Altered calcium/calmodulin kinase II activity changes calcium homeostasis that underlies epileptiform activity in hippocampal neurons in culture

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Abbreviations:
SERCA: sarco(endo)plasmic reticular calcium ATPase, TTX: tetrodotoxin
Abstract

Epilepsy is characterized by the occurrence of spontaneous recurrent epileptiform discharges (SREDs) in neurons. A decrease in calcium/calmodulin-dependent protein kinase II (CaMK-II) activity has been shown to occur with the development of SREDs in a hippocampal neuronal culture model of acquired epilepsy (AE) and altered calcium (Ca\(^{2+}\)) homeostasis has been implicated in the development of SREDs. Using antisense oligonucleotides, this study was conducted to determine if selective suppression of CaMK-II activity, with subsequent induction of SREDs, was associated with altered Ca\(^{2+}\) homeostasis in hippocampal neurons in culture. Antisense knockdown resulted in the development of SREDs and a decrease in both immunocytochemical staining and enzyme activity of CaMK-II. Evaluation of [Ca\(^{2+}\)]\(_i\) using Fura indicators revealed that antisense-treated neurons manifested increased basal [Ca\(^{2+}\)]\(_i\), while missense-treated neurons showed no change in basal [Ca\(^{2+}\)]\(_i\). Antisense suppression of CaMK-II was also associated with an inability of neurons to restore a Ca\(^{2+}\) load. Upon removal of oligonucleotide treatment, CaMK-II suppression and Ca\(^{2+}\) homeostasis recovered to control levels and SREDs were abolished. To our knowledge the results demonstrate the first evidence that selective suppression of CaMK-II activity results in alterations in Ca\(^{2+}\) homeostasis and the development of SREDs in hippocampal neurons and suggest that CaMK-II suppression may be causing epileptogenesis by altering Ca\(^{2+}\) homeostatic mechanisms.
Introduction

Epilepsy, common neurological disorder affecting approximately 1-2% of the population worldwide (Hauser and Hesdorffer, 1990; McNamara, 1999), is characterized by the occurrence of spontaneous recurrent epileptiform discharges (SREDs) in populations of neurons. Acquired epilepsy (AE) is caused by a known brain injury, such as status epilepticus (SE), stroke, or traumatic brain injury that induces long-lasting plasticity changes in previously normal brain tissue and leads to the development of SREDs (Hauser and Hesdorffer, 1990). Epileptogenesis is the process responsible for transforming normal brain tissue into the epileptic phenotype, manifesting the occurrence of seizures or SREDs (Shin and McNamara, 1994; DeLorenzo et al., 1998; Delorenzo et al., 2005). Although considerable research is being conducted on long-term changes associated with epileptogenesis, the exact cellular and molecular mechanisms involved in epileptogenesis are still not completely understood (Shin and McNamara, 1994; Delorenzo et al., 2005). Therefore, studies elucidating the underlying mechanisms mediating the plasticity changes associated with epileptogenesis are crucial to advancing both the treatment and prevention of epilepsy.

It has been suggested that chronic elevations in intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)\(_i\)]) and alterations in Ca\(^{2+}\) homeostatic mechanisms in epileptic neurons play a role in the induction and maintenance of the epileptic phenotype (DeLorenzo et al., 1998; Raza et al., 2001; Raza et al., 2004; Delorenzo et al., 2005). Calcium is an important second messenger involved in diverse cellular events, such as membrane excitability and synaptic activity (DeLorenzo et al., 2005). Elevated [Ca\(^{2+}\)\(_i\)] can lead to excitotoxicity, which precedes epileptogenesis and epilepsy (DeLorenzo et al., 1998; Raza et al., 2004; Sun et al., 2004; Delorenzo et al., 2005). In normal cells, [Ca\(^{2+}\)\(_i\)] is regulated by several Ca\(^{2+}\) homeostatic mechanisms (Carafoli et al., 1997).
Studies in epileptic neurons have shown that disruption of Ca$^{2+}$ extrusion and sequestration mechanisms can lead to elevated basal [Ca$^{2+}$]$_i$ with a decreased ability to restore resting [Ca$^{2+}$]$_i$ levels following an external Ca$^{2+}$ load (Pal et al., 2001; Raza et al., 2001; Sun et al., 2004). Although many studies have linked increases in [Ca$^{2+}$]$_i$ and altered Ca$^{2+}$ homeostatic mechanisms to epilepsy (Delorenzo et al., 2005), it remains unclear as to what molecular events during epileptogenesis cause these changes in Ca$^{2+}$ homeostasis.

Calcium-calmodulin-dependent protein kinase II (CaMK-II) plays a major role in modulating neuronal excitability and function (Kelly et al., 1984), with alterations in CaMK-II levels linked to neuronal hyperexcitability. Decreases in CaM kinase II have been reported in numerous in vivo and in vitro models of epilepsy (Bronstein et al., 1993), including kindling (Wasterlain and Farber, 1984; Taft et al., 1987), electrical stimulation SE (Perlin et al., 1992), pilocarpine (Churn et al., 2000a), and low Mg$^{2+}$ in cultured neurons (Blair et al., 1999). Additionally, CaMK-II knockout mice demonstrated the epileptic phenotype and developed spontaneous seizures (Butler et al., 1995). It has been demonstrated that knocking down CaMK-II activity in cultured hippocampal neurons with an antisense oligonucleotide resulted in epileptiform activity as evidenced by the presence of SREDs (Churn et al., 2000b). It was also shown in cultured hippocampal neurons that CaMK-II activity was decreased in association with the development of SREDs (Blair et al., 1999). These studies implicate a role for altered CaMK-II function towards the induction and maintenance of the epileptic phenotype and suggest that alterations in CaMK-II activity may play a role in the induction of epileptogenesis by altering Ca$^{2+}$ homeostatic mechanisms.

This study was initiated to evaluate whether the decrease in CaMK-II activity during epileptogenesis could represent a molecular mechanism that underlies alterations in Ca$^{2+}$
homeostasis observed in the epileptic phenotype. Antisense oligonucleotides were used to experimentally suppress CaMK-II function in hippocampal neurons in culture. Following knockdown of CaMK-II, electrophysiological studies were initiated to determine the presence or absence of SREDs and Ca\(^{2+}\) imaging studies were employed using the fluorescent calcium indicators Fura-2 and Fura-FF to evaluate [Ca\(^{2+}\)], dynamics. Similar experiments were performed on neurons that were allowed to recover from antisense knockdown of CaMK-II. Antisense oligonucleotide suppression of CaMK-II in neurons resulted in the development of SREDs and higher basal [Ca\(^{2+}\)], and a decreased ability to restore resting [Ca\(^{2+}\)], following a Ca\(^{2+}\) load when compared to control and antisense-recovered neurons. The results provide the first direct evidence that CaMK-II activity is involved in maintaining Ca\(^{2+}\) homeostasis in neurons and that the alterations in Ca\(^{2+}\) homeostasis resulting from suppression of CaMK-II may underlie the development of SREDs. Due to the pathophysiological similarities to the glutamate-injury and low Mg\(^{2+}\) in vitro models of AE, knocking down CaMK-II levels in cultured hippocampal neurons provides a novel model for studying epileptiform activity.

**Methods**

**Hippocampal Neuronal Culture**

Primary hippocampal cultures were prepared as previously described by our laboratory (Sombati and Delorenzo, 1995). Briefly, hippocampal cells were isolated from 2-day postnatal Sprague-Dawley rats (Harlan, Indianapolis, IN) and plated at a density of 2.4 x 10^4 cells/cm\(^2\) onto glial support layers previously plated onto poly-L-lysine (0.05 mg/ml)-coated Lab-Tek two-well cover glass chambers (Nalge-Nunc International, Naperville, IL). Cultures were maintained at 37°C in a 5% CO\(_2\)/95% O\(_2\) air atmosphere and fed twice weekly with Neurabasal A media.
(Invitrogen, Carlsbad, CA) enriched with B-27 serum-free supplement (Invitrogen, Carlsbad, CA) and 0.5mM L-glutamine. Cultures were exposed to 5 µM cytosine arabinoside two days following plating to inhibit non-neuronal growth. Experimental studies and \([\text{Ca}^{2+}]_i\) measurements were performed on neurons maintained for 15-18 days in vitro (DIV) to ensure adequate neuronal development.

**α CaMK-II Antisense Knockdown Analysis**

Antisense and missense oligonucleotide probes (Operon Biotechnologies, Huntsville, AL) were constructed as described previously (Churn et al., 2000b). For the α CaMK-II knockdown, an antisense oligonucleotide complimentary to the +1 to +18 nucleotides was constructed with the following sequence: 5’ GGT AGC CAT CCT GGC ACT 3’; the missense sequence for α CaMK-II was: 5’ GGT AGC CAT AAG GGC ACT 3’. Knockdown treatment for α CaMK-II involved treating hippocampal cultures for 3 days with 3.0 µM of either antisense or missense oligonucleotides every 24 hours, as determined by measuring CaMK-II activity (Churn et al., 2000b). Oligonucleotide treatment was initiated at 13 DIV to allow for analysis to occur by 17 DIV. Untreated neurons were used as controls. During the treatment regimen, hippocampal cultures were maintained in Neurobasal A media supplemented with B-27. At the end of the 3 day treatment protocol, cultures were utilized for Fura-2 (5-Oxazolecarboxylic acid, 2-(6-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-(2-(bis(2-((acetoxy(methoxy)-2-oxoethyl)amino-5-methylphenoxy)ethoxy)-2-benzofuanyl)-(acetoxy)methyl ester) and Fura-FF \([\text{Ca}^{2+}]_i\) measurements. To assess the recovery and restoration of CaMK-II activity, cultures were treated with antisense or missense oligonucleotide every 24 hours for 3 days. After the third day of treatment, oligonucleotide treatments were discontinued and the neurons were allowed to
recover in oligonucleotide-free medium. After full recovery of CaMK-II activity, cultures were utilized for [Ca$^{2+}$], measurements.

**Measurement of CaMK-II activity**

CaMK-II activity was determined measuring CaMK-II-dependent substrate phosphorylation of the synthetic peptide Syntide II (Sigma, St. Louis, MO) following the methods of Churn (Churn, 1995). Phosphorylation reaction solutions contained 41 µg of protein, 60 µM Syntide II, 10 mM MgCl$_2$, 7 µM [$\gamma$-32P]ATP, 10 mM PIPES (pH 7.4), ± 5 µM CaCl$_2$ and ± 1 µg calmodulin. Standard reactions were performed in a shaking water bath at 30 °C. The phosphorylation reactions were initiated by adding Ca$^{2+}$, continued for 1 min, and stopped by adding 20 µM EDTA. 10 µL aliquots of assay solution were blotted onto P-81 phosphocellulose filter paper (Whatman). Each reaction was quantitated in triplicate. The filter paper was then washed three times in 50 mM phosphoric acid, rinsed with acetone, and allowed to air dry. Radioactive phosphate was then quantitated by scintillation counting (Churn, 1995).

**Immunocytochemical staining of α CaMK-II**

After briefly washing in phosphate buffered saline (PBS), neuronal cultures were fixed in 4% paraformaldehyde in PBS for 10 minutes followed by 3 x 5 min wash in PBS. Fixed cultures were blocked and permeabilized in SuperBlock® blocking buffer (Pierce, Rockford, IL) containing 0.2% Triton X-100 for 60 min at room temperature. Cells were then incubated with a mouse monoclonal antibody to the CaMK-II α subunit (10 µg/ml, clone 6G9; Biomol, Plymouth Meeting, PA) in SuperBlock® blocking buffer containing 0.1% Triton X-100 overnight at 4°C. Cells were washed 4 x 5 min in PBS containing 0.1% Triton X-100. Following wash, cells were incubated with Texas Red (Vector Labs, Burlingame, CA) conjugated anti-mouse IgG (20 µg/ml) in SuperBlock® blocking buffer for 60 min at room temperature. Cells were washed 4 x
5 min in PBS containing 0.1% Triton X-100 followed by one wash in PBS alone. Labeled cells were coated with Vectashield (Vector Labs, Burlingame, CA) and cover slipped. Control staining was carried out in an identical manner with the exception of removal of the primary antibody. Fluorescence microscopy was carried out on an Olympus inverted microscope fitted with a 20X objective and a Texas Red filter cube allowing for excitation/emission of 595/615 nm. Fluorescent images were captured with a Q-fire digital camera and evaluated with Olympus MicroSuite™ imaging software (Soft Imaging System, Lakewood, CO).

**Whole-Cell Current-Clamp analysis of hippocampal neuronal cultures**

Electrophysiological analysis was performed using previously established procedures in our laboratory (Sombati and Delorenzo, 1995). Briefly, cell culture media was replaced with physiological bath recording solution (pBRS) containing 145 mM NaCl, 2.5 mM KCl, 10 mM N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid (HEPES), 10 mM glucose, 2 mM CaCl₂, 1 mM MgCl₂, 2 µM glycine, pH 7.3, osmolarity adjusted to 325 with sucrose. Cell culture plates were mounted on the stage of an inverted microscope (Nikon Diaphot, Japan), continuously perfused with pBRS and then studied using the whole-cell current-clamp recording procedure. Patch electrodes with a resistance of 2-4 MΩ were pulled on a Brown-Flaming P-80C electrode puller (Sutter Instruments, Novato, CA) and then fire polished. For whole-cell current-clamp analysis, the electrode was filled with a solution containing (in mM) 140 K⁺ gluconate, 1 MgCl₂ and 10 Na-HEPES, pH 7.2, osmolarity adjusted to 310 ± 5 mOsm with sucrose. Data was digitized and transferred to videotape using a PCM device (Neurodata, New York, NY) and then played back on a DC-500 Hz chart recorder (Astro-Med Dash II, Warwick, RI). Intracellular recordings were carried out using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in whole-cell current-clamp mode.
Intracellular free calcium measurements

Intracellular free Ca\textsuperscript{2+} measurements were carried out using previously established procedures (Pal et al., 1999; Pal et al., 2001; Raza et al., 2001; Sun et al., 2004). Hippocampal neurons were loaded with acetoxyethyl (AM) form of the membrane permeable ratiometric fluorescent Ca\textsuperscript{2+} indicators Fura-2 or Fura-FF (1 µM; Invitrogen, Carlsbad, CA) in pBRS for 30 min at 37°C. Loaded cells were washed three times with pBRS and incubated for an additional 15 min to allow for cellular esterase cleavage of the AM moiety and intracellular trapping of the free acid Fura-2 or Fura-FF indicators. Culture dishes were mounted onto a heated stage at 37°C on an Olympus IX-70 inverted microscope fitted with a 20X fluorite water immersion objective and coupled to an ultra high-speed fluorescence imaging system (Olympus America/Perkin Elmer). The fluorescence excitation source was a 75W Xenon arc lamp (Olympus America). Neutral density filters of variable opacities were used to attenuate unwanted excitation. Alternating excitation wavelengths of 340 nm and 380 nm were generated using a Lambda 10-2 filter wheel (Sutter Instruments Co., Novato CA) and 510 nm emissions were acquired using a Fura filter cube (Olympus America) with a dichroic at 400 nm. Alternating emissions from 340 nm and 380 nm excitation were captured with an ORCA-ER high-speed digital CCD camera (Hamamatsu Photonics K.K., Japan). Image acquisition and processing was computer controlled using the UltraVIEW™ Imaging system software v5.2 (Perkin Elmer). Using the image analysis software, a region of interest was selected for each pyramidal-shaped neuron in the field and intracellular free Ca\textsuperscript{2+} levels were presented as an absolute ratio value of 340/380 excitation-emissions. To calculate 340/380 ratio values, image pairs at each wavelength were captured and digitized at varying intervals and the images at each wavelength were averaged over four frames.
Background auto fluorescence values for both 340 nm and 380 nm excitations were obtained by imaging a field of neurons lacking the fluorescent indicator and were subtracted from experimental 340/380 excitation-emissions values from indicator loaded cells. Absolute ratio values of 340/380 excitation-emissions of intracellular free Ca\(^{2+}\) levels were used in this study as a quantitative measure of [Ca\(^{2+}\)]\(_i\) levels, employing established techniques (Pal et al., 1999; Pal et al., 2000; Raza et al., 2001; Sun et al., 2004).

Experimental studies were carried out on oligonucleotide-treated hippocampal cultures to evaluate [Ca\(^{2+}\)]\(_i\) dynamics. In some experiments, recording solution during Ca\(^{2+}\) imaging contained 400 nM tetrodotoxin (TTX; Sigma, St. Louis, MO) to block synaptic transmission to allow for measurement of isolated [Ca\(^{2+}\)]\(_i\) dynamics in the absence of seizure and synaptic activity. Multiple neuronal culture fields were evaluated to determine basal [Ca\(^{2+}\)]\(_i\) measurements, each for 60 seconds with image/ratio values being acquired at 5 second intervals. For evaluation of the glutamate-induced Ca\(^{2+}\) loads, basal [Ca\(^{2+}\)]\(_i\) levels were recorded for 3 minutes at 30 second intervals before glutamate treatment. The recording solution was replaced with 1 mL of pBRS containing glutamate (50 \(\mu\)M; Sigma, St. Louis, MO) and glycine (10 \(\mu\)M) was added to the cells. After 2 min of exposure, the glutamate was removed and the cells were washed twice in pBRS. The cells were monitored for up to 30 min post glutamate treatment at 30-second intervals.

**Calcium calibration**

The [Ca\(^{2+}\)]\(_i\) values in the paper are presented as 340/380 ratio values for Fura-2 and Fura-FF, since absolute [Ca\(^{2+}\)]\(_i\) concentrations can vary depending on the fluorescent indicator used (Hyrc et al., 1997; Pal et al., 2000; Raza et al., 2001). It has been reported that ratio values provide more objective presentation of [Ca\(^{2+}\)]\(_i\) (Pal et al., 2000; Raza et al., 2001). However, we
have performed Ca\textsuperscript{2+} calibration determinations on both the Fura-2 and Fura-FF data to provide estimates of absolute [Ca\textsuperscript{2+}]i concentrations from the 340/380 ratio values using established procedures (Pal et al., 2001). An in vitro calcium calibration curve was constructed for each indicator and used to convert fluorescent ratios to [Ca\textsuperscript{2+}]i concentrations using ionomycin (10\mu M) and prepared calibration buffers (Invitrogen, Carlsbad, CA). [Ca\textsuperscript{2+}]i concentrations were calculated from the background corrected 340/380 ratios using the following equation (Gryniewicz et al., 1985):

\[
[Ca^{2+}]_i = \frac{(K_d \cdot S_{f2}/S_{b2})(R - R_{min})}{(R_{max} - R)}
\]  

(Equation 1)

where R is the 340/380 ratio at any time; R\textsubscript{max} is the maximum measured ratio in saturating Ca\textsuperscript{2+} solution (39\mu M free Ca\textsuperscript{2+}); R\textsubscript{min} is the minimal measured ratio Ca\textsuperscript{2+} free solution; S\textsubscript{f2} is the absolute value of the corrected 380-nm signal at R\textsubscript{min}; S\textsubscript{b2} is the absolute value of the corrected 380-nm signal at R\textsubscript{max}; and the K\textsubscript{d} values used were 224 nM and 20 \mu M for Fura-2 and Fura-FF respectively (Pal et al., 2001; Sun et al., 2004).

For basal [Ca\textsuperscript{2+}]i experiments, Fura-2 was used as the indicator, since Fura-FF is not sensitive in the low nM range (Hyrc et al., 1997). For the experiments evaluating Ca\textsuperscript{2+} homeostasis, both Fura-2 and Fura-FF were used due to the large change in [Ca\textsuperscript{2+}]i produced by the glutamate exposure. Basal [Ca\textsuperscript{2+}]i concentrations for control, missense-treated, antisense-treated and antisense-recovered neurons were determined from the ratio values and were 107.1 ± 3.4 nM, 95.1 ± 3.4 nM, 138.4 ± 7.9 nM, and 110.3 ± 4.6 nM, respectively. For the calcium homeostasis experiments, peak glutamate-induced [Ca\textsuperscript{2+}]i values in missense-treated and antisense-treated neurons using Fura-2 were calculated to be 1.99 ± 0.16 and 1.88 ± 0.36 \mu M, respectively. Peak glutamate-induced [Ca\textsuperscript{2+}]i concentrations in missense-treated and antisense-treated neurons employing Fura-FF were determined to be 29.3 ± 1.4 and 33.8 ± 2.7 \mu M. The
values obtained in this study for peak glutamate induced $[\text{Ca}^{2+}]_i$ values using Fura-2 and Fura-FF were essentially identical to values obtained previously in both in vitro and in vivo epileptic neurons (Pal et al., 2000; Raza et al., 2001). Although the peak $[\text{Ca}^{2+}]_i$ concentration varied between Fura-2 and Fura-FF as previously observed (Pal et al., 2000), the ability of the indicators to reflect the neurons Ca$^{2+}$ buffering capability was similar. Although Fura-2 gave a lower absolute calcium concentration upon calibration, the shapes of its decay curves were almost identical to those of Fura-FF.

Data Analysis

$[\text{Ca}^{2+}]_i$ data were collected using the UltraVIEW™ Imaging system and statistically analyzed and plotted using SigmaPlot® (version 8.0) software. Experiments were repeated at least three times from different batches of neuronal cultures. The significance of the data was tested by Student’s $t$-test, one-way ANOVA, or one way repeated measures (RM) ANOVA where applicable. The Tukey’s test was used as the post hoc analysis for multiple comparisons. Statistical analysis was performed using SigmaStat 2.0 (Jandel Corp., San Rafel, CA). $P<0.05$ was considered statistically significant for all data analysis. To compare the kinetics of the $[\text{Ca}^{2+}]_i$ decay curves, ratio values from individual neurons obtained after glutamate exposure were normalized to “Percent of $[\text{Ca}^{2+}]_i$ Load” using the following equation:

$$\text{Percent of } [\text{Ca}^{2+}]_i \text{ Load}_{t=x} = \left[1 - \frac{\text{Ratio}_{t=0} - \text{Ratio}_{t=x}}{\text{Ratio}_{t=0} - \text{Ratio}_{\text{Basal}}} \right] \times 100 \quad (\text{Equation 2})$$

where Ratio$_{t=x}$ is the 340/380 ratio at any time following the glutamate exposure; Ratio$_{t=0}$ is the 340/380 ratio at the peak $[\text{Ca}^{2+}]_i$ load; and Ratio$_{\text{Basal}}$ is the average 340/380 ratio prior to glutamate exposure.
Results

**Suppression of CaMK-II activity and protein expression in hippocampal neurons in culture.**

These studies were initiated to evaluate whether the decreased activity and expression of CaMK-II caused by antisense oligonucleotide suppression specific for the α subunit of CaMK-II were reversible. Hippocampal neurons in culture were exposed to either missense or antisense oligonucleotides specific for the α subunit of CaMK-II. CaMK-II-dependent substrate phosphorylation of the synthetic peptide Syntide II was carried out to evaluate the activity of CaMK-II following oligonucleotide treatment (Fig 1A). Immunocytochemical staining was performed to determine the localization of the α subunit of CaMK-II in cultures treated with missense or antisense oligonucleotides (Fig 1B). Antisense oligonucleotide treatment significantly decreased CaMK-II activity and protein expression in comparison to control and missense-treated neurons (Fig 1A and B). Removal of the antisense oligonucleotide treatment followed by a 3-day recovery period resulted in a restoration of CaMK-II activity and protein expression to missense control levels (Fig 1A and B: bottom panel), demonstrating that the antisense oligonucleotide suppression CaMK-II was reversible.

**Suppression of CaMK-II activity correlates with the development of epileptiform activity.**

Whole-cell current-clamp recordings were performed on pyramidal neurons from control, missense-treated, antisense-treated, and antisense-recovered cultures (Fig 2). Recordings from missense-treated cultures showed the absence of SREDs and manifested similar intrinsic baseline activity as seen in control cultures (Fig 2A and B). Antisense oligonucleotide suppression of CaMK-II resulted in the expression of SREDs in hippocampal neurons (Fig 2C). Expansion of
one of the SREDs from the recording in Fig. 2C revealed the presence of paroxysmal
depolarizing shifts (PDSs) with high frequency spiking activity, characteristic of epileptiform
discharges (Fig 2C:a). The presence of intermittent SREDs remained constant during the
antisense oligonucleotide suppression of CaMK-II. Following withdrawal of antisense
oligonucleotide treatment with subsequent recovery of CaMK-II activity and expression (Fig. 1
A and B), SREDs were no longer observed and neuronal electrophysiological activity returned to
control levels (Fig 2D). These results demonstrate that recovery from antisense-oligonucleotide
treatment restored CaMK-II activity and completely abolished SREDs, strongly indicating a
causal relationship between CaMK-II suppression and the development of SREDs.

**Suppression of CaMK-II activity results in elevation of basal \([\text{Ca}^{2+}]_{i}\).**

Since alteration of \([\text{Ca}^{2+}]_{i}\) has been implicated in epileptogenesis (Delorenzo et al., 2005),
we wanted to investigate whether the suppression of CaMK-II, with subsequent induction of
SREDs, affected neuronal \([\text{Ca}^{2+}]_{i}\). To evaluate the effects CaMK-II knockdown on \([\text{Ca}^{2+}]_{i}\) in
neurons, \([\text{Ca}^{2+}]_{i}\) imaging was conducted on control, missense- treated, antisense-treated, and
antisense-recovered neurons using the fluorescent \(\text{Ca}^{2+}\) indicator Fura-2. Figure 3 shows basal
\([\text{Ca}^{2+}]_{i}\) measurements from control (n=124), missense-treated (n=164), and antisense-treated
(n=164) neurons. Treatment with missense oligonucleotide did not alter basal \([\text{Ca}^{2+}]_{i}\), with
average 340/380 ratios from control and missense-treated neurons at 0.26 and 0.25, respectively.
Neurons from antisense-treated cultures had significantly higher basal \([\text{Ca}^{2+}]_{i}\) compared to both
control and missense-treated neurons, with an average ratio value of 0.31 (p<0.001, ANOVA
with Tukey’s *post hoc* test), demonstrating that treatment with antisense oligonucleotide with
subsequent suppression of CaMK-II function caused an increase in basal neuronal \([\text{Ca}^{2+}]_{i}\) (Figure
3A).
To determine if the effect of CaMK-II knockdown on basal $[Ca^{2+}]_i$ levels was reversible, treatment with antisense oligonucleotide to $\alpha$ CaMK-II was discontinued and neurons were allowed 3 days to recover. As previously shown, SREDs were no longer observed and the activity and protein expression of CaMK-II were restored to normal levels following recovery (Fig. 1 and 2). Using Fura-2, basal $[Ca^{2+}]_i$ was measured in CaMK-II antisense-recovered neurons ($n=86$). Following the recovery period, ratio values had significantly declined to 0.27 (Fig. 3, $p<0.001$ ANOVA with Tukey’s post hoc test). These results indicate that the alterations in basal $[Ca^{2+}]_i$ induced by the antisense oligonucleotide suppression of CaMK-II were reversible upon a 3-day recovery period following removal of antisense treatment.

Figure 4 shows a histogram breaking down the percentage of neurons from each treatment group into specific ratio value ranges. The distribution of basal $[Ca^{2+}]_i$ in neurons from this study are similar to the distribution of basal $[Ca^{2+}]_i$ in neurons from the stroke-induced epilepsy model (Sun et al., 2004). A greater percentage of control (Fig 4A) and missense-treated neurons (Fig 4B) demonstrated lower basal $[Ca^{2+}]_i$, with 80.8% of control neurons and 78.1% of missense-treated neurons having a ratio value of 0.3 or less. A greater percentage of antisense-treated neurons (Fig 4C) demonstrated higher basal $[Ca^{2+}]_i$. Only 51.0% of neurons had a ratio value of 0.3 or less. A greater percentage of neurons from antisense-recovered cultures returned to the lower ratio ranges compared to non-recovered neurons (Fig. 4D), as demonstrated by a leftward shift of the histogram. 81.9% of the antisense-recovered neurons had ratio values of 0.3 or less.

**Alteration of $Ca^{2+}$ homeostasis in neurons with decreased expression of $\alpha$ CaMK-II.**

To evaluate the ability of the neurons to regulate $[Ca^{2+}]_i$ homeostasis, a $Ca^{2+}$ load was induced by brief exposure to glutamate resulting in an immediate and marked increase in $[Ca^{2+}]_i$. 


using established techniques (Pal et al., 2001; Sun et al., 2004). Both Fura-2 and Fura-FF were employed to evaluate changes in \([Ca^{2+}]_i\), caused by glutamate exposure. \([Ca^{2+}]_i\) measurements were taken from missense-treated and antisense-treated neuronal cultures. Following brief glutamate exposure (50 µM, 2 min), the glutamate-induced \(Ca^{2+}\) load and the ability of each treatment group to regulate \([Ca^{2+}]_i\) was analyzed. Analysis of the glutamate-induced \([Ca^{2+}]_i\) load in missense-treated and antisense-treated neurons indicated that the increased \([Ca^{2+}]_i\) during the glutamate exposure was not significantly different in both Fura-2 and Fura-FF-loaded cultures. The change in the 340/380 ratios before and during glutamate in cultures loaded with Fura-2, was 1.07 ± 0.05 for missense-treated cultures and 0.93 ± 0.09 for antisense-treated cultures (Fig 5A; \(p = 0.1\), Student’s \(t\)-test). In Fura-FF-loaded cultures, the change in the 340/380 ratios before and during glutamate was 0.19 ± 0.01 for missense-treated cultures and 0.20 ± 0.02 for antisense-treated cultures (Fig 5B; \(p = 0.8\), Student’s \(t\)-test). Thus the impairment of the antisense-treated neurons to buffer \([Ca^{2+}]_i\) was not due to an increased \([Ca^{2+}]_i\) load produced by glutamate.

Since the increase in 340/380 ratios during glutamate exposure was not different between missense-treated and antisense-treated neurons, we normalized the post-glutamate ratio values of individual neurons to the ratio values of their \(Ca^{2+}\) loads in order to compare \(Ca^{2+}\) decay curves between missense-treated and antisense-treated neurons (Fig 5C and D). Individual curves, normalized as percent of \(Ca^{2+}\) load were averaged and compared using the RM ANOVA (Sun et al., 2004). By 30 minutes post-glutamate, missense-treated neurons recovered to within 10% of their \(Ca^{2+}\) load (8.3% ± 1.9% for Fura-2 and 6.7% ± 3.3% for Fura-FF). Antisense-treated neurons recovered to 45% of their \(Ca^{2+}\) load (44.6% ± 5.0% for Fura-2 and 45.7% ± 14.3% for Fura-FF), which is significantly elevated over missense-treated neurons (\(p<0.001\)).
In order to determine if [Ca^{2+}]_i in antisense-treated neurons eventually returned to basal levels after the glutamate-induced Ca^{2+} load, neurons were imaged 2 h after glutamate exposure. Missense-treated and antisense-treated neurons were exposed to glutamate (50 µM, 2 min). One hour later, neurons were loaded with Fura-2 for 30 min, then washed 3 times with recording solution and incubated an additional 15 min for esterase cleavage. Neurons were then placed on the microscope and 340/380 ratio values were obtained. Two hours after glutamate exposure, ratio values from missense-treated neurons (n=21) had dropped to 0.24 whereas ratio values from antisense-treated neurons (n=30) had dropped to 0.31 (Fig 6). To ensure basal [Ca^{2+}]_i was consistent with what was previously reported, basal [Ca^{2+}]_i was obtained from the same batches of neurons and were not statistically different from the values obtained 2-h post-glutamate. However, analysis with the Student’s t-test revealed that the 2-h post-glutamate values from antisense-treated neurons were still significantly elevated over missense-treated neurons (p<0.001).

The effects of recovery of CaMK-II suppression on the ability of the neurons to handle a Ca^{2+} load were also evaluated. Neuronal cultures were treated with α CaM kinase II antisense oligonucleotide for 3 days, and then allowed to recover for 3 days, thereby restoring CaMK-II function. Neurons were loaded with Fura-2 and [Ca^{2+}]_i was measured every 30 seconds. A Ca^{2+} load was produced by brief exposure to glutamate (50 µM, 2 min) (Fig. 7). In order to compare the Ca^{2+} decay curves of missense-treated, antisense-treated and antisense-recovered neurons, the ratio values were normalized to percent of Ca^{2+} load as previously described. As previously reported, missense-treated neurons declined to 8.3% of their Ca^{2+} load whereas antisense-recovered neurons only recovered to 44.6% of their Ca^{2+} load during the 30 min of recording. Antisense-recovered neurons recovered to 13.7% ± 2.3% of their Ca^{2+} load, which is
significantly lower than antisense-treated neurons and not significantly different from missense-treated neurons (Fig 8, p<0.001, RM ANOVA). By allowing cultures to recover from the antisense oligonucleotide-dependent suppression of CaMK-II, neurons were able to completely buffer [Ca$^{2+}$], following glutamate exposure and restore basal Ca$^{2+}$ levels. The ability of the antisense-recovered neurons to buffer the Ca$^{2+}$ load indicates that both the development of SREDs and the altered Ca$^{2+}$ homeostasis were reversible. These results further indicate a relationship between the effects of CaMK-II activity on Ca$^{2+}$ homeostatic mechanisms and the development of epileptiform activity.

To exclude the possibility that ongoing SRED activity was accounting for observed changes in [Ca$^{2+}$], homeostasis, TTX was added to the cultures prior to [Ca$^{2+}$] imaging to inhibit basal electrophysiological activity and epileptiform discharges. Addition of TTX lowered basal [Ca$^{2+}$] in both missense-treated and antisense-treated cultures (0.23 and 0.26 respectively). However, with the abolishment of seizure and background activity, basal [Ca$^{2+}$] levels from antisense-treated cultures still remained significantly elevated above [Ca$^{2+}$] from missense-treated cultures (Fig 8A; p<0.001, Student’s $t$-test). Similarly, in the presence of TTX, the ability of antisense-treated neurons to recover from a glutamate-induced Ca$^{2+}$ load is altered compared to missense-treated neurons (Fig 8B). Antisense-treated neurons recovered to 40.1 ± 7.2% of the Ca$^{2+}$ load whereas missense-treated neurons recovered to 11.1 ± 4.6% of the Ca$^{2+}$ load. Thus, alterations in the [Ca$^{2+}$] homeostatic mechanisms associated with decreased CaMK-II activity and protein expression were not solely dependent on the presence of SREDs in this preparation.
Discussion

Alterations in Ca\(^{2+}\) homeostasis and CaMK-II levels have been observed in various models of epilepsy (Bronstein et al., 1993; Butler et al., 1995; DeLorenzo et al., 1998; Blair et al., 1999; Churn et al., 2000a; Delorenzo et al., 2005). Since epileptogenesis has been associated with increased [Ca\(^{2+}\)]\(_i\) and alterations in Ca\(^{2+}\) homeostatic mechanisms (reviewed in Delorenzo et al., 2005), it was important to determine if changes in CaMK-II activity were associated with changes in Ca\(^{2+}\) homeostasis and the development of epileptogenesis. The present study provides evidence that suppression of CaMK-II activity is associated with both alterations in Ca\(^{2+}\) homeostasis and the development of SREDs in a reversible manner. Fluorescence imaging of [Ca\(^{2+}\)]\(_i\) with both Fura-2 and Fura-FF was used to determine [Ca\(^{2+}\)]\(_i\) in neurons treated with an antisense oligonucleotide to knock down levels of CaMK-II. Addition of antisense oligonucleotide decreased CaMK-II activity and immunocytochemical expression and resulted in the development of SREDs, a characteristic of epilepsy. In the antisense-treated neurons, basal [Ca\(^{2+}\)]\(_i\) was elevated over both control and missense-treated neurons. Reversal of the CaMK-II knockdown following removal of the oligonucleotide restored CaMK-II activity and basal [Ca\(^{2+}\)]\(_i\) and abolished epileptiform activity (SREDs), demonstrating a cause and effect relationship between CaMK-II levels and the development of SREDs and changes in basal [Ca\(^{2+}\)]\(_i\). [Ca\(^{2+}\)]\(_i\) in antisense-treated neurons were still elevated over [Ca\(^{2+}\)]\(_i\) in missense-treated neurons incubated in TTX to abolish both basal synaptic and seizure activity, demonstrating that CaMK-II changes in [Ca\(^{2+}\)]\(_i\) was effecting the underlying Ca\(^{2+}\) homeostatic mechanisms that regulate basal [Ca\(^{2+}\)]\(_i\), independent of neuronal activity.
In addition to evaluating the effects of CaMK-II knockdown on \([\text{Ca}^{2+}]_i\), this study evaluated the effects of altering CaMK-II activity on \(\text{Ca}^{2+}\) homeostatic mechanisms that regulate the neuron’s ability to handle a \(\text{Ca}^{2+}\) load. Following a glutamate-induced \(\text{Ca}^{2+}\) load, antisense-treated neurons were not able to buffer \([\text{Ca}^{2+}]_i\) back to basal levels as efficiently as control neurons, similar to findings observed in hippocampal neuronal culture models of both SE and stroke-induced AE (Pal et al., 1999; Sun et al., 2004). Abolishing both basal synaptic and seizure activity with TTX did not affect the altered \(\text{Ca}^{2+}\) homeostatic mechanisms observed with decreased CaMK-II activity. To determine the reversibility of the CaMK-II knockdown-induced changes on \(\text{Ca}^{2+}\) homeostatic mechanisms and the development of SREDs, antisense-treated neurons were allowed to recover from the antisense oligonucleotide treatment. SREDs were abolished and \(\text{Ca}^{2+}\) homeostasis was restored and recovered neurons were as effective as controls in their ability to effectively buffer \([\text{Ca}^{2+}]_i\), back to basal levels following a glutamate-induced \(\text{Ca}^{2+}\) load. The results from this study employed both the high and lower affinity \(\text{Ca}^{2+}\) indicators Fura-2 and Fura-FF. Both missense-treated and antisense-treated neurons loaded with each indicator showed similar responses in response to the glutamate-induced \(\text{Ca}^{2+}\) load. The data suggest a correlation between the suppression of CaMK-II activity with alterations in \(\text{Ca}^{2+}\) homeostatic mechanisms and expression of SREDs in hippocampal neurons in culture, indicating that CaMK-II is playing a role in regulating \(\text{Ca}^{2+}\) homeostasis during epileptogenesis in this model of epileptiform activity.

A decrease in function of CaMK-II has been shown to occur in a number of models of epilepsy (Bronstein et al., 1993; Butler et al., 1995; Churn et al., 2000a; Churn et al., 2000b). However, this is the first study that shows a direct relationship between a specific suppression of CaMK-II activity and a loss in the ability of neurons to maintain \(\text{Ca}^{2+}\) homeostatic mechanisms.
in association with the development of SREDs. CaMK-II is a ubiquitous enzyme that phosphorylates and regulates the activity of many receptors, including NMDA, GABA<sub>A</sub>, and IP<sub>3</sub>, all of which play a role in epilepsy (Cardy and Taylor, 1998; Huang et al., 2005). CaMK-II activation has been associated with increased excitability, including LTP by potentiating excitatory synapses (reviewed by Bronstein et al, 1993). However, decreased CaMK-II activity has been consistently found in various models of epilepsy. The present study shows that decreasing the expression of CaMK-II results in a hyperexcitable state in neurons in culture. Further studies are needed to determine the role of decreased CaMK-II activity in inducing SREDs and altering Ca<sup>2+</sup> homeostasis.

It can be deduced from the results of the present study that the phosphorylation of CaMK-II substrates might be linked to the observed alterations in Ca<sup>2+</sup> homeostasis. Further studies need to be conducted to isolate the specific substrate(s) involved in these processes. Interestingly, unlike the SE induced model of AE (Sombati and Delorenzo, 1995), knocking down levels of CaMK-II does not permanently alter Ca<sup>2+</sup> homeostasis or permanently induce SREDs. Our results indicate that a 3-day recovery period is sufficient to fully restore CaMK-II activity, abolish SREDs and restore the ability of the neurons to regulate Ca<sup>2+</sup> homeostasis. Thus, this model of reversible AE may provide unique opportunities to identify the important cellular mechanisms mediating the induction and maintenance of AE and indicate that the effects of CaMK-II in regulating Ca<sup>2+</sup> homeostasis plays an important role in the development of AE in hippocampal neurons in culture. Although the using cultured hippocampal neurons is a well established model for studying epilepsy, it is important to confirm the results of studies in culture with in vivo models. Further studies are needed to determine the in vivo effects of CaMK-II knockdown to further understand the role of CaMK-II in epilepsy.
Calcium plays a pivotal role in normal neuronal function (Llinas et al., 1992; Berridge, 1998). Normal neuronal $[\text{Ca}^{2+}]_{i}$ is maintained around 100 nM (Mody and MacDonald, 1995). This concentration is less than one-tenth thousandth of the free extracellular Ca$^{2+}$ concentration ($[\text{Ca}^{2+}]_{e}$) (Putney, 1999). In light of the important signaling function of Ca$^{2+}$ and the excitotoxic implications of excess $[\text{Ca}^{2+}]_{i}$, neurons have an intricate system to regulate $[\text{Ca}^{2+}]_{i}$. Evidence indicates that Ca$^{2+}$ homeostasis is markedly altered acutely during the injury and epileptogenic phases of AE (Raza et al., 2004), and even in the chronic spontaneous recurrent seizures (epilepsy) phases of AE (Pal et al., 2000; Pal et al., 2001; Raza et al., 2001; Raza et al., 2004). These observations provide direct evidence that the alterations of normal Ca$^{2+}$ homeostasis plays a role in epileptogenesis and the maintenance of the epileptic phenotype (Delorenzo et al., 2005).

Complex regulatory processes are mediated by Ca$^{2+}$ homeostatic mechanisms in neurons (Carafoli et al., 1997). Increased or prolonged entry of extracellular Ca$^{2+}$ could contribute to the altered Ca$^{2+}$ homeostatic mechanisms in epilepsy (Carafoli et al., 1997). $[\text{Ca}^{2+}]_{i}$ homeostasis is regulated by a number of cellular mechanisms which include calcium-induced calcium release (CICR) from intracellular stores, sequestration into the endoplasmic reticulum by the sarco/endoplasmic reticulum calcium ATPase (SERCA) (Carafoli et al., 1997), mitochondrial uptake of Ca$^{2+}$ (Kunz et al., 1999) and efflux via the plasma membrane Na$^{+}$/Ca$^{2+}$ exchanger (Ryan, 1999). In addition, a number $[\text{Ca}^{2+}]_{i}$ buffering systems which include the binding proteins calbindin, calretinin and parvalbumin (Nagerl et al., 2000) are important regulators of $[\text{Ca}^{2+}]_{i}$. In the CaMK-II knockdown neurons, Ca$^{2+}$ homeostasis was disrupted as indicated by elevated basal $[\text{Ca}^{2+}]_{i}$ and the impaired ability to buffer a Ca$^{2+}$ load. These results are similar to previous observations of altered $[\text{Ca}^{2+}]_{i}$ in other models of epilepsy, including a low Mg$^{2+}$-induced SE model of AE (Pal et al., 1999) and the stroke model of AE (Sun et al., 2004).
Studies have implicated decreased activity of SERCA leading to increases in [Ca\textsuperscript{2+}]\textsubscript{i} in whole animal and in vitro models of epilepsy (Parsons et al., 2000; Pal et al., 2001). Enhanced CICR via the IP\textsubscript{3} receptor has also been shown to be a contributor to increased [Ca\textsuperscript{2+}]\textsubscript{i}, and altered Ca\textsuperscript{2+} homeostatic mechanisms in epilepsy (Pal et al., 2001). Further studies are needed to evaluate what cellular pathway(s) is involved in the loss of Ca\textsuperscript{2+} homeostasis in the CaMK-II antisense knockdown model of AE.

CaMK-II activity plays a role in maintaining Ca\textsuperscript{2+} homeostasis in both skeletal and cardiac muscle, with a positive correlation between CaMK-II activity and SERCA activity. In these tissues, CaMK-II has been shown to regulate SERCA by two different mechanisms. One mechanisms involves CaMK-II phosphorylation of the SERCA accessory protein, phospholamban, which enhances the uptake of Ca\textsuperscript{2+} by SERCA (Colyer, 1998). Although phospholamban is not present in neurons (Plessers et al., 1991), there is evidence that a protein analogous to phospholamban exists in neurons and may be required for neuronal SERCA activity (Dou and Joseph, 1996). Additionly, SERCA activity in skeletal muscle is increased by direct phosphorylation by CaMK-II (Xu et al., 1993). This relationship has yet to be studied in neurons and could provide the link between decreased CaMK-II activity and altered Ca\textsuperscript{2+} homeostasis in our model of epilepsy.

A better understanding of the molecular mechanisms that underlie the relationship between altered Ca\textsuperscript{2+} dynamics and the induction and maintenance of AE is important for understanding the process of epileptogenesis (Delorenzo et al., 2005). Although further studies are needed, it is apparent that elucidating the molecular basis contributing to altered Ca\textsuperscript{2+} regulatory mechanisms in the CaMK-II knockdown model of AE may provide an insight into the
long-term plasticity changes associated with epilepsy and offer specific molecular targets for preventing and possibly reversing the pathophysiological condition of AE.
References


Footnotes

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Legends for Figures

Figure 1. Antisense oligonucleotide knockdown of α CaMK-II causes decrease in both expression and activity of CaMK-II. Hippocampal neurons in culture were treated with CaMK-II missense and antisense oligonucleotides for 3 days (missense and antisense). To evaluate the recovery from the antisense oligonucleotide, oligonucleotides were removed from the culture media and neurons were allowed to recover for three days (antisense recovery). (A) Following these treatment paradigms, cells were harvested from the culture plates and CaMK-II-dependent phosphorylation of exogenously added Syntide II was determined (Methods). CaMK-II activity was significantly decreased in the antisense treated cultures, but not in the missense treated or antisense recovery conditions. The data represent the means ± S.E.M. and are expressed as percent of control (untreated cultures) CaMK-II activity. (*p<0.01, Student’s t-test, n=5). (B) Immunocytochemistry was performed with a mAb directed against the α subunit of CaMK-II. Exposure of the neurons to antisense oligonucleotides resulted in a substantial decrease in CaMK-II immunocytochemical staining. After 3 days of recovery from antisense oligonucleotide, immunocytochemical staining of CaMK-II returned to control levels.

Figure 2. Induction of SREDs following antisense oligonucleotide knockdown of α CaMK-II. Whole-cell current-clamp recordings were obtained from neurons undergoing four different treatment regimens in culture. Representative recordings from control (A) and missense-treated (B) neurons displayed nonsynchronous background neuronal activity characterized by intermittent action potentials. (C) Neurons treated with antisense oligonucleotides displayed SREDs. A continuous 30-min recording from this neuron revealed the presence of characteristic SREDs. The lower panel recording (a) corresponds to an expansion of the first SRED from panel

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(C) above and demonstrates the presence of individual paroxysmal depolarization shifts that comprise a high frequency discharge activity of a single SRED. (D) Neurons that recovered from antisense oligonucleotide treatment demonstrated absence of SREDs and displayed a return to control conditions with nonsynchronous background neuronal activity.

**Figure 3.** Knockdown of CaMK-II results in changes of basal \([\text{Ca}^{2+}]_i\). (A) 340/380 ratio values were obtained from control (n=124; white bar), missense-treated (n=164; black bar), antisense-treated (n=164; gray bar), and antisense-recovered neurons (n=83, checked bar). Both control and missense-treated neurons demonstrate low basal \([\text{Ca}^{2+}]_i\). Basal \([\text{Ca}^{2+}]_i\) measured from antisense-treated neurons was significantly higher than basal \([\text{Ca}^{2+}]_i\) measured from both control and missense-treated neurons. Recovery of neurons from antisense oligonucleotide treatment restored basal \([\text{Ca}^{2+}]_i\) to control levels. Data are represented by mean ratio value ± S.E.M. *p<0.001, ANOVA with Tukey’s post hoc test, control vs. missense-treated vs. antisense-treated; †p<0.001, ANOVA with Tukey’s post hoc test, antisense-recovered vs. antisense-treated neurons.

**Figure 4.** Histograms demonstrating percent distribution of basal \([\text{Ca}^{2+}]_i\) within bins (0.025 increments) of ratio values less than 0.30 in black and greater than 0.30 in white from control (A), missense-treated (B), antisense-treated (C), and antisense-recovery neurons (D). For control, missense-treated and antisense recovery groups, 80.8%, 78.1% and 81.9% fell below the 0.30 ratio value level (black) respectively. Antisense treatment resulted in a redistribution of basal \([\text{Ca}^{2+}]_i\) where only 51% of neurons had ratio values less than 0.30.
Figure 5. The altered ability of CaMK-II knockdown neurons to restore basal \([Ca^{2+}]_i\) was not due to an increased \(Ca^{2+}\) load. (A) Peak increase in Fura-2 ratio during glutamate \((50 \mu M, 2\) min) exposure in missense-treated \((n=29; \text{whit bar})\) and antisense-treated \((n=29; \text{black bar})\) neurons. No statistically significant differences were observed between missense-treated and antisense-treated neurons. (B) Peak increase in Fura-FF ratio during 50 \(\mu M, 2\) min exposure in missense-treated \((n=17; \text{white bar})\) and antisense-treated \((n=9; \text{black bar})\) neurons. No statistically significant differences were observed between missense-treated and antisense-treated neurons. (C) \(Ca^{2+}\) decay curves obtained with Fura-2 were normalized as percent of \(Ca^{2+}\) load and demonstrate the impaired ability of antisense-treated neurons \((n=28; \bullet)\) to buffer \([Ca^{2+}]_i\) compared to missense-treated neurons \((n=32; \circ)\). (D) \(Ca^{2+}\) decay curves obtained with Fura-FF were normalized as percent of \(Ca^{2+}\) load and demonstrate the impaired ability of antisense-treated neurons \((n=9; \bullet)\) to buffer \([Ca^{2+}]_i\) compared to missense-treated neurons \((n=17; \circ)\). For both indicators, the percent of \(Ca^{2+}\) load remained significantly higher for antisense-treated neurons at all time points after glutamate was washed from the neurons. *\(p<0.001\), RM ANOVA with Tukey’s post hoc test. Data are represented as mean percent \(Ca^{2+}\) load ± S.E.M..

Figure 6. Recovery of \([Ca^{2+}]_i\) to basal levels 2-h post glutamate exposure in both (A) missense-treated and (B) antisense-treated neurons. Neurons were exposed to 50 \(\mu M, 2\) min glutamate, then analyzed with Fura-2 2-h after glutamate was washed from the neurons. At 2-h, ratio values from both missense-treated and antisense-treated neurons were not significantly different from basal \([Ca^{2+}]_i\). At the 2-h time point, ratio values obtained from antisense-treated neurons \((n=30; \bullet)\) were significantly higher than those from missense-treated neurons \((n=21; \circ)\). *\(p<0.001\), Student’s t-test. Data are represented as mean ± S.E.M..
Figure 7. Restoration of Ca\textsuperscript{2+} homeostasis in neurons recovered from antisense oligonucleotide. Neurons recovered from CaMK-II antisense oligonucleotide were loaded with Fura-2 and 340/380 ratio values were obtained. Antisense-recovered neurons demonstrate the ability to buffer [Ca\textsuperscript{2+}], following a Ca\textsuperscript{2+} load produced by a 2-min exposure to 50 µM glutamate. Ca\textsuperscript{2+} decay curves were normalized to percent of Ca\textsuperscript{2+} load. Antisense-treated neurons (n=32; ●) show a decreased rate of decline of [Ca\textsuperscript{2+}], compared to missense-treated (n=28; ○) and antisense-recovered neurons (n=29; ▲) in culture. [Ca\textsuperscript{2+}], in antisense-treated neurons remained elevated for up to 30 min following the glutamate exposure, while [Ca\textsuperscript{2+}], in missense-treated and antisense-recovered neurons declined, rapidly approaching basal levels. The data represent the mean percent Ca\textsuperscript{2+} load ± S.E.M.. *p<0.001, RM ANOVA with Tukey’s post hoc test, antisense-treated vs. antisense-recovered.

Figure 8. Abolishment of SREDs did not restore [Ca\textsuperscript{2+}], homeostasis. TTX (400nM) was added to the pBRS and glutamate solutions to inhibit synaptic activity prior to and during Ca\textsuperscript{2+} imaging. (A) In the presence of TTX, basal [Ca\textsuperscript{2+}] in antisense-treated neurons (n=106, black bar) remained elevated above missense-treated neurons (n=97, white bar). (*p<0.001, Student’s t-test, antisense + TTX vs. missense + TTX). Data are represented as mean ratio value ± S.E.M. (B) Following a glutamate-induced Ca\textsuperscript{2+} load, antisense-treated neurons (n=19, ●) remained elevated above missense-treated neurons (n=24, ○) in the presence of TTX for up to 30 min after glutamate exposure. Data are represented as mean percent Ca\textsuperscript{2+} load ± S.E.M.. *p<0.01, RM ANOVA with Tukey’s post hoc test.
Figure 6
Figure 8

The top graph shows the ratio (340/380) for Missense + TTX and Antisense + TTX. The bottom graph illustrates the % Ca Load over time post-glu (min).