# Anti-GluA3 antibodies in frontotemporal dementia: effects on glutamate neurotransmission and synaptic failure

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**Abstract**

A number of evidences has put forward new players in the pathogenesis of Frontotemporal Dementia (FTD) claiming for a role of autoimmunity and altered glutamate neurotransmission in triggering disease onset. We reported the presence of autoantibodies recognizing the GluA3 subunit of AMPA receptors in about 25% of FTD cases. Here we evaluated the mechanisms involved in anti-GluA3 autoimmunity in FTD, through molecular/neurochemical analyses conducted on patients' brain specimens, corroborated by Transcranial Magnetic Stimulation (TMS) and analysis of glutamate, D-serine and L-serine levels in the CSF. We observed that GluA3 autoantibodies affect glutamatergic neurotransmission, decreasing glutamate release and altering GluA3-containing AMPA receptor levels. These alterations were accompanied by changes of scaffolding proteins involved in receptor synaptic retention/internalization. The above results were confirmed by TMS, suggesting a significant impairment of indirect measures of glutamate neurotransmission in FTD patients as compared to controls, with further add-on harmful effect in those FTD patients with anti-GluA3 antibodies. Finally, FTD patients showed a significant increase of glutamate, D-serine and L-serine levels in the CSF.

# 1 Introduction

Frontotemporal Dementia (FTD), a common cause of presenile dementia, is a clinically and neuropathologically heterogeneous disorder. It presents with behavioural abnormalities, personality change, deficits of executive functions and language impairment (Seelaar et al., 2011). Up to 40% of cases have a family history of dementia, with an autosomal dominant inheritance in around a third of patients (Stevens et al., 1998). Mutations within *microtubule-associated protein tau (MAPT)* (Hutton et al., 1998), *Granulin (GRN)* (Baker et al., 2006, Cruts et al., 2006), and *chromosome 9 open reading frame 72 (C9orf72)* (DeJesus-Hernandez et al., 2011, Renton et al., 2011) are proven major causes of genetic FTD, accounting for 10-20% of all FTD cases.

In the last years, a number of evidences has suggested a new player in FTD pathogenesis, arguing for a role of autoimmunity in triggering disease onset (Alberici et al., 2018). This hypothesis stemmed from epidemiological data and clinical studies, reporting a significantly increased risk of autoimmune disorders (Miller et al., 2013) and autoimmune system dysregulation in FTD patients (Cavazzana et al., 2018) and from genetic research which argued for immune-mediated genetic

enrichment in FTD, particularly within the HLA region (Broce et al., 2018; Ferrari et al., 2014). 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.

We have recently reported a high frequency of autoantibodies recognizing the GluA3 subunit of AMPARs in patients with FTD (Borroni et al., 2017), corroborating the role of glutamate neurotransmission in this disorder (Murley and Rowe, 2018). Furthermore, in both rat hippocampal neuronal primary cultures and in human neurons derived from iPSCs, we demonstrated that anti-GluA3 antibodies were detrimental for neurons and for AMPA function (Borroni et al., 2017).

However, it is still unclear whether anti-AMPA receptor antibodies are the trigger event for protein misfolding, or are produced as a consequence of neuronal loss, boosting, in turn, the on-going neurodegenerative process. In both cases, anti-AMPA receptors antibodies could represent a promising therapeutic target.

Accordingly, it needs to be doubtless proven *i)* whether anti-AMPA GluA3 receptors antibodies mediate a detrimental effect *in vivo* in FTD patients and *ii)* how these antibodies modify pre- and postsynaptic glutamate neurotransmission.

To this, in the present work we used a multidisciplinary approach to unravel the role of glutamate transmission and anti-AMPA GluA3 receptor autoimmunity in FTD. In particular, *a)* we evaluated glutamate receptors composition at synapses in autoptic brain specimens from patients affected by frontotemporal lobar degeneration (FTLD); *b)* we investigated the impact of anti-GluA3 antibodies on the AMPA-mediated control of presynaptic glutamate release by using cerebrospinal fluid (CSF) obtained from anti-GluA3 antibodies positive patients; *c)* we indirectly assessed *in vivo* glutamate neurotransmission integrity by Transcranial Magnetic Stimulation (TMS) in both anti-GluA3 antibodies positive and negative FTD patients, compared to controls and *d)* we measured L-glutamate and D-/L-serine levels in the CSF of FTD patients with or without anti-GluA3 autoantibodies.

## 2 Materials and methods

**2.1 Serum and CSF dosage of anti-GluA3 antibody levels.** Serum samples were frozen immediately after centrifugation and stored at −20 °C pending enzyme-linked immunosorbent assay (ELISA). The detection of anti-GluA3 antibodies (peptide A and peptide B) was performed by ELISA according to a previously published protocol (Mantegazza et al., 2002; Borroni et al., 2017). CSF stored at −80°C or in liquid nitrogen was used for anti-GluA3 antibody dosage. The same protocol used for serum was applied to test anti-GluA3 antibody peptide A in CSF, except for plates

(Immulon 4HBX 96-well plates, Dynatech, Germany) and the working dilution (1:2 for CSF and 1:4500 for the secondary antibody) (Borroni et al., 2017).

**2.2 Biochemical assays.** The temporal (n=6) and occipital (n=5) cortex from FTLD patients were obtained from The Netherlands Brain Bank, Netherlands Institute for Neuroscience, Amsterdam (open access: www.brainbank.nl). All material has been collected from donors for or from whom a written informed consent, for research purpose, had been obtained by the Netherlands Brain Bank. We considered patients with FTLD-Tau to obtain a more homogeneous sample and on the basis of our previous findings on the effect of anti-GluA3 antibodies on Tau metabolism (Borroni et al., 2017). Western blot analysis was performed in total homogenate and Triton insoluble postsynaptic fractions (TIF). Briefly, brain specimens were manually homogenized twice in lysis buffer (Sucrose 0.32 M, Hepes 1 mM, Magnesium Chloride 1 mM, Sodium carbonate 1 mM) and centrifuged at 900xg for 15 min at 4°C. The resulting supernatants were pooled and centrifuged 13,000xg for 15 min at 4°C. Pellets were then resuspended in 1 mM Hepes and ultra-centrifuged 100,000xg for 1 hr at 4°C. Precipitates were dissolved, incubated for 15 min in 150 mM potassium chloride, 0.5% Triton and ultra-centrifuged again 100,000xg for 1 hr at 4°C. The final pellets (TIF) were homogenized with a glass-glass potter in Hepes 20 mM buffer. All purification steps were performed in the presence of protease and phosphatase inhibitor cocktails (Complete$^{TM}$, Roche Diagnostics, Monza, Italy). After separation by SDS-PAGE on an 8% gel under denaturing conditions, the proteins were electrotransferred to nitrocellulose membrane. Membranes were blocked with I-block solution (Invitrogen, Thermo Scientific, Milan, Italy) and incubated overnight with primary antibodies (anti-GluA1 antibody, Cell Signaling, BK131855, 1:500; anti-GluA2 antibody, Neuromab #75-002, 1:500; anti-GluA3 antibody, Synaptic System, #182203, 1:1,000; anti-GluA1 p845, Abcam, ab76321, 1:1,000; anti-GRIP1 antibody, Abcam, Ab25963, 1:500; anti-PICK1 antibody, Abcam, Ab3420, 1:500; anti-Tubulin antibody, Sigma Aldrich, T9026, 1:5,000; anti-GAPDH antibody, Santa Cruz, Sc-25778, 1:2,500), followed by incubation with horseradish peroxidase-linked anti-rabbit or anti-mouse IgG antibody (1:5,000, Biorad, Hercules, CA, USA) in TBS containing 0.1% Tween-20 at room temperature for 1h. Finally, proteins were visualized using an electrochemical luminescence (ECL) kit (Clarity Western ECL substrate, Bio-Rad, Hercules, CA, USA or LiteAblot TURBO, Euroclone, Milan, Italy) and the images were obtained using Chemidoc Imaging System (Bio-Rad, Hercules, CA, USA). Quantification was performed using the ImageJ software, and each protein was normalized on the corresponding Tubulin/GAPDH band.

**2.3 Purification of synaptosomes and release experiments.** Mice (male, strain C57BL/6J) were obtained from Charles River (Calco, Italy) and were housed in the animal facility of DIFAR, University of Genoa, under environmentally controlled conditions (ambient temperature = 22°C,

humidity = 40%) on a 12-h light/dark cycle with food and water *ad libitum*. Mice were euthanized by cervical dislocation, and subsequently decapitated, and the hippocampi were rapidly removed. The experimental procedures followed the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC) and the ARRIVE guidelines, and they were approved by the Italian Ministry of Health (DDL 26/2014 and previous legislation; protocol number n° 50/2011-B). Experiments were performed following the Guidelines for Animal Care and Use of the National Institutes of Health and in accordance with the Society's Policies on the Use of Animals and Humans in Neuroscience Research. In line with the 3Rs rules (replacement, refinement and reduction), any effort was made to reduce the number of animals to obtain statistically reliable results. Synaptosomes were prepared by homogenizing the cortex as previously described (Summa et al., 2011). Synaptosomes were re-suspended in a physiological solution with the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; $NaH_2PO_4$, 1.2; $NaHCO_3$, 5; HEPES, 10; glucose, 10; pH 7.2-7.4 and incubated at 37° C for 30 min in the presence of the patients' sera (dilution 1:200 to 1:100). After 15 minutes, [$^3$H]D-aspartate ([$^3$H]D-ASP, (specific activity 11.3 Ci/mmol,Perkin Elmer Boston, MA, USA, f.c.: 50 nM) was added to the incubation suspension. Synaptosomes were layered on microporous filters at the bottom of parallel thermostated chambers in a Superfusion System (Pittaluga, 2016). Synaptosomes were superfused at 0.5 ml/min for 36 minutes with a standard physiological solution to equilibrate the system and then for 3 minutes fractions were collected for monitoring tritium release. At t = 39 min of superfusion, synaptosomes were exposed to 50 µM D-AMPA (Tocris Bioscience, Bristol, UK) till the end of superfusion.

**2.4 Glutamate neurotransmission assessment *in vivo* by Transcranial Magnetic Stimulation (TMS).** For all participants, informed consent in the study was obtained according to sampling protocols that were approved by Ethics Committee of Brescia Hospital, Italy. The study was conducted in accordance with the Helsinki Declaration. TMS was performed with a figure-of-eight coil (each loop diameter, 70mm) connected to a Magstim Bistim$^2$ system (Magstim Company, Oxford, UK). The magnetic stimuli had a monophasic current waveform (rise time of 100µs, decaying back to zero over 800µs). Motor evoked potentials (MEPs) were recorded from the right first dorsal interosseous (FDI) muscle through surface Ag/AgCl electrodes placed in a belly- tendon montage and acquired using a Biopac MP-150 electro- myograph (BIOPAC Systems Inc., Santa Barbara, CA). The TMS coil was held tangentially over the scalp region corresponding to the primary hand motor area contralateral to the target muscle, with the coil handle pointed 45 degrees posteriorly and laterally to the sagittal plane. The motor hotspot was defined as the location where TMS consistently produced the largest MEP size at 120% of the resting motor threshold (rMT) in

the target muscle and was marked with a felt-tip pen on the scalp to ensure stable placement of the coil throughout the experiment. rMT was defined as the minimal stimulus intensity needed to produce MEPs with an amplitude of at least 50lV in 5 of 10 consecutive trails during complete muscle relaxation, which was controlled by visually checking the absence of electromyography (EMG) activity at high-gain amplification, as previously published (Benussi et al., 2016). Intracortical facilitation (ICF), which has been shown to be likely mediated by glutamatergic receptors, was studied at rest with a paired-pulse paradigm, delivered in a conditioning-test design, as previously reported. Briefly, the conditioning stimulus (CS) was set at an intensity of 70% of the rMT, whereas the test stimulus (TS) was adjusted to evoke a MEP approximately 1mV peak-to-peak in the relaxed FDI. An interstimulus interval (ISI) of 7 ms between the CS and TS was applied (Ziemann et al., 1996). Ten stimuli were delivered for each ISI and 14 control MEPs in response to the TS alone were recorded in all participants.

**2.5 HPLC detection of D-/L-serine and L-glutamate levels.** Human CSF samples (100 µl) were mixed in a 1:10 dilution with HPLC-grade methanol (900 µl) and centrifuged at 13,000xg for 10 min. Supernatants were dried, suspended in 0.2 M TCA and then neutralized with NaOH. Samples were then subjected to pre-column derivatization with o-phthaldialdehyde/N-acetyl-L-cysteine. Diastereoisomer derivatives were resolved on a Simmetry C8 5-µm reversed-phase column (Waters, 4.6x250 mm).

**2.6 Statistical analysis.** For HPLC experiments identification and quantification of D-Ser, L-Ser, and L-Glu were based on retention times and peak areas, compared with those associated with external standards. Amino acids level was expressed as µM, while the D-/total serine ratio was expressed as percentage (%). Statistical analyses were performed by Mann-Whitney or Kruskal-Wallis test followed by Dunn's test, when required.

For release experiments the amount of radioactivity released into each superfusate fraction was expressed as fractional efflux. The AMPA-evoked release of tritium was quantified as percent increase over basal release. Analysis of variance was performed by ANOVA followed by Dunnett's multiple-comparisons test. The level of significance was set at $p < 0.05$.

For western blot experiments, significance of the differences was determined by unpaired Student's t-test.

For TMS experiments, a one-way ANCOVA was run to determine the difference in intracortical connectivity between groups, corrected for disease severity (using the frontotemporal lobar degeneration-modified clinical dementia rating scale, FTLD-CDR). When a significant main effect was reached, *post-hoc* tests with Bonferroni correction for multiple comparisons were conducted to

analyze individual group-differences. Statistical significance was assumed at p<0.05. Data analyses were carried out using SPSS 21.0 software.

**2.7 Data availability** The data that support the findings of this study are available from the corresponding author, upon reasonable request.

# 3 Results

## 3.1 Effect of GluA3 antibody on AMPA receptor subunit composition in the temporal cortex of FTLD patients

Post-mortem specimens of temporal and occipital cortex were analyzed to evaluate the effects induced by GluA3 autoantibodies on the glutamatergic synapse. To this, we firstly analyzed 10 CSF samples for which autopsy was available, and we detected the presence of anti-GluA3 antibodies in 5 out of 10 samples (see Table 1). Among these, we then considered post-mortem specimens from 3 FTLD patients with CSF anti-GluA3 antibodies and 3 FTLD patients without CSF anti-GluA3 antibodies for further analyses.

We previously reported that administration of CSF containing anti-GluA3 antibodies results in a significant decrease of the GluA3 subunit levels at postsynaptic sites both in rat primary hippocampal neurons and in cortical neurons obtained from human iPSCs (Borroni et al., 2017). Similarly, here we found that FTLD GluA3_Ab+ patients showed a significant reduction in GluA3 levels in a postsynaptic fraction purified from the temporal cortex (\*\*p=0.0038, unpaired Student's t-test, vs. GluA3_Ab-, Fig. 1A), whereas there was no difference in the postsynaptic levels of GluA1 (p=0.3702, unpaired Student's t-test, vs. GluA3_Ab-, Fig. 1A), GluA2 (p=0.3042, unpaired Student's t-test, vs. GluA3_Ab-, Fig. 1A) AMPA receptor subunits, and in the phosphorylated form of GluA1 (Ser845, p=0.1297, unpaired Student's t-test, vs. GluA3_Ab-, Fig. 1A), a well-validated marker of synaptic plasticity (Oh et a., 2006; Marcello et al., 2013; Song et al., 2017). Conversely, western blotting performed in the total homogenate reveals a significant increase in GluA2 subunit levels in GluA3_Ab+ patients (\*p<0.05, unpaired Student's t-test vs. GluA3_Ab-, Fig. 1B), and no changes in GluA1 and GluA3 subunits. We considered the occipital cortex of the same patients as an internal control area not affected by this disease. Analysis of postsynaptic fractions (Fig. S1A) and total homogenate (Fig. S1B) obtained from the occipital cortex, did not show any significant difference in AMPA receptor subunits between GluA3_Ab+ or GluA3_Ab- patients. Overall, these results argue for a biological effect of anti-GluA3 antibodies on the postsynaptic levels of GluA3-containing AMPA receptors.

The mechanisms involved in the synaptic retention of AMPA receptors have been largely elucidated and protein-protein interaction plays a key role in these events (Diering and Huganir, 2018; Pick and Ziff, 2018). Mainly, two scaffolding proteins are involved in receptor insertion/internalization at the postsynaptic membrane, namely PICK1 and GRIP1 (Diering and Huganir, 2018). The first is involved in the regulated removal of AMPA receptors from the synaptic plasma membrane (Hanley et al., 2008) while the second is necessary for the anchorage of the receptor at the postsynaptic density (Anggono and Huganir, 2012). In accordance with a reduced expression of GluA3 at the synapse, PICK1 levels were significantly increased in both fractions (+88% postsynaptic fraction, **p=0.0031, unpaired Student's t-test vs. GluA3_Ab-, Fig. 2A; +47% homogenate, *p=0.0486 unpaired Student's t-test vs. GluA3_Ab- Fig. 2B, respectively). Moreover, we observed in GluA3_Ab+ patients a decrease of GRIP1 in both the postsynaptic density and in the total homogenate from frontotemporal cortex (-55% and -76% respectively), although this effect did not reach a statistical significance (Fig. 2A, p=0.1545, unpaired Student's t-test vs. GluA3_Ab-; Fig. 2B, p=0.1147, unpaired Student's t-test vs. GluA3_Ab-). Accordingly, quantification of PICK1/GRIP1 ratio highlighted a 4-fold increase GluA3_Ab+ patients both in the postsynaptic compartment and in the total homogenate (*p<0.05, unpaired Student's t-test vs. GluA3_Ab-, Fig. 2A,B). In agreement with the absence of any alteration of AMPA receptor subunits (Fig. S1A,B), no effect was detected in the levels of both GRIP1 and PICK1 in the occipital cortex of GluA3_Ab+ (Fig. S2A,B). No significant alteration was observed in GluA3_Ab+ patients on the synaptic (Fig. 3A) and total levels (Fig. 3B) of NMDA-type receptor subunit in the temporal cortex thus suggesting that GluA3 antibodies target AMPA receptors without interfering with other glutamate receptor subtypes.

## 3.2 Effect of GluA3 antibody on the AMPA-evoked glutamate exocytosis from mice cortical synaptosomes

To further investigate the role of GluA3 autoantibodies on glutamatergic neurotransmission, we evaluated the effects of the CSF from GluA3_Ab+ patients (n=4) with low [0.100 optical density (OD), 450nm] to high (0.341 OD, 450nm) antibody titer compared to the CSF of GluA3_Ab- patients (n=4) on the (S)-AMPA-evoked glutamate exocytosis from mice cortical synaptosomes. Glutamate exocytosis was quantified as the release of preloaded [3H]D-Aspartate ([3H]D-Asp), an un-metabolizable analogue of glutamate that allows a reliable measurement of the release of glutamate from cortical nerve endings (Grilli et al., 2004). Previous data showed the existence in cortical synaptosomes of presynaptic release-regulating AMPA receptors, the activation of which elicits the $Ca^{2+}$-dependent, exocytotic-like release of glutamate (Pittaluga et al., 1997). Incubation

of synaptosomes with the CSF of GluA3_Ab- patients (1:100 to 1:200 dilutions) failed to significantly modify the AMPA-evoked release of [3H]D-Asp. Whereas, incubation of synaptosomes with GluA3_Ab+ patients CSF reduced the AMPA-evoked tritium release in a dilution-dependent fashion (Fig. 4A). These results were further analyzed by correlating the reduction of the AMPA-induced release efficiency in synaptosomes exposed to selected dilutions (1:200 and 1:100, Fig. 4B and 4C respectively) of the 8 (4 Ab+ and 4 Ab-) CSF patients and the anti-GluA3 antibody titer. In both cases, the analysis unveiled a strict correlation between the two parameters as clearly indicated by the coefficient of linear correlation analysis (1:200, $r^2 = 0.8764$; 1:100, $r^2 = 0.9072$).

Overall these data indicate that the presence of GluA3 autoantibodies affect glutamatergic neurotransmission at both pre- and post-synaptic sites, decreasing glutamate release and altering GluA3-containing AMPA receptor levels.


**3.3 Assessment of glutamatergic intracortical circuits with Transcranial Magnetic Stimulation**

It is well-known that a physiological AMPA receptor activity plays a pivotal role in the functional regulation of glutamate neurotransmission in the central nervous system (Diering and Huganir, 2018). Transcranial Magnetic Stimulation (TMS) has become a promising tool to assess specific cortical circuits in the central nervous system (Benussi et al., 2017). Specifically, TMS was used to investigate intracortical facilitation (ICF), a paired-pulse protocol which induces facilitation of the motor evoked potential, likely mediated by excitatory glutamatergic receptors, allowing the indirect and in-vivo assessment of these circuits (Benussi et al., 2017). Analysis of peak ICF (ISI 7 ms) was significantly reduced in 111 FTD patients [including both GluA3_Ab+ (n=37) and GluA3_Ab- (n= 74) patients], compared to a group of 70 age-matched healthy controls, [$F_{(1,179)}=219.5$, partial $\eta^2=0.55$, $p<0.001$; Fig. 5A] (see Table 2 for patients' demographic characteristics). Moreover, one-way ANCOVA, corrected for disease severity (frontotemporal lobar degeneration-modified clinical dementia rating scale, FTLD-CDR), showed a significant difference in ICF between groups [$F_{(2,177)}=78.5$, partial $\eta^2=0.47$, $p<0.001$; Fig. 5B]. *Post hoc* analysis with Bonferroni corrections showed that ICF was significantly reduced in both GluA3_Ab+ and GluA3_Ab- patients compared to healthy controls ($p<0.001$) and was significantly reduced in GluA3_Ab+ compared to GluA3_Ab- patients ($p=0.010$; Fig. 5B), with a decreased facilitation in GluA3_Ab+ patients (mean difference of $0.14\pm0.05$).


**3.4 Increased D-/L-serine and L-glutamate content in the cerebrospinal fluid of patients affected by FTD**

In order to investigate the homeostasis of the glutamatergic neurotransmission in FTD patients, we measured the L-glutamate (L-Glu) content as well as the levels of NMDAR co-agonist D-serine (D-Ser) and its L-enantiomer, L-serine (L-Ser) in CSF of 50 FTD patients and 23 healthy controls (see Table 2). HPLC analysis showed a significant increase of D-Ser amount in the CSF of FTD patients compared to control subjects (Mann-Whitney test, Ctrl *vs* FTD, $p = 0.0003$; Fig. 6A). Similarly, we observed higher levels of L-serine (L-Ser) in FTD patients, compared to control individuals (Mann-Whitney test, Ctrl *vs* FTD, $p = 0.0001$; Fig. 6B). Accordingly, no main changes in the D-/total serine ratio were found between the two groups (Mann-Whitney test, $p = 0,7104$; Fig. 6C). Remarkably, we found a significant increase of L-Glu levels in FTD patients compared to control subjects (Mann-Whitney test, Ctrl *vs* FTD, $p = 0.0004$; Fig. 6D).

Next, we evaluated the effect induced by the expression of GluA3 autoantibodies on D-/L-Ser and L-Glu levels in the CSF samples of FTD patients. To this aim, we subdivided the FTD group in two experimental groups composed by FTD subjects positive (GluA3_Ab+, n = 24) and FTD negative (GluA3_Ab-, n = 26) for the anti-GluA3 antibody (see Table 2). We found significant increased levels of D-/L-Ser and L-Glu in both GluA3_Ab+ and GluA3_Ab- FTD patients, as compared to controls, but we failed to find out significant differences between GluA3_Ab+ and GluA3_Ab- subgroups (see Fig 6, E-H; Fig. 6E, Kruskal-Wallis test, $p = 0,0017$; Dunn's test, Ctrl *vs* FTD GluA3_Ab+, $p = 0.0038$; Ctrl *vs* FTD GluA3_Ab-, $p = 0.0010$; Fig. 6F, Kruskal-Wallis test, $p = 0,0010$; Dunn's test, Ctrl *vs* FTD GluA3_Ab+, $p = 0.0007$; Ctrl *vs* FTD GluA3_Ab-, $p = 0.0021$; Fig. 6G, Kruskal-Wallis test, $p = 0,7792$; Fig. 6H, Kruskal-Wallis test, $p = 0,0024$; Dunn's test; Ctrl *vs* FTD GluA3-Ab+, $p = 0.0029$; Ctrl *vs* FTD GluA3-Ab-, $p = 0.0022$).

**4 Discussion**

In the present study, we demonstrated that anti-AMPA antibodies mediate *in vivo* detrimental effects inducing a complex alteration of glutamatergic neurotransmission in FTD (see Fig. 7). In particular, results here presented demonstrate that anti-GluA3 antibodies lead to a reduction in the postsynaptic expression of GluA3-containing AMPA receptors in the temporal cortex of FTLD patients. This evidence is in accordance with our previous *in vitro* studies demonstrating that administration of human anti-GluA3 antibodies results in a significant decrease of the GluA3 subunit levels at postsynaptic sites in both rat primary neurons and in neurons derived from human iPSCs (Borroni et al., 2017).

GluA3 is a highly relevant subunit of AMPA receptors in the brain and a high proportion of cortical AMPA receptors contain this subunit (Schwenk et al., 2014). Importantly, GluA3-containing AMPA receptors are, in general, uniformly enriched in the synapse, and only rarely are distributed

peri-synaptically (Jacob and Weinberg, 2015). From a functional point of view, GluA2/GluA3 AMPA receptors are recruited in a constitutive manner to synapses, where they can replace GluA1-containing receptors that are usually added to synaptic membranes during plasticity (Shi et al., 2001). Previous reports obtained in mice models addressed a specific role for GluA3 in Alzheimer's Disease. GluA3 ko mice are protected against Aβ-driven synaptic deficits and memory impairment. In particular, Aβ trigger the synaptic removal of GluA3-containing AMPA receptors (Reinders et al., 2016). Moreover, knocking out of the *GRIA3* gene encoding the GluA3 subunit produces alteration of social and aggressive behavior in mice (Adamczyk, 2012).

Similar to our data on anti-GluA3 antibodies, a very recent study from Haselmann and coworkers (2018) showed that human GluA2 antibodies induce AMPA receptor internalization and a consequent decrease of the synaptic abundance GluA2-containing AMPA receptors. This mechanism leads also to an impairment of long-term synaptic plasticity and affects learning and memory. Furthermore, administration of human anti-NMDA antibodies to mice, induces with a similar mechanism a progressive and selective decrease of NMDAR synaptic clusters (Planagumà et al., 2015; Olivero et al., 2019).

It is well known that the synaptic pool of AMPA receptors is highly dynamic, undergoing activity-dependent endo/exocytosis events through PDZ-mediated interaction with GRIP1 and PICK1 (Anggono and Huganir, 2012; Diering and Huganir 2018). PICK1 mediates the depletion of GluA2/3 AMPARs from synapses (Kim et al., 2001; Terashima et al., 2008), while GRIP1 anchors the receptors in the postsynaptic density (Anggono and Huganir, 2012). Here we show that the reduced GluA3 localization at synapses in the temporal cortex of anti-GluA3 positive FTLD patients is accompanied by a 4-fold increase PICK1/GRIP1 ratio. This observation indicates that GluA3 antibodies promote endocytosis of the receptor subunit, probably through interaction with PICK1. Moreover, we observed that the presence of GluA3_Ab+ decreases glutamate release from synaptosomes in a dose-dependent manner. Accordingly, it is possible to state that the presence of GluA3 antibodies can affect glutamate neurotransmission acting both at the presynaptic terminal, by reducing glutamate release, and at dendritic spines lowering the availability in the postsynaptic membranes of AMPA-type glutamate receptors.

The above results were further corroborated by neurophysiological techniques, confirming the harmful effect of anti-GluA3 antibodies in FTD patients. Taking into account the key role of AMPA receptors in the regulation of glutamate neurotransmission (Diering and Huganir, 2018), in the present study we used TMS to investigate intracortical facilitation (ICF) and to perform an *in vivo* assessment of excitatory glutamatergic circuits (Benussi et al., 2017). 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). Here, we observed 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. This observation may be predicting of a more pronounced impairment of glutamatergic neurotransmission in presence of the GluA3 antibody.

Finally, we carefully characterized L-Glu, L-Ser and D-Ser levels in the CSF of FTD patients. A recent report from Madeira and coworkers (2018), performed on a limited number (N=14) of FTD patients, found a mild increase in D- and L-serine levels in Alzheimer's disease patients but not in FTD affected subjects. Conversely, in the present work, we detect that the altered synaptic AMPA receptor composition and the impaired glutamate neurotransmission observed in FTD patients were accompanied by a significant increase in the CSF levels of D-Ser, L-Ser and L-Glu. Notably, the presence of anti-GluA3 antibodies does not induce any significant difference in the levels of L-Glu, D-Ser, and L-Ser. These results may indicate a compensatory process aimed to balance the reduced AMPA-mediated transmission in FTD patients. However, even if these neurochemical results combined with the TMS analysis can represent a novel potential biomarker in FTD, further studies are surely needed to evaluate the mechanisms involved in these events.

Overall, our results are in agreement with several recent preclinical (Udagawa et al., 2015; Decker et al., 2016; Longhena et al., 2017) and clinical studies (Leuzy, 2015; Benussi et al., 2017, 2019) indicating a key role of glutamate receptors and glutamate neurotransmission in the pathogenesis of FTD and assign a specific role for anti-GluA3 antibodies in a subgroup of these patients. As in the other autoimmune disorders of the Central Nervous System, as Rasmussen or anti-NMDA encephalitis (Esposito et al., 2019), here we observe a selective neuronal vulnerability confined to the temporal cortex, while the occipital cortex is spared. However, the mechanism(s) leading to autoimmune response to AMPA receptors in FTD needs further investigations: it might be hypothesized an immune-mediated genetic enrichment, particularly within the HLA region, or a predisposition related to specific protein misfolding. Despite this, restoration of a physiological glutamatergic transmission should be taken into account and might be obtained by acting at the AMPA-type glutamate receptors as well as at the immune system.

In conclusion, we can hypothesize that an immune system dysregulation might result into an abnormal production of autoantibodies directed against the GluA3 subunit, causing a complex dysfunction in glutamatergic transmission, potentially associated to tau or TDP-43 deposition in FTLD. Accordingly, the role of glutamate in the brain circuits represents an interesting and innovative approach to: (i) better understand the neurodegenerative process in FTD; (ii) discover

new strategies to revert or slow disease progression through the modulation of glutamatergic pathway via immune system.

## Conflict of interest

The authors declare that they have no competing interests.

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**Figure Legends**

**Figure 1. Neurobiological effect of GluA3 antibody on AMPA receptor's composition in temporal cortex of FTLD patients.**

A, B   Western blot quantification of GluA1, GluA2, GluA3 and phosphorylated ser845 GluA1 subunit in TIF fraction (A) and total homogenate (B) obtained from frontotemporal cortex of patients with (Ab+, closed bars) or without (Ab-, open bars) anti-GluA3 antibodies. Left panel: representative blot; right panel: densitometric quantification. Tubulin was used for normalization. *p<0.05, **p<0.01, unpaired Student's t-test (mean of 3 experiments with n=3).

**Figure 2. Neurobiological effect of GluA3 antibody on AMPA receptor subunit-interacting proteins in frontotemporal cortex of FTD patients.**

A, B   Western blot quantification of PICK1, GRIP1, and their ratio, in TIF fraction (A) and in total homogenate (B) obtained from frontotemporal cortex of patients with (Ab+, closed bars) or without (Ab-, open bars) anti-GluA3 antibodies; left panel representative blot; right panel densitometric quantification. Tubulin was used for normalization. *p<0.05, **p<0.01, unpaired Student's t-test (mean of 3 experiments with n=3).

**Figure 3. Neurobiological effect of GluA3 antibody on NMDA receptor's composition in frontotemporal cortex of FTD patients.**

A, B   Western blot quantification of NMDAR1, NMDA2A, NMDA2B and NMDA2D subunit in TIF fraction (A) and total homogenate (B) obtained from frontotemporal cortex of patients with (Ab+, closed bars) or without (Ab-, open bars) anti-GluA3 antibodies. Left panel: representative blot; right panel: densitometric quantification. Tubulin was used for normalization. (mean of 3 experiments with n=3).

**Figure 4. Neurobiological effect of GluA3 antibody on the AMPA-evoked glutamate exocytosis from mice cortical synaptosomes.**

A       (S)AMPA (50 µM) evoked $^3$H[D-Asp exocytosis from synaptosomes incubated in the absence (grey bar) or in the presence of CSFs (dilution as indicated) from patients without (Ab-,

black bar, n=4) and with Ab+, (white bar, n=4) anti-GluA3 antibodies. Results are expressed as AMPA-evoked tritium overflow. Data are the means ± SEM of three experiments (run in triplicate) for each CSF. *p<0.05, one-way ANOVA analysis followed by Dunnett's test.

B, C    Correlation between the Ab- (closed symbols) and Ab+ (open symbols, 1:200 dilution) CSF-induced changes to the AMPA-evoked release of tritium (expressed as % of inhibition) and the respective anti-GluA titer for each CSF. The linear regression analysis coefficient ($r^2$) is reported within the plot. (C) as for B but the CSF is diluted 1:100.

## Figure 5. Indirect assessment of glutamatergic intracortical circuits with Transcranial Magnetic Stimulation.

A       Peak ICF (7 ms interstimulus interval) in FTD patients (both GluA3+ and GluA3-) and age-matched healthy controls (Ctr).

B       Peak ICF in FTD GluA3+ and GluA3- patients compared to age-matched healthy controls (Ctr). ***$p < 0.001$; **$p<0.005$.

## Figure 6. Increased D-/L-serine and L-glutamate content in the cerebrospinal fluid of patients affected by Frontotemporal Dementia.

A-D     Content of (A) D-serine, (B) L-serine (C) D-/total serine ratio and (D) L-glutamate in the cerebrospinal fluid of the entire cohort of FTD patients (FTD, n = 50) and control subjects (Ctrl, n= 23). ** $p<0.01$, *** $p<0.0001$, compared to control group (Mann-Whitney test).

E-H     Amount of (e) D-serine, (F) L-serine (G) D-/total serine ratio and (H) L-glutamate in the cerebrospinal fluid of anti-GluA3 positive (+) and negative (-) FTD patients (FTD GluA3_Ab[+], n = 24; FTD GluA3_Ab[-], n = 26) and control individuals (Ctrl, n= 23). ** $p<0.01$, compared to control group (Dunn's test). In each sample, all free amino acids were detected in a single run by HPLC and expressed as μM, while the ratio is expressed as percentage (%). Dots represent the single subjects' values while bars illustrate the means ± SEM.

## Figure 7. Schematic representation of the molecular and functional effects induced by the presence of anti-GluA3 antibodies.

A       Effect of GluA3 antibody on synaptic AMPA receptor subunit composition and glutamate release at excitatory glutamatergic synapses.

B       Reduced Intracortical Facilitation (ICF) as measured by Transcranial Magnetic Stimulation in FTD patients with anti-GluA3 antibodies as compared to FTD patients without anti-GluA3 antibodies.

**Table 1.** Demographic and clinical characteristics of autopsy FTLD-Tau patients

| Patient | GluA3_Ab+ | | | | | GluA3_Ab- | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Age at death, (years) | 60 | 66 | 71 | 53 | 63 | 52 | 64 | 46 | 54 | 49 |
| Age at onset, (years) | 48 | 57 | 62 | 42 | 55 | 46 | 59 | 39 | 52 | 39 |
| Disease duration, (years) | 12 | 9 | 9 | 11 | 8 | 6 | 5 | 7 | 2 | 10 |
| Gender | M | F | F | M | F | M | F | M | F | M |
| Onset symptom | Ex. dysf. | n.a. | behaviour | memory | personal. | memory | behaviour | personality changes | n.a. | personality changes |
| Clinical diagnosis | AD | Pick's | Pick's | AD | Pick's | AD | Pick's | Pick's | Pick's | Pick's |
| CSF GluA3 autoantiboides titer (OD) | 0.032* | 0.510 | 0.041 | 0.022* | 0.241* | 0.009 | 0.001 | 0.001* | 0.001* | 0.001* |

GluA3_Ab+: patients with GluA3 autoantibodies; GluA3_Ab-: patients without GluA3 autoantibodies; M: male; F: female; n.a.: not available; ex. dysf.: executive dysfunction; behavior: behavioural abnormalities; AD: Alzheimer Disease; CSF: cerebrospinal fluid; OD: optical density (internal cut-off>0.019).

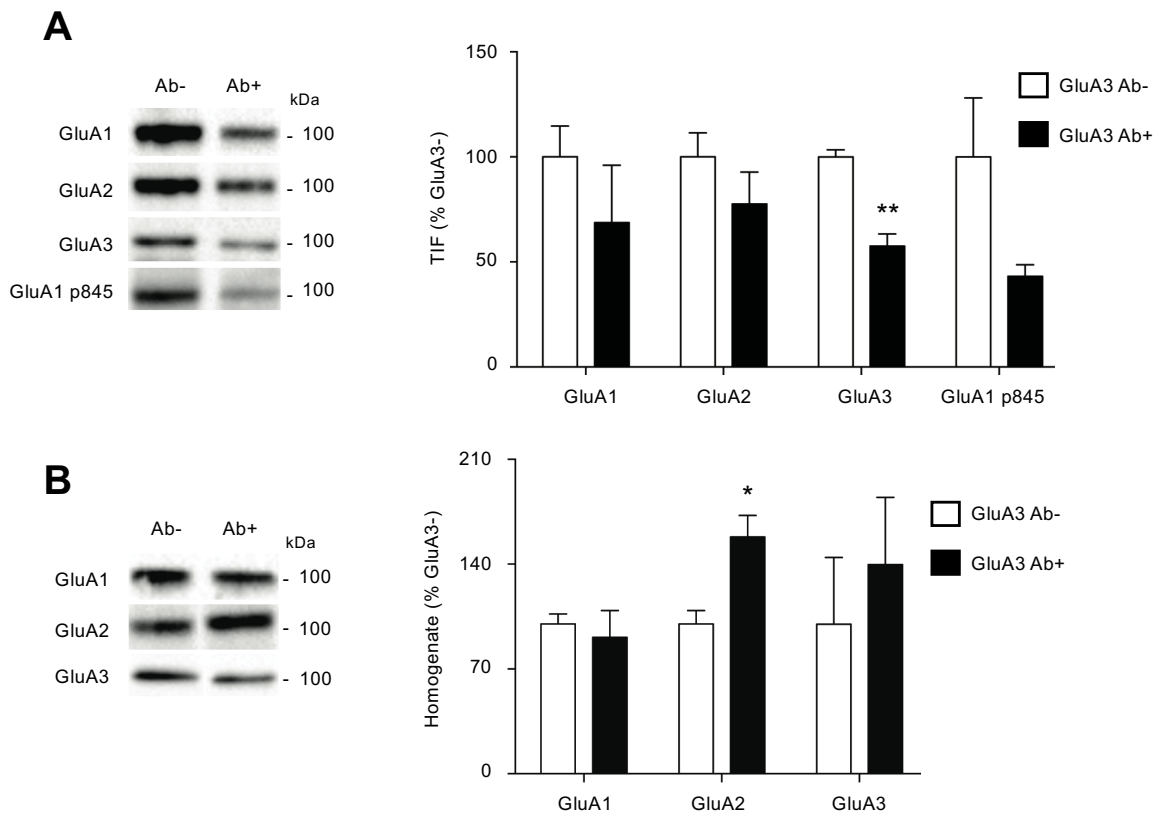*used on autopsy specimens of temporal and occipital cortex (see text for details).

**Table 2.** Demographic and clinical characteristics of autopsy FTLD-Tau patients.

| Variable | Controls | Frontotemporal dementia | | |
|---|---|---|---|---|
| | Total | Total | GluA3_Ab+ | GluA3_Ab- |
| ***TMS parameters evaluation*** | | | | |
| Subjects (N) | 70 | 111 | 37 | 74 |
| Age (mean±SEM of years) | 68.0 ± 1.1 | 65.2 ± 0.8 | 65.0 ± 1.6 | 65.3 ± 0.9 |
| Gender | 27 M, 43 F | 61 M, 50 F | 20 M, 17 F | 41 M, 33 F |
| ***CSF dosages*** | | | | |
| Subjects (N) | 23 | 50 | 24 | 26 |
| Age (mean±SEM of years) | 72.3 ± 2.8 | 68.0 ± 1.1** | 67.5 ± 1.9** | 68.5 ± 1.3** |
| Gender | 17 M, 6 F | 30 M, 20 F | 16 M, 8 F | 14 M, 12 F |
| Smoke | 0 S, 13 NS, 10 N/A | 7 S, 40 NS, 3 N/A | 5 S, 18 NS, 1 N/A | 2 S, 22 NS, 2 N/A |
| Alcohol | 8 U, 5 NU, 10 N/A | 26 U, 21 NU, 3 N/A | 11 U, 12 NU, 1 N/A | 15 U, 9 NU, 2 N/A |

Abbreviations: N=number; M=males; F=Females; S=smokers; NS=non-smokers; U=users; NU=non-users; N/A=not available information. Age and storage duration effects were evaluated by Kruskall-Wallis test, followed by Dunn's test. **$p<0.01$, compared to Controls; ##$p<0.01$, compared to GluR3_Ab+ FTD. Gender, smoke and alcohol effects were evaluated by chi-square test.

# Figure 1

## A



## B

# Figure 2

**A**



**B**

# Figure 3

## A



## B
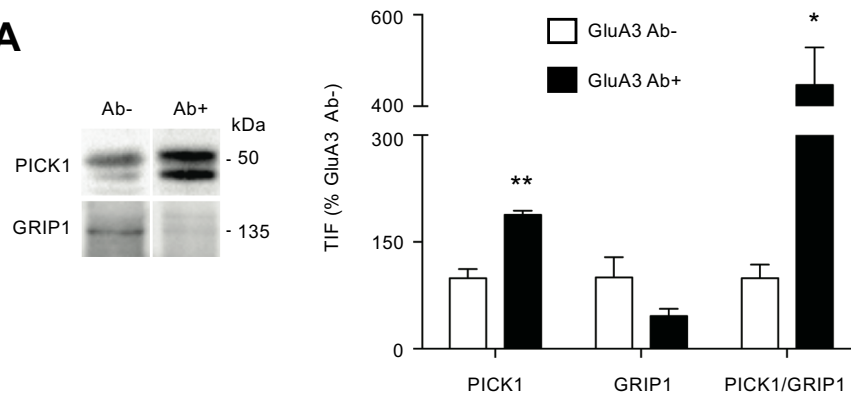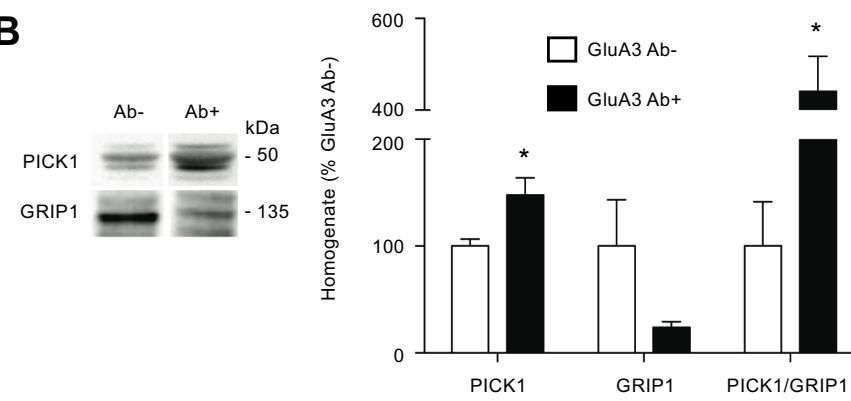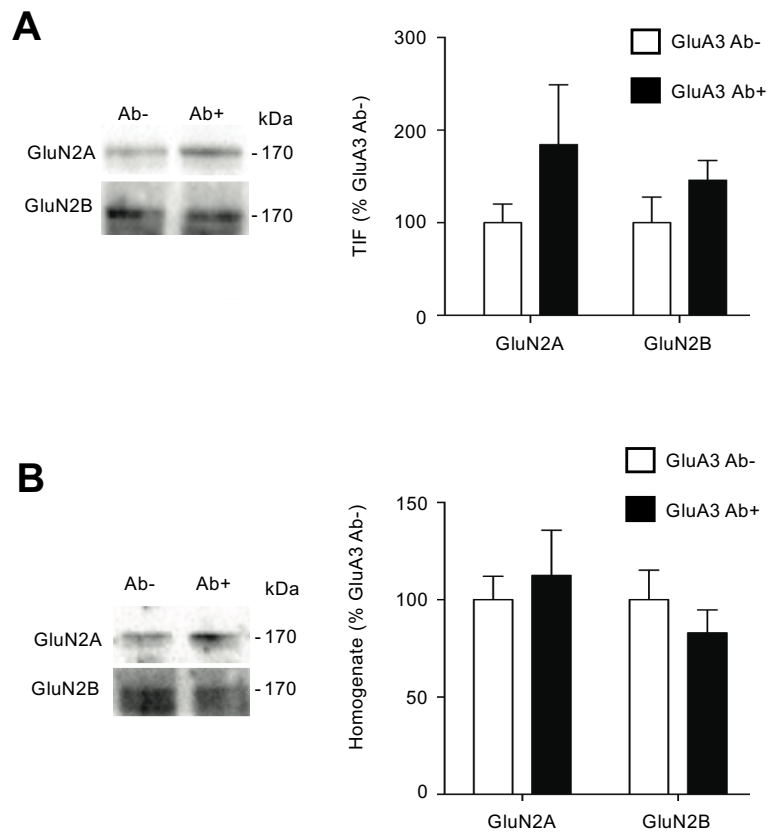
# Figure 4

## A
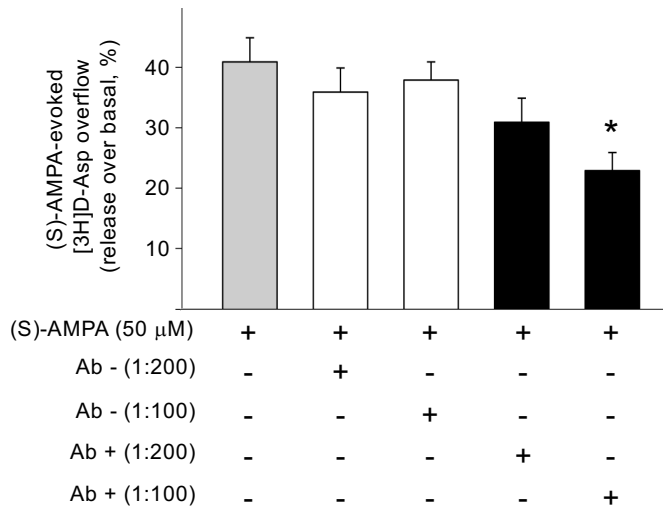


## B
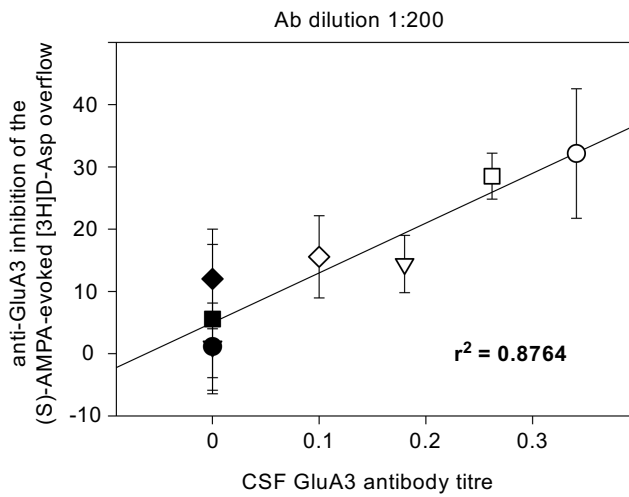


## C
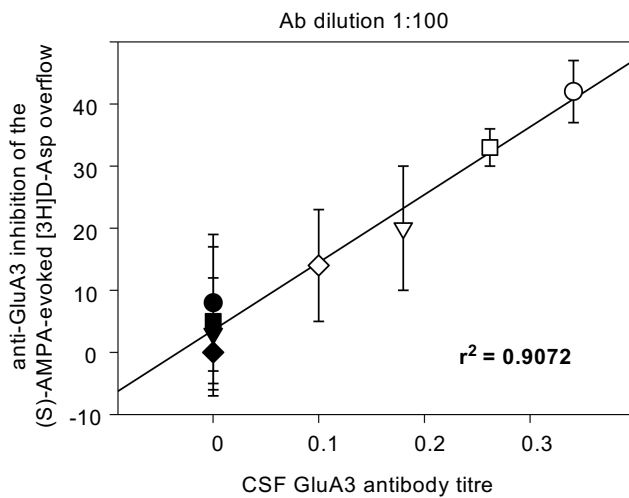
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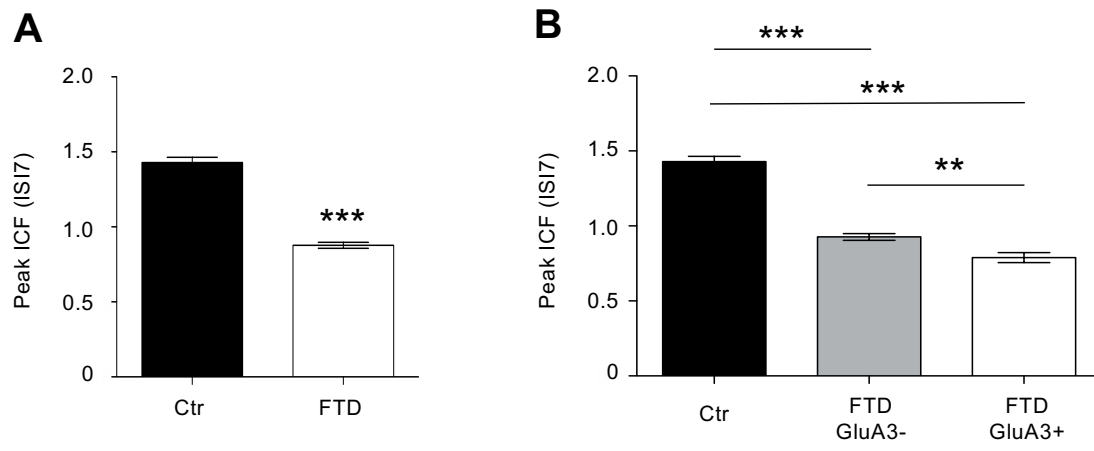
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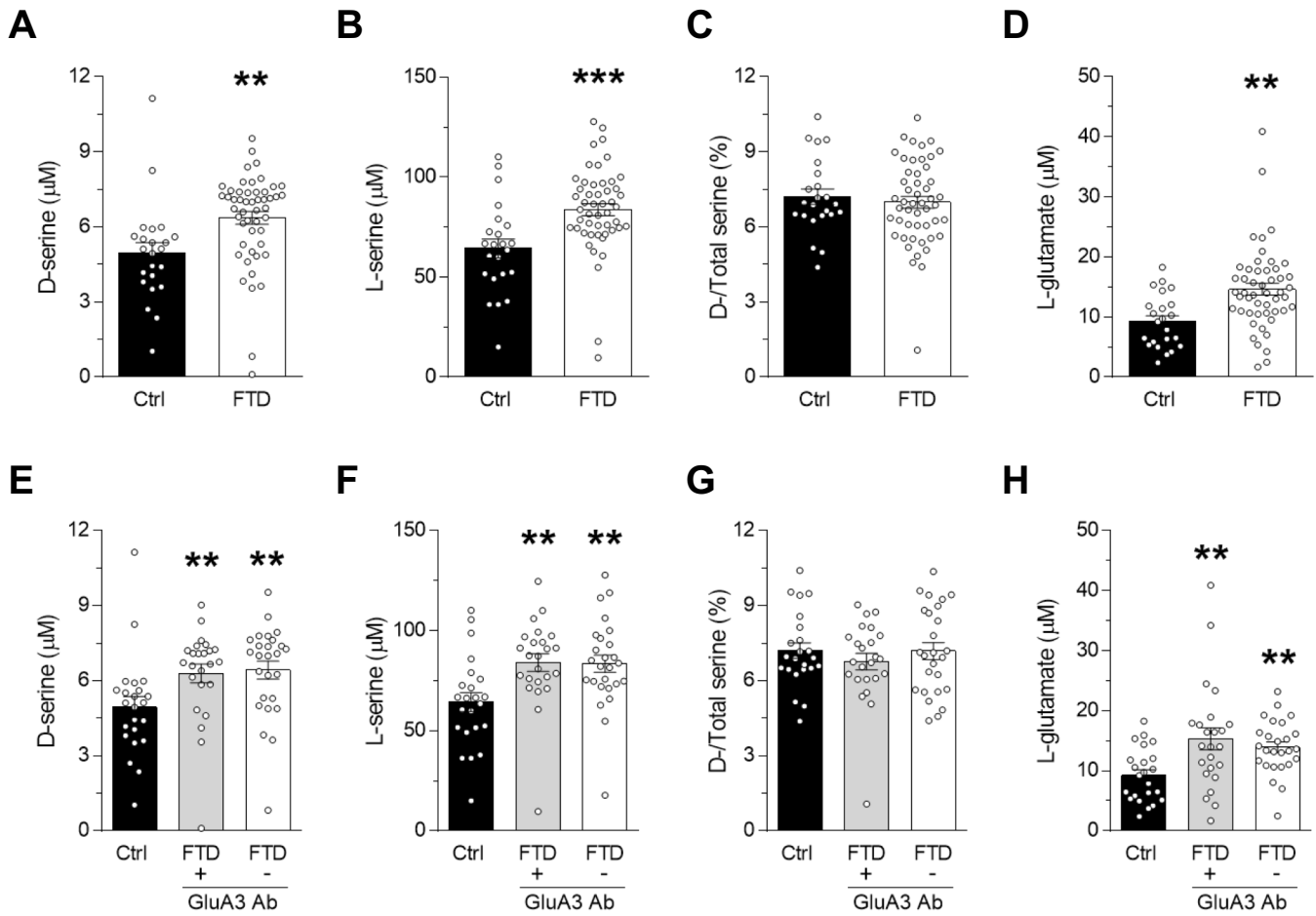
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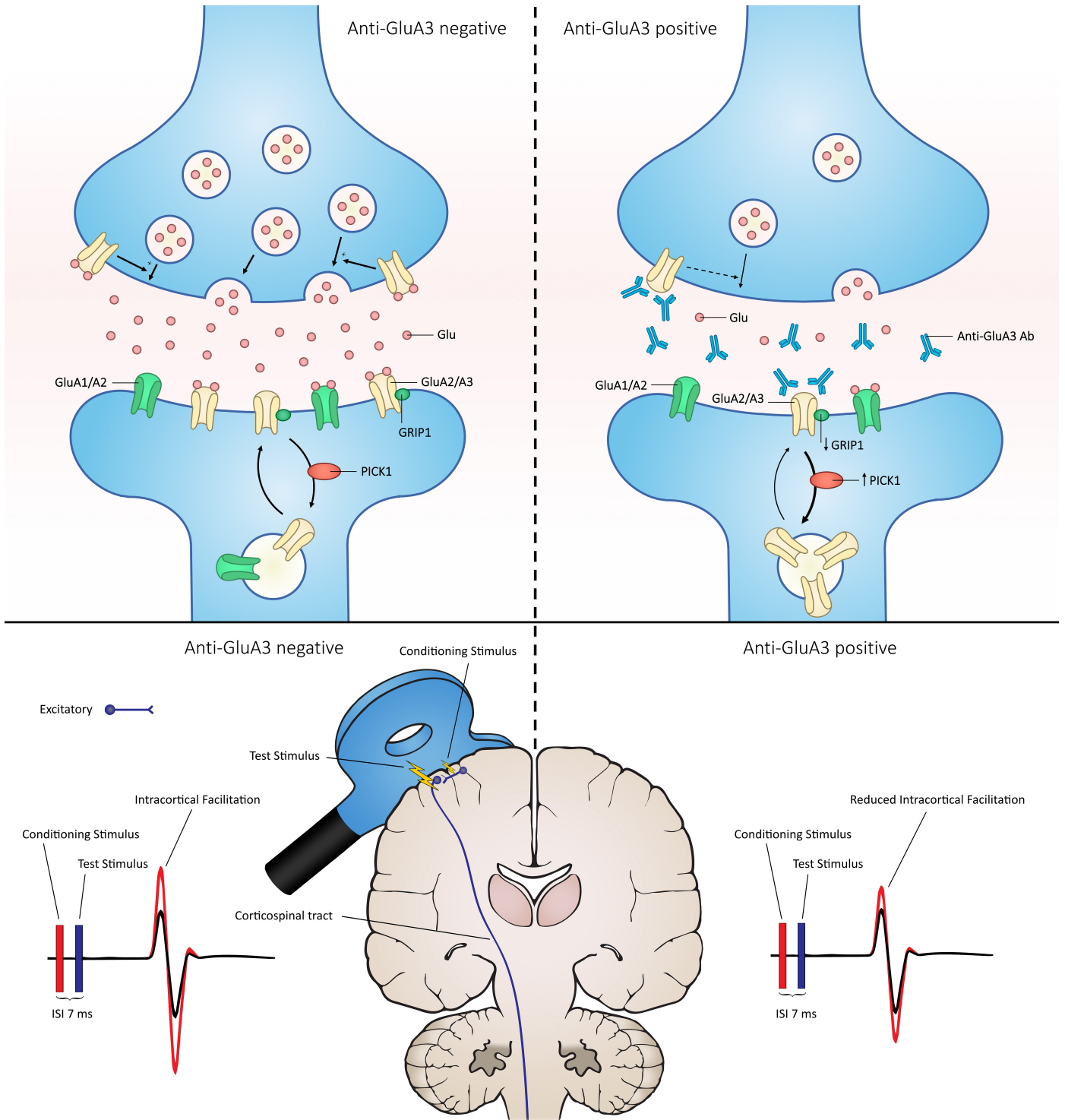


Anti-GluA3 negative

Anti-GluA3 positive

Glu

Anti-GluA3 Ab

GluA1/A2

GluA2/A3

GluA1/A2

GluA2/A3

GRIP1

↓GRIP1

PICK1

↑PICK1

Anti-GluA3 negative

Anti-GluA3 positive

Conditioning Stimulus

Test Stimulus

Excitatory

Intracortical Facilitation

Reduced Intracortical Facilitation

Conditioning Stimulus

Test Stimulus

Conditioning Stimulus

Test Stimulus

Corticospinal tract

ISI 7 ms

ISI 7 ms

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