation (p-tau) promotes tau migration into the post-synaptic compartment. In this compartment, p-tau negatively affects the synapses' functionality, interfering with ionotropic glutamate receptors (Italia et al. 2022). We measured the levels of tau and its hyperphosphorylated form in the post-synaptic compartment and in the total homogenate isolated from mice PFCs. We found selective accumulation of the p-tau (Ser202, Thr205) at post-synaptic sites (Fig. 4.18A, B) but not in the total homogenate of anti-GluA3 hIgG-treated mice (data not shown). Furthermore, we did not detect any differences in total tau or pTDP43 levels in the post-synaptic fraction or in the total homogenate following anti-GluA3 hIgG administration (Fig. 4.18A, B; data not shown). This last result suggests the specific involvement of tau-related pathological intracellular cascades in anti-GluA3 hIgG-treated mice.

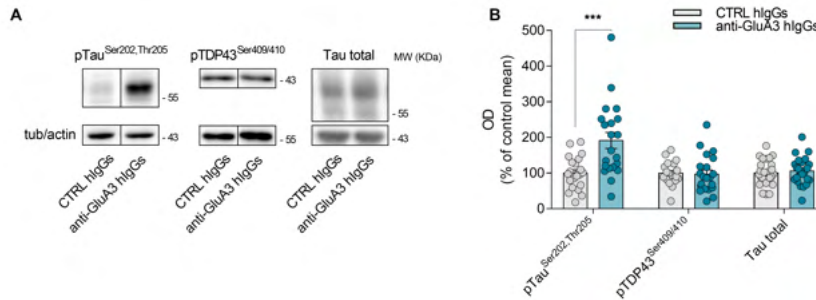


Figure 4.18: *Effects of chronic injection of anti-GluA3 hIgGs on FTD-related neuropathological markers.* (A) Western blot representative images and (B) bar graph of densitometric quantification of p-Tau (Ser202, Thr205, AT8), pTDP43 (Ser409,410) and tau total in Triton-insoluble postsynaptic fraction (TIF) obtained from mice PFC at the end of chronic treatment (two-tailed Mann-Whitney test or two-tailed unpaired t-test; p-Tau: $U=91$, $p=0.0004$, $n=22-21$ /group; pTDP43: $t(40)=0.218$, $p=0.8285$, $n=21-21$ /group; Tau total: $t(48)=0.5962$, $p=0.5538$, $n=26-24$ /group). *** $P < 0.001$.

Having detected selective accumulation of p-tau in the post-synaptic compartment and altered GluA3-containing AMPAR retention in the membrane of anti-GluA3 hIgG-treated mice, we hypothesised that these events could negatively influence dendritic spines' morphology and structure. Ex-vivo spine morphology analysis revealed that mice chronically infused with anti-GluA3 hIgGs were characterised by a significant decrease in dendritic spine density in the PFC (Fig. 4.19A, B). For a more detailed morphological anal-

ysis, we measured dendritic spine's length and head and neck width, which allowed spine categorisation according to shape (mushroom, stubby, or thin). However, no differences in spine length or head width were found between anti-GluA3 hIgG-treated mice and CTRL hIgG-treated mice (Fig. 4.19C, D); accordingly, no differences in the relative percentage of spine subtypes were detected (data not shown).

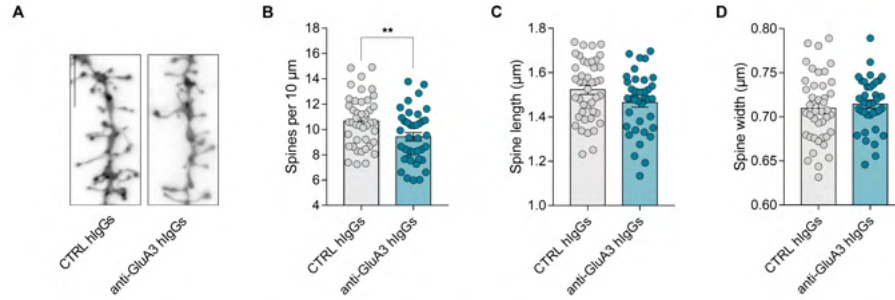


Figure 4.19: *Effects of chronic injection of anti-GluA3 hIgGs on spine morphology.* (A) Representative images showing dendrites of adult mice PFC at the end of the chronic treatment with either anti-GluA3 hIgGs and CTRL hIgGs (scale bar = 5 μm); bar graphs representing (B) protrusion densities (two-tailed unpaired t-test; $t(80)=2.794$, $p=0.0065$, $n=43-39/\text{group}$), (C) spine length (two-tailed unpaired t-test; $t(80)=1.931$, $p=0.0570$, $n=43-39/\text{group}$), and (D) spine head width (two-tailed unpaired t-test; $t(78)=0.6222$, $p=0.5356$, $n=43-37/\text{group}$). n = number of neurons. ** $P < 0.01$.

Altogether, these results indicate that the chronic presence of anti-GluA3 hIgGs leads to synapse loss that may represent an early sign of a neurodegenerative process.

We further investigated whether the presence of anti-GluA3 hIgGs could trigger a neuroinflammatory process. We evaluated microglial status and activation. First, we analysed the expression of Iba1, a Ca^{2+} -binding protein constitutively expressed by both surveillant and activated microglia, and CD11b and CD68, two markers of microglial activation in the mouse PFC. Through immunohistochemical analysis, we quantified the number of Iba1+ cells and their average size (Fig. 4.20B, C), and we evaluated the expression of CD68 (Fig. 4.20D). Furthermore, we investigated the complexity of Iba1+ arborisation through Sholl analysis (Fig. 4.20F, G). Finally, we analysed CD11b/c levels in total homogenate obtained from mice PFCs (Fig. 4.20H, I). None of these parameters were affected by anti-GluA3 hIgG administration with the exception of the mean area of Iba1+ cells, which appeared to be larger in anti-GluA3 hIgG-treated mice (Fig. 4.20C). Accordingly, anti-GluA3 hIgG administration did not mediate profound alterations in microglial status.

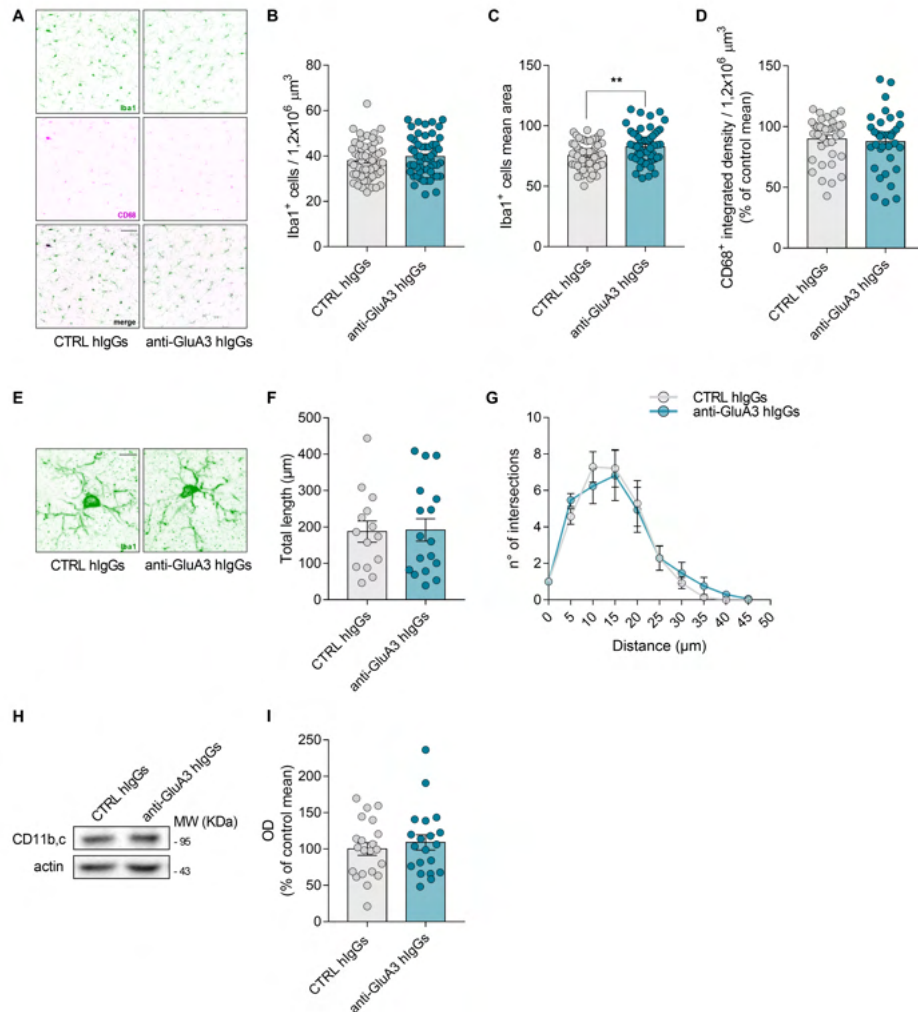


Figure 4.20: *Effects of chronic anti-GluA3 hIgG administration on microglia.* (A) Representative images of Iba1 and CD68 staining in PFC slices at 20x magnification (scale bar = 50 μm). Iba1 staining quantification as (B) total number of Iba1⁺ cells (two-tailed unpaired t-test; $t(115)=1.217$, $p=0.2261$, $n=60-57/\text{group}$) and (C) Iba1⁺ cells mean area (two-tailed unpaired t-test; $t(115)=3.126$, $p=0.0022$, $n=60-57/\text{group}$) in $1.2 \times 10^6 \mu\text{m}^3$ of PFC slices. CD68 staining quantification as (D) integrated density of CD68 signal in $1.2 \times 10^6 \mu\text{m}^3$ of PFC slices (two-tailed unpaired t-test; $t(70)=0.4233$, $p=0.6733$, $n=37-35/\text{group}$). (E) Representative images of Iba1 staining in PFC slices at 63x magnification (scale bar = 10 μm); results of morphological Sholl analysis on Iba1 stained microglial arbor in PFC slices at 63x magnification expressed as (F) total length of ramifications (two-tailed unpaired t-test; $t(29)=0.1107$, $p=0.9126$, $n=14-17/\text{group}$) and (G) complexity of arborization (multiple t-tests; $df=29$, $p=\text{ns}$, $n=14-17/\text{group}$). (H) Western blot representative images and (I) bar graph of densitometric quantification of CD11b,c (two-tailed unpaired t-test; $t(39)=0.6781$, $p=0.5017$, $n=21-20/\text{group}$) in total homogenate obtained from mice PFC. ** $P < 0.01$.

4.2.2 Chronic anti-GluA3 hIgGs administration reduced interest for novelty

To evaluate the in-vivo effects of chronic anti-GluA3 hIgG administration, we first measured the locomotor activity of the mice in an open field (OF) arena, and we observed that the anti-GluA3 hIgG-treated mice exhibited a reduction in the total distance travelled compared with CTRL mice (Fig. 4.21A). Because this effect could be related to reduced propensity to explore the environment, we stimulated exploration by introducing two identical objects in the OF. Despite this, anti-GluA3 hIgG-treated mice still showed reduced locomotor activity in the OF (Fig. 4.21B).

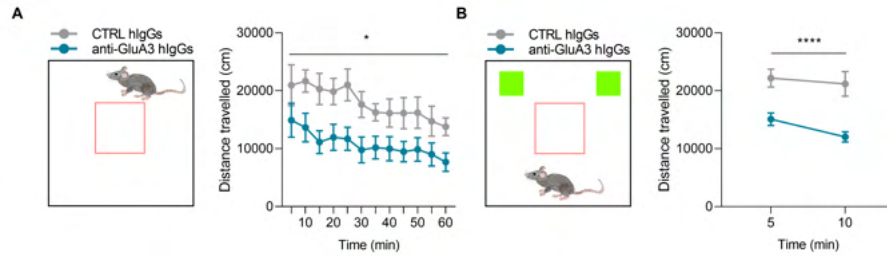


Figure 4.21: *Reduced interest for exploration after chronic anti-GluA3 hIgG injection.* Locomotor activity measured as total distance travelled in the OF arena in (A) absence (2way ANOVA; treatment: $F(1,11)=6.268$, $p=0.0293$, $n=6-7$ /group) or (B) presence of objects (2way ANOVA; treatment: $F(1,28)=23.62$, $p<0.0001$, $n=12-12$ /group). * $P < 0.05$; **** $P < 0.0001$.

We did not observe any motor impairments in the Rotarod task (Fig. 4.22A) or anxiety-like behaviour in the Elevated Plus Maze (Fig. 4.22B, C) and in the OF arena (Fig. 4.22D).

Therefore, we hypothesised that anti-GluA3 hIgG administration could result in reduced interest in exploration. To further investigate this finding, we evaluated mice via the Novel Object Recognition Task (Fig. 4.23A), which measures the capability of mice to discriminate between a familiar and a newly presented object. The introduction of a new object represents an even stronger stimulus to motivate exploration in mice. In addition, the capability to recognise the new object requires the functioning of higher cortical areas, including the PFC (Barker et al. 2011), and this capability is known to be severely impaired in FTD patients (Hornberger et al. 2010, Hornberger et al. 2012). Anti-GluA3 hIgG-treated animals showed a reduced discrimination index 5 minutes after the acquisition phase (Fig. 4.23B), suggesting that mice failed or were not interested to recognise the novel object. However, anti-GluA3 administration did not impair recognition memory after 2 hours (Fig. 4.23C).

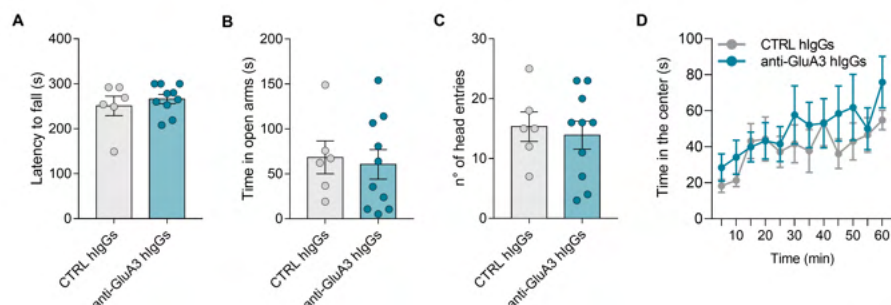


Figure 4.22: *Chronic administration of anti-GluA3 hIgGs did not affect locomotor activity or motor coordination and did not trigger anxiety-like behaviour* (A) Performance of mice in the rotarod task expressed as the latency to fall from the rotarod bar (two-tailed Mann-Whitney test; $U=24$, $p=0.5415$, $n=6-10$ /group). Performance of mice in the Elevated Plus Maze expressed as (B) time spent in open arms (two-tailed unpaired t-test; $t(14)=0.3052$, $p=0.7647$, $n=6-10$ /group) and (C) number of head entries (two-tailed unpaired t-test; $t(14)=0.4012$, $p=0.6944$, $n=6-10$ /group). (D) Performance of mice in OF expressed as time spent in the centre (2way ANOVA; treatment: $F(1,12)=0.6339$, $p=0.4414$, $n=6-8$ /group).

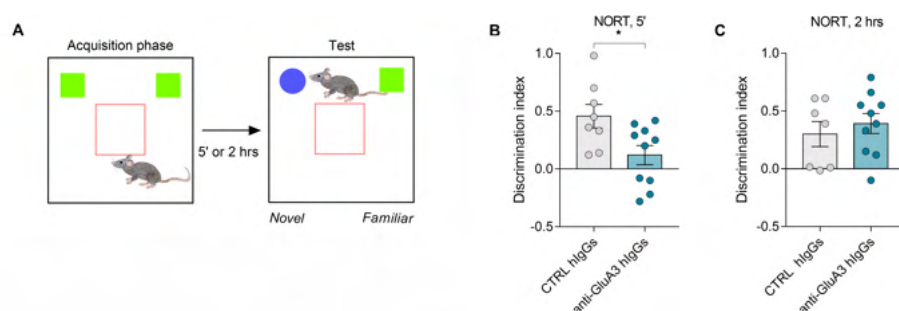


Figure 4.23: *Reduced interest for newly presented objects after chronic anti-GluA3 hIgG injection.* (A) Novel Object recognition task (NORT) schematic representation; performance of mice in NORT expressed as discrimination index after (B) 5 minutes (two-tailed unpaired t-test; $t(16)=2.582$, $p=0.0201$, $n=8-10$ /group) or (C) 2 hours (two-tailed unpaired t-test; $t(15)=0.6697$, $p=0.5132$, $n=7-10$ /group) from the acquisition phase. * $P < 0.05$

To investigate whether the reduced interest was limited to exploration or could be generalised to other natural stimuli, we assessed the mice's propensity for social stimuli and food via the Affective Discrimination Task and the Free Reward Consumption Task, respectively. The Affective Discrimination Task measures the ability of mice to discriminate conspecifics based on their affective state. In this task, the mice were presented with two demonstrator mice: one that underwent fear conditioning ('demonstrator fear') or was alternatively subjected to stress ('demonstrator stress') and one that did not receive any manipulation ('demonstrator neutral') (Fig. 4.24A, B). While CTRL mice preferred to spend more time with the emotionally altered demonstrators, mice injected with anti-GluA3 hIgGs spent a similar amount of time with both demonstrators (Fig. 4.24C, D).

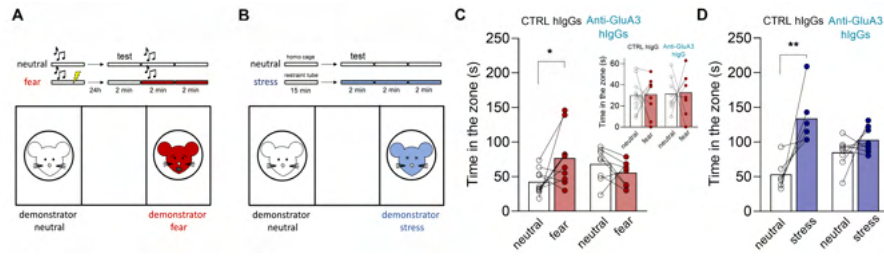


Figure 4.24: *Reduced interest for an emotionally altered conspecifics after chronic anti-GluA3 hIgG injection.* Schematic representation of Affective Discrimination Task (ADT) with 'neutral' and (A) 'fear' or (B) 'stress' demonstrators; performance of mice in the ADT expressed as the time spent in the chamber with a neutral demonstrator and in the chamber with (C) fear (2way ANOVA; Interaction: $F(1,16)=6.471$, $p=0.0217$, $n=10-8$ /group; Bonferroni correction) or (D) stress (2way ANOVA; Interaction: $F(1,12)=6.076$, $p=0.0298$, $n=6-8$ /group; Bonferroni correction) demonstrator during 4 minutes after the presentation of the conditioned tone (fear) or during the entire 6 minutes of test (stress). (C) Inset, performance of mice in the ADT expressed as the time spent in the chamber with a neutral demonstrator and in the chamber with fear (2way ANOVA; Interaction: $F(1,16)=4.884e-005$, $p=0.9945$, $n=10-8$ /group; Bonferroni correction) during the initial 2 minutes of the test that is before the presentation of the conditioned tone. * $P < 0.05$; ** $P < 0.01$.

In the Free Reward Consumption Task, the mice were habituated to a novel cage where they received 50 palatable reward pellets for 2 hours. At the end of the 2 hours, we measured the number of pellets eaten by the mice. We evaluated mice both under the conditions of satiety and after a period of food restriction, and we found that anti-GluA3 hIgG-treated mice exhibited a reduced interest in food reward consumption compared with CTRL mice under both conditions (Fig. 4.25A).

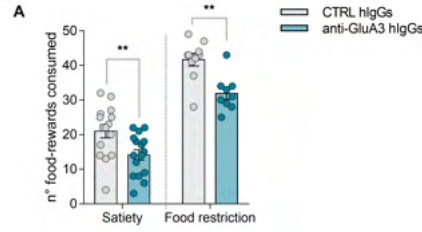


Figure 4.25: *Reduced interest for food consumption after chronic anti-GluA3 hIgG injection.* (A) Number of palatable pellets of food consumed in the free reward consumption task by mice in both a condition of satiety (two-tailed unpaired t-test; $t(29)=2.838$, $p=0.0082$, $n=15-16/\text{group}$) and after a period of food restriction (Mann-Whitney test; $U=12$, $p=0.0053$, $n=10-9/\text{group}$). $**P < 0.01$.

Overall, these data indicate that chronic anti-GluA3 hIgG administration affects locomotor activity in mice, their capability to discriminate a novel object and an emotionally altered conspecific, and their interest in food.

Taken together, these results suggest a reduction of interest and preference for novelty following chronic anti-GluA3 hIgG administration.

4.2.3 Chronic anti-GluA3 hIgGs administration can trigger hyper-oralilty or binge-like eating in mice

To further explore the propensity of the mice for food reward, we evaluated their food-seeking behaviour using an operant conditioning task. Mice were presented with two nose-poke holes: an active hole, which delivered one food reward pellet upon nose-poking (fixed ratio = 1), and an inactive hole, which did not produce any effect (Fig. 4.26A).

Under the condition of food restriction, both groups showed a positive preference index (Fig. 4.26B), suggesting that they preferred the food-related response; however, anti-GluA3 hIgG-treated mice exhibited a higher number of food-related responses (Fig. 4.26D). Moreover, anti-GluA3 hIgG-treated mice exhibited food-associated responses at shorter time intervals, as indicated by reduced latencies between responses (Avg. food latency, Fig. 4.26C). Analysing the number of food responses as a function of time, we found that the difference in this parameter at the end of the session was entirely attributed to the behaviour of anti-GluA3 hIgG-treated mice during the first 10 minutes (Fig. 4.26E). Similarly, plotting the time as a function of food-related responses, it was clear that anti-GluA3 hIgG-treated mice required much less time to achieve the same result in terms of food rewards (Fig. 4.26F). We obtained similar results evaluating mice under conditions of satiety (data not showed).

These results suggest that anti-GluA3 hIgG-treated mice search for food in a more compulsive way than CTRL mice.

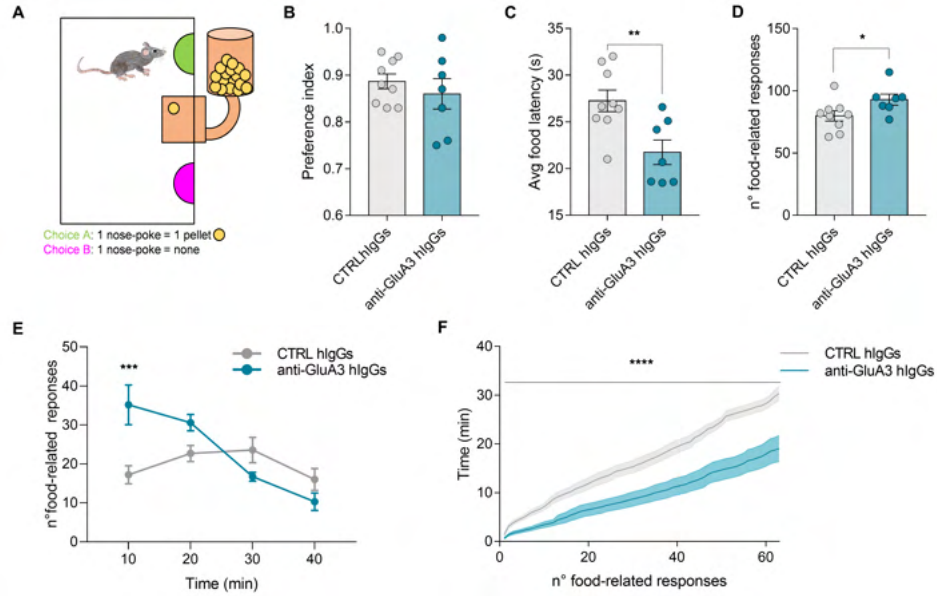


Figure 4.26: *Binge-like eating and impulsivity after chronic anti-GluA3 hIgG injection (1/2)*. (A) Schematic representation of the Food Seeking task in the operant boxes: over a period of 40', mice can choose between a nose-poke that delivers one palatable pellet of food (choice A) and a nose-poke that does not produce any effect (choice B); mice performance in the food seeking task expressed as (B) preference index (two-tailed unpaired t-test; $t(14)=0.7939$, $p=0.4405$, $n=9-7/\text{group}$), (C) average of time between one food-related response and the subsequent one (i.e., avg food latency) (two-tailed unpaired t-test; $t(14)=3.146$, $p=0.0072$, $n=9-7/\text{group}$) and (D) number of food-related responses (choice A) (two-tailed unpaired t-test; $t(14)=2.169$, $p=0.0478$, $n=9-7/\text{group}$); in (E-F) the number of food-related responses within the session in operant boxes is reported: in (E), the number of food-related responses is reported as a function of time (2way ANOVA; interaction: $F(3,42)=7.561$, $p=0.0004$, $n=9-7/\text{group}$; Bonferroni correction) whereas in (F) is highlighted the time required to perform a certain number of food-related responses (2way ANOVA; interaction: $F(62,868)=7.925$, $p<0.0001$, $n=9-7/\text{group}$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

We then increased the cost of food-related actions by reinforcing the responses from a fixed ratio of 1 to 4 or 8. Under these conditions, 4 or 8 nose pokes were required to make a food-related response, whereas only 1 nose-poke was necessary for the other response. However, in this case, we did not detect any differences between the two groups (Fig. 4.27A, B).

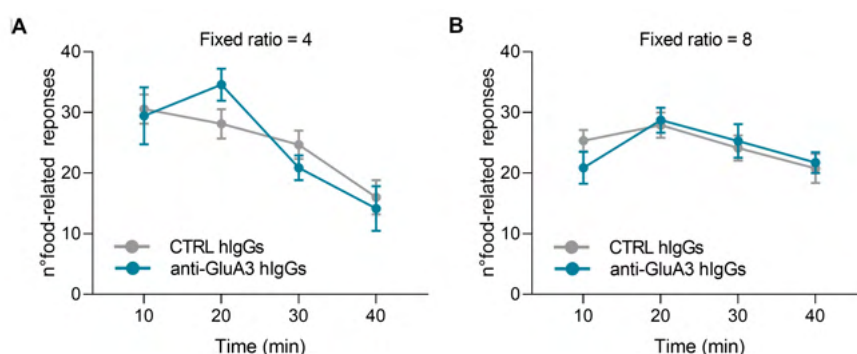


Figure 4.27: *No differences between CTRL group and anti-GluA3 hlgGs treated animals when the fixed ratio is increased.* Mice performance in the Food-Seeking task with food restriction and (A) fixed ratio = 4 (2way ANOVA; interaction: $F(3,42)=1.383$, $p=0.2613$, $n=9-7/\text{group}$) or (B) fixed ratio = 8 (2way ANOVA; interaction: $F(3,42)=1.209$, $p=0.3184$, $n=9-7/\text{group}$) expressed as number of food-related responses as a function of time.

A temporal pattern of reward consumption similar to the one obtained in the Food Seeking Task with fixed ratio of 1 was evident in the Sucrose Preference Task. In this task, the mice can choose between water and a solution of water supplemented with 1% sucrose. We observed no differences between the two groups at the end of the task (Fig. 4.28A). However, we observed temporal effects during this task: anti-GluA3 hlgG-treated mice showed an increased preference for the sucrose solution compared with the CTRL group in the first 12 hours (Fig. 4.28A). Finally, to determine whether reward-seeking behaviour could have an impact on mouse diet, we weighed mice at the end of chronic treatment (Fig. 4.28B). Anti-GluA3 hlgG-treated mice, compared with CTRL mice, had increased weight following the chronic administration. Monitoring mouse weight during treatment, we found that the difference at day 29 was most likely attributed to the eating behaviour of the mice during the final weeks that is when the chronic model of anti-GluA3 hlgG administration had already been established (Fig. 4.28C). These results suggest that chronic anti-GluA3 hlgG treatment can trigger hyperorality or binge-like eating.

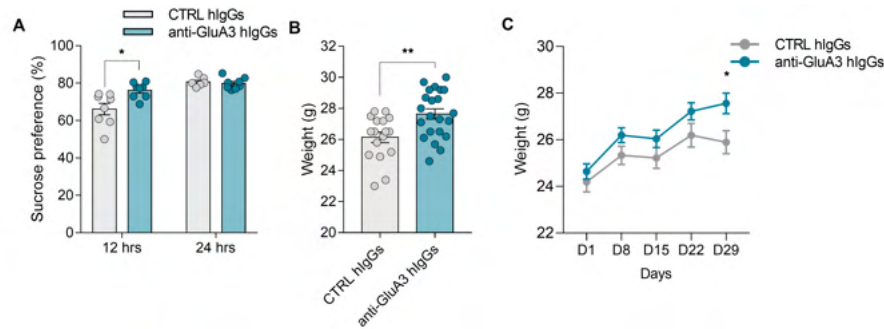


Figure 4.28: *Binge-like eating and impulsivity after chronic anti-GluA3 hIgG injection (2/2)*. (A) Mice performance in the Sucrose Preference Task expressed as Sucrose preference in percentage (two-tailed unpaired t-test; 12 hours: $t(14)=2.907$, $p=0.0115$, $n=8-8/\text{group}$; 24 hours: $t(13)=0.5992$, $p=0.5594$, $n=7-8/\text{group}$). Mice weight (B) at the end of the chronic treatment (D29) (two-tailed unpaired t-test; $t(37)=3.125$, $p=0.0034$, $n=17-22/\text{group}$) and (C) monitored once a week (2way ANOVA; treatment: $F(1,23)=4.54$, $p=0.0440$, $n=11-14/\text{group}$; Bonferroni correction). * $P < 0.05$; ** $P < 0.01$.

4.2.4 Behavioural and molecular features in frontotemporal dementia patients

Note. The results reported into this section were performed by Professor Barbara Borroni's team at the Spedali Civili of Brescia.

Previous clinical data from our group suggest that anti-GluA3-Ab+ FTD patients have marked impairments in glutamatergic neurotransmission (Borroni et al. 2017; Palese et al. 2020). In the present study, we demonstrated that chronic administration of anti-GluA3 hIgGs elicited similar detrimental effects in mice. Moreover, in mice, these defects were paralleled by other specific molecular and behavioural alterations. Therefore, we decided to go back to clinics to investigate whether the anti-GluA3 hIgG-mediated alterations identified in the pre-clinical model were also present in GluA3-Ab+ FTD patients.

With this aim, 260 FTD patients were enrolled in the present study, of which 86 (33.1%) were GluA3-Ab+ (see table 4.1). Behavioural symptoms, evaluated with the frontal behaviour inventory and corrected for age and disease duration, showed significant differences between groups regarding poor judgement and impulsivity, personal neglect, hyperorality/food fads, logopenia, asponaneity, inattention, inflexibility, and apathy (see table 4.1). We did not observe significant differences in other behavioural features or in cognitive tests between groups. One-way ANCOVA, corrected for age and disease duration, indicated significant differences in CSF p-tau181, but not total tau or amyloid- β 1-42. Notably, the p-tau results were in

parallel with what we identified in the pre-clinical model, where, in the post-synaptic compartment, chronic anti-GluA3 hIgG administration triggered the accumulation of p-tau without affecting total tau levels.

Table 4.1: Clinical and biological characteristics of anti-GluA3-Ab+ and GluA3-Ab- FTD patients.

	GluA3+ (n=86)	GluA3- (n=174)	p-value
Sex (female) n (%)	36 (41.9%)	72 (41.4%)	1.000
Age at onset (years)	60.9 (59.1-62.7)	63.1 (61.8-64.3)	0.052
Disease duration (years)	2.6 (2.2-3.0)	2.4 (2.1-2.7)	0.388
Education (years)	10.0 (9.1-10.9)	9.3 (8.6-9.9)	0.214
Frontal behavioural inventory			
Apathy	1.6 (1.3-1.8)	1.1 (0.9-1.3)	0.039
Aspontaneity	1.5 (1.2-1.8)	1.0 (0.8-1.2)	0.034
Indifference/emotional flatness	1.0 (0.7-1.3)	0.7 (0.6-0.9)	0.177
Inflexibility	1.0 (0.8-1.3)	0.6 (0.4-0.8)	0.034
Personal neglect	0.9 (0.7-1.2)	0.5 (0.3-0.6)	0.034
Disorganization	1.1 (0.9-1.4)	0.8 (0.6-1.0)	0.087
Inattention	1.2 (1.0-1.5)	0.8 (0.7-1.0)	0.034
Loss of insight	1.1 (0.8-1.4)	0.9 (0.7-1.1)	0.254
Logopenia	1.2 (0.9-1.4)	0.7 (0.5-0.9)	0.034
Comprehension deficit	0.4 (0.2-0.6)	0.3 (0.1-0.4)	0.297
Aphasia and verbal apraxia	1.0 (0.7-1.3)	0.9 (0.7-1.1)	0.533
Alien hand and/or apraxia	0.3 (0.1-0.5)	0.3 (0.1-0.4)	0.843
Perseveration/obsessions	0.7 (0.4-0.9)	0.5 (0.4-0.7)	0.533
Irritability	1.0 (0.8-1.3)	0.7 (0.5-0.9)	0.094
Excessive jocularity	0.2 (0.0-0.3)	0.1 (0.0-0.2)	0.739
Poor judgment and impulsivity	0.9 (0.7-1.2)	0.4 (0.2-0.6)	0.024
Hoarding	0.1 (0.0-0.2)	0.1 (0.0-0.2)	0.781

Continued on next page

Table 4.1: Clinical and biological characteristics of anti-GluA3-Ab+ and GluA3-Ab- FTD patients. (Continued)

	GluA3+ (n=86)	GluA3- (n=174)	p-value
Inappropriateness	0.6 (0.4-0.8)	0.4 (0.2-0.5)	0.117
Restlessness/roaming	0.3 (0.2-0.5)	0.1 (0.0-0.3)	0.094
Aggression	0.5 (0.3-0.7)	0.2 (0.2-0.4)	0.061
Hyperorality/food fads	1.0 (0.7-1.2)	0.5 (0.3-0.7)	0.034
Hypersexuality	0.1 (0.0-0.2)	0.1 (0.0-0.2)	0.967
Utilization behaviour	0.1 (0.0-0.1)	0.0 (0.0-0.1)	0.176
Incontinence	0.4 (0.2-0.5)	0.2 (0.1-0.3)	0.079
Cerebrospinal fluid			
Total-tau	362.1 (296.4- 427.9)	322.8 (273.4- 372.1)	0.193
Phospho-tau181	82.0 (59.7-104.3)	46.7 (29.9-63.5)	0.042
Amyloid beta1-42	898.1 (795.4- 1000.7)	814.2 (739.4- 889.0)	0.193

Overall, these data indicate that anti-GluA3-hIgG-treated mice show high overlap with the disease symptomatology of FTD patients positive for anti-GluA3 Abs. The anti-GluA3-Ab+ patients exhibited accentuated hyperorality, inattention, and apathy, which are the main behavioural traits that characterised our pre-clinical model of chronic anti-GluA3 hIgG administration. Furthermore, this correlation with clinical data indicates the high face validity of our pre-clinical model, which makes it a valuable tool for gaining insights into human pathology and testing new therapeutic personalised medicine approaches.

4.2.5 The administration of PAM S 47445 rescued anti-GluA3 hIgG-mediated detrimental effects

Chronic administration of anti-GluA3 hIgGs led to a significant reduction in the surface retention of GluA3/GluA2 AMPARs (Fig. 4.16C). Considering these results and the reduction of glutamatergic transmission previously observed in anti-GluA3-Ab+ patients by neurophysiological techniques (Palese et al. 2020), we hypothesised that the stimulation of AMPARs still available at the post-synaptic membrane could counteract the detrimental effects of anti-GluA3 hIgG on the glutamatergic system. To test this hypothesis, we treated mice with a well-validated AMPAR positive allosteric modulator (PAM),

namely S 47445 (tulampator, CX-1632) (Bretin et al. 2017). This molecule produces a robust increase in AMPAR activation in different pre-clinical *in vivo* studies (Bretin et al. 2017, Giralt et al. 2017) and has previously been assessed in a clinical trial (Clinicaltrials.gov identifier NCT number: NCT02805439). We administered S 47445 daily from day 15 to day 29 through intraperitoneal injection (Fig. 4.29). Indeed, the behavioural results suggested that the detrimental effects mediated by chronic anti-GluA3 hIgG administration were present from at least day 15.

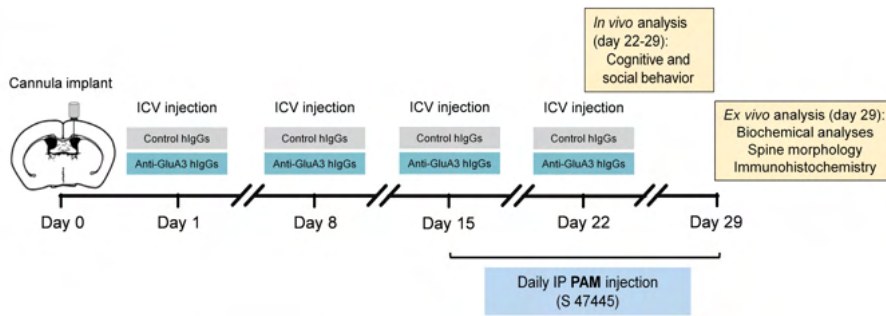


Figure 4.29: Scheme representing the timeline of rescue strategy administration to chronic animal model. Mice receive daily intraperitoneal (IP) injection of PAM S 47445 from day 15 to day 29.

S 47445 administration fully rescued anti-GluA3 hIgG-mediated accumulation of p-tau in the post-synaptic compartment (Fig. 4.30A, B). In addition, we observed total recovery of spine density in the anti-GluA3 hIgG-treated animals that received daily administration of S 47445 (Fig. 4.30C, D). Therefore, the chronic stimulation of AMPAR-mediated glutamatergic neurotransmission by S 47445 administration was sufficient to rescue the molecular and morphological detrimental effects induced by anti-GluA3 hIgGs.

Importantly, this event was paralleled by recovery of anti-GluA3 hIgG-mediated behavioural defects. Mice treated with S 47445 recovered their interest in novelty both in terms of exploration (Fig. 4.31A, C, D) and food consumption (Fig. 4.31B). Furthermore, we detected partial recovery of binge-like eating behaviour assessed in operant boxes (Fig. 4.32A-F; Fig. 4.33A-C). As reported in Fig. 4.32E, F, the administration of S 47445 to anti-GluA3 hIgG-treated mice defined a new group of animals with a food-seeking behaviour (expressed as the number of food-related responses) that was in the middle between the CTRL mice and anti-GluA3 hIgG-treated mice.

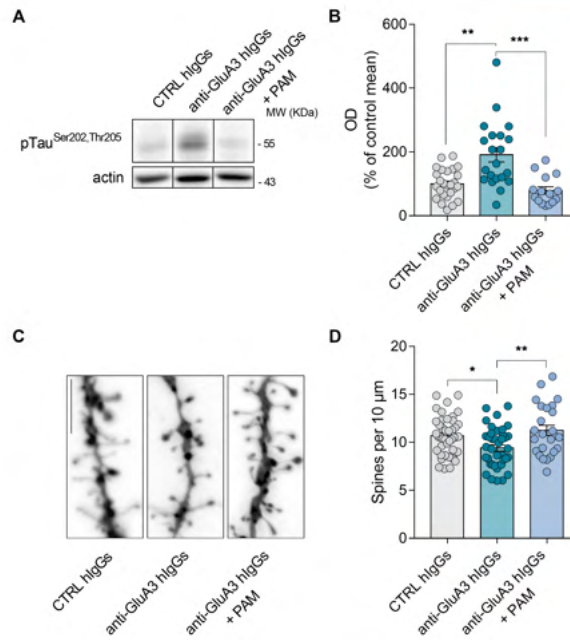


Figure 4.30: *PAM administration totally rescued anti-GluA3-hIgG-mediated detrimental effects in terms of p-tau accumulation and loss of dendritic spines.* (A) Western blot representative images and (B) bar graph of densitometric quantification of p-Tau (Ser202, Thr205, AT8) in TIF obtained from mice PFC at the end of chronic treatment with CTRL hIgGs and anti-GluA3 hIgGs ± PAM (Kruskal-Wallis; $p=0.0001$, $n=26-21-15$ /group; Dunn's correction). (C) Representative images showing dendrites of adult mice PFC at the end of the chronic treatment with CTRL hIgGs or anti-GluA3 hIgGs ± PAM and bar graphs representing (D) protrusion densities (ordinary one-way ANOVA; treatment: $F(2.104)=6.034$, $p=0.0033$, $n=43-39-25$ /group; Tukey correction). In (C), n = number of animals; in (E), n = number of neurons. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

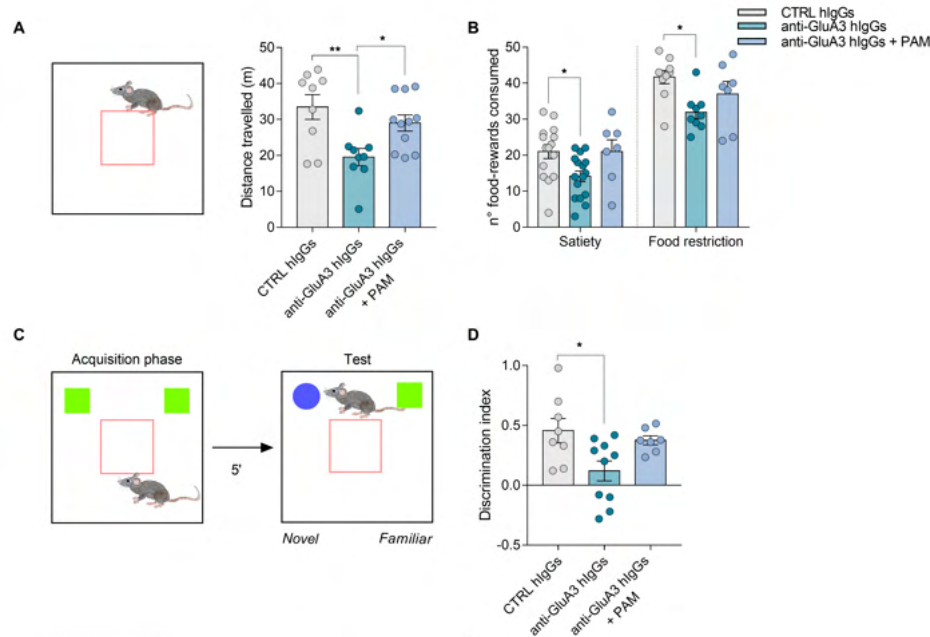


Figure 4.31: *PAM administration partially or totally rescued anti-GluA3 hIgG-mediated behavioral effects in term of interest for novelty.* (A) Locomotor activity measured as the total distance travelled in the OF arena in the first 10 minutes of exploration (ordinary one-way ANOVA; treatment: $F(2,26)=6.537$, $p=0.0050$, $n=9-9-11$ /group; Tukey correction). (B) Number of palatable pellets of food consumed in the Free Reward Consumption task by mice in a condition of satiety (ordinary one-way ANOVA; treatment: $F(2,35)=4.344$, $p=0.0206$, $n=15-16-7$ /group; Tukey correction) or after a period of food restriction (ordinary one-way ANOVA; treatment: $F(2,23)=5.126$, $p=0.0144$, $n=10-9-7$ /group; Tukey correction). (C) NORT schematic representation; performance of mice in NORT expressed as discrimination index after (D) 5 minutes from the acquisition phase (ordinary one-way ANOVA; treatment: $F(2,22)=4.856$, $p=0.0179$, $n=8-10-7$ /group; Tukey correction). * $P < 0.05$; ** $P < 0.01$.

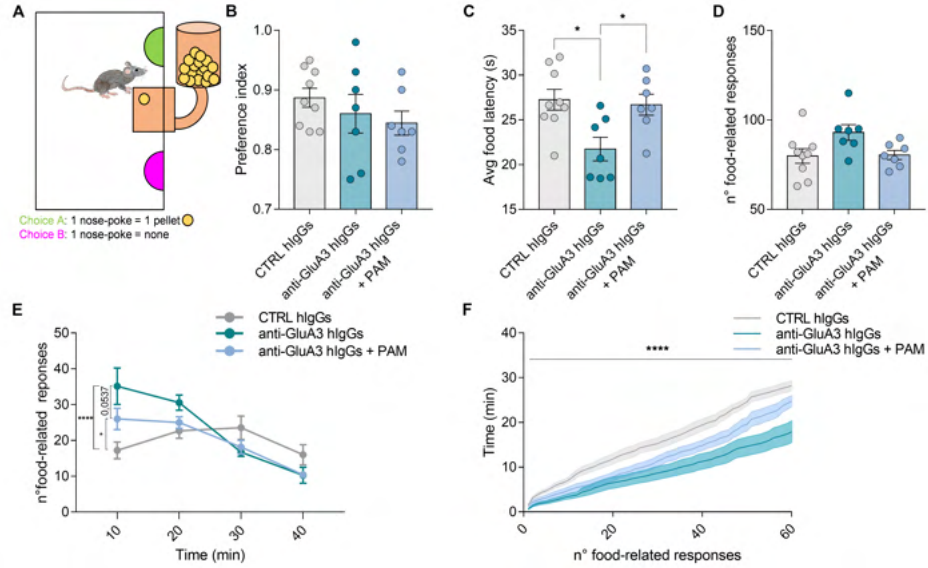


Figure 4.32: *PAM administration partially or totally rescued anti-GluA3 hIgG-mediated behavioral effects in term of binge-like eating assessed in operant boxes under food restriction.* (A) Schematic representation of the food seeking task; mice performance in the food seeking task under food restriction expressed as (B) preference index (ordinary one-way ANOVA; treatment: $F(2,20)=0.9339$, $p=0.4095$, $n=9-7-7/\text{group}$; Tukey correction), (C) average of time between one food-related response and the subsequent one (i.e., avg food latency) (ordinary one-way ANOVA; treatment: $F(2,20)=5.992$, $p=0.0091$, $n=9-7-7/\text{group}$; Tukey correction) and (D) number of food-related responses (choice A) (ordinary one-way ANOVA; treatment: $F(2,20)=3.535$, $p=0.0485$, $n=9-7-7/\text{group}$; Tukey correction); in (E-F) number of food-related responses during the session in operant boxes are reported ((E): 2way ANOVA; interaction: $F(6,60)=4.668$, $p=0.0006$, $n=9-7-7/\text{group}$; Tukey correction; (F): 2way ANOVA; interaction: $F(124,1240)=5.067$, $p<0.0001$, $n=9-7-7/\text{group}$)). * $P < 0.05$; *** $P < 0.0001$.

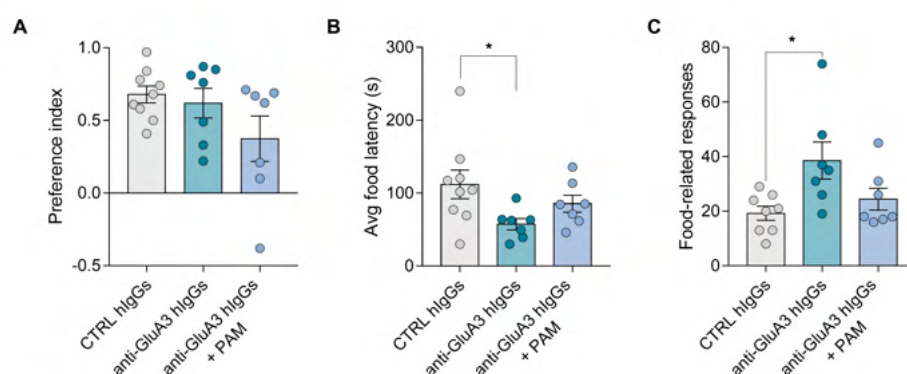


Figure 4.33: *PAM administration partially rescued anti-GluA3 hIgG-mediated behavioural effects in term of binge-like eating assessed in operant boxes in a condition of satiety.* Performance in the Food Seeking task without food restriction expressed as (A) preference index (ordinary one-way ANOVA; treatment: $F(2,20)=2.292$, $p=0.1270$, $n=9-7-7$ /group; Tukey correction), (B) average of time between one food-related response and the subsequent one (ordinary one-way ANOVA; treatment: $F(2,20)=3.29$, $p=0.0582$, $n=9-7-7$ /group; Tukey correction) and (C) number of food-related responses (ordinary one-way ANOVA; treatment: $F(2,19)=4.625$, $p=0.0231$, $n=8-7-7$ /group; Tukey correction). * $P < 0.05$. To apply one-way ANOVA, normal distribution was checked using Shapiro-Wilk normality test.

Overall, both *ex vivo* and behavioural data indicated that chronic S 47445 administration counteracted anti-GluA3 hIgG-mediated detrimental effects.

4.2.6 Effects of anti-GluA3 chronic administration on the activity of mPFC glutamatergic neurons that project to NAc

Note. The experiments reported below were performed during my 6-months period in professor Camilla Bellone's laboratory at the University of Geneva.

As reported above, both anti-GluA3 Ab+ patients and the chronic animal model showed a marked impairment in glutamatergic neurotransmission at the PFC level. We also detected deficits in reward-seeking behaviour. Thus, we hypothesize that anti-GluA3 hIgGs alter the modulation exerted by mPFC glutamatergic neurons on downstream brain regions and, specifically, on NAc (Fig. 4.34). In our hypothesis, this putative altered modulation of NAc by mPFC is at the root of the observed behavioural deficits in reward seeking. Notably, a reduction of glutamatergic signalling to the medium spiny neurons (MSNs) of NAc has already been associated with maladaptive

attitudes toward natural stimuli such as food (Reed et al. 2018; Morgan et al. 2022; Domingo-Rodriguez et al. 2020).

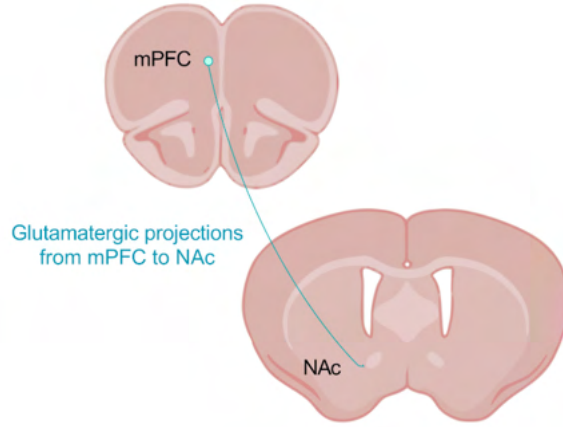


Figure 4.34: *Schematic representation of mPFC projections to NAc.*

To monitor the neuronal activity of mPFC glutamatergic neurons projecting to NAc in freely moving anti-GluA3 hIgGs treated mice or control ones, I exploited *in vivo*-calcium imaging through GCaMP-based fiber photometry. With this aim, the day of ICV cannula implantation (day -7) into the lateral ventricle, we injected a retrograde AAV-expressing the calcium indicator GCaMP (ssAAV-retro/2-mCaMKII α -jGCaMP7s-WPRE-bGHp(A)) in the NAc and then implanted an optic fiber in the mPFC. Thanks to its retrograde activity, this virus guarantees the expression of GCaMP only in the mPFC glutamatergic neurons that project to NAc. On day 1, we started the weekly administration of anti-GluA3 hIgGs or control hIgGs. Once established the chronic model of hIgGs administration (day 22), we evaluated the activity of mPFC glutamatergic neurons projecting to NAc with fiber photometry during behavioural tasks (Fig. 4.35).

Specifically, we tested anti-GluA3 hIgGs-treated mice and control ones in a free interaction task. In sessions of 20 minutes, actor mice were let free to interact into an open field arena with stimuli that entail, respectively, a neutral valence (an object), an increasing positive valence (a palatable food or social stimuli such as juvenile males or females in oestrus) or a negative valence (CD1 aggressive mice). Contextually, we monitored the activity of mPFC glutamatergic neurons projecting to NAc. Actor mice were tested with these different stimuli on different days with at least one day of rest between one session and the subsequent one (Fig. 4.36). We chose this task since it allows to evaluate, both in term of behaviour and neuronal activity, whether the chronic treatment with anti-GluA3 hIgGs affects the ability of mice to assign the right valence to the selected stimuli.

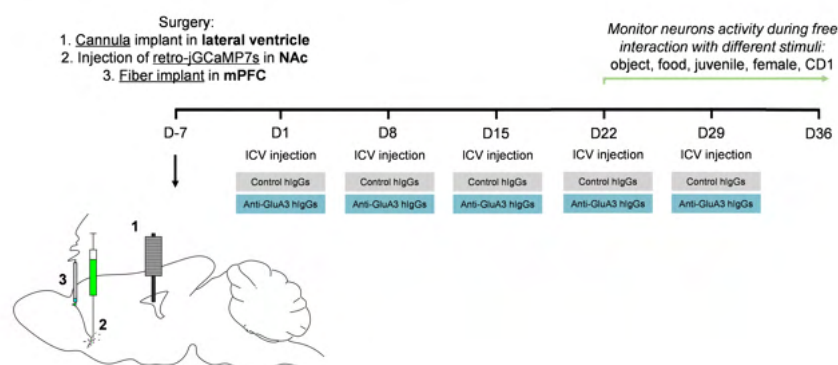


Figure 4.35: *Schematic representation of the experimental timeline.* At day -7 (D-7), mice are implanted with a ICV cannula into the lateral ventricle and a fiber in mPFC; contextually, they are injected with a retro-jGCaMP7s in NAc. Starting from day 1 (D1), mice are weekly treated (at D1, 8, 15, 22, 29), through the ICV cannula, with purified anti-GluA3 hIgGs or control hIgGs. From day 22 to day 36, mice are tested in free interaction task. At day 36, mice are sacrificed.

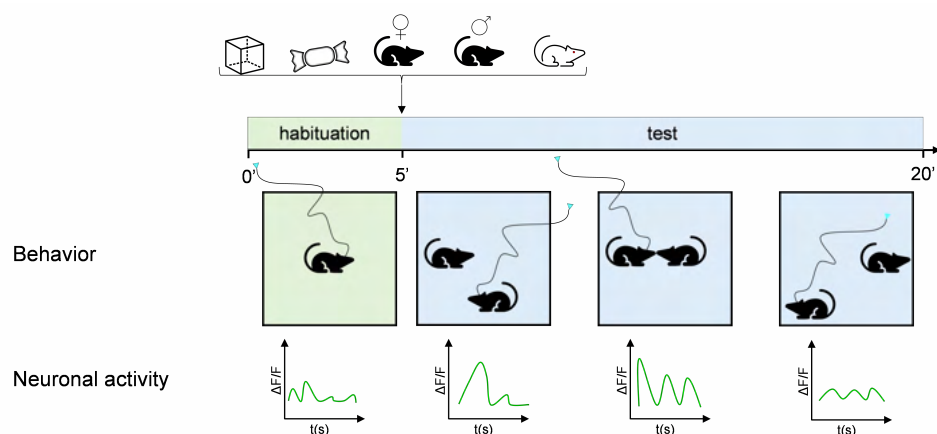


Figure 4.36: *Schematic representation of the free interaction task.* The task consists in a 20-minutes session into an open field arena where the actor mouse can freely move. After 5 minutes of habituation to the arena, we introduce a stimulus (an object, a palatable food, a juvenile male, a female in oestrus or an aggressive CD1 mouse), and the actor mouse can interact with it for the remaining 15 minutes of the session. During the entire task, the activity of the glutamatergic neurons projecting from mPFC to NAc is monitored through fiber photometry.

At the end of the tasks, we sacrificed mice to verify the position of the fiber and the infection with the GCaMP virus through fluorescent microscopy

(Fig. 4.37).

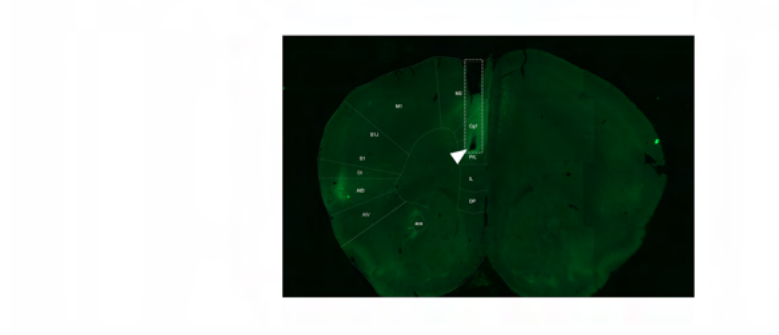


Figure 4.37: *Representative image of fiber position and viral infection in a brain slice.* The image shows the correct optic fiber position in the prelimbic cortex (PrL). The position of the fiber is highlighted by the dotted rectangle and the fiber tip can be clearly distinguished (white triangle). Viral infection with retro-GCaMP is evident, thanks to GCaMP green signal, in the regions that project to NAc including the PrL cortex under the tip of the fiber.

I am now analyzing the data I have collected during these experiments.

5. Discussion

Several studies initially detected anti-GluA3 in the serum of patients with autoimmune encephalitis and in up to 20% to 30% of patients affected by different types of epilepsy (Ganor et al. 2005a; Ganor et al. 2005b; Gardoni et al. 2021; Rogers et al. 1994). Interestingly, the presence of anti-GluA3 antibodies correlated with learning, attention and psychiatric symptoms (Goldberg-Stern et al. 2014). More recently, anti-GluA3 IgG have gained further attention in the field of neurology: indeed, our research group identified autoantibodies directed against the GluA3 sub-unit of AMPARs in 20%–25% FTD patients (Borroni et al. 2017; Palese et al. 2020).

Although previous *in vitro* studies showed that anti-GluA3 autoantibodies affect glutamatergic neurotransmission through different mechanisms (Borroni et al. 2017; Palese et al. 2020; Day et al. 2023; Malina et al. 2006; Levite et al. 1999a), the impact of these autoantibodies on cognitive and social functions *in vivo* is still unknown, as well as the biological substrates of these putative modifications.

To address this gap of knowledge and better dissect the contribution of anti-GluA3 autoantibodies to the above-mentioned neurological conditions, during my PhD, I have developed two models of *in vivo* administration of anti-GluA3 antibodies isolated from patients (anti-GluA3 hIgGs) to mice.

5.1 Acute model

During the first part of my PhD, I characterized the effects mediated by a single acute intracerebral injection of purified anti-GluA3 hIgGs in WT mice (see section 4.1). In this first set of experiments, we demonstrated that anti-GluA3 hIgGs lead to significant molecular and morphological changes at dendritic spine in the PFC as well as modifications of cognitive and social behavior *in vivo* in mice.

In the acute model, we observed that anti-GluA3 hIgGs affects synaptic levels of the GluA3 sub-unit in the PFC but not in the hippocampus. Even if AMPARs are widely distributed throughout the brain and represent a key player for activation of the glutamatergic synapse, their relative abundance varies in the different areas. In particular, hippocampal synapses contains

the highest density of AMPARs (Petrulia et al. 1992; Schwenk et al. 2014). Assuming a widespread brain diffusion of ICV injected anti-GluA3 hIgGs, the increased AMPAR levels in the hippocampus compared to the PFC could account for the observed resilience of hippocampal synaptic AMPAR to molecular modifications induced by the autoantibodies.

From the morphological point of view, we detected a significant increase of protrusion density and dendritic spine size in the PFC 24 h after intracerebral injection of anti-GluA3 hIgGs. Different explanations can be put forward for this apparently unexpected morphological data. Firstly, previous *in vitro* studies demonstrated that anti-GluA3 hIgGs can activate GluA3-containing AMPARs (Malina et al. 2006; Levite et al. 1999a): this event may lead to a subsequent fast internalization of this pool of AMPARs (Borroni et al. 2017) that are probably replaced by GluA1/GluA2 AMPARs (Shi et al. 2001). Indeed, it is well-known that, following synaptic activation, GluA3-containing AMPARs are physiologically replaced by AMPARs containing GluA1/GluA2 at the postsynaptic membrane. Considering the strict correlation between AMPAR activation, enlargement of pre-existing spines and formation of new ones, antibody-dependent activation can explain the observed morphological alterations.

Our behavioral results showed significant alteration of short-term recognition memory following single injection of anti-GluA3 antibodies. In agreement with previous studies in GluA3-deficient mice we found no effects on spatial (Adamczyk et al. 2012) and contextual memories (Humeau et al. 2007), which are cognitive functions that highly depend on the hippocampus. This is in line also with our molecular findings showing that anti-GluA3 antibodies produced effects in the PFC, while sparing the hippocampus. Indeed, mice injected with anti-GluA3 antibodies displayed marked alterations in cognitive tasks in which the role of the cortex is crucial. The cognitive alterations that we observed particularly involving memory functions could have a translational meaning towards those pathologies in which neuronal antibodies have been shown to impact cognition such as autoimmune encephalitis and FTD. Notably, short-term memory loss and working memory deficits are considered hallmarks of autoimmune encephalitis (Graus et al. 2016) and recognition memory deficits are often observed in FTD patients (Hornberger et al. 2010; Hornberger et al. 2012). Further, although our data showed alterations of short-memory functions, the impairment on novel object recognition following anti-GluA3 antibodies injection could also indicate problems in the processing of novelty. This could involve a lack of attention to novel stimuli that recall the executive dysfunctions typical of FTD patients (Hornberger et al. 2008). Thus, further research to dissect the exact role of anti-GluA3 antibodies in these cognitive functions is warranted.

In addition to cognitive alterations, we found modifications of social cognitive functions, such as empathy-like behaviors and emotion discrimination, whose processing critically depends on the key role of the PFC (Jeon

et al. 2010; Scheggia et al. 2019). Moreover, we expanded previous findings reporting a modulation of social behavior in GluA3-deficient mice (Humeau et al. 2007) by showing that anti-GluA3 antibodies modified social preference. Although our results are in contrast with findings obtained in GluA3-deficient mice they further indicate a possible crucial contribution of GluA3 subunits in social behavior and social cognition. Deficits in theory of mind loss of empathy and emotion processing represent the most prominent symptoms of both autoimmune encephalitis, i.e. limbic encephalitis, and FTD (Henry et al. 2015; Russell et al. 2020; Sprengelmeyer et al. 2010), which clearly have an impact on social daily life of the patients. Here we showed that anti-GluA3 antibodies modified mice social preference and altered social cognitive functions. In particular we found that discrimination of emotional states of fear and social transmission of fear were impaired following anti-GluA3 antibodies injections. These results might suggest that individual differences in empathy-like behaviors and perception of social cues and emotional states might shape social preference. Thus, our results could indicate an important role of an overlooked system involving GluA3-containing AMPAR synaptic transmission in social cognition.

Previous studies related to the *in vivo* infusion in mice of anti-NMDAR antibodies indicated that all antibody-dependent effects resolved one week after the last administration (Planagumà et al. 2014; Planagumà et al. 2016). In agreement with these results, here we observed anti-GluA3-dependent effects starting 24 h after injection and leading to behavioral alterations at days 2–4. However, we did not see behavioral, molecular and morphological alterations when experiments were performed 10–14 days after GluA3 injection, thus suggesting that antibody-induced effects at the excitatory glutamatergic synapse are transient and reversible.

With this first set of experiments, we showed that the abnormal presence of anti-GluA3 hIgGs in mouse brain causes a complex dysfunction in glutamatergic synapses thus potentially playing a role in the behavioral dysregulation proper of patients carrying these autoantibodies. However, a single acute injection of anti-GluA3 antibodies can not recapitulate the phenotype proper of anti-GluA3-Ab+ patients—Thus, we decided to develop a second animal model where anti-GluA3 hIgGs were chronically administered.

5.2 Chronic animal model

Given the negative effects of acute GluA3 autoantibodies administration on synapses, behavior, and cognition, whether and how the chronic presence of autoantibodies triggers a neurodegenerative process and the association between this process and the appearance of FTD-related neuropathological and behavioral signature should be further investigated. Thus, we developed a chronic mice model of autoimmunity by infusing mice with anti-GluA3

hIgGs or control hIgGs for one month, once a week, through an intracerebroventricular cannula (see section 4.2).

Thanks to this model, we demonstrated that chronic anti-GluA3 hIgGs administration in mice mediated a selective increase in the intracellular levels of GluA3-containing AMPAR and a consequent decrease in their surface retention. As we did not detect any differences in GluA3-containing AMPAR levels in the post-synaptic fraction, we hypothesized that anti-GluA3 hIgGs induced an endocytic process of GluA3-containing AMPARs within the dendritic spine compartment. *Ex-vivo* morphological evaluation showed that mice chronically infused with anti-GluA3 IgG are characterized by early signs of synapse loss in the prefrontal cortex with a decrease of dendritic spine density. This data is apparently in contrast to what observed into the acute animal model; however, as already discussed into the previous section and in section 1.1.4.4, these contrasting results may represent two temporally distinct events of the same phenomenon.

We found that chronic anti-GluA3 hIgGs administration in mice led to selective accumulation of the p-tau in the post-synaptic compartment. Remarkably, we also detected an increase in the level of p-tau in the CSF of GluA3-Ab+ FTD patients compared with FTD patients not carrying the autoantibody. A large body of literature suggests that a variety of toxic stimuli can trigger aberrant tau phosphorylation. In its hyperphosphorylated form, tau is more prone to aggregate to form the toxic inclusions that characterise tauopathies (Alonso et al. 2018; Bodea et al. 2016). However, recent studies have indicated that tau hyperphosphorylation also promotes its migration into the postsynaptic compartment (Italia et al. 2022). In this compartment, p-tau negatively affects synapse functionality as it interferes with ionotropic glutamate receptors: several studies reported a reduction in the activity and in the synaptic expression of AMPAR and NMDAR in the presence of tau mislocalisation and hyperphosphorylation (Alfaro-Ruiz et al. 2022; Hoover et al. 2010; Miller et al. 2014; Prikas et al. 2022; Regan et al. 2021; Teravskis et al. 2019; Li et al. 2019). Other studies also suggested that acting on glutamatergic signalling, and specifically potentiating AMPAR, prevents tau-mediated toxic synaptic effects (Monteiro-Fernandes et al. 2020), suggesting that the toxic cross-talk between AMPAR and tau could also be the explained the other way around.

Along with molecular and morphological alterations, anti-GluA3 hIgGs induce a peculiar behavioral signature in mice characterized by a reduction in interest for novelty (which was partially evident also into the acute animal model) in addition to binge-like eating and impulsivity. We assessed the reduction in interest for novelty through different stimuli. In the tasks, mice were presented with a series of stimuli entailing a progressive increase in the required cognitive load. Anti-GluA3 hIgG-treated mice failed to discriminate between salient stimuli involving newly presented objects, emotionally altered conspecifics, and palatable pellets of food. Therefore, these features could

be interpreted as a reduction of interest or inattention for novelty rather than a deficit in recognition, which is consistent with the reduced interest for exploration in different environmental conditions.

We found contrasting behavioral responses when food rewards were presented to home caged mice or following operant conditioning. However, it must be highlighted that these two conditions entailed rather different cognitive processing; under the first condition, mice had free access to palatable pellets of food, whereas, in the second, they actively sought out food. The latter scenario triggered binge-eating-like behaviour and impulsivity in anti-GluA3 hIgG-treated mice. A similar impulsive behaviour was observed in the sucrose preference test. However, we did not observe any differences when we increased the fixed ratio schedule. As previously described in the literature for a murine model of obesity (Bickel et al. 2021; Matikainen-Ankney et al. 2023), this may be explained by the fact that anti-GluA3-treated mice exhibited increased intolerance towards delayed rewards (consequently, it could be a matter of time rather than effort). Overall, our results suggest that anti-GluA3 hIgGs can affect the reward system in general by negatively influencing mice's attitudes towards natural stimuli/rewards.

Anti-GluA3-Ab+ FTD patients exhibited significant differences in some behavioural traits, such as poor judgment and impulsivity, hyperorality/food fads, inattention, and apathy. Because these behavioral traits reflect those identified in our pre-clinical model, we concluded that anti-GluA3 hIgG-treated mice exhibited a high degree of overlap with the disease symptomatology of these patients. In our pre-clinical model, the presence of anti-GluA3 hIgGs alone induced the appearance of signs and symptoms that resembled the ones exhibited by the patients; this correspondence suggests a link of causation between anti-GluA3 hIgG positivity and the appearance of a specific symptomatology. In addition, it indicates the high face validity of our model, making it a valuable tool not only for gaining insights into human pathophysiology, but also as a tool to evaluate new pharmacological approaches of personalized medicine for anti-GluA3 hIgG-Ab+ patients.

Finally, of primary relevance, we used our model to evaluate the efficacy of a well-validated AMPAR PAM (S 47445) whose safety was previously approved in a clinical trial (Clinicaltrials.gov, identifier NCT number: NCT02805439) and whose efficacy was already validated in different pre-clinical models (Bretin et al. 2017; Calabrese et al. 2017; Mendez-David et al. 2017; Morley-Fletcher et al. 2018). The stimulation of the remaining available AMPAR at the post-synaptic membrane with S 47445 counteracted almost all the deficits induced by anti-GluA3 hIgG. This result represents a proof of concept that the defects in glutamatergic neurotransmission mediated by anti-GluA3 hIgGs are at the root of the observed molecular and behavioural abnormalities. Moreover, the observed efficacy of S 47445 in our pre-clinical model can pave the way for further studies aimed at investigating the putative clinical use of this class of compounds for precision medicine

therapy in GluA3-Ab+ patients. The partial rescue that we observed in some behavioural traits could be due to the dosage of 3 mg/kg used in the present study instead of the 10 mg/kg described in some pre-clinical studies (Mendez-David et al. 2017; Pilar-Cuellar et al. 2019) and due to the timing. The ex-vivo analyses were all performed after 15 days of daily PAM administration (i.e., on day 29); the behavioural tasks were performed from day 22 to day 29 on mice that had been treated with PAM for fewer days.

In conclusion, thanks to these two *in vivo* studies, we clarified the contribution of anti-GluA3 autoantibodies to central nervous system symptoms and pathology and identified a specific subgroup of FTD patients with distinct clinical features. In addition, the *on going* analysis of the activity of the mPFC glutamatergic neurons that project to NAc will allow to shed more light on the functional effects mediated by anti-GluA3 hIgGs. Finally, our findings will be instrumental in the development of a therapeutic personalized medicine strategy for patients positive for anti-GluA3.

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6. Scientific Career and Research Activity

Experiences abroad

6-months (from May 2023 to October 2023) internship in Professor Camilla Bellone's laboratory at University of Geneva, Department of Basic Neurosciences.

Research project: Dissecting the neuronal correlates of impaired reward-seeking behaviour induced by anti-GluA3 hIgGs administration.

Papers published or under revision in peer-reviewed journals

1. Rubino E., **Italia M.**, Giorgio E., Boschi S., Dimartino P., Pipucci T., Roveta F., Cambria C. M., Elia G., Marcinnò A., Gallone S., Rogaeva E., Antonucci F., Brusco, A., Gardoni F., Rainero I.
Association of a GRIN2C missense variant with Familial Late-Onset Alzheimer's Disease
Submitted for publication
2. Carrano N., Ribeiro A., **Italia M.**, La Greca F., Genova F., D'Andrea L., Zianni E., Ponzoni L., Barthet G., Collins S. C., Sala M., Mitro N., Mulle C., Yalcin B., Marcello E., Gardoni F., DiLuca M., Scheggia D.
RNF10 as an NMDA receptor-dependent signaling protein necessary for cognitive flexibility
Submitted for publication
3. **Italia M.**, Salvadè M., La Greca F., Zianni E., Ferrari E., Archetti S., Alberici A., Benussi A., Solje E., Haapasalo A., Hoffmann D., Katisko K. L., Krüger J., Facchinetti F., Scuderi C., Padovani A., DiLuca M., Scheggia D., Borroni B., Gardoni F.
Anti-GluA3 autoantibodies define a new sub-population of Frontotemporal dementia patients with distinct neuropathological features

Under second revision to Brain, Behavior and immunity

4. Fontana E., Bongiani M., Benussi A., Bronzato E., Scialo C., Sacchetto L., Cagnin A., Castriciano S., Buratti E., Gardoni F., **Italia M.**, Schreiber A., Ferracin C., Fiorini M., Legname G., Newell K.L., Cracco L., Garringer H.J., Cecchini M.P., Polymenidou M., Padovani A., Monaco S., Legname G., Ghetti B., Borroni B., Zanusso G.
Detection of TDP-43 seeding activity in the olfactory mucosa from patients with Frontotemporal Dementia
Alzheimer's & Dementia, 2023; doi: 10.1002/alz.13541
5. Scheggia D., La Greca F., Maltese F., Chiacchierini G., **Italia M.**, Molent C., Bernardi F., Coccia G., Carrano N., Zianni E., Gardoni F., Di Luca M., Papaleo F.
Reciprocal cortico-amygdala connections regulate prosocial and selfish choices in mice
Nature Neuroscience, 2022; doi: 10.1038/s41593-022-01179-2
6. Ferrari E., Scheggia D., Zianni E., **Italia M.**, Brumana M., Palazzolo L., Parravicini C., Pilotto A., Padovani A., Marcello E., Eberini I., Calabresi P., Di Luca M., Gardoni F.
Rabphilin-3A as a novel target to reverse α -synuclein-induced synaptic loss in Parkinson's disease
Pharmacological Research, 2022; doi: 10.1016/j.phrs.2022.106375
7. **Italia M.**, Ferrari E., Di Luca M., Gardoni F.
NMDA and AMPA Receptors at Synapses: Novel Targets for Tau and α -Synuclein Proteinopathies
Biomedicine, 2022; doi: 10.3390/biomedicines10071550
8. Forastieri C., **Italia M.**, Toffolo E., Romito E., Bonasoni M.P., Ranzani V., Bodega B., Rusconi F., Battaglioli E.
Evolution Increases Primates Brain Complexity Extending RbFOX1 Splicing Activity to LSD1 Modulation
Journal of Neuroscience, 2022; doi: 10.1523/JNEUROSCI.1782-21.2022
9. **Italia M.**, Ferrari E., Di Luca M., Gardoni F.
GluA3-containing AMPA receptors: From physiology to synaptic dysfunction in brain disorders
Neurobiology of Disease, 2021; doi: 10.1016/j.nbd.2021.105539
10. Scheggia D., Stanic J., **Italia M.**, La Greca F., Zianni E., Benussi A., Borroni B., Di Luca M., Gardoni F.
GluA3 autoantibodies induce alterations in dendritic spine and behavior in mice
Brain, Behavior, and Immunity, 2021; doi: 10.1016/j.bbi.2021.07.001

11. **Italia M.**, Forastieri C., Longaretti A., Battaglioli E., Rusconi F.
Rationale, Relevance, and Limits of Stress-Induced Psychopathology in Rodents as Models for Psychiatry Research: An Introductory Overview
 International Journal of Molecular Sciences, 2020; doi:10.3390/ijms21207455

Congresses

Oral presentations

1. December 4th, 2022, Symposium of the Italian-Israeli Neuroscience Societies on Neurodegenerative Diseases, Eilat, Israel
Anti-GluA3 antibodies in Frontotemporal Dementia
2. June 11th, 2022, SINS PhD National meeting, Brescia, Italy
Insights into Frontotemporal Dementia pathogenesis: the role of anti-GluA3 antibodies

Posters

1. September 14th-17th 2023, 20th SINS National Congress, Turin, Italy
Anti-GluA3 autoantibodies define a new sub-population of Frontotemporal dementia patients with distinct neuropathological features
 Italia M., Salvadè M., La Greca F., Zianni E., Spinola A., Ferrari E., Archetti S., Alberici A., Benussi A., Padovani A., DiLuca M., Scheggia D., Borroni B., Gardoni F.
2. November 2nd-5th, 2022, ISFTD, Lille, France
Anti-GluA3 antibodies in Frontotemporal Dementia: an in vivo approach
 Italia M., Scheggia D., Zianni E., Salvadè M., La Greca F., Alberici A., Benussi A., Archetti S., Padovani A., Borroni B., DiLuca M., Gardoni F.
3. October 20th-21st, 2022, VIII European Synapse Meeting, Coimbra, Portugal
Anti-GluA3 antibodies in Frontotemporal Dementia: an in vivo approach
 Italia M., Scheggia D., Zianni E., Salvadè M., La Greca F., Alberici A., Benussi A., Archetti S., Padovani A., Borroni B., DiLuca M., Gardoni F.
4. July 9-13th, 2022, FENS forum, Paris, France
Anti-GluA3 antibodies in Frontotemporal Dementia: an in vivo approach
 Italia M., Scheggia D., Zianni E., Salvadè M., La Greca F., Stanic J., Borroni B., DiLuca M., Gardoni F.

Awards and achievements

1. September 2023, SINS 2023 Best Poster Award
2. June 2023, Italian Society of Pharmacology (SIF) Grant to visit Prof. Camilla Bellone's laboratory in Geneva from May 1st to October 31st, 2023
3. June 2023, Travel Grant to attend the SINS 2023 National Congress in September 2023
4. March 2023, EMBO Scientific Exchange Grant to visit Prof. Camilla Bellone's laboratory in Geneva from May 1st to October 31st, 2023
5. September 2022, Travel Grant to attend the ISFTD meeting in November 2022
6. August 2022, Scholarship to attend the 8th European Synapse Meeting (ESM) in October 2022

Memberships

From 2022 member of the Società Italiana di Farmacologia (SIF).

Training Courses

1. July - October 2023: Crash course on Python, Coursera online course, Google Career Certificates
2. May – June 2023: Module 1, Introductory online Course in Laboratory Animal Science (mouse and rat), RESAL, Lausanne, Switzerland
3. November – December 2022: Small Animals (rodents-zebrafish-xenopus): Specific Training For Personnel Involved In Animal Experimentation For Scientific Purposes, University of Milan (UNIMI), Milano, Italy

Teaching activities

1. 2020 - 2023: tutoring and teaching activities for “Recupero OFA di Biologia” (University of Milan)
2. 2021 - 2023: co-promoter of bachelor's and master's students during their research internships
3. March 2021: tutoring for the courses of Genetic and molecular bases of diseases (LM-9)