Supplemental Information

Autonomous CaMKII Activity as a Drug Target for Histological and Functional Neuroprotection after Resuscitation from Cardiac Arrest

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SUPPLEMENTAL TABLES & FIGURES

| Experimental Groups | tatSCR | tatCN21 | Hypothermia | Hypo + tatCN21 |
|---------------------|--------|---------|-------------|----------------|
| N                   | 11     | 11      | 6           | 6              |
| Body weight (BW, g) | 23.3 ± 0.5 | 22.5 ± 0.3 | 24.2 ± 1.0 | 22.3 ± 0.4     |
| Total ischemia time (sec) | 457 ± 6 | 462 ± 7 | 476 ± 8 | 452 ± 11       |
| Epinephrine (µg/g BW) | 0.31 ± 0.02 | 0.31 ± 0.03 | 0.38 ± 0.04 | 0.40 ± 0.03 |
| Surviving animals (%) | 84.6(11/13) | 91.6(11/12) | 100.0(6/6) | 85.7(6/7)     |
| Health Assessment Score | | | | |
| POD 1 | 1.9 ± 0.3 | 1.5 ± 0.2 | 1.5 ± 0.5 | 1.2 ± 0.5 |
| POD 2 | 1.1 ± 0.2 | 0.6 ± 0.2 | 1.3 ± 0.6 | 0.3 ± 0.2 |
| POD 3 | 0.4 ± 0.2 | 0.3 ± 0.1 | 0.8 ± 0.5 | 0.3 ± 0.2 |

**Table S1.** Related to Figure 1; Body weight, cardiac arrest parameters, survival rate, and general health assessment. Total ischemia represents time from induction of cardiac arrest to successful resuscitation. Postoperative day (POD) refers to day following CA/CPR.

| Experimental Groups | tatSCR | tatCN19o |
|---------------------|--------|----------|
|                    | 0.001 mg/kg | 0.01 mg/kg | 0.1 mg/kg | 1 mg/kg |
| N                   | 11     | 8        | 8         | 8       | 7       |
| Body weight (BW, g) | 23.3 ± 0.5 | 23.9 ± 0.6 | 23.4 ± 0.6 | 22.0 ± 0.5 | 22.3 ± 0.7 |
| Total ischemia time (sec) | 457 ± 6 | 475 ± 8 | 443 ± 6 | 454 ± 8 | 459 ± 10 |
| Epinephrine (µg/g BW) | 0.31 ± 0.02 | 0.51 ± 0.02 | 0.37 ± 0.02 | 0.43 ± 0.34 | 0.41 ± 0.03 |
| Surviving animals (%) | 84.6(11/13) | 100%(8/8) | 88.9(8/9) | 88.9(8/9) | 87.5(7/8) |
| Health Assessment Score | | | | | |
| POD 1 | 1.9 ± 0.3 | 1.8 ± 0.3 | 1.5 ± 0.3 | 0.8 ± 0.3 | 1.9 ± 0.6 |
| POD 2 | 1.1 ± 0.2 | 0.4 ± 0.2 | 0.8 ± 0.2 | 0.3 ± 0.2 | 1.0 ± 0.0 |
| POD 3 | 0.4 ± 0.2 | 0.3 ± 0.2 | 0.6 ± 0.2 | 0.0 ± 0.0 | 0.6 ± 0.2 |

**Table S2.** Related to Figure 3; Body weight, cardiac arrest parameters, survival rate, and general health assessment. Total ischemia represents time from induction of cardiac arrest to successful resuscitation. Postoperative day (POD) refers to day following CA/CPR.
|                              | Wild Type | T286A Mutant |
|------------------------------|-----------|--------------|
| N                            | 8         | 8            |
| Body weight (BW, g)          | 22.0 ± 0.8| 20.2 ± 0.6   |
| Total ischemia time (sec)    | 466 ± 6   | 481 ± 9      |
| Epinephrine (µg/g BW)        | 0.50 ± 0.03| 0.50 ± 0.04 |
| Surviving animals (%)        | 100.0(8/8)| 100.0(8/8)   |
| Health Assessment Score      |           |              |
| POD 1                        | 1.8 ± 0.4 | 1.5 ± 0.2    |
| POD 2                        | 1.3 ± 0.2 | 1.0 ± 0.2    |
| POD 3                        | 0.8 ± 0.3 | 0.5 ± 0.3    |

Table S3. Related to Figure 2; Body weight, cardiac arrest parameters, survival rate, and general health assessment between T286A mutant and its wild-type control groups. Total ischemia represents time from induction of cardiac arrest to successful resuscitation. Postoperative day (POD) refers to day following CA/CPR.
Figure S1. Related to Figure 1; tatCN21 has no effect on hypothermia-induced temperature regulation. Mice were randomly assigned to receive hypothermia or hypothermia + tatCN21 treatment after resuscitation. Rectal and brain temp were monitored during the recovery and showed no difference between two groups.
Figure S2. Related to Figure 2; tatCN19o has no effect on CaMKII phosphorylation or distribution.

Injection of CN inhibitors after CA/CPR was expected to block autonomous CaMKII activity without affecting the T286 phosphorylation that generates autonomous activity or the distribution of CaMKII. This was here formally tested using the optimized inhibitor version tatCN19o; the results further support that tatCN19o acts by inhibiting autonomous CaMKII activity and not other functions of autonomous activity. (A) CaMKII autophosphorylation at T286, total CaMKII were assessed in synaptosome (P2) membrane fraction 3 hrs following resuscitation by Western analysis. (B) Quantification of the ratio of phosphorylated CaMKII to total CaMKII in P2 fraction showed a significant increase after CA/CPR (all groups increased compared to Sham, p<0.05; Student’s t-test) that was unaffected by tatCN19o treatment. (C) CaMKII amount and T286 autophosphorylation in the cytosolic (S3) fraction 3 h after resuscitation assessed by Western analysis. (D) Quantification of the amount of CaMKII in the cytosolic relative to membrane fraction showed that this distribution was unaltered by tatCN19o. (E) Injection of 0.01 mg/kg tatCN19o after CA/CPR significantly reduced the CaMKII autonomy present in the synaptosomal P2 fraction (*: p < 0.05; two tailed t-test; nine biochemical assays with protein extract from three different animals for each condition), demonstrating target engagement by tatCN19o in vivo. Notably, the protein dilution within these assays compared to intact brain tissue was ~3,000fold (with 40 μg/ml compared to ~120 mg/ml). Dilution of free inhibitor is even higher, due to the preparation of the P2 fraction by differential centrifugation. Even low levels of continued inhibition after such a dilution are detectable only for extremely high-affinity and tight-binding inhibitors.
Figure S3. Related to Figure 1, 3; CaMKII inhibition kinetics by CN peptides. (A) For tight-binding inhibitors, kinetic parameters have to be determined by the Morrison equation, with Morrison plots visualizing the mode of inhibition (as Michaelis-Menten kinetics that can use Lineweaver-Burke plots for visualization are not applicable) (Morrison, 1969). The dependence of the apparent Ki (Ki app) on the substrate concentration is plotted for different modes of inhibition. (B-D) All CN inhibitors inhibit CaMKII by a mixed noncompetitive mode of inhibition. Plots on the left show CaMKII inhibition by the peptides at different substrate concentrations (color coded as indicated). On the right, Morrison plots show the dependence of the apparent Ki on substrate concentration to visualize the mode of inhibition. The actual Ki and the α-factor (which indicates the degree of the competitive component) are indicated. Consistent with increased binding of CN19o also to the CaMKII substrate binding site (in addition to the neighboring T-site)(Coultrap and Bayer, 2011), the α-factor for this peptide appeared to be slightly higher compared to the parent compounds. However, this increase was not statistically significant and the parent compounds CN19 and CN19a2v also showed a substrate-competitive component in their mixed inhibition (a component previously not detected by traditional Michaelis-Menten analysis; (Chang et al., 1998).
Figure S4. Related to Figure 4; CA/CPR or drug treatment did not affect performance in the open field test. Open field test was performed 7 days after resuscitation and there were no differences in total distance or velocity among each group.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cardiac arrest and cardiopulmonary resuscitation model
As previous described (Grace et al., 2015; Orfila et al., 2014; Shimizu et al., 2016), mice were anesthetized with 3% isoflurane and maintained with 1.5–2% isoflurane in oxygen enriched air via facemask. Temperature probes were placed into the left ear canal (tympanic) and rectum. Rectal temperature was controlled at near 37 °C during surgery with a heating lamp and heating pad. For drug administration, a PE-10 catheter was inserted into the right internal jugular vein and flushed with heparinized 0.9% saline solution. Animals were endotracheally intubated using an intravenous catheter, connected to a mouse ventilator (Minivent, Hugo Sachs Elektronik, March-Hugstetten, Germany). Electrocardiogram (EKG) was monitored throughout the experimental procedures. Cardiac arrest was induced by injection of 50 μL 0.5 M KCl via the jugular catheter. Then endotracheal tube was disconnected from the ventilator and anesthesia was stopped. During cardiac arrest, the pericranial temperature was maintained at 37.5 ± 0.2 °C. Body temperature was allowed to fall spontaneously during the arrest to 36 °C. CPR was begun 6 min after induction of cardiac arrest, by slow injection of 0.5–1.0 mL of epinephrine (16 μg epinephrine/mL 0.9% saline), chest compressions at a rate of approximately 300 min⁻¹, and ventilation with 100% oxygen. If return of spontaneous circulation (ROSC) could not be achieved within 2 min of CPR, resuscitation was stopped and the animal was excluded from the study.

After ROSC was achieved, mice were randomized into 2 groups: normothermic and hypothermic. In animals of the hypothermic groups, rapid cooling was started by using a water-filled pad placed underneath the animal, which was chilled by running through an ice-water bath. The body temperature dropped to 34 °C quickly and was maintained at 34 ± 0.5 °C for 1 hr. Then the animal was rewarmed by the heating lamp and pad for about 10–15 min until body temperature reached 36 °C. In animals of the normothermic group, the body was rewarmed to reach 36 °C by using the heating lamp and pad at a rate of 0.3–0.5 ºC/min. Rectal and tympanic temperature were monitored closely during the experiment. Mechanical ventilation was stopped and the endotracheal tube was removed when spontaneous breathing reached a rate of 60 breaths/min. Temperature probes and catheters were removed, and the skin wounds were closed. The animal was then placed into its home cage for recovery.
Health assessment score
Mice were weighed daily, and a health assessment score was calculated for each mouse daily for three days after CA/CPR. The graded scoring systems ranged from 0 to 2, 0 to 3, or 0 to 5 depending on the behavior assessed, with 0 indicating no deficit and the upper limit indicating the most impaired. The behaviors assessed included consciousness (0–3), interaction (0–2), ability to grab wire top (0–2), motor function (0–5), and activity (0–2) (Deng et al., 2014). Scores in each category were summated to generate an overall health assessment score.

Hematoxylin & eosin staining
Three days after CA/CPR, animals were anesthetized with 3% isoflurane and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed, post-fixed with paraformaldehyde and embedded in paraffin. Coronal sections 6 μm thick were serially cut and stained with hematoxylin and eosin (H&E). The hippocampal CA1 region was analyzed, three levels (100 μm apart), beginning from −1.5 mm bregma. Nonviable neurons were determined by the presence of hypereosinophilic cytoplasm and pyknotic nuclei. The percentage of nonviable neurons was calculated for each brain region (average of 3 levels per region). The investigator was blinded to treatment before analyzing neuronal damage.

Hippocampal slice preparation
Hippocampal slices were prepared from young adult male C57BL/6 mice at 7 days after recovery from CA/CPR. Animals were anesthetized with 3% isoflurane in an O₂-enriched chamber. Mice were decapitated and the brains quickly extracted and placed in ice-cold (2–5 °C) oxygenated (95% O₂/5% CO₂) artificial cerebral spinal fluid (ACSF) composed of the following (in mmol/L): 125 NaCl, 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 2.0 CaCl₂, 1.0 MgCl₂, and 12 glucose. Horizontal hippocampal slices (300-350 μm thick) were cut with a Vibratome 1000 (Leica) and transferred to a holding chamber containing ACSF for 1.5–2 h before recording.

Electrophysiology
For synaptically evoked field potentials, hippocampal slices were placed on a temperature-controlled (32 ± 1 °C) interface chamber perfused with oxygenated ACSF at a rate of 1.5 mL/min as previously described (Orfila et al., 2014). Extracellular field recordings were performed by
stimulating the Schaffer collaterals and recording the field excitatory post-synaptic potential (fEPSP). The fEPSPs were adjusted to 50% of the maximum slope and test pulses were evoked at a rate of 0.05 Hz. A 20 min stable baseline period was established before delivering a theta burst stimulation (TBS) train [four pulses delivered at 100 Hz in 30 ms bursts, repeated 10 times with 200 ms interburst intervals]. Following TBS, the fEPSP was recorded for 60 min. Analog fEPSPs were amplified (1000 × ) and filtered through a pre-amplifier (Model P511, Grass Instruments) at 0.03 Hz–1.0 kHz, digitized at 10 kHz and stored on computer for later off-line analyses. The derivative (dV/dT) of the initial 2–3 ms onset of the fEPSP was measured (fEPSP slope), and the amount of potentiation calculated as the percent change from baseline (the averaged 10 min slope value from 50–60 min post-TBS divided by the averaged slope value at 10 min prior to TBS). For the time-course graphs, normalized fEPSP slope values were averaged and plotted as the percent change from baseline and referenced to 100%.

CaMKII preparations
Rodent CaMKIIα was expressed in a baculovirus/Sf9 cell system and purified as described (Bayer et al., 2001; Singla et al., 2001). Human CaMKIIα was expressed in HEK-293 cells; cells were transfected with the expression vector by the calcium phosphate method (on 10 cm plates, 1 day after a 1:5 split from confluent plates), and harvested 72 h later. Cells were rinsed, scraped into ice-cold PBS, and collected by low speed centrifugation (5 min at 1,000 g at 4°C). The cells were homogenized with a motorized pellet pestle (Kontes) for 10 sec in 0.4 ml ice cold 50 mM PIPES pH 7.2, 10% glycerol, 1 mM EDTA, 1 mM DTT, and complete protease inhibitor (Roche). Debris was removed by centrifugation (20 min at 16,000 g at 4°C). Human CaMKII concentrations in the extracts were derived by comparison to purified rat CaMKII standard by quantitative Western analysis (Coultrap et al., 2012; Coultrap et al., 2010). Briefly, purified rat CaMKII (150 – 1200 fmol) was diluted into untransfected HEK cell extract and gel loading buffer run on 10% PAGE gels. Human CaMKII extracts were loaded in an equal total protein amount as the standard. Western blots were performed using anti-CaMKIIα antibody (CBα2).

CaMKII activity assays
Phosphate incorporation into peptide substrates was assessed as described (Coultrap et al., 2010). For the comparison of mouse and human CaMKII, the kinase reactions were done at 30
°C for 1 min and contained 2.5 nM CaMKII kinase subunits (purified from HEK cell extracts), 50 mM PIPES, pH 7.1, 0.1% bovine serum albumin, 10 mM MgCl₂, 100 μM [γ-32P]ATP (~1 mCi/μmol), and 75 μM Syntide-2 substrate peptide. For assessing the Ki of CaMKII inhibition, the substrate concentration was varied as indicated; CaMKII concentration was 0.1 nM for the optimized CN19o and 2.5 nM for the less potent inhibitors CN19 and CN19a2v. For assessing CaMKII autonomy in the synaptosomal P2 fraction from hippocampus, 2 μg of total protein was used in 50 μl reaction volumes. Stimulated activity was measured for naïve CaMKII in presence of 1 mM CaCl₂ and 1 μM CaM; autonomous activity assays contained instead 1.5 mM EGTA. T286 prephosphorylation was done by reacting CaMKII (60 – 200 nM) in stimulation buffer, but without substrate and 32P, for 5 min on ice. Before activity assays, autophosphorylation stopped and CaM dissociation was induced by addition of 5 mM EDTA for at least 5 min on ice. CaMKII activity was calculated as pmol of phosphorylated substrate per pmol of CaMKII subunits per min (resulting in the unit product/kinase/min). CaMKII activity in presence of tatCN19o (0.75, 2.5, 5 or 20 nM) was normalized to activity without inhibitor.

**Analysis of the kinetics of CaMKII inhibition**

The data were analyzed using the Morrison Ki model for tight-binding inhibitors (Morrison, 1969), essentially as we have described previously (Barcomb et al., 2013). For this analysis the initial reaction velocity was plotted as a function of inhibitor concentration for the different substrate concentrations. In GraphPad Prism, the Morrison Kᵢ model is designed specifically for competitive inhibitors, therefore a modified version of the Morrison Kᵢ was created in the program to model a tight binding mixed noncompetitive inhibitor (equations shown below). The mixed noncompetitive model incorporates both the competitive (where α = ∞) and noncompetitive (where α = 1) models. From this equation, the mode of inhibition was determined as was the Kᵢ and the α value (ratio of Kᵢ for enzyme-substrate complex to the Kᵢ for enzyme alone). The two models were compared by an extra sum-of-squares F-test to determine the better fit (which was the mixed noncompetitive model for all inhibitors tested). Inhibitory properties were then determined with the below equations (vᵢ = reaction velocity with inhibitor, vₒ = reaction velocity without inhibitor, [E] = enzyme concentration, [I] = inhibitor concentration, [S] = substrate concentration, Kᵢ = inhibitory constant, Kᵢapp = apparent inhibitory constant, α = cooperativity parameter). The cooperativity parameter, α, represents the
degree to which the binding of inhibitor changes the affinity of the enzyme for substrate. If \( \alpha = 1 \) then binding does not change the affinity, therefore the mechanism is noncompetitive; if \( \alpha >> 1 \) then binding of inhibitor prevents substrate binding, therefore binding is competitive; if \( 0 < \alpha < 1 \) then binding of inhibitor enhances substrate binding, therefore binding is uncompetitive. For all inhibitors tested, \( \alpha \) was between 2 and 5, indicating a mixed noncompetitive mode of inhibition in all cases.

Morrison equation (Morrison, 1969):

\[
v_i = v_o \left( 1 - \frac{([E] + [I] + K_i^{app}) - \sqrt{([E] + [I] + K_i^{app})^2 - 4[E][I]}}{2[E]} \right)
\]

For competitive inhibitors:

\[
K_i^{app} = K_i \left( 1 + \frac{[S]}{K_m} \right)
\]

For noncompetitive (and mixed) inhibitors:

\[
K_i^{app} = \frac{[S] + K_m}{K_m + \frac{[S]}{\alpha K_i}}
\]

Subcellular fractionation
Frozen hippocampi was placed in ice-cold buffered sucrose solution (250 µl per hippocampus), containing 10 mM Tris, pH 7.4, 320 mM sucrose, phosphatase and proteinase inhibitors (Thermo
Samples were homogenized with a pestle and drill homogenizer. Homogenates were centrifuged for 10 minutes at 1,000 g. Supernatant (S1) was collected and transferred to fresh tube and centrifuged for 15 minutes at 10,000 g. The pellet (P2), which contains synaptosomal plasma membrane, was suspended in 60 µL of Neuronal Protein Extraction Reagent (Thermo Scientific), boiled. The supernatant (S2) was centrifuged for 60 minutes at 100,000 g, and the supernatant (S3) which is the cytosolic fraction was precipitated with cold acetone for 12hr at -20C, and centrifuged for 10 minutes at 15,000 g, supernatant was removed. Pellet was re-suspended with 60 µL Neuronal Extraction Reagent, boiled. Protein concentrations were quantified with Pierce BCA Protein Assay Kit (Thermo Scientific), and then kept in -80 °C until used.

**Western blot analysis**

The total 40 µg protein from each sample were resolved via electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels at 80V for stacking gel and 160 V for running gel for about 1 h total. Protein was transferred to polyvinylidene difluoride membranes for 1 h at 300 mA at 4 °C, and incubated at room temperature (22 °C) with gentle rocking in 10% BSA in Tris-buffered saline with Tween [140 mM NaCl, 20 mM Tris (pH 7.4) and 0.1% Tween 20. Blots were incubated with anti-phospho-Thr^{286} CaM Kinase II (1 : 3000 dilution in 1% BSA; PhosphoSolutions, Aurora, USA), anti-CaM Kinase II α (1 : 2000 dilution in 1% BSA; Sigma Aldrich, St. Louis, MO, USA), or anti-β-actin (1 : 5000 in 1% BSA; Sigma-Aldrich, St Louis, MO, USA) overnight at 4 °C, and washed three times for 10 min each in Tris-buffered saline with Tween, followed by a 1 h incubation with a horseradish peroxidase-conjugated second antibody (1 : 5000; Thermo Scientific) at room temperature. Blots were then washed in Tris-buffered saline with Tween three times for 10 min each, and bands were detected using SuperSignal® chemiluminescent substrate kits (Thermo Scientific) and a ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA). Quantification of western blots was performed using image lab software version 4.0 (Bio-Rad).

**Behavioral tests**
Open Field

The open field test was used to assess locomotor activity levels at 7 days after CA/CPR, as previously described by our group (Allen et al., 2011). Mice were allowed to habituate to the testing room 30 minutes prior to commencing the test. The apparatus consisted of a 52 cm diameter circular arena enclosed by 28 cm walls. Each mouse was placed in the center of the field and allowed to explore the arena for 10 minutes while being continuously recorded from a video camera placed directly above the center of the arena. At the end of the task mice were returned to their home cage. Locomotor activity was assessed as total distance traveled, measured by ANY-maze automated tracking software (Stoelting, Inc., Wood Dale, IL, USA).

Contextual fear conditioning

The contextual fear conditioning paradigm was utilized as a hippocampal-dependent memory task (Rudy and O’Reilly, 2001). The apparatus consisted of two fear conditioning chambers with shock grid floors, consisting of 16 stainless steel rods connected to a shock generator (Colbourn Instruments, Model H13-15, Whitehall, PA, USA). Mice were transported in white buckets during the training and testing sessions. During training on day 8th after CA/CPR, mice were allowed to habituate the conditioning chamber for two separate 2 minute pre-exposure sessions followed by a foot shock (2-sec/1.0 mA electric shock) immediately after the second exposure. Following shock, mice were returned to their home cages. Testing occurred 24 hours later, mice were transported in white buckets and placed back into the fear conditioning chambers. Freezing behavior was measured in 10 sec intervals across a 5 minute test by a blinded observer, and was defined as the absence of movement except for heart beat/respiration.

SUPPLEMENTAL REFERENCES

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