Group I metabotropic glutamate receptor-mediated long term depression is disrupted in the hippocampus of WAG/Rij rats modelling absence epilepsy

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Abstract

Absence epilepsy is frequently associated with cognitive dysfunction, although the underlying mechanisms are not well understood. Here we report that some forms of hippocampal synaptic plasticity are abnormal in symptomatic Wistar Albino Glaxo/ Rijswijk (WAG/Rij) rats. Metabotropic Glu1/5 receptor-mediated long term depression (LTD) at Schaffer collateral CA1 synapses is significantly reduced in symptomatic, 5-6 months old WAG/Rij rats compared to age-matched non epileptic control rats. There were no significant changes in mGlu1/5-dependent LTD in pre-symptomatic, 4-6 weeks old WAG/Rij rats compared to age matched controls. The changes in LTD found in symptomatic WAG/Rij forms are not indicative of general deficits in all forms of synaptic plasticity as long term potentiation (LTP) was unchanged. Immunoblot analysis of hippocampal tissue showed a significant reduction in mGlu5 receptor expression, a trend to an increase in pan Homer protein levels and a decrease in GluA1 receptor expression in the hippocampus of symptomatic WAG/Rij rats vs non-epileptic control rats. There were no changes in mGlu1α receptor or GluA2 protein levels. These findings suggest that abnormalities in hippocampal mGlu5 receptor-dependent synaptic plasticity are associated with the pathological phenotype of WAG/Rij rats. This lays the groundwork for the study of mGlu5 receptors as a candidate drug target for the treatment of cognitive dysfunction linked to absence epilepsy.

Key words: Group I mGlu receptors; hippocampal synaptic plasticity; Absence epilepsy; WAG/Rij rats

Key points

- mGlu1/5 receptor-mediated long term depression (LTD) at Schaffer collateral CA1 synapses is significantly reduced in symptomatic WAG/Rij rats.

- Variations in expression of mGlu5 receptors and Homer proteins in the hippocampus of WAG/Rij rats might contribute to the deficits in LTD

- No difference in mGlu receptor-mediated LTD between pre-symptomatic and age matched control Wistar rats

- There were no changes in NMDA receptor-dependent LTP in WAG/Rij rats compared to age matched control Wistar rats

- Hippocampal mGlu5 receptor-dependent synaptic plasticity are associated with the pathological phenotype of WAG/Rij rats

Introduction

Absence epilepsy is a generalized, non-convulsive, type of epilepsy characterized by sudden and transient decrease in the level of consciousness and concomitant synchronous bilateral 3-4 Hz spike-and-wave discharges (SWD) in the electroencephalogram (EEG). Seizures, as occurring in childhood absence epilepsy (CAE), start between 3 and 7 years of age, last for 3 to 30 seconds, and may be highly frequent (up to 100 times per day) (Panayiotopoulos, 2001; Jallon et al., 2005; Berg et al. 2013). SWD, as observed in genetic models of absence epilepsy (van Luijtelaar and Zobeiri, 2014), are generated by pathological oscillations within cortico-thalamo-cortical networks comprising the somatosensory cortex (SSCtx), ventrobasal thalamic (VBT) nuclei, and the thalamic reticular nucleus (RTN) (Meeren et al., 2002; Blumenfeld 2005). The RTN functions as a metronome of the circuit, and a pathological enhancement of GABAergic neurotransmission at the synapses between RTN and VBT neurons sustains the activity of T-type voltage-sensitive calcium channels underlying SWDs (Meeren et al., 2002; Blumenfeld 2005). T-type channel inhibitors, such as ethosuximide, are first-line drugs in the treatment of CAE. Although these and other drugs (e.g., clonazepam and to a lesser extent lamotrigine) are efficient in reducing SWDs, >30% of patients with absence epilepsy are resistant to medication (Modi et al., 2011; Tenney and Glauser, 2013; van Luijtelaar et al., 2017).

This drives the search for new drugs in the treatment of absence epilepsy. Metabotropic glutamate (mGlu) receptors modulate synaptic transmission within the cortico-thalamo-cortical circuit and are therefore candidate drug targets (reviewed by Ngomba et al., 2011; Ngomba and van Luijtelaar, 2018). We have studied mGlu1, -2/3, -4 and -5 receptors in Wistar Albino Glaxo/ Rijswijk (WAG/Rij) rats, which develop spontaneous SWDs around 2-3 months of age, and have a high incidence of daily SWDs at 6 months of age (Coenen and van Luijtelaar, 1987; 2003; Schridde and van Luijtelaar, 2004). SWDs in WAG/Rij rats are associated with transient behavioural arrest, and are reduced by conventional anti-absence drugs (Peeters et al., 1988; Depaulis and van Luijtelaar, 2006). Expression and function of mGlu1 and mGlu5 receptors are reduced in the thalamus of symptomatic WAG/Rij rats, and systemic administration of selective mGlu1 or mGlu5 positive allosteric modulators reduces SWD incidence in these animals (Ngomba et al., 2011; D'Amore et al., 2013; 2014). In particular, pharmacological activation of mGlu5 receptors up-regulates the high affinity GABA transporter, GAT-1, and restrains tonic inhibition in the VBT of WAG/Rij rats (Celli et al., 2020), a mechanism that may protect against absence seizures by reducing hyperpolarization in VBT neurons.

One aspect that has not been addressed so far is whether group-I mGlu receptors are involved in the pathogenesis of behavioural and cognitive abnormalities that are associated with absence seizures. Patients with absence epilepsy show impairments in different cognitive domains, such as visuospatial memory, verbal memory, attention, and executive functions (Pavone et al., 2001; Caplan et al., 2008; Seidenberg, 2008; Vega et al., 2010; Bhise et al., 2010, Glauser et al., 2010; Masur et al., 2013; Cnaan et al., 2017). Memory deficits may have a negative impact on the quality of life of patients with absence epilepsy, and are associated with difficulties in academic achievement (Harrison et al., 2013). The association between absence seizures and cognitive function has been investigated in WAG/Rij rats, which show an impairment in spatial memory, short term memory, working memory and reversal learning as compared to non-epileptic control rats (van Luijtelaar et al., 1989; Karson et al., 2012; Jafarian et al. 2015; Malyshev et al., 2012; Leo et al., 2019). In one study, the defective performance

of WAG/Rij rats in the passive avoidance test and Morris water maze test with respect to non-epileptic controls was age-dependent (Karson et al., 2012). Because the incidence or hourly number of the pathological SWDs in WAG/Rij rats increased with age, it is reasonable to hypothesize a cause-to-effect relationship between absence seizures and cognitive impairments. However, the molecular and electrophysiological determinants of cognitive dysfunction in WAG/Rij rats are unknown, and knowledge of these mechanisms is necessary for the study of the pathological link between absence seizures and cognitive impairment. This gave us impetus to study mechanisms of synaptic plasticity in the dorsal hippocampus, a brain region that plays a key role in the formation and retrieval of contextual memory (Nadel et al., 2012; Basu et al., 2015). Group-I mGlu receptors are involved in mechanisms of induction and expression of long-term potentiation (LTP) and long-term depression (LTD) of excitatory synaptic transmission in the CA1 region of the hippocampus and other hippocampal subfields (Bashir et al., 1993; Manahan-Vaughan and Reymann, 1995; Manahan-Vaughan, 1997; Anwyll, 1999; Bortolotto et al., 1999; Manahan-Vaughan and Braunewell, 2005; Bikbaev et al., 2008). Abnormalities in mGlu5 receptor-dependent LTD have been found in mice modelling different forms of monogenic autism (Huber et al., 2002; Bear et al., 2004; Dolen and Bear, 2008; Ronesi et al., 2012; Pignatelli et al., 2014; Gogliotti et al., 2016; Tao et al., 2016; Kelly et al., 2018), and the discovery that mGlu5 receptor-dependent LTD was enhanced in the hippocampus of *fmr-1* gene knockout mice paved the way to the clinical development of mGlu5 receptor negative allosteric modulators for the treatment of fragile-X syndrome (Zeidler et al., 2017). Recent studies have shown that alterations in mGlu5 receptor-dependent LTD change reversal learning and modify cognitive flexibility (Wall et al., 2018; Privitera et al., 2019).

To our knowledge, there are no studies on glutamate-mediated activity-dependent synaptic plasticity in the hippocampus of WAG/Rij rats or other rat or mouse models of absence epilepsy. Here we report that symptomatic WAG/Rij rats exhibit defects in group-I mGlu receptor-mediated LTD in the

CA1 region and reduced hippocampal mGlu5 receptor protein levels as compared to age-matched control Wistar rats.

Materials and Methods

Animals

All experiments were approved by the local Animals Welfare and Ethics Board (AWERB) at the University of Warwick. In this study we used male WAG/Rij rats and male non-epileptic control Wistar rats of 4-6 weeks and at 5-6 months of age (Charles River, UK). Although SWD were not measured, clear, frequent absence episodes could be observed in the WAG/Rij rats at 5-6 months of age. The rats were housed under standard conditions (T = 22°C) with inverted light-dark cycle (light on from 8.00 p.m. to 8.00 a.m.) and food and water *ad libitum*. Animals were culled between 9.00 to 10.00 a.m. for electrophysiological experiments and to obtain tissue for the biochemical experiments.

Electrophysiological recording and analysis

Hippocampal slice preparation

Hippocampal slices (400 μm) were obtained from 4 to 6 weeks-old and from 5 to 6 months-old WAG/Rij and Wistar rats. In accordance with the UK Animals (Scientific Procedures) Act (1986). 4-6 week old rats (which weigh less than 250 grams) were sacrificed by cervical dislocation and decapitated. However at 5-6 months of age, the rats weighed more than 250 grams, and thus were sacrificed by anaesthetic overdose (with isoflurane) and then decapitated. The brain was rapidly removed and placed in ice-cold high Mg^{2+}, low Ca^{2+} artificial CSF (aCSF), consisting of the following (in mM): 127 NaCl, 1.9 KCl, 8 $MgCl_2$, 0.5 $CaCl_2$, 1.2 KH_2PO_4, 26 $NaHCO_3$, 10 D-glucose (pH 7.4

when bubbled with 95% O_2 and 5% CO_2, 300 mOsm/l). Parasagittal brain slices were then prepared using a Microm HM 650V microslicer in ice-cold aCSF (2-4 °C). For LTD experiments, slices were trimmed with the CA3 region removed (to prevent seizure activity). Slices were allowed to recover at 34 °C for 3-6 hr in aCSF (1 mM $MgCl_2$, 2 mM $CaCl_2$) before use.

Extracellular recording from hippocampal slices

Field excitatory postsynaptic potentials (fEPSPs) were recorded from hippocampal slices from WAG/Rij rats and Wistar rats. An individual slice was transferred to the recording chamber, submerged in aCSF (composition as above), maintained at 32 °C, and perfused at a rate of 6 ml/min. The slice was placed on a grid allowing perfusion above and below the tissue and all the tubing was gas tight (to prevent loss of oxygen). To record fEPSPs, an aCSF filled microelectrode was placed on the surface of *stratum radiatum* in the CA1 region. A bipolar concentric stimulating electrode (FHC) controlled by an isolated pulse stimulator, model 2100 (AM Systems, WA) was used to evoke fEPSPs in the Schaffer collateral-commissural pathway. Recordings of mGlu receptor-mediated long term depression (LTD) were made in the presence of 50 μM picrotoxin to block $GABA_A$ receptors (Tocris) and the NMDA receptor antagonist L-689,560 (trans-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline; 5 μM; Tocris). Field EPSPs were evoked at 0.1 Hz (200 ms stimulus), with a 20-min baseline recorded at a stimulus intensity that gave 40% of the maximal response. Metabotropic glutamate receptor mediated-LTD was induced by the group 1 MGluR agonist (RS)-3,5-DHPG (3,5-dihydroxyphenylglycine, Tocris). DHPG (100 μM) was applied for 10 min and then washed off for at least one hour as previously described (Wall et al., 2018). We choose this method to induce LTD rather than synaptic low frequency stimulation as it is more reliable, particularly in the older rats and thus reduced experimental animal numbers. LTP was

induced by using two different stimulation protocols: tetanic stimulation consisting (1s 100 Hz) and theta-burst stimulation which was composed by three trains of 10 bursts at 5 Hz, with inter-train intervals of 20 s. Recordings of fEPSPs were made using a differential model 3000 amplifier (AM systems, WA, USA) with signals filtered at 3 kHz and digitized online (10 kHz) with a Micro CED (Mark 2) interface controlled by Spike software (Vs 7.08), Cambridge Electronic Design, (Cambridge UK). Field EPSPs were analysed using Spike software and graphs prepared using Origin (Microcal), with the slope of fEPSPs measured from a 1 ms linear region following the fibre volley.

Immunoblotting

Western blot analysis was performed as previously described (Zuena et al., 2018). Membranes were probed with the following antibodies: rabbit anti-mGlu5 receptor (EMD Millipore, AB5675, 1:2000), rabbit anti-mGlu1 receptor (Upstate, 07-617, 1:1000), mouse anti-Homer (Santa Cruz, sc-17842, 1:1,000), rabbit β-tubulin (Cell-signalling, 2146S, 1:2000). The following secondary antibodies were used: anti-Mouse IgG (Cell signalling, 7076s, 1:5000) anti-Rabbit IgG (Cell signalling, 7074s, 1:5000).

Statistical Analysis

Statistical analyses applied were the post hoc Mann-Whitney test, Two way ANOVA with Tukey's multiple comparisons. In all studies, n indicates the number of samples per group, and cases in which P-values<0.05 were considered statistically significant. Data presented in figures are means (±SEM).

Results

1. There is a significant reduction in the amplitude of mGlu receptor LTD in the hippocampus of symptomatic WAG/Rij rats compared to age matched non-epileptic control rats

Before measuring synaptic plasticity, we first compared basal synaptic transmission in 5-6 month old symptomatic, WAG/Rij rats with age-matched non epileptic Wistar rat controls. Field excitatory postsynaptic potentials (fEPSPs) were recorded at the Schaffer collateral (SC)-CA1 synapses in the presence of $GABA_A$ and NMDA receptor antagonists (50 μM picrotoxin and 5 μM L689,560 respectively). Neither the stimulus input/output curves (Fig 1A) nor the degree of paired pulse facilitation (Fig 1B) were significantly different between the two strains of rats. Thus basal synaptic transmission in the hippocampus is not affected by the neural changes that underlie the absence symptoms present in the WAG/Rij rats.

Following a 20 minute baseline, mGlu receptor-mediated LTD was induced with the mGlu1/5 orthosteric agonist, DHPG (100 μM) applied for 10 minutes followed by 1 hour of wash. In contrast to the lack of difference in basal synaptic transmission, the DHPG-induced LTD was significantly reduced in WAG/Rij rats (Fig. 1C, two-tailed unpaired Mann-Whitney test $p = 0.0303$, $U = 4$; Fig. 1D, two-tailed unpaired Mann-Whitney test $p = 0.0101$, $U = 2$). At 55-60 minutes after DHPG wash, the mean reduction in fEPSP slope for Wistar rats was 65.46 ± 10.45 % and for WAG/Rij rats was 29.26 ± 4.76%.

2. There is no difference in mGlu receptor-mediated LTD between pre-symptomatic WAG/Rij rats (4-6 weeks of age) and age matched Wistar rats

We first investigated whether there were any differences in basal synaptic transmission between pre-symptomatic (4-6 weeks old) WAG/Rij rats and age-matched Wistar rats. Under these conditions, (in the presence of picrotoxin and L689,560) WAG/Rij rats exhibited a significant reduction in the

input/output relationship, particularly at higher stimulus strengths (above 3 V, two-tailed unpaired Mann Whitney test, p = 0.0411, Fig 2A). This change in the input/output relationship was not a consequence of a reduction in the probability of release, as there was no significant difference in the degree of paired pulse facilitation (Fig 2A, B). It was also not a consequence of a smaller number of presynaptic fibres being activated in the slices from WAG/Rij rats (volley amplitude at 5V stimulation strength: Wistar rats 0.345 ± 0.02 mV vs 0.293 ± 0.03 mV in WAG/Rij rats, p = 0.2019, two-tailed unpaired Mann Whitney test). Thus the difference may stem from a reduction in postsynaptic receptor number.

There was no significance difference in either the amplitude of the peak inhibition produced by DHPG and the induced LTD (Fig. 2C) between WAG/Rij and Wistar rats. At 55-60 minutes after DHPG wash the mean reduction in fEPSP slope was 51.48% in WAG/Rij rats and 49.17% in Wistar rats (two-tailed unpaired Mann Whitney test p = 0.9242).

We then compared the DHPG induced depression in the same strains of rats at the two ages. There was a large and significant reduction in both short term depression (STD, peak inhibition during DHPG application) and LTD in symptomatic vs. pre-symptomatic WAG/Rij rats (STD, two-tailed unpaired Mann-Whitney test, p = 0.0136, U = 10; LTD, two-tailed unpaired Mann-Whitney test, p = 0.0317, U = 13) (Fig. 3A, B). In contrast there were no significant changes in either LTD or STD between young and 5-6 month old Wistar rats (Fig. 3C, D). Therefore, the reduction in mGlu receptor mediated-LTD only occurs in symptomatic WAG/Rij rats and may be a consequence of the SWD.

3. There are no significant changes in NMDA receptor-dependent LTP in WAG/Rij rats compared to age matched control rats

To examine whether the reduction in mGlu receptor dependent-LTD observed in symptomatic WAG/Rij rats could reflect a more general impairment in all forms of activity-dependent synaptic

plasticity, we examined NMDA receptor-dependent, long term potentiation (LTP). We first examined basal synaptic transmission in the absence of the $GABA_A$ and NMDA receptor antagonists (that were used for the LTD experiments) at 4-6 weeks of age. Neither the input/output relationships generated in the absence of the $GABA_A$ and NMDA receptor antagonists, nor paired-pulse facilitation showed significant differences (Fig. 4A, B). LTP was induced with a tetanic stimulation. There was no significant difference between the two strains of animals in the amplitude of LTP expression (Fig. 4C, D)

In rats aged 5-6 months, there was no significant differences in the input-output curves or in paired-pulse facilitation (Fig. 5A, B). There was also no significant difference in the amplitude of LTP induced with theta bursts stimulation (TBS). We used TBS (rather than a tetanus) as we found it a more reliable stimulus for inducing LTP in the older animals. Thus the changes in plasticity observed in the symptomatic WAG/Rij rats are specific to mGlu receptor-mediated LTD and do not affect all forms of plasticity.

4. Changes in the hippocampal expression of mGlu5 receptors, Homer protein and AMPA receptor subunits in symptomatic WAG/Rij rats could contribute to the deficits in LTD.

Using western blots, we first examined whether there are changes in the expression of mGlu1α and mGlu5 proteins in the hippocampus of WAG/Rij rats as this could contribute to the deficits in LTD (Fig 6). Changes in mGlu receptor expression have been reported in the cerebral cortex and thalamic nuclei of WAG/Rij rats in comparison to age-matched non-epileptic controls (Ngomba et al., 2011; D'Amore et al. 2013). This is to our knowledge, the first time that mGlu receptor expression has been investigated in the hippocampus of pre-symptomatic and symptomatic WAG/Rij rats.

Under our experimental conditions, immunoblot analysis of mGlu1α and mGlu5 receptors showed a major band at 140 and 150 kDa corresponding to the respective receptor monomers (Fig. 6A, B).

A faint higher molecular size band (>220 kDa) was occasionally observed and may correspond to receptor dimers (not illustrated). This higher molecular weight band was not included in the densitometric analysis. The hippocampal expression of mGlu1α receptors did not significantly differ between WAG-Rij and Wistar rats regardless of the age of the animals (Two way ANOVA: genotype, $F_{3,18} = 0.7872$, $p = 0.5166$; age, $F_{6,18} = 3.201$, $p = 0.0256$).

In contrast, hippocampal mGlu5 receptor protein levels were significantly reduced in 5-6 month old symptomatic WAG/Rij rats, as compared to age matched Wistar rats (Two way ANOVA: genotype, $F_{3,18} = 10.10$, $p = 0.0004$; age, $F_{6,18} = 0.4682$, $p = 0.8228$, Fig 6B). There was however no significant difference in mGlu5 receptor expression between symptomatic and pre-symptomatic WAG/Rij rats.

We extended the analysis to investigate Homer protein because of its established role in group1-mGlu receptor signalling (Brakeman et al., 1997) and LTP/LTD expression (Collingridge et al., 2004; Bliss et al., 2014; Park et al., 2018 Fig 7). Homer proteins were detected with an anti-pan-Homer antibody as a single band at about 45 kDa, (Fig 7) corresponding to the expected molecular size of the long isoforms of Homer (Brakeman et al., 1997). Levels of Homer protein showed a trend to an increase in the hippocampus of 5-6 months old WAG/Rij rats, as compared to age matched Wistar rats, although the differences are not statistically significant (Two way ANOVA: genotype, $F_{3,18} = 2.488$, $p = 0.0933$; age, $F_{6,18} = 0.3290$, $p = 0.9129$)

We then investigated the expression of GluA1 and GluA2 AMPA receptor subunits, as they maybe internalised by Arc during mGlu5 receptor mediated LTD (for example see da Silva et al 2016). GluA1 and GluA2 were detected as single bands at 100 and 98 kDa, respectively. GluA1 protein expression was significantly reduced in symptomatic WAG/Rij rats compared to 5-6 month old non-epileptic control Wistar rats (Two way ANOVA: genotype, $F_{3,18} = 5.994$, $p = 0.0051$; age, $F_{6,18} = 1.828$, $p = 0.15$, Fig 8A). There were no significant differences in GluA2 protein expression (Two way ANOVA: genotype, $F_{3,18} = 0.9473$, $p = 0.4386$; age, $F_{6,18} = 4.004$, $p = 0.0101$, Fig. 8B).

In summary, there is a significant reduction in hippocampal mGlu5 receptor expression in WAG/Rij rats and a trend to an increase in Homer protein expression (Fig 6B and 7) which could contribute to the changes in mGlu receptor-dependent LTD.

Discussion

Here, we have provided the first demonstration that a form of activity-dependent synaptic plasticity, group-I mGlu receptor-mediated LTD, is altered in the hippocampus of an established rat model of absence epilepsy. This change in plasticity was not general for all the forms of synaptic plasticity as no significant changes in LTP were observed. This deficit in mGlu1/5-dependent LTD was found in 5-6 month-old WAG/Rij rats. At this age, WAG/Rij rats exhibit a high incidence of SWD, characteristic of absence seizures (see Introduction and References therein), these SWD are not observed in 4-6 week-old, pre-symptomatic, WAG/Rij rats. Although in this study we did not directlty measure the SWD we did observe clear absence symptoms in the 5-6 month old WAG/Rij rats. This suggests that the impairment of hippocampal synaptic plasticity develops in parallel with the worsening of the epileptic phenotype.

An increased functional connectivity between the thalamus and dorsal hippocampus has been associated with the occurrence of SWD in the γ-butyrolactone rat model of absence seizures (Mousavi et al., 2017). If a similar association exists in 5-6 months old WAG/Rij rats, then the high incidence of SWD might cause maladaptive changes in hippocampal synaptic plasticity, resulting in defective mGlu1/5 receptor-mediated LTD. However, it cannot be excluded that seizure-independent, age-related mechanisms disrupt LTD in WAG/Rij rats. Experiments in which WAG/Rij rats are chronically maintained under constant antiepileptic medication since the presumed time-at-onset of absence seizures (approximately at 2-3 months of age) are needed to examine the cause-to-effect

relationship between seizures and abnormalities in hippocampal synaptic plasticity, as was done in experiments in which the age-dependent increase in SWD, the depressive phenotype, the thickness of the *corpus callosum*, and the expression of cortical HCN1 and sodium channels were partly prevented by early and long-term administration of ethosuximide (Blumenfeld et al., 2008; van Luijtelaar et al., 2013).

While it was originally believed that LTD had a complementary role in the regulation of signal-to-noise and was involved in forgetting, it is now established that LTD has a direct role in hippocampal information storage (Kemp and Manahan-Vaughan, 2007). Hippocampal LTD plays a key role in spatial learning (Ge et al., 2010; Goh et al., 2012), novelty acquisition (Manahan-Vaughan and Braunewell, 1999; Lemon and Manahan-Vaughan, 2006), and novelty exposure-induced memory enhancement (Dong et al., 2012). Recent experiments have shown that alterations in group-I mGlu receptor mediated-LTD leads to disruptions in reversal learning and a reduction in cognitive flexibility (Wall et al., 2018; Privitera et al., 2019).

We induced group-I mGlu receptor-dependent LTD in the hippocampal CA1 pyramidal neurons by 10 minute application of the mGlu1/5 receptor agonist, DHPG (Kemp and Bashir, 1999; Huber et al., 2000; Volk et al., 2006). DHPG-induced LTD in hippocampal CA1 region is not mediated by the canonical signaling pathway activated by mGlu1/5 receptors, i.e., $G_{q/11}$-dependent activation of phospholipase Cβ (PLCβ), formation of inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG), intracellular Ca^{2+} release and activation of protein kinase C (Fitzjohn et al., 2001; Kleppisch et al., 2001; Moult et al., 2006), but rather depends on tyrosine dephosphorylation and activation of tumor necrosis factor-α converting enzyme (TACE), which stimulate AMPA receptor endocytosis (Moult et al., 2006; Cho et al., 2008; Chang et al., 2008; Luscher and Huber, 2010). We found that mGlu5 receptor expression is reduced, whereas expression of the long isoforms of Homer protein showed a trend to an increase in the hippocampus of 5-6 months old WAG/Rij rats. We can speculate that while the overall hippocampal expression of mGlu5 receptors is reduced, both mGlu1 and mGlu5

receptors have increased coupling to long Homer proteins in symptomatic WAG/Rij rats, and this leads to a bias in the receptor signalling towards the canonical PLCβ/$InsP_3$/Ca^{2+} pathway (long Homer isoforms link mGlu1/5 receptors to $InsP_3$ receptors) at the expense of the pathways involved in LTD induction and maintenance. The observed significant reduction in GluA1 AMPA receptor subunits found in symptomatic WAG/Rij rats is difficult to reconcile with the blunted DHPG-induced LTD found in these animals. Determinations of surface-expressed AMPA receptors subunits and AMPA receptor trafficking in postsynaptic elements of CA1 pyramidal neurons are necessary before drawing any concrete conclusions. One caveat to our westen blot data is that we did not dissect the hippocampal subfields, in particular the CA1 region where the LTD was induced. This may reduce the significance of some of the data and could explain some of the changes which are not consistent with some of the findings, such as the change in GluA1 AMPA receptor subunits.

Changes in group-I mGlu receptor-mediated LTD in the hippocampus have been linked to cognitive dysfunction associated with autism spectrum disorders. The evidence that DHPG-induced LTD was amplified in the hippocampus of *fmr1* gene knockout mice paved the way to the clinical development of mGlu5 receptor antagonists in the treatment of Fragile-X syndrome (Huber et al., 2002; Bear et al., 2004; Ronesi and Huber, 2008; Dolen and Bear, 2008; Wuang and Huber, 2009). mGlu1/5 receptor-mediated LTD was also found to be amplified in the hippocampus of mice modelling Angelman syndrome, CYF1P1 and SUNGAP1 haploinsufficiency, and chromosome 16p11.2 microdeletion (Bozdagi et al., 2012; Pignatelli et al., 2014; Barnes et al., 2015; Tian et al., 2015), dysregulated in mice modeling Rett's syndrome (Tao et al., 2016), and reduced in mice modeling tuberous sclerosis (Auerbach et al., 2011). Most of these models are characterized by convulsive seizures, e.g., audiogenic seizures in Fragile-X and Angelman mice (Musumeci et al., 2000; Mandel-Brehm et al., 2015). Interestingly, methyl-CpG-binding protein 2 (MeCP2)-deficient mice modeling Rett syndrome show seizure-like events associated with delta frequency power of the recorded local

field potentials, which share similarities with absence seizures (Colic et al., 2013). Thus, MeCP2-deficient mice and WAG/Rij rats represent two unrelated models in which absence-like seizures are associated with abnormalities in mGlu1/5-dependent LTD in the hippocampus. This further strengthens the hypothesis that pathological oscillations within the cortico-thalamo-cortical circuit generating absence seizures may cause alterations in mGlu1/5 receptor-dependent synaptic plasticity in the hippocampus resulting into intellectual disability and cognitive dysfunction.

Reductions in the expression and function of mGlu5 receptors have been found in the thalamus and somatosensory cortex of WAG/Rij rats, and pharmacological enhancement of mGlu5 receptors with a selective positive allosteric modulator (PAM) was found to reduce SWD frequency in these rats without the development of tolerance (Ngomba et al., 2011; D'Amore et al., 2013; 2014; Celli et al., 2020). Present findings suggest that, in WAG/Rij rats, mGlu5 receptors are dysfunctional also in the hippocampus, and this may underlie some cognitive abnormalities observed in these animals (van Luijtelaar et al., 1989; Karson et al., 2012; Jafarian et al. 2015; Malyshev et al., 2012; Leo et al., 2019). It will be interesting to examine whether mGlu5 receptor PAMs are able to correct learning and memory deficits in symptomatic WAG/Rij rats, and they do so independently of their therapeutic effect on absence seizures. This is an important step towards the development of mGlu5 receptor PAMs for the treatment of absence epilepsy and associated cognitive dysfunction.

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701

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707 Author Contributions

708 Conceived the project: RTN FN; Designed the experiments LI FN RTN MJW; Performed the
709 experiments: GDC EM LI RC MJW. Analysed the data: GDC LI FN MJW. Contributed
710 reagents/materials/analysis tools: FN GVL RTN MJW; Wrote the paper: GDC FN LI (initial draft),
711 all the authors read and commented on the manuscript and GDC FN RTN MJW (final version).

712

Figure legends

Fig. 1 – mGlu receptor-mediated LTD is reduced in hippocampal slices from symptomatic WAG/Rij rats

A, Mean fEPSP slope plotted against stimulus strength for Wistar rats (n = 3 animals, 8 slices) and WAG/Rij rats (n = 3 animals, 10 slices). Inset, examples of superimposed fEPSPs at different stimulus strength (1 to 5 V) from Wistar and WAG/Rij rats. There was no significant difference in the stimulus input/output relationships between WAG/Rij and Wistar rats ($p = 0.6706$). **B**, The mean paired pulse ratio (PPR) plotted against paired pulse interval for WAG/Rij rats (n = 3 animals, 7 slices) and Wistar rats (n = 3 animals, 6 slices). No significant difference was observed between the strains ($p = 0.4127$). Inset, averaged fEPSPs traces at 50 ms interval from Wistar and WAG/Rij rats. **C,** Normalised mean fEPSP slope plotted against time for WAG/Rij (n= 3 animals; 7 slices) and Wistar rats (n = 4 animals, 5 slices). Following a 20 minute baseline, DHPG (100 μM, 10 minutes) was used to induce LTD with fEPSPs recorded for at least 1 hr after washing DHPG. LTD is expressed as percentage depression of baseline (100%) and statistical significance was determined between 55 and 60 min after DHPG wash ($p = 0.0303$); WAG/Rij: (29.26 ± 5.14 %) and Wistar (65.46 ± 11.68 %) rats. Representative fEPSP waveforms taken between 10-15 min (1) and LTD at 40-50 min (2). **D**, Bar graph summarizing mean reduction in fEPSP slope for WAG/Rij and Wistar rats. Data are represented as mean ± SEM with each point data from an individual slice. Statistical comparisons were performed with post hoc Mann-Whitney test. All experiments were carried out in the presence of 50 μM picrotoxin and 5 μM L689,560.

Fig. 2 –There is no change in Group I mGlu receptor mediated LTD in hippocampal slices of pre-symptomatic 4-6 weeks old WAG/Rij and Wistar rats

736 **A**, Mean fEPSP slope plotted against stimulus strength for Wistar rats (n = 5 animals, 9 slices) and
737 WAG/Rij rats (n = 4 animals, 11 slices). Inset, examples of superimposed fEPSPs at different stimulus
738 strength (1 to 5 V) from Wistar and WAG/Rij rats. A significant reduction in the fEPSP slope was
739 seen in WAG/Rij rats compared to Wistar rats (at stimuli from 3 to 5 V, p = 0.0411). **B**, The mean
740 paired pulse ratio (PPR) plotted against paired pulse interval for WAG/Rij rats (n = 4 animals, 9
741 slices) and Wistar rats (n = 5 animals, 9 slices). No significant differences were observed between the
742 strains (p = 0.944). Inset, averaged paired fEPSP waveforms at 50 ms interval from Wistar and
743 WAG/Rij rats. **C,** Normalised mean fEPSP slope plotted against time for WAG/Rij rats (n = 4
744 animals, 10 slices) and Wistar rats (n =5 animals, 9 slices). Following a 20 minute baseline, DHPG
745 (100 μM, 10 minutes) was used to induce LTD with fEPSPs recorded for at least 1 hr after washing
746 DHPG. LTD is expressed as a percentage depression of baseline (100%) 55-60 minutes after DHPG
747 wash and is not significantly different (p = 0.9242); WAG/Rij (51.48 ± 5.59 %) and Wistar rats
748 (49.18 ± 5.42 %). Representative fEPSP waveforms taken between 10-15 min (1) and LTD at 50-60
749 min (2). **D**, Bar graph summarizing mean reduction in fEPSP slope for WAG/Rij and Wistar rats.
750 Data are represented as mean ± SEM with each point data from an individual slice. Statistical
751 comparisons were performed with post hoc Mann-Whitney test. All experiments were carried out in
752 the presence of 50 μM picrotoxin and 5 μM L689,560.

753

754 **Fig. 3 – There are significant differences in DHPG induced STD and LTD in symptomatic vs.**
755 **pre-symptomatic WAG/Rij rats**

756 **A**, Normalised mean fEPSP slope plotted against time for 4-6 week old pre-symptomatic WAG/Rij
757 rats (n= 4 animals; 10 slices) and 5-6 month old symptomatic WAG/Rij rats (n = 3 animals, 10 slices).
758 Following a 20 minute baseline, DHPG (100 μM, 10 minutes) was used to induce LTD with fEPSPs
759 recorded for at least 1 hr after washing DHPG. Short term depression (STD) was measured as a
760 percentage depression of baseline (100%) during the application of DHPG whereas LTD was

761 measured 55-60 minutes after DHPG wash. **B**, Bar charts summarising mean STD and LTD in 4-6
762 week and 5-6 month old Wistar rats. There was significant differences in the amplitude of both STD
763 and LTD. **C**, Normalised mean fEPSP slope plotted against time for 4-6 week old Wistar rats (n= 5
764 animals, 9 slices) and 5-6 month old Wistar rats (n = 4 animals, 5 slices). Following a 20 minute
765 baseline, DHPG (100 μM, 10 minutes) was used to induce LTD with fEPSPs recorded for at least 1
766 hr after washing DHPG. **D**, Bar charts summarising mean STD and LTD in 4-6 week and 5-6 month
767 old Wistar rats. Data are represented as mean ± SEM with each point data from an individual slice.
768 There was no significant differences in the amplitude of either STD or LTD. The data used in this
769 figure comes from figures 1 and 2.

770

771 **Fig. 4 – There are no significant differences in NMDA receptor-dependent LTP in**
772 **presymptomatic WAG/Rij rats and age matched controls**

773 **A**, Mean fEPSP slope plotted against stimulus strength for 4-6 week old Wistar rats (n =5 animals, 6
774 slices) and 4-6 week old pre-symptomatic WAG/Rij rats (n = 7 animals, 8 slices). Inset, examples of
775 superimposed fEPSPs at different stimulus strength (1 to 5 V) from Wistar and WAG/Rij rats. There
776 was no significant difference in the fEPSP slope in WAG/Rij rats compared to Wistar rats (p =
777 0.6706). **B**, The mean paired pulse ratio (PPR) plotted against paired pulse interval for 4-6 week old
778 pre-symptomatic WAG/Rij rats (n =7 animals, 8 slices) and Wistar rats (n = 5 animals, 6 slices). No
779 significant difference was observed between the strains (p = 0.6667). Inset, averaged fEPSPs traces
780 at 50 ms interval from Wistar and WAG/Rij rats. **C,** Normalised mean fEPSP slope plotted against
781 time for 4-6 week old pre-symptomatic WAG/Rij rats (n= 7 animals; 8 slices) and 4-6 week old
782 Wistar rats (n =4 animals, 5 slices). Following a 20 minute baseline, LTP was induced (tetanus
783 stimulation) with fEPSPs recorded for at least 1 hr after induction. LTP is expressed as percentage
784 increase over baseline (100%) and statistical significance was determined between 55 and 60 min (p
785 = 0.5828). Inset, average waveforms before and after LTP **D**, Bar charts summarising mean

magnitude of LTP in Wistar and WAG/Rij rats at 4-6 weeks of age. Statistical comparisons were performed with post hoc Mann-Whitney test. Data are represented as mean ± SEM with each point data from an individual slice.

Fig. 5 – **There are no significant differences in NMDA receptor-dependent LTP in symptomatic WAG/Rij rats and age matched controls**

A, Mean fEPSP slope plotted against stimulus strength for 5-6 month old Wistar rats (n = 4 animals, 9 slices) and 5-6 month old symptomatic WAG/Rij rats (n = 4 animals, 13 slices). Inset, examples of superimposed fEPSPs at different stimulus strength (1 to 5 V) from Wistar and WAG/Rij rats. There was no significant difference in the fEPSP slope in WAG/Rij rats compared to Wistar rats ($p = 0.7243$). **B**, The mean paired pulse ratio (PPR) plotted against paired pulse interval for 5-6 month old symptomatic WAG/Rij rats (n = 4 animals, 13 slices) and age-matched Wistar rats (n = 4 animals, 9 slices). No significant difference was observed between the strains ($p = 0.4127$). Inset, averaged fEPSPs traces at 50 ms interval from Wistar and WAG/Rij rats. **C**, Normalised mean fEPSP slope plotted against time for 5-6 month old symptomatic WAG/Rij rats (n= 6 animals; 9 slices) and age-matched Wistar rats (n =4 animals, 6 slices). Following a 20 minute baseline, LTP was induced (Theta bursts) with fEPSPs recorded for at least 1 hr after induction. LTP is expressed as percentage increase over baseline (100%) and statistical significance was determined between 55 and 60 min. There was no significant difference in the fEPSP slope in WAG/Rij rats compared to Wistar rats ($p = 0.9083$). Inset, average waveforms before and after LTP **D**, Bar charts summarising mean magnitude of LTP in Wistar and WAG/Rij rats at 4-6 weeks of age. Statistical comparisons were performed with post hoc Mann-Whitney test. Data are represented as mean ± SEM with each point data from an individual slice.

Fig. 6- Changes in mGlu5 receptor protein expressions but not mGlu1 receptor expression in the hippocampus of WAG/Rij and non-epileptic control rats.

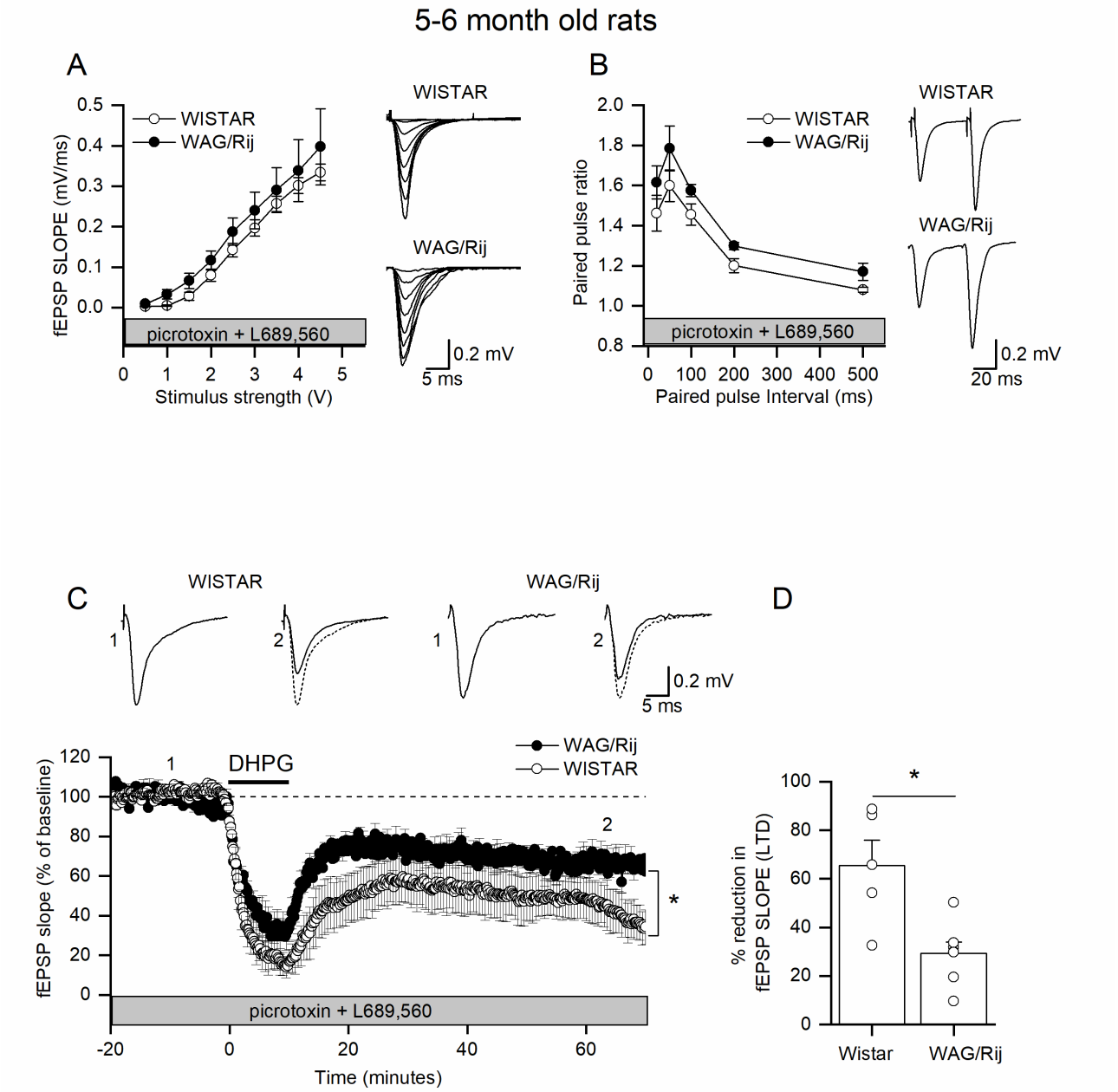
Western blot analysis of mGlu1 and mGlu5 receptors is shown in panels A and B, respectively. Data are mean ± SEM of 7 determinations per group (data points are shown for each sample). *p < 0,05 vs. 4-6 weeks old Wistar rats and 5-6 months old Wistar rats in B (Two way ANOVA + Tukey's test). Representative immunoblots are shown.

Fig. 7 – Homer protein levels in the hippocampus of WAG/Rij rats and non-epileptic controls.

Western blot analysis of Homer protein expression. Data are mean ± S.E.M of 7 determinations per group (data points are shown for each sample). A representative immunoblot is shown.

Fig. 8 – Reduced expression of GluA1 AMPA receptor subunits in in the hippocampus of symptomatic WAG/Rij rats.

Western blot analysis of GluA1 and GluA2 receptors subunits is shown in A and B, respectively. Data are means ± S.E.M of 7 determinations per group. *p<0,05 vs. 5-6 months old Wistar rats. (Two way ANOVA + Tukey's test). Representative immunoblots are shown.



829

830 Figure 1

831

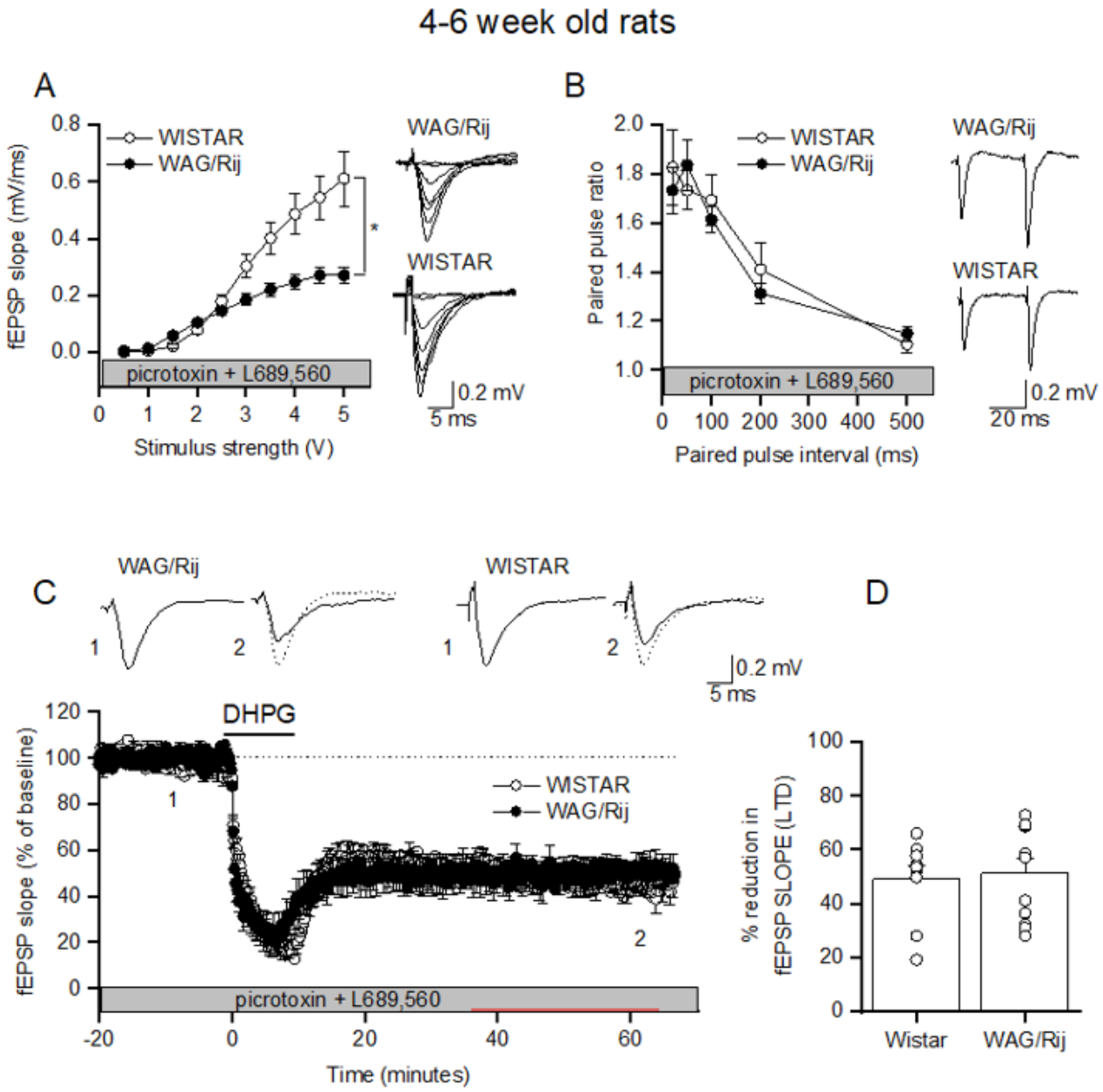

4-6 week old rats
A
WISTAR
WAG/Rij
fEPSP slope (mV/ms)
picrotoxin + L689,560
Stimulus strength (V)
WAG/Rij
WISTAR
0.2 mV
5 ms
B
Paired pulse ratio
Paired pulse interval (ms)
20 ms
C
DHPG
fEPSP slope (% of baseline)
Time (minutes)
D
% reduction in fEPSP SLOPE (LTD)
Wistar
WAG/Rij

Figure 2.

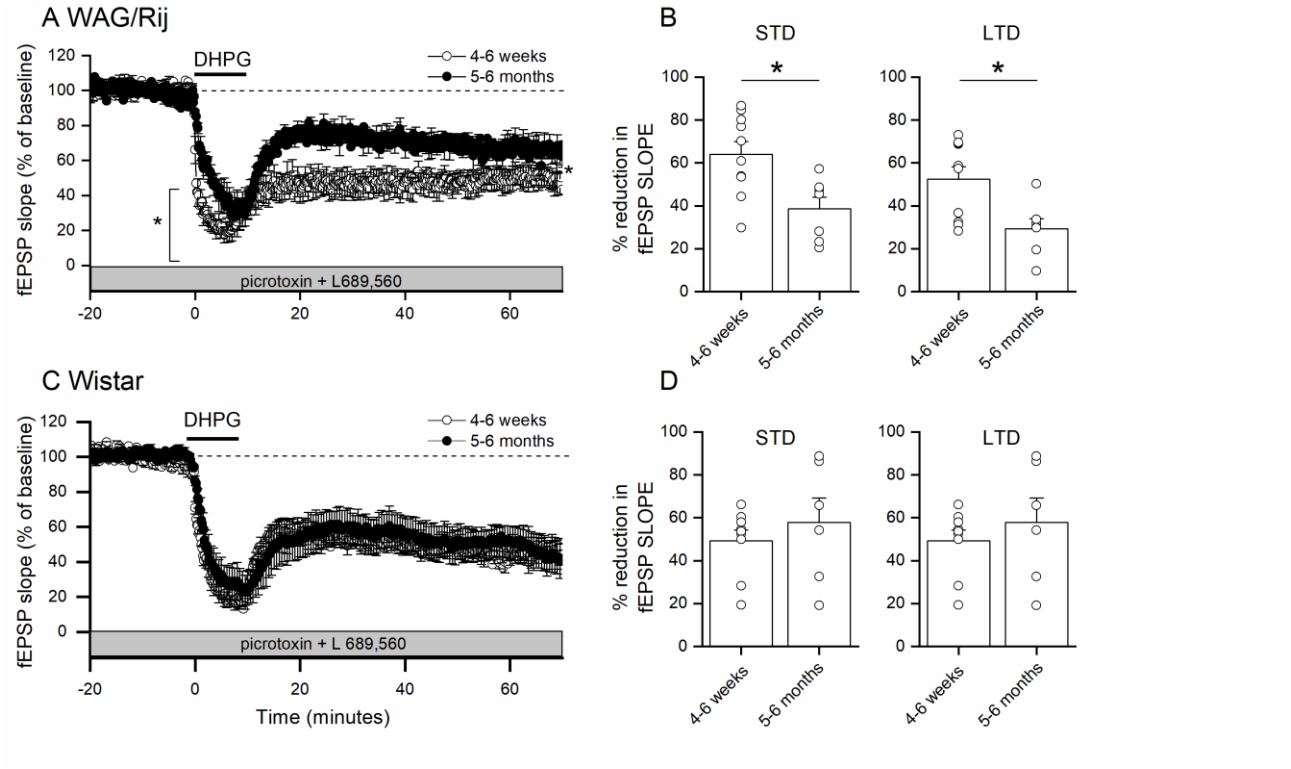

A WAG/Rij
DHPG
4-6 weeks
5-6 months
fEPSP slope (% of baseline)
picrotoxin + L689,560
B
STD
LTD
% reduction in fEPSP SLOPE
4-6 weeks
5-6 months
C Wistar
picrotoxin + L 689,560
Time (minutes)
D

Figure 3

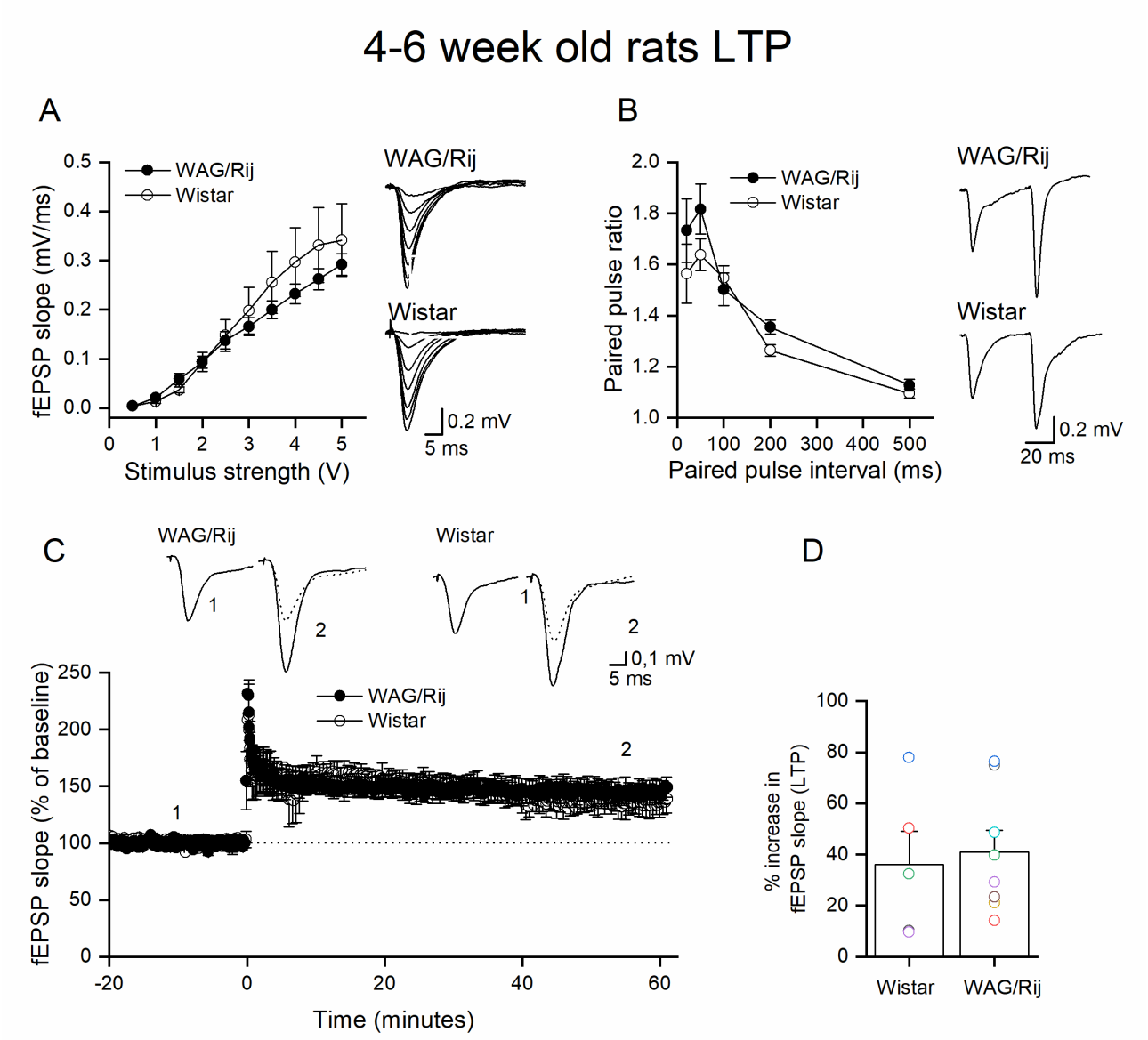
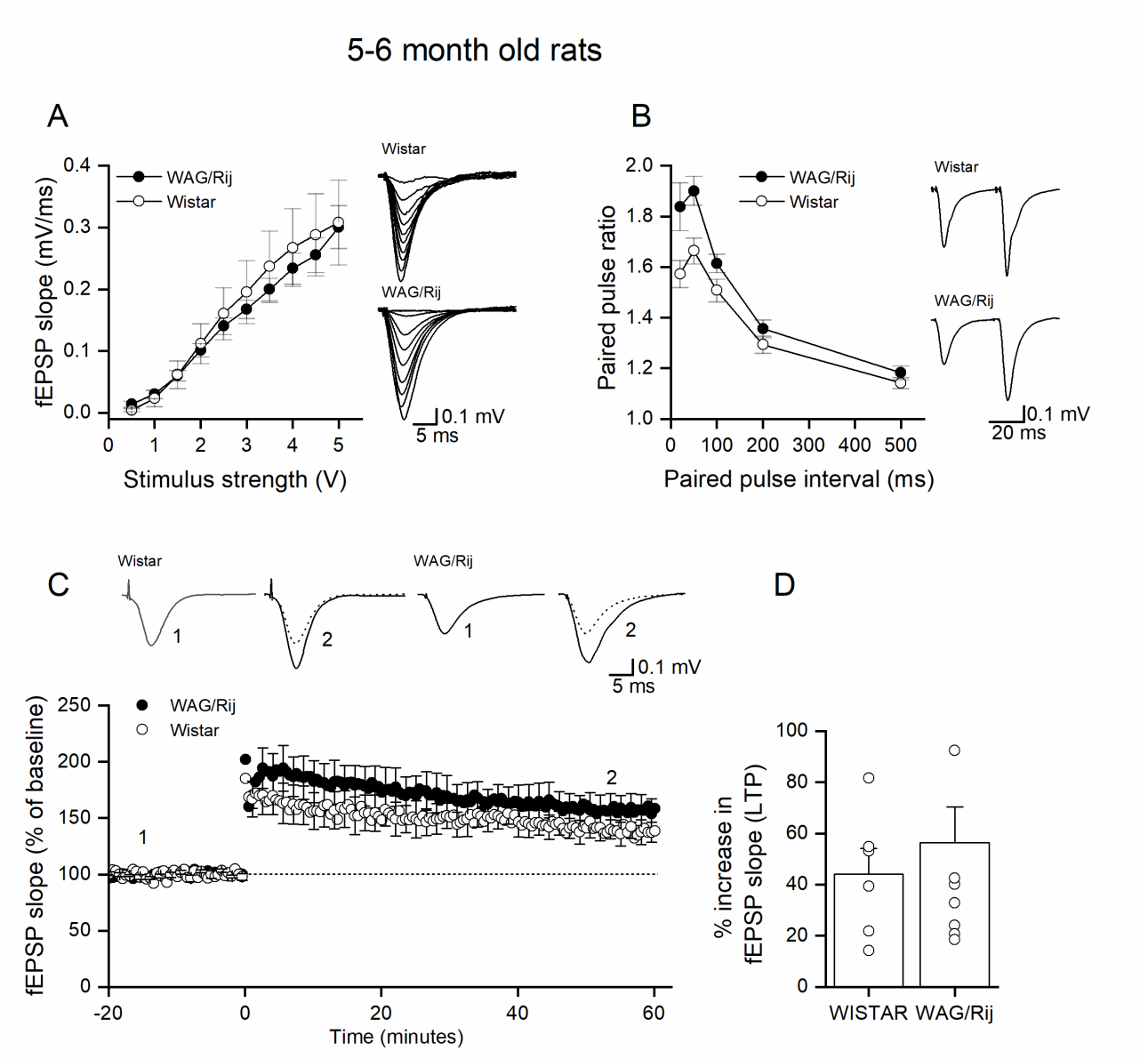

4-6 week old rats LTP
A
fEPSP slope (mV/ms)
WAG/Rij
Wistar
Stimulus strength (V)
0.2 mV
5 ms
B
Paired pulse ratio
Paired pulse interval (ms)
20 ms
C
fEPSP slope (% of baseline)
Time (minutes)
0,1 mV
D
% increase in fEPSP slope (LTP)

Figure 4



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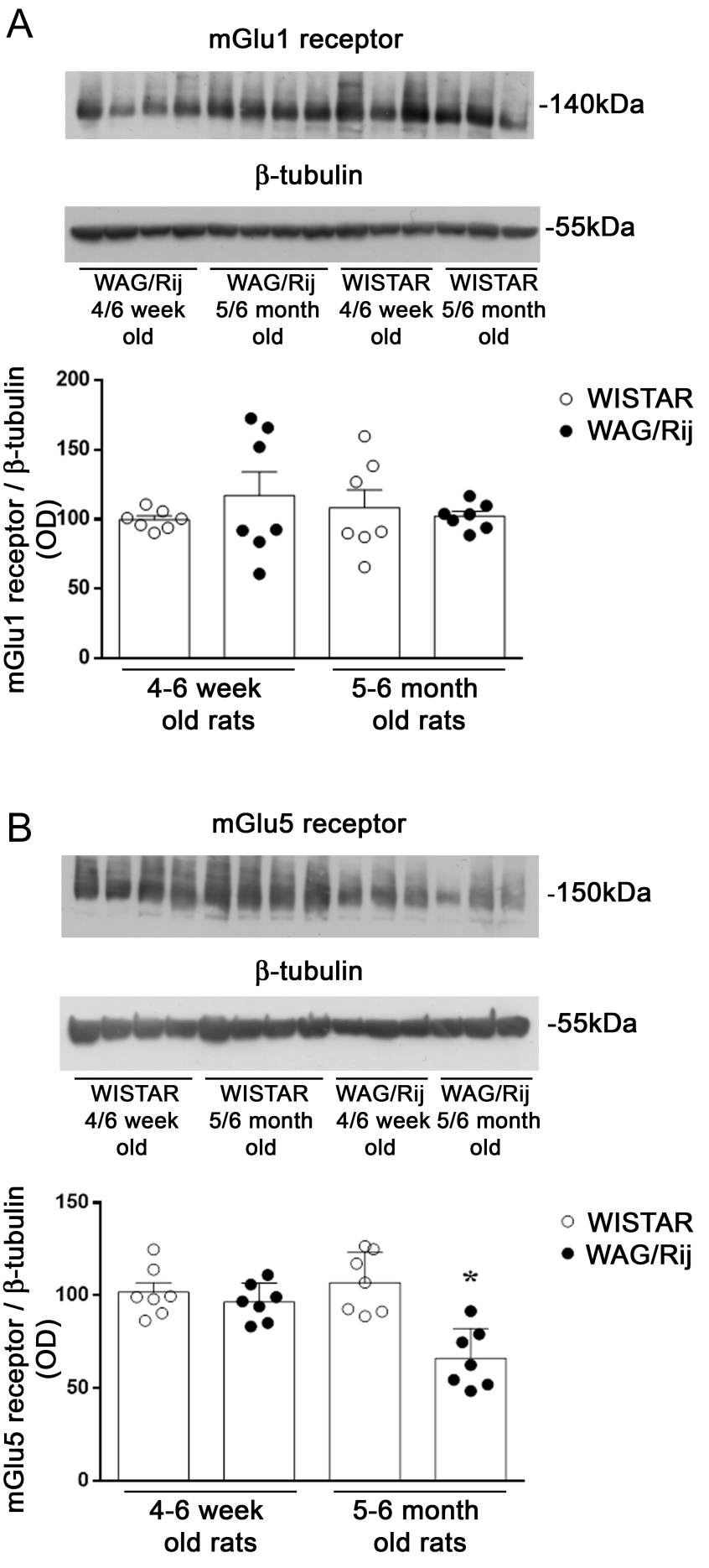
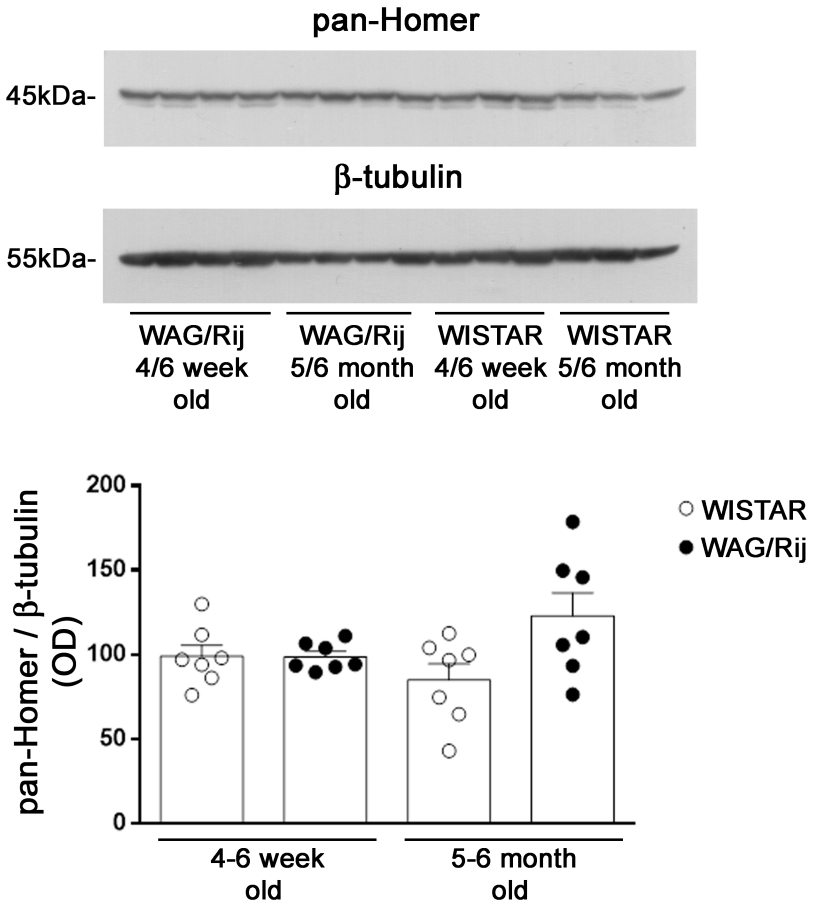
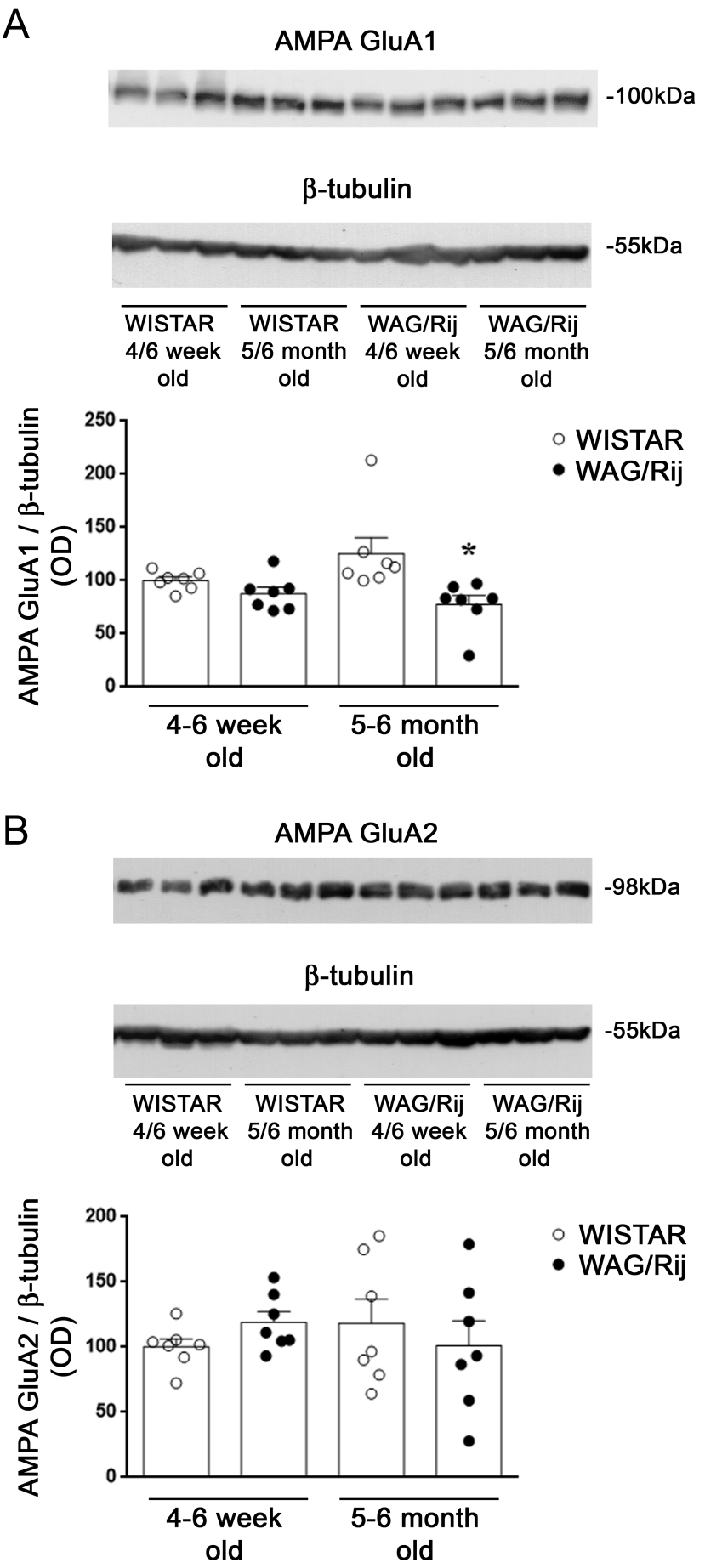
858 Figure 5.


A
mGlu1 receptor
-140kDa
β-tubulin
-55kDa
WAG/Rij 4/6 week old
WAG/Rij 5/6 month old
WISTAR 4/6 week old
WISTAR 5/6 month old
mGlu1 receptor / β-tubulin (OD)
200
150
100
50
0
WISTAR
WAG/Rij
4-6 week old rats
5-6 month old rats
B
mGlu5 receptor
-150kDa
β-tubulin
-55kDa
WISTAR 4/6 week old
WISTAR 5/6 month old
WAG/Rij 4/6 week old
WAG/Rij 5/6 month old
mGlu5 receptor / β-tubulin (OD)
150
100
50
0
*
WISTAR
WAG/Rij
4-6 week old rats
5-6 month old rats


pan-Homer
45kDa-
β-tubulin
55kDa-
WAG/Rij 4/6 week old
WAG/Rij 5/6 month old
WISTAR 4/6 week old
WISTAR 5/6 month old
pan-Homer / β-tubulin (OD)
200
150
100
50
0
WISTAR
WAG/Rij
4-6 week old
5-6 month old


A
AMPA GluA1
-100kDa
β-tubulin
-55kDa
WISTAR 4/6 week old
WISTAR 5/6 month old
WAG/Rij 4/6 week old
WAG/Rij 5/6 month old
AMPA GluA1 / β-tubulin (OD)
250
200
150
100
50
0
WISTAR
WAG/Rij
*
4-6 week old
5-6 month old
B
AMPA GluA2
-98kDa
β-tubulin
-55kDa
WISTAR 4/6 week old
WISTAR 5/6 month old
WAG/Rij 4/6 week old
WAG/Rij 5/6 month old
AMPA GluA2 / β-tubulin (OD)
200
150
100
50
0
WISTAR
WAG/Rij
4-6 week old
5-6 month old