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Differential changes in mGlu2 and mGlu3 gene expression following pilocarpine-induced *status epilepticus*: A comparative real-time PCR analysis

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Abstract

Group II metabotropic glutamate (mGlu II) receptors subtype 2 and 3 (mGlu2 and mGlu3) are subtle regulators of neuronal excitability and synaptic plasticity in the hippocampus. In recent years, researchers have investigated the potential neuroprotective and anticonvulsant effects of compounds acting on mGlu II receptors. However, abnormal expression and function of mGlu2 and mGlu3 have been reported in temporal lobe epilepsy, a phenomena that may limit the therapeutic effectiveness of these potentially new antiepileptic drugs. Here, we investigated seizure-induced changes in mGlu2 and mGlu3 mRNA following pilocarpine-inducted *status epilepticus* (SE) and subsequent epileptogenesis. Relative changes in gene expression were assessed by comparative analysis of quantitative real-time PCR (qrtPCR) by the delta-delta CT method. Pilocarpine-treated and control rats were sacrificed at different periods (24h, 10 days, one month and more than two months) following SE. Total RNA was isolated from microdissected dentate gyrus and processed for RT-PCR and qrtPCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control gene. Analysis of relative quantification (RQ) ratios of mGlu2 and mGlu3 mRNA expression revealed a significant down-regulation of both targets at 24h after SE. Gene expression partially recovered at 10 days following SE reaching control levels at one month after SE. Two month after SE, mGlu2 mRNA expression was significantly down-regulated to ~41% of control expression whereas mGlu3 mRNA was comparable to control levels. Our data indicate that mGlu2 and mGlu3 expression is dynamically down-regulated or selectively enhanced during critical periods of epileptogenesis. Seizure-induced differential dysregulation of mGlu2 and mGlu3 receptors may affect the availability of these molecular targets for therapeutic compounds in epilepsy.

Keywords

pilocarpine; epilepsy; presynaptic; granule cells; metabotropic glutamate receptors

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1. Introduction

Compelling evidences indicate that G-protein coupled group II metabotropic glutamate (mGlu II, mGlu2/3) receptors subtype 2 (mGlu2) and 3 (mGlu3) play a major role in regulating glutamatergic transmission at both presynaptic and postsynaptic sites in the hippocampus (Kamiya et al., 1996; Kew et al., 2001; Lea et al., 2001; Manzoni et al., 1995; Sanabria et al., 2004; Scanziani et al., 1997; Toth et al., 2000; Yokoi et al., 1996). Immunohistochemical and electron microscopy studies have revealed that mGlu2 is preferentially located at presynaptic sites, especially in mossy fiber axons of granule cells in the dentate gyrus (Petralia et al., 1996; Shigemoto et al., 1997). Upon activation, presynaptic mGlu2 act as feedback regulators of excessive glutamate release by reducing the release probability of glutamatergic synapses and inducing a long-term depression (LTD) of synaptic transmission (Kamiya et al., 1996; Sanabria et al., 2004; Scanziani et al., 1997). In contrast, mGlu3 receptors are mainly positioned at postsynaptic sites in the dentritic arborizations of granule cells (Petralia et al., 1996; Tamaru et al., 2001) where they are thought to modulate plasticity of medial perforant path-dentate gyrus synapses (Lea et al., 2001; Poschel et al., 2005).

Despite major breakthroughs in pharmacological drug development, about 30% of patients suffering from epilepsy remain medically intractable (Kwan and Brodie, 2006). Accordingly, novel therapeutic compounds are necessary to battle pharmacoresistance (Majores et al., 2007). Hyperexcitability has been associated with anomalous glutamate release, especially in mesial temporal lobe epilepsy (MTLE) (Liu et al., 1995). Hence, compounds that reduce glutamate release may be strategically important as antiepileptic (potentially neuroprotective), because of their ability to attenuate exaggerated glutamate release. Accordingly, pharmaceutical research has devoted special attention to novel drugs that can reduce presynaptic release of glutamate (Fink et al., 2002; Macdonald, 1996; Omori et al., 1999; Wheeler, 2002). In this line, the modulatory action of mGlu2 and mGlu3 in synaptic transmission has revived interest in targeting these glutamate receptors for the treatment of epilepsy. However, a down-side of this strategy is that expression of these receptors can be abnormally regulated by seizures. For instances, in pups and adult rats, kainic acid-induced *status epilepticus* (SE) provoke a reduction of mGlu2 mRNA in granule cells of dentate gyrus (Aronica et al., 1997b). During the chronic period of the rat pilocarpine model of MTLE, mGlu2/3 immunostaining was dramatically attenuated in mossy fibers and at the *stratum lacunosum/moleculare* of the hippocampus while an apparent compensatory increase was detected at the supragranular layers (middle and upper molecular layers) of the dentate gyrus (Pacheco Otalora et al., 2006). This rearrangement of mGlu2/3 expression was also observed in the pilocarpine mice model of MTLE and in patients suffering MTLE (Tang et al., 2004). Moreover, previous study indicates that hippocampal kindling can produce a persistent up-regulation of mGlu2/3 in the supragranular layer of the dentate gyrus (Aronica et al., 2000b). Despite these preliminary data, epileptogenesis-dependent relative changes in mGlu2 and mGlu3 transcript levels have not been adequately investigated in MTLE. In this study, we used a comparative ΔΔCt paradigm of quantitative real-time PCR (qrtPCR) to assess changes in relative gene expression of mGlu2 and mGlu3 at different time-points of epileptogenesis following pilocarpine-induced SE.

2. Results

2.1. Pilocarpine-induction of SE and subsequent epilepsy

In this study, we investigated changes in mGlu2 and mGlu3 gene expression at different stages of epileptogenesis following SE in rats (acute, “latent” and chronic periods). For these purpose, we isolated total RNA from microdissected dentate gyrus area of rats that were sacrificed at four time points after pilocarpine-induced SE. The SE duration in all groups (24h, 10 days, 1 month and <2 month) was limited to ~3h by administration of diazepam to minimize variability

in the development of the pilocarpine model considering that the duration of SE positively correlate with late neuropathological sequalae and epileptogenesis (Lemos and Cavalheiro, 1995; Lemos and Cavalheiro, 1996). Animals that experienced less than 3h of SE were not included in the study. To determine whether expression of target genes mGlu2 and mGlu3 is modified at critical stages of epileptogenesis we developed this study in the following experimental groups: (a) acute period, rats (n=5) that were sacrificed 24h immediately after the termination of SE (24h group); (b) "latent period", rats (n=4) that were sacrificed 10 days following SE (these animals didn't exhibited any detectable Racine Scale 3 spontaneous seizures by 8h/day video-monitoring with automatic detection SeizureScan System); (c) chronic period, these epileptic rats exhibiting spontaneous recurrent seizures were assigned to two separate groups sacrificed at 1 month (n=5) (early chronic epileptogenesis) and more than 2 month (n=5) after SE (late chronic epileptogenesis). As previously reported, chronically epileptic rats exhibited in average 2–3 seizures/day and no differences were found between chronic epileptic groups in seizure frequency. Control group included rats that were injected with methyl-scopolamine and with saline instead of pilocarpine (n=4) and rats that received both methyl-scopolamine and pilocarpine but did not developed SE (n=4). No seizures were detected in this last group after at least 10 sections (8h each) of video-monitoring.

2.2. Analysis of changes in gene expression of mGlu2 and mGlu3 following pilocarpine-induced SE using comparative real-time PCR

In this study, we applied the comparative CT quantification (ΔΔCt method) of qrtPCR for comparing changes in gene expression of mGlu2 and mGlu3 following pilocarpine-induced SE. Relative quantification was performed using *GAPDH* as endogenous control gene after analyzing the gene expression profile of four candidate reference genes (i.e. *RpL10*, *RpL28*, eukaryotic *18S* and *GAPDH*) in randomly selected twenty different samples (cDNA from control and several epileptic groups). The rationale was to select the more stable gene that varied the less even during the epileptogenic process. *GAPDH* exhibited the less variability among the samples as determined by the analysis of the standard deviation of the threshold cycles (CT) values (see below).

Since the analysis of gene expression included different time-points after SE and animals with different age, we first assessed age-dependent changes in mGlu2 and mGlu3 by performing relative quantification in material obtained from age-matched control rats of different survival times. Our analysis of the relative quantification (RQ) values using the ΔΔCt approach of qrtPCR revealed not significant changes in mGlu2 and mGlu3 expression in control rats sacrificed a different survival periods corresponding to 24h, 10 days, 1 month and >2 month experimental groups (ANOVA, $P>0.05$). Accordingly, data from these control rats were grouped together for statistical analysis.

Our analysis revealed a significant change in gene expression of mGlu2 (ANOVA, $P<0.001$, $F=6.3$) and mGlu3 (ANOVA, $P<0.01$, $F=4.7$) at different stages of epileptogenesis following pilocarpine-induced SE (Table 1, Fig. 2). A significant down-regulation of both mGlu2 (56% reduction compared to controls, *post-hoc* $P<0.05$) and mGlu3 (63 % reduction compared to controls, *post-hoc* $P<0.01$) was detected via by *post-hoc* Dunnett 's test at 24h following SE. Gene expression partially recovered for both mGlu2 (21% reduction of controls) and mGlu3 (26% reduction of controls) transcripts at 10 days after SE where no significant differences were revealed when compared to control levels (*post-hoc* Dunnett's test, $P>0.05$). Gene expression completely recovered during chronic epileptogenesis at 1 month following SE where a non-significant 10% increase was detected for mGlu2 (*post-hoc* Dunnett's test, $P>0.05$) and a 11% reduction was noticed in mGlu3 (*post-hoc* Dunnett's test, $P>0.05$). In marked contrast, gene expression of these target genes diverged after 2 month of epileptogenesis in such a way that mGlu2 expression was significantly down-regulated at 41%

of control levels (*post-hoc* Dunnett's test, $P<0.05$) while mGlu3 remained increased although no significant changes were detected when compared to controls (by *post-hoc* Dunnett's test, $P>0.05$) (Fig. 2).

3. Discussion

Our data indicate that pilocarpine-induced SE provokes an epileptogenesis-related dysregulation of mGlu2 and mGlu3 expression in the dentate gyrus. A significant down-regulation of these transcripts was observed at 24h after the SE insult as previously reported for mGlu2 in kainic acid-induced SE (Aronica et al., 1997a). In this *in situ* hybridization study, SE induced a significant reduction in the expression of mGlu2 mRNA in granule cells of the dentate gyrus in pups and adult rats but did not alter the expression of mGluR1, mGlu3 and mGluR5 mRNA. In a separate study, mGlu2/3 and mGluR5 protein expression was found to be markedly increased in glial cells of CA3 and hilus by 1 week after electrical stimulation-induced SE, and persisted up to 3 months after SE (Aronica et al., 2000a). Interestingly, we detected a compensatory increase in both mGlu2 and mGlu3 transcripts at 10 days after SE that reach not significant differences than control. The difference among these data can be explained by the fact that we are measuring changes in mRNA levels in total RNA extracted from entire dentate gyrus while in the first study mRNA was quantified via *in situ* hybridization on granule cells and in the second study, protein expression was analyzed instead of changes in transcript levels. It is also possible that mGlu3 is preferentially expressed at glial cells playing a major role in epilepsy-related hippocampal gliosis. Consistent with this notion, increased of mGlu2/3 protein expression was reportedly enhanced in hippocampal immunoreactive astrocytes in epileptic patients suggesting that mGlu2 and/or mGlu3 may be involved in the process of reactive gliosis during the course of epileptogenesis (Tang and Lee, 2001). In line with these findings, we observed a complete and persistent recovery on mGlu3 expression during late epileptogenesis (> 2 month after SE). Therefore, the functional implications of relative changes in mGlu2 and mGlu3 mRNA expression on total RNA during epileptogenesis had to be considered in the context of cell-specific changes. Additional approaches including single-cell qrtPCR are necessary to assess cell-specific changes in mGlu2 and mGlu3 gene expression in epilepsy.

After initial reduction in the acute phase of the pilocarpine model, relative expression of mGlu2 recovered near control values at 1 month, but a significant down-regulation was again detected during the late phases of the chronic period. These data correlate with previous immunohistochemical studies (Pacheco Otalora et al., 2006; Tang et al., 2004) reporting a marked deficit in mGlu2/3 expression in chronically epileptic rats in the pilocarpine model of epilepsy and in patients suffering MTLE (Pacheco Otalora et al., 2006; Tang et al., 2004). It has been demonstrated that mGlu2 play a critical role in regulating presynaptic excitatory transmission in dentate gyrus mossy fiber-CA3 synapses (XXX). Hence, deficient expression of mGlu2 mRNA and proteins may provoke an exaggerated glutamate release, hyperexcitability and neurodegenration during epileptogenesis.

In previous studies, strong mGlu2/3 immunoreactivity was observed in the molecular layers of dentate gyrus in experimental models of MTLE (Pacheco Otalora et al., 2006; Tang et al., 2004). Since mGlu3 is preferentially localized in postsynaptic sites in this area, it was suggested that such increase was dependent on a preferential up-regulation of mGlu3. At this location, mGlu3 is considered to play in regulatory role modulating the excitatory perforant path glutamatergic inputs from entorhinal cortex. For instance, *in vivo* electrophysiological studies revealed that mGlu3 in the perforant path play and essential role in LTD, and a modulatory role in activity-dependent long-term potentiation (LTP), via distinct pre- and post-synaptic mechanisms (Kilbride et al., 2001; Poschel et al., 2005). Hence, increased expression of mGlu3 protein and normalized expression of mGlu3 transcripts in dentate gyrus may represent a

compensatory phenomenon to counter the hyperexcitability of entorhinal cortex-dentate gyrus pathway in chronic epileptogenesis. In addition, mGlu3 high expression in glial cells is considered to exert a neuroprotective action by regulating glial-neuronal pathways and glutamate metabolism (Bruno et al., 1997). As discussed above, mGlu3 is increased in reactive glial cells in epilepsy (Aronica et al., 2000a). Therefore, seizure-related changes in mGlu3 may represent an additional compensatory change in epilepsy. Further studies are necessary to elucidate the functional role of mGlu3 in chronic epileptogenesis.

Recent gene profiling studies have been designed to provide a comprehensive assessment on epilepsy progression and to discover new pharmacological targets (Aronica and Gorter, 2007; Elliott and Lowenstein, 2004; Hatazaki et al., 2007; Hunsberger et al., 2005; Lee et al., 2007; Lukasiuk et al., 2003; Majores et al., 2007). Analysis of a large number of genes via the Affymetrix Gene Chip System revealed a differential gene expression in total RNA extracted from CA3 and entorhinal cortex at three different time points after electrically induced SE representing acute, latent and chronic phases of this model of acquired epilepsy (Gorter et al., 2006). This study also investigated whether mGlu3 expression change during acute, latent and chronic epileptogenesis. Consistent with our data, the authors detected a significant down-regulation of mGlu3 in the acute phase following SE (Cornus Ammonis and entorhinal cortex) and in the latent period (entorhinal cortex). Moreover, gene expression levels apparently return to control levels in the chronic phase. In addition, similar pattern of expression was observed for most glutamate signaling-related genes that were acutely down-regulated (approximately twofold) during the acute phase but recovered to control levels after the latent period. Furthermore, gene expression remained below control levels in some genes including mGluR1 in the entorhinal cortex. As confirmed in our qrtPCR results for mGlu2 and mGlu3, seizure-induced deficits, wave of expression, and compensatory changes are a common gene expression pattern during the course of epileptogenesis. The Seizure-related changes in gene expression exhibited different patterns that may indicate dynamic reorganization of molecular machinery (transcriptional, post-transcriptional, etc) accompanying structural and functional modifications in epilepsy. The mechanisms leading to the decline in mGlu2 during early or late (chronic) phases of epileptogenesis are currently unknown. It is possible that the hyperexcitability and metabolic distress associated with SE induce an acute deficit in the transcription machinery that can explain the down-regulation 24 h following SE. After an apparent recovery, a pervasive deficiency may recur on the transcription process as a consequence of spontaneous recurrent seizures. Further experiments are necessary to elucidate the molecular mechanisms underlying transcriptional channelopathies in acquired epilepsy.

Advances in genomic and proteomic approaches will undoubtedly provide a better understanding in the molecular determinants of epileptogenesis when associated to functional studies. These studies can shed light in seizure-related modifications of current antiepileptic targets, a form of seizure-dependent plasticity that may interfere with the effectiveness of antiepileptic drugs. Epilepsy-associated post-transcriptional changes in ion channels, receptors and transporters (acquired channelopathies) may alter availability of molecular targets or generate dysfunctional “epileptic” targets with deficient pharmacological properties via abnormal splicing or additional post-translational modifications (*e.g.* phosphorylation).

Experimental Procedure

4. Material and methods

4.1. Animals and pilocarpine model of epilepsy—All experiments were performed in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and with the approval of The University of Texas at Brownsville Institutional Animal Care and Use Committee (Protocol#: 2007-001-IACUC). Wistar rats were kept in acclimatized temperature-controlled *vivarium* with water and food *ad libitum*. All

efforts were made to minimize the number of animals in the study. Chronically epileptic rats were obtained by the pilocarpine model of temporal lobe epilepsy following described procedures (Cavalheiro, 1995; Mello et al., 1993; Pacheco Otalora et al., 2008). At the time of inducing SE animals were approximately 20–30 days (180–250g). Methyl-scopolamine nitrate (0.1 mg/kg in saline, s.c.) (Sigma-Aldrich, St. Louis, MO) was administered 30 min prior pilocarpine to minimize the systemic side effects of cholinergic overstimulation (Turski et al., 1984). Animals were then injected with 4% pilocarpine hydrochloride (Sigma-Aldrich) (350 mg/kg in saline, i.p.). Controls included (a) animals that received methyl-scopolamine but were injected with saline instead of pilocarpine, (b) pilocarpine-injected animals that did not exhibit seizures. Behavioral SE was limited to ~3 hr by administering two doses of diazepam (10–12 mg/kg, s.c. 6h interval). This procedure helps to increase the survival rate (Danzer and McNamara, 2004; Mello et al., 1993; Pacheco Otalora et al., 2006; Pacheco Otalora et al., 2008). All animals that suffered SE were given a subcutaneous injection of 20 ml Ringer-lactate solution and diet was enriched with Nutra-Gel® soft food (Bio-Serv, Frenchtown, NJ) for at least one week. After SE, rats were monitored for detection of at least two spontaneous seizures (8h/day) using a JVC MiniDV digital video-camera and researcher-assisted SeizureScan software (Clever Sys., Inc, Reston, VA). Seizures were confirmed *off-line* by a trained researcher. Only seizures graded ≥ 3 in the Racine's scale (Racine, 1972) per week were computed (according to sensitivity of the detection system). In average, SE-suffering rats experienced approximately 2–3 per 8h period/day regularly during the observation period. For isolation of total RNA pilocarpine-treated rats were sacrificed at 24h, 10 days, one month and at more than two month (late chronic period) following induction of SE.

4.2. Isolation of total RNA isolation and RT-PCR

Dentate gyrus RNA isolation: A set of age-matched control and epileptic rats were anesthetized as above and rapidly decapitated to prepare 600 μm horizontal hippocampal slices in ACSF (2–4°C). Sections were obtained from an area corresponding to a depth of 3–4.5 according to the Paxinos's Atlas. Epileptic rats were sacrificed at different post-SE time points. The region of dentate gyrus was carefully microdissected with 27 gauge needles assisted via stereomicroscope (10–20x) and cold light transillumination. Under transillumination, a thin C-shaped dark line corresponding to the granule cell layer can be observed in the dentate gyrus. The dissection was performed along an imaginary line corresponding to the hippocampal fissure. A perpendicular cut to this line was performed at the level of the anterior border of dentate gyrus (C-shaped dark line) to separate the dentate gyrus from the Ca3 and part of the Ca4 area. This material also include some interneurons and other hilar neurons, however the majority of the cells are represented by granule cells. Sections were collected, weighed (~20 mg), homogenized, and processed for total RNA isolation at 4°C using the RNAqueous®-4PCR Kit (Foster City, CA), following manufacturer's instructions. The concentration and purity of total RNA for each sample was determined by the Quant-iT™ RNA Assay Kit and the Q32857 Qubit™ fluorometer (Carlsbad, Invitrogen, CA) and confirmed by optical density measurements at 260 and 280nm using a BioMate 5 UV-visible spectrophotometer (Thermo Spectronic, Waltham, Mass). For each sample, the RNA integrity was assessed clear, sharp bands for 28S:18S ribosomal RNA (rRNA) by denaturating agarose gel electrophoresis of RNA. Briefly, total RNA (5 μg) was denatured, and subjected to electrophoresis in 2.2 M formaldehyde-1% agarose gel in morpholinepropanesulfonic acid (MOPS) containing ethidium bromide (5 mg/ml). The agarose was prepared in RNase free ultrapure water (pH=7.0) (Sambrook and Russell, 2001).

4.3. Reverse transcription and endpoint polymerase chain reaction (RT- PCR)

RNA reverse transcription: RNA samples from each set of control and epileptic rats where processed in parallel under the same conditions. RT and PCR reactions were performed on an iCycler Thermal Cycler PCR System (Bio-Rad Laboratories, Hercules, CA) with 96-well

reaction module using the High Capacity cDNA Reverse Transcription Kit (P/N: 4368814, Applied Biosystems, CA, USA) for synthesis of single stranded cDNA. The cDNA synthesis was carried out by following manufacturer's protocol. Each RT reaction contained 1000 ng of extracted total RNA template, 50 nM random RT primer, 1 × RT buffer, 0.25 mM each of dNTPs, 3.33 U/μl Multiscribe reverse transcriptase and 0.25 U/μl RNase Inhibitor. The 20 μl reactions were incubated in the iCycler Thermocycler in thin-walled 0.2-μl PCR tubes for 10 min at 25°C, 120 min at 37°C, 5 sec at 85°C and then held at 4°C.

Determining the efficiency of the RT reaction and amount of input RNA template: Exceeding the capacity of the RT reactions may lead to significant errors in the RNA quantification process. Hence, input amount of total RNA was set (normalized for each sample) to 1000 ng after determining the capacity, linearity and RT efficiency of the RT Kit using serial dilutions of input RNA. Briefly, each RNA concentration was reverse transcribed using the same RT reaction volume. The resulting cDNA template was added to the real-time PCR Master Mix and PCR reactions were amplified using the StepOne™ Real-Time PCR System (Applied Biosystems) using Taqman-based Applied Biosystems gene expression assays Hs99999901_s1 for eukaryotic 18S rRNA (a common RNA mass normalizer) and Rn99999916_s1 for endogenous control Glyceraldehyde 3 phosphate dehydrogenase 1 (*GADPH*). The amount of input RNA (1000 ng) was comprised within the dynamic range of the amplification.

Primers design for mGlu2 and mGlu3 mRNA: Primers for qrtPCr analysis of mGlu2 and mGlu3 expression levels were designed using the Primer Express software v2.0 (Applied Biosystems) and/or vectorNTI Primer Design For PCR (Invitrogen). The primers for each amplicon were selected so as to contain minimal internal secondary structure (i.e. hairpins and primer–dimer formation) as determined by OligoAnalyzer 3.0 webserver available at IDT SciTools and to have compatible T_m values (~60°C). Information about specific primers for the rat mGlu2 and mGlu3 mRNA related sequences and housekeeping gene GAPDH is provided in Table 1. The Housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GADPH), served as an internal control and simultaneously assessed in separate reaction tubes.

End-point PCR analysis: Each PCR amplification (20 μl of final volume) contained 10μM of sense and antisense transcript-specific primers, 10mM deoxynucleotides (dNTP), 10X PCR buffer, 50mM $MgCl_2$, 1 unit of AmpliTaq Gold® DNA Polymerase (P/N 4338857, Applied Biosystems), and 0.25μl of cDNA template. PCR was initiated with a denaturation step at 95° C (for 10 min) followed by 30 cycles at 95°C (for 30 s), 60°C for 30s (annealing), and 72°C for 30s (polymerization). PCR amplification conditions were the same for these primer sets. The PCR-amplified products (10 μL) were analyzed using standard 2% agarose electrophoresis and visualized with ethidium bromide staining (0.5 μg/ml). Images were digitally acquired using Bio-Rad ChemiDoc XRS. Product bands were identified according to DNA ladders (Fig. 1 B2).

***4.4. Quantitative real-time PCR analysis of* mGlu2 and mGlu3 expression—**Real-time PCR reactions were carried out in a StepOne™ Real-Time PCR System using the SYBR Green I chemistry and primers for mGlu2 and mGlu3 previously validated in end-point PCR assays. All primer pairs were optimized to ensure the specific amplification of the PCR product and the absence of any primer dimer. Because SYBR green I binds indiscriminately to double-stranded DNA, other nonspecific products may be detected along with the target gene. To verify that only specific product was amplified, a melting point analysis was performed after the final cycle (Fig 1A). Before running qrtPCR experiments, the efficiency of amplification was determined for each primer set by using serial dilutions of input DNA (Fig. 1 B1). All primer sets exhibited an efficiency >90% as required for the ΔΔCt relative quantification

algorithm (Fig. 1 B1) (Pfaffl, 2006). Primers were also validated via end-point PCR analysis using as template rat genomic DNA (DtRAN, normal brain, BioChain, Hayward, CA) and calibrator cDNA (total RNA from control rat dentate gyrus), followed by gel electrophoresis analysis. As shown in Fig. 1 B2, all primers resulted in a sharp single band around the estimated product size. Bands of different intensities were observed after RT-PCR on initial same amount of genomic DNA. After treatment with DNA intercalating agent ethidium bromide, intensity of the fluorescent band under UV is proportional to the amount and size of amplicons in the gel. Assuming that the total amount of the amplicons are almost the same under the same PCR conditions (*i.e.* cycles and similar primer efficiencies), the larger amplicon as for mGlu2 (229 bp) will result in larger fluorescent signal when compared to smaller size amplicons mGlu3 (188bp) and GAPDH (104bp) (Fig. 1 B2). For qrtPCR analysis, each sample was run in triplicates. Some of the reactions were repeated to evaluate variation between assays. Replicates were omitted if Ct standard deviation was greater than 1.5. Each run included a no-template control to test for contamination of assay reagents. Each standard PCR reaction contains 0.33 μl of RT product as template, 0.1 μM of each primer, and SYBR Green Master mix: 0.25×SYBR Green I, 0.2 mM dNTP mixture, 5 mM $MgCl_2$, 1 U/reaction AmpliTaq Gold DNA polymerase and 1×buffer. Following a 94°C denaturation for 10 min, the reactions were cycled 40 times with a 94°C denaturation for 15s, and a 60°C annealing for 1 min. During the initial optimization procedures, specific amplification was occasionally confirmed by electrophoresis of PCR products on 2% Tris borate ethylenediamine tetraacetic acid (TBE) agarose gel. Primers for mGlu2, mGlu3 and GAPDH were designed to be suitable for qrtPCR as describe above. Three types of controls aimed at detecting genomic DNA contamination in the RNA sample or during the RT or PCR reactions were always included: a RT mixture without reverse transcriptase, a RT mixture including the enzyme but no RNA, and a PCR mixture with no cDNA template were subjected to PCR.

The data was collected using OneStep Software and relative quantification was performed using the comparative threshold (CT) method after determining the CT values for reference (*GAPDH*) and target genes (mGlu2 or mGlu3) in each sample sets according to the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2006) as described by the manufacturer (Applied Biosystems; User Bulletin 2). Changes in mRNA expression level were calculated after normalization to *GAPDH*.

As calibrator sample we used cDNA from arbitrarily selected control rat. The program calculates the ΔCts and the ΔΔCt with the formulas below:

$$\Delta Ct = Ct\_Mean(GAPDH) - Ct\_Mean(Target)$$

$$\Delta\Delta Ct = \Delta Ct - \Delta Ct\_Mean$$

$$\text{Gene expression level} = 2^{-\Delta\Delta Ct}$$

Values of fold changes in the control sample versus the post-SE samples represent averages from triplicate measurements. Changes in gene expression were reported as fold changes relative to controls. Data were analyzed by ANOVA (followed by post-hoc analysis) or via paired *t*-test to check for statistically significant differences among the groups (significance *P*-value was set at < 0.05).

Evaluation of reference genes and data analysis: We assessed the stability of 4 different commonly used housekeeping genes in a diverse set of 20 different samples (cDNA from control and epileptic tissue obtained after different post-SE survival period). For this purpose, we used TaqMan® Gene Expression Assays (Applied Biosystems) for: (1) ribosomal protein L10 (Rpl10, Rn00821252_g1), (2) ribosomal protein L28 (Rpl28, Rn00821143_g1), (3) Eukaryotic 18S rRNA (18S, Hs99999901_s1), and (4) GAPDH (Rn99999916_s1). Additional information is provided in the Applied Biosystem's website by searching by the assays ID number. All TaqMan Assays, with the exception of those designated with the _H suffix in the

Assay ID, have a specificity of greater than 30,000 fold higher sensitivity for the Target compared to the most homologous homolog in that species. Applied Biosystems publically displays as much bioinformatics information as possible on their website. Actual primer and probe sequences can be obtained from Applied Biosystems after a proper confidentiality agreement. Same cDNA pool was used for qrtPCR analysis of each of the 4 genes using the TaqMan validated assays. Real-time PCRs were performed in triplicate for each of the 20 cDNA samples along with a no template control in parallel for each gene. The amplification curves for each gene were generated, grouped across all the tissue samples and cycle threshold (CT) determined at the threshold fluorescence value of 0.2. We calculate the average CT and the standard deviation of the CT values for each gene to measure the expression stability as follows: GAPDH: 21.3 ± 0.65; Rpl10: 26.3 ± 2.1; Rpl28: 23.7 ± 2.2; and 18S: 11.7 ± 9.1. Expression levels of these genes did not vary significantly in any of the control and epileptic samples. GAPDH was selected as our internal housekeeping gene (most stable) because it exhibited minimal variability (lowest standard deviation) across samples.

Statistical analysis of data: For means comparisons the statistical significance of the difference was assessed using paired, non-paired Student's *t*-test or one-way ANOVA. Statistics and graphs were prepared using the software package Statistica (Statsoft, Inc), SPSS and Sigmaplot (Systat Software, Inc. San Jose, CA). The level of statistical significance was set as $P<0.05$.

Abbreviations

CT, Cycle threshold
mGlu2, metabotropic glutamate receptor type 2
mGlu3, metabotropic glutamate receptor type 3
mGluR II, group II metabotropic glutamate receptors
Rpl10, gene for the ribosomal protein L10
Rpl28, gene for ribosomal protein L28
18S, gene for eukaryotic 18S ribosomal RNA
MTLE, Mesial temporal lobe epilepsy
RFU, Relative fluorescence units
SE, status epilepticus
dT, oligodeoxythymidine
dNTP, deoxynucleotides
GADPH, gene for glyceraldehyde-3-phosphate dehydrogenase
dNTP, deoxynucleotides
RQ, Relative quantification

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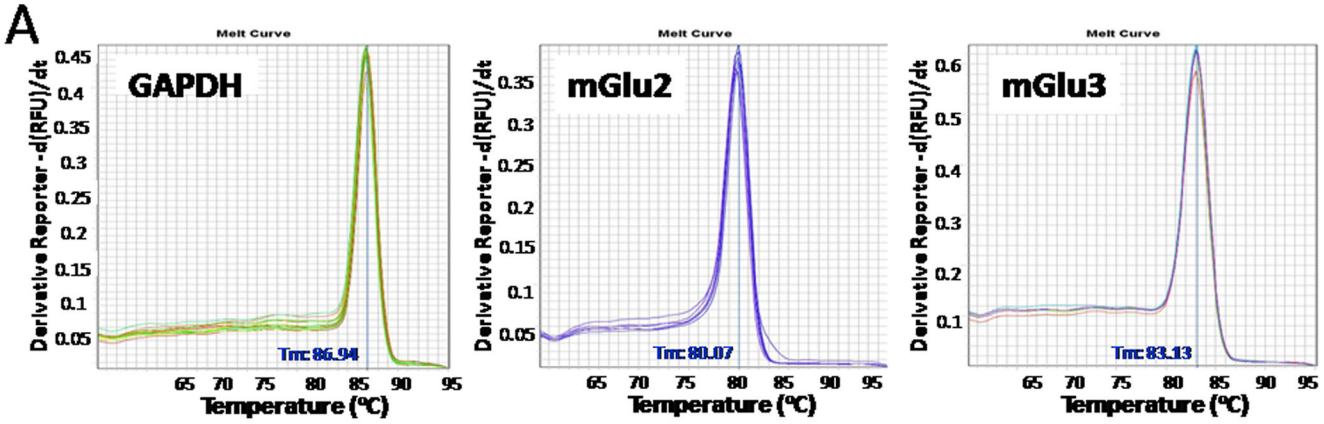
A



B1

Target	Slope	R2	Eff (%)
GAPDH	-3.26	0.99	100.1
mGlu2	-3.33	0.99	99.28
mGlu3	-3.41	0.99	96.39

B2

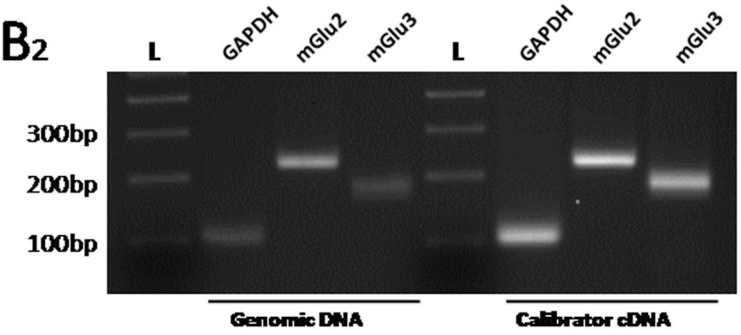


Figure 1.

Validation of primers for qrtPCR and relative quantification (RQ) using the $2^{-\Delta\Delta Ct}$ method. **A**. Melting curves revealing a single product for each primer sets. **B1**. Calculated efficiency (expressed as %) for each target was above 90%. **B2**. Primers were also validated via RT-PCR and gel electrophoresis analysis using genomic rat DNA as template and cDNA template from calibrator used in $2^{-\Delta\Delta Ct}$ analysis. L=DNA ladder.

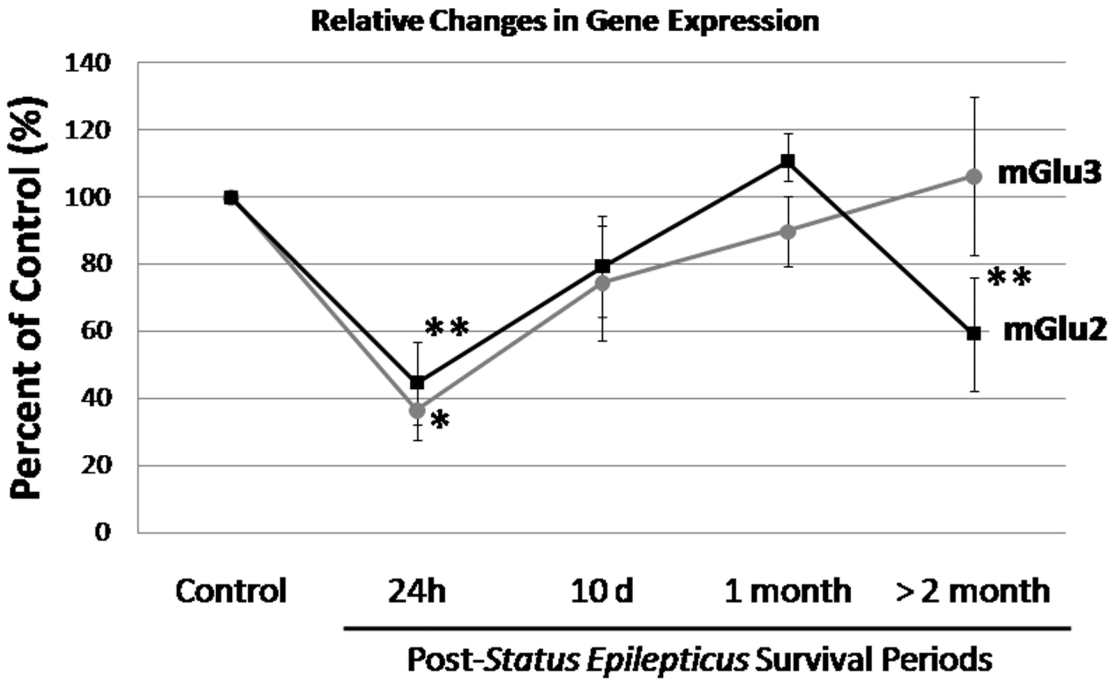


Figure 2.

Graph representing relative changes in mGlu2 (black symbols) and mGlu3 (gray symbols) at different time-points after pilocarpine-induced SE (relative to controls). A significant down-regulation was detected 24h after SE in both genes. Expression of mGlu2 transcripts partially recovered close to control values at 10 days and 1 month after SE, but then significantly declined (down-regulation) during late phases of epileptogenesis (> 2 months). In contrast, mGlu3 progressively recovered reaching values above control levels (but not significantly different than controls). * $P<0.01$ and ** $P<0.05$ by post-hoc Dunnett's test.

TABLE 1

Primers for PCR amplification of target (mGlu2 and mGlu3) and 'Housekeeping' (GAPDH) genes.

Target Gene	Gene Symbol	Accession Number	Primer Sequences	Bases	Amplicon size
mGlu2	*Grm2*	XM_343470	Sense: 5'-AGTCCTTAGCTGGGGAGCCT-3'	3008-3027	229 bp
			Antisense: 5'- AACCATCCTCTCTATCCCAGAGTAAC-3'	3211-3236	
mGlu3	*Grm3*	NM_001105712	Sense: 5'-TAGGCTGTTAGACAAAGTGCTCA-3'	2894-2916	188 bp
			Antisense: 5'- GAAGGGGCTGTTAATTAGGGCA -3'	3060-3081	
GAPDH	*Gapdh*	NM_017008	Sense: 5'-TCCCATTCTTCCACCTTTGATGCT-3'	1711-1734	104 bp
			Anitsense: 5'-ACCCTGTTGCTGTAGCCATATTCAT-3'	1790-1814	

TABLE 2

Statistical analysis of gene expression changes relative to controls

	Post-Status Epilepticus Period					
Target	**Control**	**24h**	**10 d**	**1 month**	**> 2 month**	**ANOVA**
mGlu2	1	0.44 ± 0.08	0.79 ± 0.10	1.10 ± 0.03	0.59 ± 0.11	$P<0.001$, F=6.3
mGlu3	1	0.36 ± 0.09	0.74 ± 0.08	0.89 ± 0.03	1.06 ± 0.23	$P<0.01$, F=4.7

This table shows data for relative gene expression changes compared to control levels at different post-*status epilepticus* survival period. Data (RQ values) were normalized to control levels for statistical comparisons. Values are shown as means ± SEM.