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Enhanced Long Term Potentiation and Decreased AMPA Receptor Desensitization in the Acute Period Following a Single Kainate Induced Early Life Seizure

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Abstract

Neonatal seizures are associated with long term disabilities including epilepsy and cognitive deficits. Using a neonatal seizure rat model that does not develop epilepsy, but develops a phenotype consistent with other models of intellectual disability (ID) and autism spectrum disorders (ASD), we sought to isolate the acute effects of a single episode of early life seizure on hippocampal CA1 synaptic development and plasticity. We have previously shown chronic changes in glutamatergic synapses, loss of long term potentiation (LTP) and enhanced long term depression (LTD), in the adult male rat ~50 days following kainic acid (KA) induced early life seizure (KA-ELS) in post-natal (P) 7 day old male Sprague-Dawley rats. In the present work, we examined the electrophysiological properties and expression levels of glutamate receptors in the acute period, 2 and 7 days, post KA-ELS. Our results show for the first time enhanced LTP 7 days after KA-ELS, but no change 2 days post KA-ELS. Additionally, we report that ionotropic α -amino-3-hydroxy-5-methyl-isoxazole-propionic acid type glutamate receptor (AMPA) desensitization is decreased in the same time frame, with no changes in AMPAR expression, phosphorylation, or membrane insertion. Inappropriate enhancement of the synaptic connections in the acute period after the seizure could alter the normal patterning of synaptic development in the hippocampus during this critical period and contribute to learning deficits. Thus, this study demonstrates a novel mechanism by which KA-ELS alters early network properties that potentially lead to adverse outcomes.

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Keywords

Early life seizures; Intellectual disability; autism; hippocampal dependent learning; long term potentiation; AMPA receptors; NMDA receptors

Introduction

The incidence of seizures in the first month of life (early life seizures), a period identified as the highest risk for seizures in humans, is approximately 3 out of 1000 infants (Mizrahi, 1999), and can lead to adverse neurological and developmental outcomes including intellectual disability (ID) (McBride et al., 2000; Aldenkamp et al., 1999; Vanderlinden and Lagae, 2004). Autism spectrum disorders (ASD) and epilepsy are co-morbid, and early life seizures may increase the risk for autism (Buchmayer et al., 2009; Saemundsen et al., 2007; Saemundsen et al., 2008; Tuchman, 2009). About half of early life seizures are due to hypoxia, with the remainder due to stroke, other injuries and genetic causes (Vasudevan and Levene, 2013). We have used the KA-ELS rat model to investigate the effects of a single neonatal seizure on the processes underlying learning and memory in the acute period following seizure. This model mimics excessive glutamate, thought to be the primary mechanism causing seizures in neonates due to hypoxia and stroke (Yager, et al., 2002), without associated cell-loss (Nitecka et al., 1984), synaptic reorganization (Cornejo et al., 2007), recurrent spontaneous seizures (epilepsy) (Bernard et al., 2013), or side effects of therapeutic intervention (Turski and Ikonomidou, 2012). Thus the KA-ELS model uniquely allows us to determine seizure specific changes rather than those associated with genetic causes, hypoxia, stroke, or other injury.

Prior characterization of the adult KA-ELS rat found impairment in synaptic plasticity and hippocampal dependent memory (Cornejo et al., 2007; Bernard et al., 2013; Sayin et al., 2004). The behavioral phenotype of the adult KA-ELS rat includes abnormal working memory, fear conditioning, socialization, and increased anxiety (Cornejo et al., 2007; Bernard et al., 2013; Castelhana et al., 2013; Cornejo et al., 2008; Moreira et al., 2011; Sayin et al., 2004). This behavior profile is thought to be representative of ASD and ID, and similar behavior profiles have been seen in other rodent models of early life seizures (Lugo et al., 2014; Lippman-Bell et al., 2013; Talos et al., 2012) and in genetic rodent models of ID with associated ASD (Bakker and Oostra, 2003; LaSalle and Yasui, 2009; Waltereit et al., 2011). Strides have been made in identifying the mechanisms that underlie deficits in learning and ASD phenotype in the adult KA-ELS rat; however, the developmental pathogenesis has not been clearly defined. In adult KA-ELS rats, LTP of hippocampal CA1 synapses is decreased, presumably due to a decrease in the membrane pool of the GluA1 subunit of the AMPAR, decreased total expression of the GluN2A subunit of the N-methyl-D-aspartate type glutamate receptor (NMDAR), increased expression of post synaptic density-95 (PSD-95) protein, and increased expression of striatal enriched protein tyrosine phosphatase (STEP) (Cornejo et al., 2007; Bernard et al., 2013). Additionally, metabotropic glutamate receptor (mGluR) dependent LTD is enhanced in the adult KA-ELS rat associated with alterations in the localization and phosphorylation of fragile $\times$ mental retardation protein and ribosomal protein p70-S6 kinase (S6K), however, S6K is not activated 2 days

after KA-ELS (Bernard et al., 2013). Therefore the mechanisms that occur in the acute period following early life seizures that alter this critical developmental period to result in deficits are currently unknown and are investigated in the present work.

AMPA expression, phosphorylation, and localization underlie the expression of synaptic plasticity while NMDAR are necessary for the induction of LTP and NMDAR dependent LTD. Between birth and P28 many developmental changes occur in the expression and function of AMPARs and NMDARs (Kerchner and Nicoll, 2008), that if altered by early life seizures, may explain the changes in synaptic plasticity in the adult KA-ELS rat. The amount of LTP is precisely set, such that it is first observed at P5, peaks at P15 and then settles to adult levels (Harris and Teyler, 1984; Harris et al., 1992; Jackson et al., 1993; Teyler et al., 1989). This developmental specificity in the magnitude of LTP at P15 is thought to correlate with a critical developmental period specifically related to network organizational changes at this time (Harris et al., 1992). Perturbations in LTP at this time are likely to critically impact how the future network will function (Hensch, 2004). We hypothesized that KA-ELS alters the normal patterning of synaptic development of hippocampal CA1 pyramidal cells in the acute period following the seizure, thus we examined LTP and the expression profiles and electrophysiological properties of AMPARs and NMDARs in the CA1 region of the hippocampus 2 and 6-8 days after KA-ELS. We expected to see similar changes in the acute period as those seen in the adult KA-ELS rat. However, in contrast to decreased LTP seen in the adult KA-ELS rat, here we show KA-ELS induced increase in LTP expression in the acute period following the seizure. Surprisingly, no changes in total expression, phosphorylation, or internalization of AMPARs basally are detected. Additionally, no differences were observed in NMDAR expression or function between control and KA-ELS rats. However, AMPAR desensitization was decreased by KA-ELS 6-8 days post seizure. These results highlight a mechanism by which the network properties are altered by KA-ELS and could potentially propagate later life deficits.

Materials and Methods

Animals

All studies conformed to the requirements of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use subcommittee of the University of Colorado, Denver AMC. Timed-pregnant Sprague Dawley rats (Charles Rivers Labs, Wilmington, MA) gave birth in house. Dams were housed in micro-isolator cages with water and chow available *ad libitum*. All experiments in this study were performed with rats before recommended weaning age.

Materials

All chemicals were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise specified.

Seizure Induction

Seizure induction was performed as described (Cornejo et al., 2007). Briefly, male Sprague-Dawley rats were subcutaneously injected with KA (Tocris, Bristol, UK) (2 mg/kg) or saline on P7. KA injection at this age induces a mild discontinuous seizure characterized by generalized tonic-clonic jerks, or “swimming” 30 min post injection that lasts nearly 3 hrs. KA-ELS mimics human neonatal seizures such as hypoxia, in which excessive glutamate accumulations occur (Yager et al., 2002). Additionally, seizure induction on P7 correlates with human neonatal seizures biochemically (Talós et al., 2006) and electrographically (Dzhala et al., 2005). Two hours after KA injection, rats were implanted with Avid microchips to ensure that experimenters were blinded to the condition of the animal. Pups were returned to their dam after a 3 hour observation period post seizure induction. Seizures are not observed outside of this window or in adulthood (Bernard et al., 2013).

Hippocampal Slice Preparation

P9 and P13-15 rats (2 and 6-8 days post seizure) were decapitated and the brain rapidly removed. P13-15 are collectively referred to as P14, and 6-8 days post seizure are collectively referred to as 7 days post seizure in the following sections. Parasagittal slices (400 μ m) were prepared on a Leica VT 1200 microtome in ice cold solution containing (in mM) 206 Sucrose, 2.8 KCl, 1.25 NaH_2PO_4 , 26 NaHCO_3 , 10 Glucose, 10 MgSO_4 , 2 NaAscorbate, 0.4 CaCl_2 , and 2.5 N-acetyl L-cysteine. Scalpel cuts were made to remove CA3 while retaining the CA1 region of the hippocampus with the overlying cortex and dentate gyrus intact for electrophysiology. Slices were then recovered > 60 min at room temperature in a submersion chamber in standard artificial Cerebral Spinal Fluid (aCSF), containing (in mM) 124 NaCl, 26 NaHCO_3 , 1.2 NaH_2PO_4 , 10 D-glucose, 3 KCl, 2 NaAscorbate, 1 MgSO_4 , 2 CaCl_2 , and 2.5 N-acetyl L-cysteine) prior to all experiments. All solutions were oxygenated with carbogen (95% O_2 - 5% CO_2). The CA1 region of the hippocampi were further isolated for protein expression assays by removal of the overlying cortex and dentate gyrus.

Electrophysiological Recording, Whole-cell patch-clamp recordings

CA1 pyramidal neurons were visually identified by characteristic position and shape in submerged slices using infrared differential interference contrast (DIC) optics under magnification (40x) (BX51-WI, Olympus) and patch-clamped in the whole-cell configuration. Borosilicate glass (Harvard Instruments, Holliston, MA) recording electrodes were pulled with a Sutter P-97 electrode puller (Sutter Instruments, Novato, CA) with resistance 5-9 $\text{M}\Omega$ when filled with internal patch solution comprising (in mM) 135 CsMeSO₄, 10 HEPES, 10 BAPTA, 5 Qx314, 0.3 NaGTP, 4 Na_2ATP , 4 MgCl_2 , and 0.1 spermine, pH 7.25 with 1 M CsOH. For recording, picrotoxin (100 μM) was added to the recording solution to block γ -amino butyric acid (GABA) receptors. Recording solution was exchanged at a flow rate of 3-4 ml/min at room temperature. Responses were evoked with an insulated tungsten (AM Systems, Sequim WA) bipolar stimulating electrode placed in stratum radiatum to stimulate Schaffer collateral-commissurals (SCC) near apical dendrites, approximately 80 μM from the cell soma in which the recording electrode was placed. A constant current source (WPI, Sarasota, FL) (100 μs) was used to evoke excitatory post

synaptic currents (eEPSCs) and asynchronous EPSCs (aEPSCs). Access resistance was monitored by a -5 mV voltage step, and any experiments in which access resistance changed more than 20% between the onset and completion of the experiment were not used for experimental analyses. Series resistance was less than $13\text{ M}\Omega$. Responses were amplified and filtered at 5 kHz (Multiclamp 700B) and digitized at 20 kHz (Digidata 1440A and Clampex 10.2). No series resistance cancellation or junction potential corrections were performed.

AMPA-mediated peak current-voltage experiments were performed in the presence of $100\text{ }\mu\text{M}$ D,L-APV to block NMDARs. AMPARs that are calcium permeable have inwardly rectifying IV curves that are blocked in the presence of polyamines at depolarizing membrane potentials (Bowie and Mayer, 1995; Kamboj et al., 1995; Rozov et al., 1998; Stubblefield and Benke, 2010). Peak current-voltage (I-V) relationships were obtained for the determination of rectification by recording at various holding potentials (-70 , -40 , -20 , 0 , $+20$ and $+40\text{ mV}$), sample traces are shown for each holding potential in Figure 2A; currents were normalized to average currents obtained at -70 mV for comparisons. Rectification indices were calculated by dividing the mean absolute peak current obtained at -70 mV by the mean absolute peak current obtained at $+40\text{ mV}$.

AMPA paired pulse ratios were obtained by applying two stimulations with inter-stimulus intervals of 20 , 40 , 60 , 80 , 100 , and 140 ms every 20 s and recording responses at -70 mV to measure changes in pre-synaptic activity. These responses were additionally collected in the presence of 4-Aminopyridine (4-AP) ($50\text{ }\mu\text{M}$) and increased extracellular CaCl_2 (5 mM) (desensitizing aCSF) to increase the probability of release (Hjelmstad et al., 1999). Short term potentiation experiments were performed in standard aCSF and applying a train of 5 stimuli with an intra-event interval of 50 ms (20 Hz) and an inter-event (time between sweeps) interval of 20 s at holding potential -70 mV . AMPAR desensitization experiments were additionally performed in desensitizing aCSF, and applying a train of 5 stimuli with an intra-event interval of 20 ms (50 Hz) and an inter-event interval of 20 s at holding potential of -70 mV . In a separate series of experiments, AMPAR desensitization was blocked with $100\text{ }\mu\text{M}$ cyclothiazide (CTZ) (Enzo Life Sciences, Farmingdale, NY); comparisons were made to measurements without CTZ. τ_{rise} and τ_{decay} for eEPSCs were obtained by fits to the rising phase and decay phase of average eEPSCs according to $I(t) = a \cdot \exp(-t/\tau_{\text{rise}}) - a \cdot \exp(-t/\tau_{\text{decay}}) + C$ using a non-linear least-squares iterative method.

For an additional evaluation of pre-synaptic activity NMDAR currents were recorded at $+40\text{ mV}$. Baseline was collected for 5 min and then the potent, non-competitive irreversible NMDAR antagonist MK-801 ($40\text{ }\mu\text{M}$) (Tocris, Bristol UK) that binds within the ion channel pore was perfused and responses collected for 20 minutes . These were also performed in the presence of desensitizing aCSF to ensure that this solution increased release probability. For each recording, % current was plotted against time and then curve fits were performed to determine the $\log\text{EC}_{50}$ for the time at which 50% of the current was blocked. Additional experiments to evaluate changes in NMDAR subunit-specific synaptic expression and function were also performed using $5\text{ }\mu\text{M}$ GluN2B specific antagonist RO 25-6981 (Tocris, Bristol UK).

Electrophysiological Recording, Extracellular field recordings

Two twisted-tungsten bipolar stimulating electrodes were offset in the CA1 to stimulate two independent Schaffer collateral-commissural pathways using a constant current source with a fixed duration (100 μ s), each at a rate of 0.033 Hz. Field excitatory post-synaptic potentials (fEPSPs) were recorded from the stratum radiatum region of CA1 for P14 rats, and from the CA1 cell body layer for P9 rats, using a borosilicate glass microelectrode (pulled 6 - 9 M Ω when filled with 3M NaCl), amplified 1000x (WPI, Sarasota, FL and Warner, Hamden, CT), and digitized (National Instruments, Austin, Texas) at 10 kHz using winLTP-version 2.4 (Anderson and Collingridge, 2001) to follow fEPSP slope (averaged over 4 EPSPs), measured using 20% to 80% rise times, expressed as percent of baseline, during the course of an experiment. Field EPSPs were obtained at 28°C. In order to ensure only “healthy” slices were included in our studies, responses had to meet several criteria: fiber volleys less than 1/3 of response amplitude and peak responses larger than 0.6 mV; responses and fiber volley must be stable (<5% drift). Following baseline stabilization of fEPSP slope at approximately 40% of maximal slope for at least 30 min, LTP was induced in one pathway (100 Hz $\times$ 1 s). Responses were then recorded for 60 minutes. The last ten minutes of the recordings were used for statistical comparison of the magnitude of LTP obtained in control compared to KA-ELS rat slices. Experiments in which the unstimulated pathway responses either increased or decreased 20% above baseline were not considered stable, healthy slices, and not included in the analysis.

Cross-linking

Cross-linking experiments were performed as described previously (Cornejo et al., 2007). Briefly, hippocampal slices were prepared and recovered as described, with additional cuts made to isolate the CA1 region. The slices from individual animals were separated into two groups, one for total protein concentration and the other for intracellular protein concentration. The slices for total protein concentration were incubated in ice-cold aCSF for 40 min on ice at 4°C on a horizontal shaker. The slices for intracellular protein concentration were incubated for 40 min on ice at 4°C on a horizontal shaker in ice-cold aCSF containing 1mg/ml BIS-[sulfosuccinimidiyl] suberate, (BS³) (Thermo Scientific, Rockford IL). All slices were then washed three times in ice cold aCSF containing 20 mM Tris, pH 7.4, then processed as for Western Blotting. Cross-linking with membrane impermeable cross-linker, BS³, bonds the extracellular domains of membrane protein, allowing discernment between the plasma membrane bound fraction of receptors and the intracellular pools. The molecular weight of the plasma membrane bound fraction of the receptors is greater than the detection range of the SDS-PAGE, therefore the internal fraction of the receptors are presented as percentage of the total expression of receptors, thus the membrane pool and surface expression are implied.

Western Blotting

Hippocampal slices were prepared and recovered as for electrophysiology. Additional cuts were made to isolate CA1 (as done for cross-linking, above). The tissue was sonicated in aCSF (20 μ l per slice) containing 10% SDS homogenization buffer (10% sodium dodecyl sulfate, 10 mM EDTA, 100 mM Tris, pH 8), then heated at 100°C for 5 min. Total protein

concentration was determined by BCA Protein Assay (Thermo Scientific, Rockford IL). Proteins were separated by SDS-PAGE, then transferred to PVDF membranes, and detected using the primary antibodies: ADAR2 (Abcam), GluA1, GluA2/3, Stargazin/TARP pan (Millipore), GluA1 phospho S831, GluA1 phospho S845, GluA2 phospho S880, GluN2A, GluN2B (Phosphosolutions), GluA4 (Novus), GluN1 (BD Pharmagen), and PSD-95 (Affinity Bioreagents). Immunodetection was accomplished using a chemiluminescence substrate kit (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific) and Alpha Innotech (Alpha Innotech, San Leandro, CA) imaging system. Total protein concentration were determined relative to a 5 point standard curve to ensure protein concentrations were in the linear range of detection and for quantification of immunoreactivity/ μg protein (Davies et al., 2007), and are normalized to age matched controls. Total protein expression and phosphorylated protein expression were assessed from the same blots by stripping (Restore PLUS Western Stripping Buffer, ThermoScientific) and re-probing. Phosphorylated proteins are presented as ratios of phosphorylated proteins to total protein expression. Cross-linked proteins are presented as ratios of internal protein to total protein expression.

Statistical Analyses

Statistical Analysis were performed using SigmaPlot 12.5 and GraphPad Prism 5. Data are presented as mean $\pm$ SEM. On data sets where two groups were compared, significance was determined using a two tailed t-test unless equal variance or normality were not met. In cases where normality was not met, Mann-Whitney Rank sums was performed. In cases where equal variance was not met, a Welch's correction was used. Significance for the AMPAR desensitization experiments were determined using two-way repeated measures analysis of variance (ANOVA) with Holm-Sidak post hoc analysis for multiple comparisons. For all Western blot experiments "n" refers to the number of rats. For all whole cell electrophysiology experiments "n" refers to the number of cells; however only one cell was used for each slice. For all field electrophysiology experiments "n" refers to the number of slices. For all experiments a minimum number of 3 rats per condition were used.

Results

LTP was increased 7 days post seizure

Adult KA-ELS rats show alterations in hippocampal dependent memory and a decrease in CA1 LTP. To determine when this decrease in LTP develops following seizure, we measured changes in LTP between control and KA-ELS at 2 and 7 days post seizure induction. Contrary to decreased LTP in the adult KA-ELS rats, LTP expression was not changed 2 days post seizure ($F(1,12) = 0.548$, $p = 0.473$, Two-way repeat measures ANOVA). Remarkably, LTP was significantly enhanced 7 days post seizure ($F(1, 14) = 13.692$, $p = 0.002$, Two-way repeat measures ANOVA, Holm-Sidak post hoc analysis, control vs KA-ELS: 50 min $p < 0.001$; 52 min $p = 0.008$; 54 min $p = 0.017$; 56 min $p = 0.004$; 58 min $p = 0.001$; 60 min $p = 0.002$).

The decrease in LTP seen in adult KA-ELS rats has been attributed to a decrease in the membrane pool of the GluA1 subunit of the AMPAR, decreased total expression of the

GluN2A subunit of the N-methyl-D-aspartate type glutamate receptor (NMDAR), and increased expression of post synaptic density-95 (PSD-95) protein (Cornejo et al., 2007). On the other hand, in the HI-ELS model decreased LTP has been attributed to an increase in the GluN2A subunit compared to the GluN2B subunit of the NMDAR and increased expression of AMPARs (Zhou et al., 2011; Zhou et al., 2015). To determine whether changes in glutamate receptor subunits and PSD-95 expression that account for changes in LTP in the adult KA-ELS rat and in the HI-ELS model could also account for opposing changes in the KA-ELS model in the acute period, we probed hippocampal CA1 2 and 7 days post seizure, and found no significant differences in the total or surface expression (Tables 1-2, and Supplementary Table 1). Additionally we do not see changes in phosphorylation of sites associated with AMPAR trafficking and conductance changes associated with LTP (Banke et al., 2000; Benke et al., 1998; Oh et al., 2006; Chung et al., 2000) 7 days post seizure (Table 1).

To assess whether there might be changes in the expression of synaptic receptors we used whole cell voltage clamp electrophysiology. NMDAR current blockade by GluN2B specific inhibitor, RO 25-6981, was more pronounced in controls at P9 compared to P14, indicative of normal developmental subunit expression; however, there was not a significant difference in the mean GluN2B mediated current blocked for P9 control compared to P9 KA-ELS, nor P14 controls compared to P14 KA-ELS (Supplemental Figure 1). We also used A/N ratios to determine if the expression of synaptic AMPAR were altered, and found no significant differences at either developmental time examined (Supplemental Figure 2). Additionally, we measured the amplitudes of quantal currents in the presence of 8 mM strontium to asynchronize release upon stimulation as a measure of the activated synaptic AMPA receptors (Bekkers and Clements, 1999; Oliet et al., 1996; Xu-Friedman and Regehr, 1999; Stubblefield and Benke, 2010), and found no differences between control and KA-ELS (Supplemental Figure 3).

Calcium permeable AMPARs were not recruited following KA-ELS

Changes in individual AMPAR subunit expression have been shown to contribute to epileptogenesis, where the AMPARs that are expressed are calcium permeable (Grooms et al., 2000; Sanchez et al., 2001). AMPARs in the postnatal rat hippocampus are made of tetramers of GluA2 in combination with either GluA1 or GluA3 subunits (Wenthold et al., 1996). The presence of the GluA2 subunit confers calcium impermeability on the receptors (Hollmann et al., 1991). Calcium permeable GluA1 homomers are present in hippocampal CA1 pyramidal neuron synapses only prior to P7 (Stubblefield and Benke, 2010); however studies have shown that calcium permeable AMPARs are present in adult animal models of epilepsy (Pollard et al., 1993; Rajasekaran et al., 2012) and after hypoxia induced early life seizures (HI-ELS) (Sanchez et al. 2001; Rakhade et al., 2008). Additionally, calcium permeable AMPARs are thought to be transiently inserted into the synaptic membrane during the induction of LTP (Plant et al., 2006). Thus, we next sought to determine if KA-ELS could also induce a change in synaptic calcium permeable AMPARs.

We obtained current voltage (I/V) relationships of pharmacologically isolated AMPAR-mediated eEPSCs in CA1 hippocampal pyramidal neurons and calculated rectification

indices as done previously (Stubblefield and Benke, 2010). There was not a significant difference in the rectification indices when comparing P9 control (2.312 ± 0.356 ; $n = 8$ cells), to KA-ELS (2.114 ± 0.202 ; $n = 9$ cells; $p = 0.626$, two-tailed t-test) (Figure 2B, D). Interestingly, we found a small, but significant difference in the rectification indices when comparing P14 control (2.496 ± 0.135 ; $n = 8$ cells) to KA-ELS (2.016 ± 0.171 ; $n = 8$ cells; $p = 0.045$, two-tailed t-test) (Figure 2 D). P14 control actually showed slightly decreased normalized eEPSC at +20 mV, and +40 mV than P14 KA-ELS, however this was not significant ($F(1,14) = 3.352$, $p = 0.088$; Two-way repeat measures ANOVA), (Figure 2C). In distinction to findings after HI-ELS, these results suggested that there were not any changes in GluA2 lacking, calcium permeable, AMPARs after KA-ELS. Alternatively, these results indicate that there could be increased expression of transmembrane AMPA regulatory proteins (TARPs) after KA-ELS, as it has been previously shown that when TARPs are present there is a reduction in inward rectification (Jackson and Nicoll, 2011). Thus we also examined the expression of TARPs. Although there was a surprising 40% decrease in $\gamma 2$ -4 expression 7 days post seizure, the difference was not significant (Table 2). Recent evidence however, suggests that AMPARs that are less associated with TARPs are more mobile in the synapse, and therefore less likely to display desensitization due to a replenished supply incorporated into the synapses upon stimulation (Constals et al., 2015). Thus, overall, the results indicated that synaptic calcium permeable AMPARs are likely not present 2 nor 7 days post KA-ELS, but instead AMPAR desensitization may be altered following KA-ELS.

AMPA desensitization was decreased 7 days post KA-ELS

We assessed synaptic AMPAR desensitization using a short term facilitation paradigm (Constals et al., 2015; Khodosevich et al., 2014). AMPAR responses in slices from control rats ($n = 10$ cells) showed an initial facilitation, but did not continue to facilitate with subsequent stimulation indicative of desensitization of AMPARs. However, AMPAR responses in slices from KA-ELS rats ($n = 8$ cells) continue to facilitate beyond the second stimulating pulse (Figure 3A₁-A₂); ($F(1,16) = 4.589$; $p = 0.048$, Two way repeat measures ANOVA, Holm-Sidak post hoc analysis, control vs KA-ELS: Pulse # 4 $p = 0.038$; Pulse # 5 $p = 0.003$).

We additionally assessed AMPAR desensitization in the presence of 4-AP and 5 mM CaCl₂ (desensitization aCSF) to increase release probability and ensure that the receptors desensitize via a post-synaptic process with subsequent stimulation, rather than facilitate (Hjelmstadt et al., 1999). We found that AMPAR desensitization was not significantly altered 2 days following KA-ELS as compared to control (Figure 3B₁-B₂). There were also no significant differences in AMPAR desensitization developmentally between P9 and P14 ($F(1,16) = 0.472$, $p = 0.502$, Two way repeat measures ANOVA). However, consistent with our other findings at P14, we found that AMPAR desensitization was significantly decreased 7 days following KA-ELS ($n = 9$ cells) compared to control ($n = 9$ cells) ($F(1, 16) = 6.373$, $p = 0.023$, Two way repeat measures ANOVA, Holm-Sidak post hoc analysis control vs KA-ELS: Pulse #3 $p = 0.033$; Pulse #4 $p = 0.017$; Pulse #5 $p = 0.013$) (Figure 3C₁-C₂).

Cyclothiazide (CTZ) is a positive allosteric modulator of AMPARs that results in the elimination of rapid desensitization of the receptors. To confirm that KA-ELS indeed results

in a change in the AMPAR desensitization, we performed AMPAR desensitization experiments in the presence of CTZ. As expected, AMPAR desensitization was significantly decreased by CTZ in P14 controls ($F(1,8) = 7.161$, $p = 0.028$, Two way repeat measures ANOVA, Holm-Sidak post hoc analysis control vs KA-ELS: Pulse # 3 $p = 0.015$; Pulse #4 $p = 0.012$; Pulse #5 $p = 0.022$) (Figure 3D₁-D₂). However, the effect of CTZ on AMPAR desensitization following KA-ELS was occluded ($F(1, 7) = 0.0790$, $p = 0.787$, Two way repeat measures ANOVA) (Figure 3E₁-E₂), and CTZ eliminated the difference between control and KA-ELS AMPAR desensitization ($F(1, 16) = 0.454$, $p = 0.510$, Two way repeat measures ANOVA). KA-ELS did not alter AMPAR-mediated eEPSC kinetics; however, CTZ similarly altered AMPAR kinetics for both KA-ELS and control (Supplemental Table 2).

No pre-synaptic differences between control and KA-ELS

To determine whether changes in pre-synaptic activity could account for differences seen in AMPAR desensitization, we tested paired pulse intervals ranging from 20-140 ms in standard aCSF (Figure 4A, C). Paired-pulse facilitation is largely a measure of the probability of pre-synaptic vesicle release, thus any changes in paired pulse facilitation indicates a pre-synaptic change. We did not find a difference in facilitation between control and KA-ELS at any interval tested (Figure 4C), ($F(1,20) = 2.181$; $p = 0.154$, Two way repeat measures ANOVA). These results were confirmed by comparing NMDAR responses between control and KA-ELS upon the application of the irreversible open channel blocker MK-801 (Figure 4E) (Rosenmund et al., 1993). Additionally, we did not find alterations in paired-pulse facilitation at P9 (not shown).

To confirm that desensitization aCSF indeed increased the probability of release we compared NMDAR responses from slices after application of MK-801 for control in standard aCSF to those for control in desensitization aCSF (Figure 4G). Our results indicated that indeed the desensitization aCSF increased the probability of release. There is a significant difference in the $\log EC_{50}$ between control in standard aCSF (13.163 ± 0.881 , $n = 7$ cells) and control in desensitization aCSF (9.172 ± 0.877 , $n = 9$ cells) ($p = 0.007$, two-tailed t-test). To confirm that our desensitization aCSF effected both control and KA-ELS release probability to the same extent, we compared paired pulse ratios for all intervals previously tested for paired pulse facilitation in the presence of 4-AP and 5 mM $CaCl_2$ (Figure 4B, D). We found no significant differences in the paired pulse ratios between control and KA-ELS for any of the intervals tested ($F(1,18) = 1.510$, $p = 0.235$, Two way repeat measures ANOVA). Additionally there was not a significant difference between control (9.172 ± 0.877 , $n = 9$ cells) and KA-ELS (10.627 ± 1.133 , $n = 7$ cells) in the $\log EC_{50}$ of NMDAR responses after MK-801 application ($p = 0.319$, two-tailed t-test) (Figure 4F). These results indicate that KA-ELS indeed induces a post-synaptic change, rather than a pre-synaptic change, that results in decreased AMPAR desensitization.

Discussion

Autism spectrum disorders (ASD) and epilepsy are co-morbid, and early life seizures may increase the risk for autism (Buchmayer et al., 2009; Saemundsen et al., 2007; Saemundsen

et al., 2008; Tuchman, 2009). Currently, clinical opinions by leaders in the field vary widely (Korff et al., 2015), with some recognizing that early life seizures add insult to injury and favoring the aggressive treatment of early life seizures (Chapman et al., 2015). However, without longitudinal clinical studies directly testing effect of seizures on ASD/ID, the cause will continue to remain elusive in the clinic (Deonna and Roulet, 2006). The current study helps to fill this gap by allowing us to understand how early life seizures add insult to injury across multiple clinical scenarios. To this end, we have taken advantage of the KA-ELS rat model that develops an ASD/ID phenotype to examine the early developmental alterations in synaptic function induced by early life seizures.

We show here, contrary to LTP deficits in the adult KA-ELS rat, LTP is enhanced 7 days after seizure in the P14 KA-ELS rat. In the adult KA-ELS rat, we reported increased expression of PSD-95, and of STEP, decreased surface expression of GluA1, and a net reduction in GluN2A expression (Cornejo et al., 2007; Bernard et al., 2013), each of which could independently account for reduced LTP (Hayashi et al., 2000; Migaud et al., 1998; Liu et al., 2004; Massey et al., 2004; Kim et al., 2005; Pelkey et al., 2002). In comparison, we found minimal alterations in the expression of glutamate receptor subunits and scaffolds acutely after KA-ELS. Consistent with this, standard measures of synaptic glutamate receptor function, both AMPAR and NMDAR mediated, were unchanged acutely after KA-ELS. AMPAR trafficking and phosphorylation are associated with the expression of LTP; however, we did not observe any changes in these traditional hallmarks of LTP, suggesting no changes in the dynamic range of plasticity. This suggested a potential novel disease-triggered mechanism to alter the expression of LTP. Indeed, for the first time, here we present decreased AMPAR desensitization 7 days following KA-ELS in correlation with enhanced LTP. Decreased AMPAR desensitization should lead to an increase in the overall open time, thus enhancing total charge carried through the receptors. Expectedly, this was not evident with single evoked responses where kinetics are mediated by deactivation rather than desensitization (Traynelis et al., 2010), but only became apparent following a train of responses. Thus AMPARs, especially during a stimulus train, will pass greater current and total charge accumulation leading to greater depolarization and excitability following KA-ELS. Importantly, reduced desensitization has already been demonstrated to result in greater LTP in multiple studies (Arai et al., 2004; Chang et al., 2014; Lin et al., 2002). Further, desensitization of AMPARs is thought to be involved in the expression of LTP (Ambros-Ingerson and Lynch, 1993; Arai et al., 2004), but see also (Rammes et al., 1999). Thus it is possible, although not shown here, that there is a link between changes in AMPAR desensitization and LTP following KA-ELS.

AMPA desensitization is determined by subunits, auxiliary subunits, and alternative splicing and editing (Traynelis et al., 2010; Penn and Greger, 2009). R/G editing and flip/flop splicing influence receptor assembly (Penn and Greger, 2009), dendritic trafficking (La et al., 2013; Coleman et al., 2006), and desensitization (Penn et al., 2012). Recombinant AMPARs that are R/G edited recover from desensitization more than two times faster, and half as many channels maximally desensitized (Lomeli et al., 1994). We show here an increase in the protein responsible for editing AMPAR subunits GluA2-4 mRNA at the R/G site, Adenosine deaminase acting on RNA 2 (ADAR2) (Table 1). It is unlikely that this site is 100% edited at the developmental time point examined (Lomeli et al., 1994; Balik et al.,

2012); however, whether or not a 40% increase in the expression of ADAR2 can account for changes in the editing of the AMPAR has not been shown. ADAR2 expression is regulated by multiple mechanisms (Rueter et al., 1999; Marcucci et al., 2011; Balik et al., 2013; Yang et al., 2012); however, whether these mechanisms can account for increased ADAR2 expression in KA-ELS CA1 has yet to be clarified. ADAR2 also has many targets beyond AMPARs (Keegan et al., 2004), many of which have altered editing in response to neuronal activity (Sanjana et al., 2012), and unexplored roles in plasticity. Thus, mRNA expression studies to confirm this suggestion and explore other ADAR2 targets are currently underway.

TARP presence has also been reported to reduce AMPAR desensitization and increase the rate of recovery from desensitization (Priel et al., 2005). However, more recent data suggests that AMPARs are more mobile when not associated with TARPs, thus are less likely to display desensitization (Constals et al., 2015). Total expression of TARPs following KA-ELS, although not significant, displayed trends toward a decrease in the expression of TARPs, γ 2-4. Whether or not the decrease seen in TARPs could account for the decreased AMPAR desensitization is unclear due to the lack of changes seen in AMPAR subunits crosslinked in the membranes.

CTZ differentially affects desensitization of subunits depending on the isoforms present, particularly for GluA1 subunits (Partin et al., 1994; Mosbacher et al., 1994; Koike et al., 2000; Grosskreutz et al., 2003; Partin et al., 1994; Johansen et al., 1995). Here, AMPAR desensitization was blocked by CTZ in control whereas AMPAR desensitization was not further blocked by CTZ following KA-ELS. Thus, overall the decreased AMPAR desensitization following KA-ELS supports the hypothesis that AMPAR subunits expressed after seizure are potentially GluA1 flop in combination with edited GluA2 flip isoforms. Similar proposed changes in AMPAR assembly and function have been observed in cultured neurons and associated with reduced activity (Penn et al., 2012). Importantly, here we link these changes to increased activity in the developing whole animal.

Interestingly, some of the changes that have been observed in the HI-ELS rat model in the acute period following early life seizures were not found in the KA-ELS model. These differences were not entirely unexpected because the KA-ELS rat model used here does not develop epilepsy (Bernard et al., 2013), whereas the HI-ELS model develops spontaneous seizures chronically (Rakhade et al., 2011). The similarities between the findings may suggest mechanisms for the development of cognitive dysfunction, whereas changes observed in the HI-ELS that were not found in the KA-ELS may better predict epileptogenesis. The HI-ELS model induces transient increases in the phosphorylation of GluA1 S831/S845 and GluA2 S880 that are associated with transient increases in kinase activity (Rakhade et al., 2008) and increased LTP in the same time frame following HI-ELS (Jensen et al., 1998). The increase in LTP in HI-ELS is transient, as the opposite is observed 48-72 hours post hypoxia seizure, attributed to a decrease in the number of silent synapses and an increase in the GluN2A subunit compared to the GluN2B subunit (Zhou et al., 2011; Zhou et al., 2015). Consistent with the transient nature of the phosphorylation in the HI-ELS model, we also did not observe any changes in the phosphorylation of AMPAR subunits 7 days post KA-ELS. Additionally we did not find differences in the number of silent synapses 2 nor 7 days following KA-ELS. These results were confirmed by the lack of

expression and membrane insertion changes in the AMPAR or NMDAR subunits after KA-ELS. The results highlight important differences between the HI-ELS model and the KA-ELS model, and may implicate decreases in silent synapses and increases in AMPARs and NMDARs in epileptogenesis, rather than cognitive dysfunction. Changes induced by seizure in the KA-ELS model include a decrease in AMPAR desensitization. This has not been previously reported, nor to our knowledge studied in any other animal model of early life seizures or other neurocognitive disorder. This data represent a novel mechanism by which a single episode of early life seizures can potentially lead to deficits in the adult KA-ELS rat. It is unlikely that decreased AMPAR desensitization in the acute period following seizure contributes to epileptogenesis as the KA-ELS rat model does not develop spontaneous seizures.

Several genetically modified mouse models have demonstrated the behavioral consequences of enhanced LTP and are consistent with our findings. Knock out of LIMK1, a protein kinase underlying Williams syndrome (characterized by developmental delays and learning deficits), results in enhanced LTP, increased locomotor activity in open field test, enhanced cued fear response in contextual fear conditioning, and impaired learning reversal in the Morris water maze (Meng et al., 2002). Frontal lobe ablation of Stromal Interaction Molecule (STIM) results in enhanced CA3-CA1 hippocampal LTP and learning delays in the Morris water maze (Garcia-Alvarez et al., 2015). Heterozygous mice for Dyt1, underlying DYT1 dystonia, display enhanced hippocampal LTP and motor deficits (Yokoi et al., 2015). Mice heterozygous for Neurobeachin, previously identified as an autism gene candidate, have enhanced CA1 LTP, altered self-grooming, social behaviors, contextual fear, and spatial learning and memory (Nuytens et al., 2013). Additionally, an adult rat model of depression showed a similar pattern of changes in LTP as seen in the KA-ELS rat after seizure, with no change in LTP 7 days, enhanced LTP 14 days, and impaired LTP 21 days following exposure to chronic unpredictable mild stress (Qiao et al., 2014). Thus, although traditionally, impaired LTP is thought to underlie learning deficits, any disruption in normal levels of LTP can result in a variety of deficits, and enhanced LTP, especially during the critical periods of development can be detrimental.

While several studies demonstrate the consequences of enhanced LTP on learning, whether changes in AMPAR desensitization and enhanced LTP in the acute period following KA-ELS contribute to the development of adverse outcomes during a developmentally sensitive critical period (Hensch, 2004) have yet to be fully tested. It would be interesting to block desensitization in vivo as a means to determine if it causes the same cognitive and behavioral changes in KA-ELS rats. While not specifically addressing desensitization, previous studies have blocked AMPARs with NBQX resulting in a reversal of the cognitive deficits and epilepsy in the HI-ELS model (Lippman-Bell et al., 2013). Modulators, opposite to CTZ, that instead enhance AMPAR desensitization are currently non-existent, but may represent a novel therapeutic approach in this and other disease models.

The current study found changes 7 days after KA-ELS, and did not find any differences 2 days post seizure, thus we still do not know how the seizure induced changes in AMPAR desensitization or LTP. Changes in desensitization can affect summation of synaptic currents to in turn affect overall network properties, thus potentially triggering alterations in

the function of the developing network. While it is unclear how long that this change persists into adulthood, it is possible that it propagates later life changes. The results suggest that although the seizure itself likely enhances activity while it is occurring there may be a “dormant” period where the seizure may be altering molecular mechanisms, perhaps epigenetically, prior to a physiological effect. However, the physiological effect observed 7 days post seizure, decreased AMPAR desensitization, may be a target for therapeutic intervention of future deficits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

KA-ELS	kainic acid induced early life seizure
HI- ELS	hypoxia induced early life seizure
CTZ	cyclothiazide

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Highlights

- In KA-ELS rats LTP is enhanced 7 days post seizure, but no change in LTP is seen 2 days post seizure.
- AMPA receptor desensitization is decreased 7 days post seizure, but unaltered at 2 days post seizure.
- No changes in basal synaptic transmission mediated by AMPA and NMDA receptors, total and surface expression, nor phosphorylation of AMPA or NMDA receptors is detected 2 and 7 days post seizure in KA-ELS rats.
- No changes in pre-synaptic activity between control and KA-ELS rats are detected 7 days post seizure.

Figure 1.

LTP was enhanced only at 7 days following KA-ELS (P14). A, mean fEPSPs for P9 control (closed circles) ($141.7 \pm 6.4\%$, $n = 6$ slices) and KA-ELS ($141.4 \pm 2.6\%$, $n = 9$ slices) (open circles) are plotted for stimulated pathways. Unstimulated pathways are also shown (control: closed triangles, KA-ELS: open triangles). Error bars represent SEM. Sample traces with scale bar are shown to the right of the graph. The top traces are from P9 KA-ELS, and bottom traces from P9 control. Trace 1 are representative baseline responses, and trace 2 represents responses after LTP. The traces are additionally overlaid (1 + 2). # denotes stimulus artifacts. B, mean fEPSPs for P14 control ($129.2 \pm 1.599\%$, $n = 9$ slices) (closed circles) and KA-ELS ($153.9 \pm 0.1722\%$, $n = 7$ slices) (open circles) are plotted for stimulated pathways. Unstimulated pathways are also shown (control: closed triangles, KA-ELS: open triangles). Error bars represent SEM. Sample traces and scale bar are shown to the right of the graph. The top traces are from P14 KA-ELS, and the bottom traces are from P14 control. Trace 1 are representative baseline responses, and trace 2 represents responses after LTP. The traces are additionally overlaid (1 + 2). # denotes stimulus artifacts.

Figure 2.

Calcium permeable AMPARs are not recruited following KA-ELS. A, sample traces for all holding potentials used to obtain current/voltage (I/V) relationships. # denotes stimulus artifact. B, I/V curves for P9 control and KA-ELS. Mean peak currents for all voltages are normalized to mean peak currents collected at -70 mV. There were no significant differences in the normalized mean peak currents between control and KA-ELS. C, I/V relationship for P14 control and KA-ELS. There was not a significant difference in the normalized mean peak currents between control and KA-ELS. D, there was not a significant difference in the rectification indices for P9 control, compared to P9 KA-ELS. There was a small, but significant reduction in the rectification indices for the P14 control compared to P14 KA-ELS. Statistics are presented in the text.

Figure 3.

AMPA desensitization was decreased only at 7 days following KA-ELS (P14). A₁, in standard aCSF, sample traces of 5 pulse AMPAR responses (20 Hz) for P14 control (black) and KA-ELS (gray). A₂, there is a significant difference in AMPAR responses between P14 control compared to P14 KA-ELS. B₁, in desensitization aCSF, sample traces of 5 pulse AMPAR responses (50 Hz) for P9 control (black) and KA-ELS (gray) overlaid. KA-ELS is scaled to control. B₂, there was not a significant difference in AMPAR desensitization between P9 control (n = 9 cells) and KA-ELS (n = 6 cells) ($F(1,13) = 0.369$, $p = 0.554$, Two

way repeat measures ANOVA). C_1 , in desensitization aCSF, sample traces of 5 pulse AMPAR responses (50 Hz) for P14 control (black) and KA-ELS (gray). KA-ELS is scaled to control. C_2 , AMPAR desensitization was significantly decreased in P14 KA-ELS compared to control. D_1 , in desensitization aCSF, sample traces of 5 pulse AMPAR responses (50 Hz) for P14 control with and without CTZ. Before CTZ is scaled to after CTZ. D_2 , AMPAR desensitization was significantly decreased by 100 μ M CTZ in P14 controls ($n = 9$ cells). E_1 , in desensitization aCSF, sample traces of 5 pulse responses for P14 KA-ELS with and without CTZ. Before CTZ is scaled to after CTZ. E_2 , CTZ did not significantly reduce AMPAR desensitization further in KA-ELS ($n = 8$ cells). All stimulus artifacts have been removed. Data in A_2 , B_2 , C_2 , D_2 , and E_2 are presented as normalized mean peak currents, where the mean peak currents of responses from stimulus 1-5 are normalized to the mean peak current response from stimulus 1. Error bars represent SEM.

Figure 4.

Presynaptic function is not affected 7 days following KA-ELS (P14). A, sample traces for paired pulse intervals 20, 40, 60, 80, 100, and 140 ms in a standard aCSF. Stimulus artifacts have been removed. B, sample traces for paired pulse intervals 20, 40, 60, 80, 100, and 140 ms in desensitization aCSF containing 50 μ M 4-AP, and 5 mM CaCl_2 . Stimulus artifacts have been removed. C, paired pulse ratios for control (n's for 20, 40, 60, 80, 100, and 140 ms = 12, 12, 11, 11, 10, and 10 cells, respectively) and KA-ELS (n's for 20, 40, 60, 80, 100, and 140 ms = 10, 10, 9, 9, 9, and 9 cells, respectively) in standard aCSF. D, paired pulse

ratios for control (n's for 20, 40, 60, 80, 100, and 140 ms = 10, 11, 11, 11, 11, and 11 cells, respectively) and KA-ELS (n's for 20, 40, 60, 80, 100, and 140 ms = 9, 9, 9, 9, 9, and 9 cells, respectively). E, NMDAR current as a percentage of normalized baseline current (collected for 5 min) after application of 40 μ M MK-801 for control and KA-ELS in standard aCSF. For each recording, % current was plotted against time and then curve fits were performed to determine the logEC₅₀ for the time at which 50% of the current was blocked. We found no differences in the mean logEC₅₀ between control (13.163 ± 0.881 , n = 7 cells) and KA-ELS (10.018 ± 1.254 , n = 7 cells) (p = 0.063, two-tailed t- test). F, percentage of NMDAR current before and after application of MK-801 for control and KA-ELS in desensitization aCSF. G, desensitization aCSF increases the release probability. Percentage of NMDAR current before and after application of MK-801 for control in standard aCSF and control in desensitization aCSF.

Table 1

Summary of CA1 protein expression at P14.

Antibody	Control Mean $\pm$ SEM	<i>n</i> Value	KA-ELS Mean $\pm$ SEM	<i>n</i> Value	<i>P</i> value	Statistical test
ADAR2	100 $\pm$ 15.3	7	144 $\pm$ 10.3	10	0.026	Two-tailed t-test
GluA1	100 $\pm$ 10.3	8	91 $\pm$ 22.9	8	0.234	Mann Whitney Rank Sums
GluA1 phospho S845	100 $\pm$ 27.7	8	99.3 $\pm$ 25.1	8	1.0	Mann Whitney Rank Sums
GluA1 phospho S831	100 $\pm$ 20.7	8	107.8 $\pm$ 19	8	0.878	Two-tailed t-test
GluA2/3	100 $\pm$ 11.2	8	103 $\pm$ 18.2	8	0.900	Two-tailed t-test
GluA2 phospho S880	100 $\pm$ 23.1	8	98.8 $\pm$ 21.0	8	0.959	Mann Whitney Rank Sums
GluA4	100 $\pm$ 19.8	10	73 $\pm$ 16.9	6	0.365	Two-tailed t-test
GluN1	100 $\pm$ 36.9	8	100 $\pm$ 36.8	8	1.0	Mann Whitney Rank Sums
GluN2A	100 $\pm$ 9.1	8	109 $\pm$ 16.1	8	0.644	Two-tailed t-test
GluN2B	100 $\pm$ 24.1	8	80 $\pm$ 7.7	8	0.458	Two-tailed t-test with Welch's Correction
PSD-95	100 $\pm$ 15.2	8	105 $\pm$ 10.4	8	0.770	Two-tailed t-test
Stargazin/TARP γ 2, 3, 4	100 $\pm$ 15.2	12	61.4 $\pm$ 12.9	12	0.070	Two-tailed t-test

Table 2

Summary of the percent of internal protein in P14 CA1.

Antibody	Control Mean $\pm$ SEM	<i>n</i> Value	KA-ELS Mean $\pm$ SEM	<i>n</i> Value	<i>P</i> value	Statistical test
GluA1	27.9 $\pm$ 2.1%	7	27.6 $\pm$ 3.5%	8	0.934	Two-tailed t-test
GluA2/3	34.6 $\pm$ 2.4%	7	32.7 $\pm$ 2.9%	8	0.615	Two-tailed t-test
GluN1	44.9 $\pm$ 6.1%	7	45.5 $\pm$ 6.0%	8	0.952	Two-tailed t-test
GluN2A	44.7 $\pm$ 4.0%	7	38.3 $\pm$ 5.7%	8	0.389	Two-tailed t-test
GluN2B	49.1 $\pm$ 3.2%	7	52.8 $\pm$ 2.8%	8	0.405	Two tailed t-test