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

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SUMMARY

The Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a major mediator of physiological glutamate signaling, but its role in pathological glutamate signaling (excitotoxicity) remains less clear, with indications for both neurotoxic and neuro-protective functions. Here, the role of CaMKII in ischemic injury is assessed utilizing our mouse model of cardiac arrest and cardiopulmonary resuscitation (CA/CPR). CaMKII inhibition (with tatCN21 or tatCN19o) at clinically relevant time points (30 min after resuscitation) greatly reduces neuronal injury. Importantly, CaMKII inhibition also works in combination with mild hypothermia, the current standard of care. The relevant drug target is specifically Ca^{2+}-independent "autonomous" CaMKII activity generated by T286 autophosphorylation, as indicated by substantial reduction in injury in autonomy-incompetent T286A mutant mice. In addition to reducing cell death, tatCN19o also protects the surviving neurons from functional plasticity impairments and prevents behavioral learning deficits, even at extremely low doses (0.01 mg/kg), further highlighting the clinical potential of our findings.

In Brief

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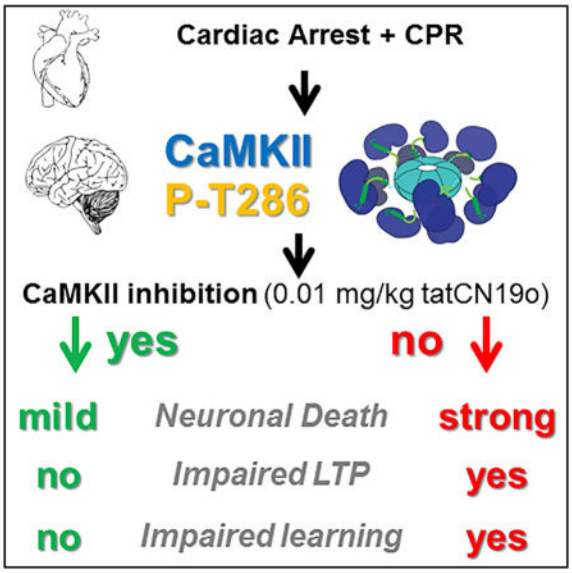
AUTHOR CONTRIBUTIONS

G.D., J.E.O., R.M.D., M.M.-G., K.M.R., S.J.C., and N.Q. conducted the experiments. R.J.T. provided expertise and feedback. K.U.B. and P.S.H. wrote the manuscript, conceived of experiments, and secured funding.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.01.011.



Deng et al. find that CaMKII and its phospho-T286-induced autonomous activity are a promising therapeutic drug target for global cerebral ischemia (induced by cardiac arrest followed by CPR in a mouse model). Pharmacological inhibition or T286A mutation leads to neuroprotection and improves synaptic and functional recovery following cardiac arrest.

INTRODUCTION

The Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII) is well established as a major mediator of synaptic plasticity that underlies learning and memory (for review see Coultrap and Bayer, 2012; Hell, 2014; Lisman et al., 2012). Ca^{2+} entry through NMDA-type glutamate receptors (NMDARs) can trigger two forms of CaMKII activity: the direct activation by Ca^{2+}/CaM termed “stimulated” activity and the subsequent Ca^{2+}-independent “autonomous” activity resulting from autophosphorylation at T286 (Coultrap et al., 2010; Miller and Kennedy, 1986). Both forms of CaMKII activity are required for NMDAR-dependent long-term potentiation (LTP) and depression (LTD) of synaptic strength, making CaMKII a crucial mediator of physiological NMDAR-dependent glutamate signaling (Coultrap et al., 2014; Giese et al., 1998). In addition, NMDARs are central in pathological glutamate signaling (excitotoxicity) that is involved in ischemic cell death (Szydlowska and Tymianski, 2010). However, the role of CaMKII in this process is less clear: inhibitor studies have suggested functions both in promoting neuronal survival (Bok et al., 2007; Hansen et al., 2003; Mabuchi et al., 2001; Waxham et al., 1996) and in promoting death (Ashpole and Hudmon, 2011; Fan et al., 2006; Gao et al., 2005; Hajimohammadreza et al., 1995; Laabich and Cooper, 2000; Takano et al., 2003; Vest et al., 2010). Overall, these inhibitor studies appear to more convincingly support a death-mediating function of CaMKII in excitotoxicity, especially in hippocampal neurons (Coultrap et al., 2011). However, the only genetic study indicates the opposite, with CaMKIIα knockout mice showing increased infarct size in a stroke model (Waxham et al., 1996).

Here, we utilized our mouse model of global ischemia, cardiac arrest and cardiopulmonary resuscitation (CA/CPR), which closely mimics the human condition with the use of epinephrine, chest compressions, and oxygen for resuscitation following asystolic cardiac arrest (Figure 1A). Importantly, this CA/CPR model allows us to take advantage of genetically altered mice to model cardiac arrest; a condition that is understudied in relation to neuroprotection and a promising clinical condition due to the necessity for rapid emergency treatment, making early intervention strategies viable. We examined whether acutely inhibiting CaMKII activity provides neuroprotection following CA/CPR and improves long-term functional recovery. We found that inhibitor timing, dosing, and effective combination with therapeutic hypothermia (the current standard of care) makes CaMKII inhibition an attractive therapeutic strategy in global cerebral ischemia. Analysis of CaMKII T286A mutant mice indicated that the neuroprotective target was specifically the autonomous activity of CaMKII that was found to be elevated in the synaptic fraction after ischemia. Thus, our results support an overall model in which long-term block of all CaMKII activity sensitizes neurons for damage, while acute post-insult inhibition of autonomous CaMKII activity provides highly efficient anatomical and functional neuroprotection.

RESULTS

CaMKII Inhibition Provides Neuroprotection Also in Combination with Hypothermia

Currently, there are no pharmacological agents available to reduce ischemic brain injury following cardiac arrest. The standard of care involves mild therapeutic hypothermia, making it imperative that new clinically relevant treatments provide additional protection when combined with hypothermia; this was tested in our CA/CPR model for the CaMKII-specific peptide inhibitor tatCN21. Immediate asystole was observed in all mice. Body weight, CPR duration, epinephrine dose, survival rate, and general health scores were not different among any of groups in this study (Tables S1–S3). Ischemia caused significant hippocampal CA1 neuronal injury, analyzed using H&E staining 3 days after resuscitation (Figures 1B and 1C). Mice administered tatCN21 (1 mg/kg, intravenously [i.v.]) 30 min after CA/CPR had significantly less hippocampal CA1 neuronal injury compared to mice treated with the scrambled control peptide tatSCR, reducing damage from 53.2% ± 3.9% ($n = 11$) for tatSCR-treated mice to 28.9% ± 5.6% ($n = 11$, $p < 0.05$) for tatCN21-treated mice (Figure 1D). To test the synergistic effect of CaMKII inhibition and hypothermia, we developed a mild hypothermia paradigm. As expected, mild hypothermia immediately after CA provided significant neuro-protection (to a similar level as seen after tatCN21 injection 30 min after CA/CPR) (Figure 1E). Importantly, combining tatCN21 with mild therapeutic hypothermia further reduced injury: neuronal cell death was reduced from 31.9% ± 10.5% ($n = 6$) for control hypothermia alone to 6.0% ± 2.7% ($n = 6$, $p < 0.05$) in tatCN21-treated hypothermic mice (Figure 1F) (i.e., to a level that is barely above the background of neuronal cell death without ischemic injury). Control experiments revealed that tatCN21 had no effect on head or body temperature (Figure S1). Thus, CaMKII inhibition can protect from brain injury under both normothermic and hypothermic conditions, demonstrating the ability to be combined with current standard of care.

Autonomous CaMKII Mediates Ischemic Cell Death

The fact that tatCN21 was neuroprotective when injected after the insult suggested that the relevant drug target was autonomous, rather than stimulated, CaMKII activity. Thus, we decided to test if CA/CPR-induced ischemia causes a corresponding long-lasting increase in CaMKII phosphorylation at T286. Indeed, ischemia caused a robust increase in T286 phosphorylation (T286-P) in synaptosome-rich fraction (P2) obtained from hippocampus 3 hr after resuscitation from CA (Figure 2A). Total CaMKII remained unchanged (Figure 2C). Interestingly, in the cytosolic fraction (S3), CA/CPR caused a decrease in both T286 phosphorylation and total CaMKII (Figures 2D–2F). Mild hypothermia did not alter the ischemia-induced change in phosphorylation or total CaMKII in either synaptosome-rich or cytosolic fractions (Figure 2). This is consistent with the additive neuroprotection by CaMKII inhibition and mild hypothermia, indicating neuroprotection by different, independent mechanisms. These data implicate ischemia-induced autonomous CaMKII activity in the progression of neuronal injury. To directly assess the role of autonomous CaMKII activity in neuronal injury, we used the autonomy-deficient CaMKII T286A mutant mice (Giese et al., 1998). T286A mutant mice exhibited much less neuronal injury (4.0% ± 0.9%, n = 8) compared to their litter-mate wild-type controls (28.9% ± 10.9%, n = 8, $p < 0.05$) (Figures 2G–2I), indicating that sustained autonomous CaMKII activity after CA/CPR significantly contributes to neuronal injury and deficits.

Injection of the CaMKII inhibitor after CA/CPR is expected to block the autonomous activity generated by T286 phosphorylation, but not to reverse the T286 phosphorylation itself. Indeed, no effect on T286 phosphorylation (or on CaMKII localization) was observed for our improved inhibitor (tatCN19o) compared to control tatSCR-treated animals (Figure S2). In contrast, CaMKII autonomy in the P2 fraction obtained from mice treated with tatCN19o was significantly reduced compared to tatSCR-treated animals (Figure S2E). The control tatSCR peptide has been previously demonstrated to have no inhibitory effect on CaMKII (Buard et al., 2010). These results further support autonomous CaMKII activity as the relevant drug target for neuroprotection (rather than other effects that could be caused by T286 phosphorylation).

Characterization of an Improved Cell-Penetrating CaMKII Inhibitor, tatCN19o

We have recently developed an optimized inhibitory peptide, CN19o, which has >250-fold enhanced potency and selectivity for stimulated CaMKII activity (Coultrap and Bayer, 2011). CN19o is a tight binding inhibitor; thus its IC_{50} depends on the CaMKII concentration, and Michaelis-Menten kinetics are not applicable (Morrison, 1969). Therefore, we varied inhibitor and substrate concentrations to determine the kinetics of inhibition by the Morrison equation (Figure S3). Inhibition was by a mixed non-competitive mode that had a substrate-competitive component, with a K_i of 0.1 ± 0.02 nM and an α-factor of 4.3 ± 1.7, consistent with the CN19o binding region on CaMKII that includes regions both outside and within the substrate binding site (Coultrap and Bayer, 2011). Compared to the parent compounds, the K_i of CN19o was dramatically improved, with minimal effect on the α-factor (the measure of the competitive component) (Figure S3). Additionally, we tested the effect of a cell-penetrating version, tatCN19o, on stimulated and autonomous CaMKII activity. As expected, tatCN19o efficiently blocked both stimulated

(Figure 3A) and autonomous (Figure 3B) CaMKII activity. Notably, tatCN19o inhibited both mouse and human CaMKII with equal potency (Figures 3A–3C), supporting the translational potential of this compound.

Efficient Neuroprotection by tatCN19o at Extremely Low Doses

Therapeutic efficacy and potency of tatCN19o was tested in a dose-response experiment. Quantification of ischemic CA1 neurons 3 days after resuscitation showed that 1 mg/kg tatCN19o protected hippocampal CA1 neurons from ischemia injury to a similar extent seen with 1 mg/kg tatCN21, reducing neuronal injury from 53.2% ± 3.9% ($n = 11$) for control tatSCR to 23.6% ± 10.8% ($n = 7$, $p < 0.05$). Most importantly, our data show that tatCN19o had at least the same level of neuroprotection at 10- to 100-fold lower doses (0.1 and 0.01 mg/kg), reducing damage to 25.8% ± 8.8% ($n = 8$, $p < 0.05$) and 10.9% ± 3.7% ($n = 8$, $p < 0.05$), respectively (Figure 3). No significant differences in cell death were observed across these doses of tatCN19o, and neuroprotection was lost only at the 1,000-fold lower dose of 0.001 mg/kg. These results indicate that CaMKII inhibition by tatCN21 and tatCN19o are similarly efficacious, but tatCN19o has higher potency, both in vitro and in vivo. From a therapy development standpoint, this is especially important for peptide inhibitors, here potentially enabling a dose of <1 mg in humans.

tatCN19o Protects Surviving Neurons from Long-Lasting Plasticity Impairments

The aim of any relevant therapeutic intervention is improved functional recovery. Notably, ischemic insults not only kill neurons, but also cause long-lasting functional impairments in the surviving neurons. In our mouse model, we have previously found that hippocampal LTP remains impaired on day 7 (and even day 30) after CA/CPR (Orfila et al., 2014). This functional impairment is not due to presence of dying neurons, as neurons dying from the insult are already eliminated at these time points (Deng et al., 2014). Thus, we decided to test the ability of acute tatCN19o treatment to prevent hippocampal dysfunction after ischemia (Figure 4A). Extracellular field recordings of CA1 neurons were performed in hippocampal slices prepared 7 days following CA/CPR and compared with slices prepared from sham-operated control mice. In sham control slices, a brief theta burst stimulus (TBS; 40 pulses) resulted in LTP after 60 min that was completely abolished in mice 7 days after CA/CPR (Figures 4B and 4C). Acute treatment after CA/CPR with low dose (0.01 mg/kg) tatCN19o completely prevented ischemia-induced loss of LTP; we observed a level that was undistinguishable from the sham-operated controls (Figures 4B and 4C). Input-output functions for fEPSPs versus stimulus intensity were obtained at the beginning of each recording to assess the effect of CA/CPR on CA3 axonal excitability (Figure 4D). The slope of each recording was measured and linear regression was used to compare across groups, with no differences observed. We examined the paired-pulse ratio (PPR) to assess the effect of CA/CPR and treatment on pre-synaptic neurotransmitter release probability. Figure 4E shows that no differences in PPR was observed.

tatCN19o Protects from Long-Term Learning Impairments

Hippocampal LTP is thought to be required for learning and memory. Indeed, long-lasting impairments in learning and memory are observed both in patients (Lim et al., 2004; Roine et al., 1993) and in our mouse model of CA/CPR (Allen et al., 2011; Orfila et al., 2014). In

order to assess if our treatment with tatCN19o prevents ischemia-induced loss of memory function, the hippocampus-dependent neurobehavioral task of contextual fear conditioning (CFC) was used. The percent time exhibiting freezing behavior when exposed to an environment associated with a noxious stimulus (foot shock) was measured. Decreased freezing behavior was observed in mice 9 days after CA/CPR (tatSCR-treated) compared to sham-control group, indicating impaired memory function. Consistent with our histology and synaptic plasticity data, treatment with tatCN19o completely restored freezing behavior to a level indistinguishable from sham-operated control (Figure 4F), indicating full protection of the memory function. Importantly, there was minimal freezing during the training phase while mice were exposed to the novel environment (≤5% freezing in all groups). Open field showed no difference in the total distance or velocity of movement among groups (Figures 4G and S4), indicating that the freezing behavior was neither due to motor deficits nor basal anxiety levels. Thus, tatCN19o treatment at clinically relevant timing after cardiac arrest not only reduces the number of neurons dying after ischemia but also improves synaptic and memory function after ischemia.

DISCUSSION

The results of this study indicate that acute inhibition of CaMKII autonomy by a single post-insult treatment at a clinically relevant time point provides sustained functional benefit. In contrast to ischemic stroke, surviving cardiac arrest requires immediate medical attention, making early pharmacological intervention feasible and the results of our study extremely promising. This clinical promise is further enhanced by the successful combination with mild therapeutic hypothermia, the current standard of care, as well as by the high efficacy at extremely low doses (0.01 mg/kg tatCN19o). While our results do not rule out that inhibition of stimulated CaMKII activity may also participate in the post-insult neuroprotection by tatCN19o, the results in the autonomy-incompetent T286A mutant mice demonstrate that the blocking of stimulated activity is not necessary and that targeting CaMKII autonomy is sufficient for neuroprotection.

Previous pharmacological studies on the role of CaMKII in glutamate excitotoxicity in vitro have been somewhat conflicting, but overall, they arguably better-support the death-promoting function of CaMKII autonomy found here in our in vivo study (Coultrap et al., 2011). However, the strongest evidence to the contrary came from the fact that CaMKIIα knockout mice have increased infarct sizes in a stroke model (Waxham et al., 1996), indicating, instead, a neuroprotective function. This apparent conflict could be explained either by non-selectivity of the pharmacology or by opposing functions of the different CaMKII isoforms, with the α-isoform being protective while other isoforms are death-promoting. However, either of these possibilities are counter-indicated by our finding that genetic CaMKIIα T286A mutation (that selectively prevents autonomous, but not Ca^{2+}-stimulated activity of the CaMKII α-isoform) provides dramatic neuroprotection. Instead, this finding supports another model proposing that long-term inhibition of CaMKII activity sensitizes neurons to injury, while acute inhibition during or after insults is neuro-protective (Ashpole and Hudmon, 2011; Coultrap et al., 2011; Vest et al., 2010). A similar dual role of CaMKIIα has been recently found for two opposing forms of synaptic plasticity, LTP and LTD (Coultrap et al., 2014). However, in this case, both LTP and LTD required autonomous

CaMKII activity generated by T286 phosphorylation. By contrast, the death-promoting versus death-protective functions of CaMKII instead appear to be differentiated by T286 phosphorylation.

The current study provides important mechanistic insights regarding the role of CaMKII in injury and functional recovery following global cerebral ischemia. The use of autonomy-incompetent T286A CaMKII mutant mice demonstrates that stimulated CaMKII activity is not sufficient to induce injury; instead, the sustained autonomous activity is required. Further, we made the observation that cerebral ischemia induced by cardiac arrest significantly increases the CaMKII T286 autophosphorylation level specifically in the synaptic membrane fraction. The increased CaMKII autonomy at synapses, but not in the cytosol, indicates that sustained synaptic CaMKII activity after ischemia plays an important role in neuronal damage. This is in contrast to several in vitro studies that implicate extrasynaptic NMDA receptor activation in cell death, while synaptic NMDA receptor activation was observed to be protective (Hardingham et al., 2002; Martel et al., 2009). However, we previously demonstrated that indirect inhibition of synaptic NMDA receptors using activators of small-conductance calcium-activated potassium channels reduced neuronal injury in our mouse model of cardiac arrest (Allen et al., 2011), thus emphasizing the importance of using clinically relevant animal models to assess mechanisms of injury and protection. Further work is warranted to assess the subcellular localization after in vivo ischemia and downstream signaling engaged in autonomy-mediated injury.

Mild therapeutic hypothermia has been shown to be protective both in preclinical and clinical global cerebral ischemic studies and is recommended as an effective treatment by the American Heart Association for adult CA/CPR and neonatal hypoxic-ischemic encephalopathy (Arrich et al., 2012; Choi et al., 2012; Moler et al., 2011; Polderman, 2009). Importantly, we show that post-arrest inhibition of CaMKII protects hippocampal neurons from ischemic insult additively with mild hypothermia, suggesting independent and additive mechanisms for hypothermia and tatCN protection. This observation is further supported by the fact that hypothermia did not affect T286 phosphorylation. Therefore, the data presented suggests that our newly developed peptide inhibitors of CaMKII are viable agents for combination therapies with therapeutic hypothermia to enhance functional recovery for CA/CPR patients in the future.

Finally, we demonstrated in this study that a single dosage 30 min after resuscitation is sufficient to rescue LTP and recover memory function after cardiac arrest and CPR. Hence, our finding indicates that a single acute dose of CaMKII inhibitor can cause sustained benefit, making this agent suitable for post-arrest patients who are in the Emergency Room/ Intensive Care Unit (ER/ICU). Interestingly, our results indicate that our optimized CaMKII inhibitor tatCN19o remains at least as efficacious as the parent compound, tatCN21. Notably, the 0.01 mg/kg dose appeared to show even more neuroprotection than the higher doses. While this was not statistically significant in the appropriate analysis by ANOVA, this low dose of tatCN19o was the only one different from tatCN21 by individual t test. Interestingly, the dual role of CaMKII in neuronal survival combined with the specific properties of tatCN19o may provide a biological basis for this observation: tatCN19o is not only tight-binding and extremely potent, but is also an active-kinase inhibitor, as its binding

site on CaMKII is made accessible only upon activation (Chao et al., 2010; Coultrap and Bayer, 2011; Coultrap et al., 2011). Consistent with being an active-kinase inhibitor, our data indicates that tatCN19o specifically targets the pool of CaMKII that is over-activated after ischemia; a lower dose may then reduce inhibition of additional, subsequently Ca^{2+}-stimulated CaMKII pools that can promote neuronal survival.

In conclusion, we have developed an optimized inhibitory peptide that targets excessive CaMKII activity and provides both neuroprotection and long-term functional benefit when administered after cardiac arrest. Our data further identified a mechanism of protection, demonstrating the critical role for autonomous CaMKII activity at hippocampal synapses as the mediator of injury, independent of stimulated CaMKII activity.

EXPERIMENTAL PROCEDURES

Experimental Animals

All experimental protocols were approved by the Institutional Animal Care and Use Committee and conformed to the NIH guidelines for the care and use of animals in research. Male C57BL/6 adult, 8- to 12-week-old, mice (Charles River) and CaMKII T286A mutant mice (Giese et al., 1998) that were back-crossed to C57BL/6 were kindly provided by Dr. Ryohei Yasuda (Max Planck Florida), with permission from Dr. Alcino Silva (UCLA) and Dr. Karl-Peter Giese (University College London). As described previously (Coultrap et al., 2014), the T286A mice were further backcrossed with C57BL/6 and genotyped using the described PCR primers (Giese et al., 1998). Mice had access to food and water ad libitum. Experiments were performed in a blinded manner in accordance with the ARRIVE guidelines, with a separate investigator generating the code.

Cardiac Arrest and Cardiopulmonary Resuscitation Model

Cardiac arrest and cardiopulmonary resuscitation (CA/CPR) was performed as previously described (Grace et al., 2015; Orfila et al., 2014; Shimizu et al., 2016). Briefly, asystolic cardiac arrest was induced by KCl injection via jugular catheter. During cardiac arrest, the pericranial temperature was maintained at 37.5°C ± 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 ~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. See the Supplemental Information for a detailed description of the methods.

H&E Staining

Three days after CA/CPR, brains were removed, post-fixed with paraformaldehyde, and embedded in paraffin. Coronal sections 6 μm thick were serially cut and stained with H&E. Three levels of the hippocampal CA1 region were analyzed (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 three levels per region). The investigator was blinded to treatment before analyzing neuronal damage.

Hippocampal Slice Preparation

Hippocampal slices were prepared 7 days after recovery from CA/CPR. Brains were quickly extracted and placed in ice-cold (2°C–5°C) oxygenated (95% O_2/5% CO_2) artificial cerebral spinal fluid (ACSF) composed of the following (in mmol/L): 125 NaCl, 2.5. KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.0 $CaCl_2$, 1.0 $MgCl_2$, and 12 glucose. Horizontal hippocampal slices (300–350 μm thick) were cut with a Vibratome VT 1000S (Leica) and transferred to a holding chamber containing ACSF for 1.5–2 hr before recording.

Electrophysiology

For synaptically evoked field potentials, hippocampal slices were placed on a temperature-controlled (32°C ± 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 ten times with 200-ms interburst intervals). Following TBS, the fEPSP was recorded for 60 min. 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 Activity Assays

CaMKIIα was prepared from HEK cell extracts, and 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, 50 mM PIPES pH 7.1, 0.1% BSA, 10 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP(~1 mCi/μmol), 75 μM Syntide-2 substrate peptide, and varying concentrations of tatCN19o. Stimulated activity was measured for naive CaMKII in presence of 1 mM $CaCl_2$ and 1 μM CaM; autonomous activity assays contained 1.5 mM EGTA instead. T286 prephosphorylation was done by reacting CaMKII (60–200 nM) in stimulation buffer, but without substrate and ^{32}P, for 5 min on ice. Before activity assays, autophosphorylation was stopped and CaM dissociation was induced by addition of 5 mM EDTA for at least 5 min on ice. CaMKII activity in presence of tatCN19o (0.75, 2.5, 5, or 20 nM) was normalized to activity without inhibitor.

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 Scientific). Samples were homogenized with a pestle and drill

homogenizer. Homogenates were centrifuged for 10 min at 1,000 ×*g*. Supernatant (S1) was collected and transferred to fresh tube and centrifuged for 15 min 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 min at 100,000 × *g*, and the supernatant (S3) that is the cytosolic fraction was precipitated with cold acetone for 12 hr at −20°C and centrifuged for 10 min at 15,000 × *g*, supernatant was removed. Pellet was resuspended with 60 μL Neuronal Extraction Reagent, boiled. Protein concentrations were quantified with Pierce BCA Protein Assay Kit (Thermo Scientific) and then kept at −80°C until used.

Behavioral Tests

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).

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). Mice were transported in white buckets during the training and testing sessions. During training on day 8 after CA/CPR, mice were allowed to habituate the conditioning chamber for two separate 2-min pre-exposure sessions followed by a foot shock (2 s/1.0 mA electric shock) immediately after the second exposure. Following shock, mice were returned to their home cages. Testing occurred 24 hr later, mice were transported in white buckets and placed back into the fear-conditioning chambers. Freezing behavior was measured in 10-s intervals across a 5-min test by a blinded observer and was defined as the absence of movement except for heart beat/respiration.

Statistical Analysis

All data are presented as mean ± SEM. Statistical analysis of all data was determined using the Student's t test for two group comparisons and one-way ANOVA and post-hoc analysis for comparison of multiple groups. Linear regression analysis was performed to compare input-output curves and one-way ANOVA to compare maximal slope values. Differences were considered statistically significant with $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- CaMKII T286A mutation dramatically reduces neuronal injury after cardiac arrest
- CaMKII inhibition is neuroprotective when administered after cardiac arrest
- CaMKII inhibition protects in combination with therapeutic hypothermia
- Very low tatCN19o doses lead to anatomical and functional neuroprotection

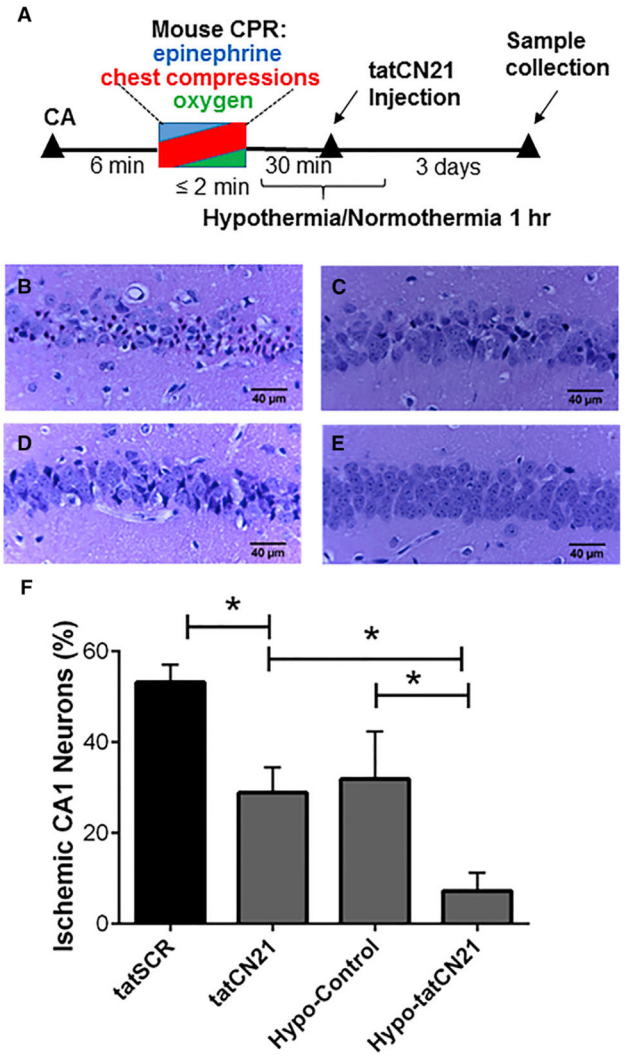

Figure 1. CaMKII Inhibition Reduces Neuronal Injury

(A) Experimental timeline.

(B–E) Representative photomicrographs of hippocampal CA1 neurons from mice injected with 1 mg/kg scrambled control peptide tatSCR (B), 1 mg/kg tatCN21 (C), or hypothermia-treated (D) and hypothermia + tatCN21 treatment (E).

(F) Quantification of ischemic neurons in CA1 region of hippocampus 3 days after CA/CPR. tatSCR injury, 53.2% ± 3.9% (n = 11); tatCN21, 28.9% ± 5.6% (n = 11); hypothermia, 31.9% ± 10.5% (n = 6); and tatCN21 + hypothermia, 6.0% ± 2.7% (n = 6). *$p < 0.05$ compared to tatSCR control; #$p < 0.05$ compared to tatCN21 or hypothermia-treated group. Error bars indicated SEM.

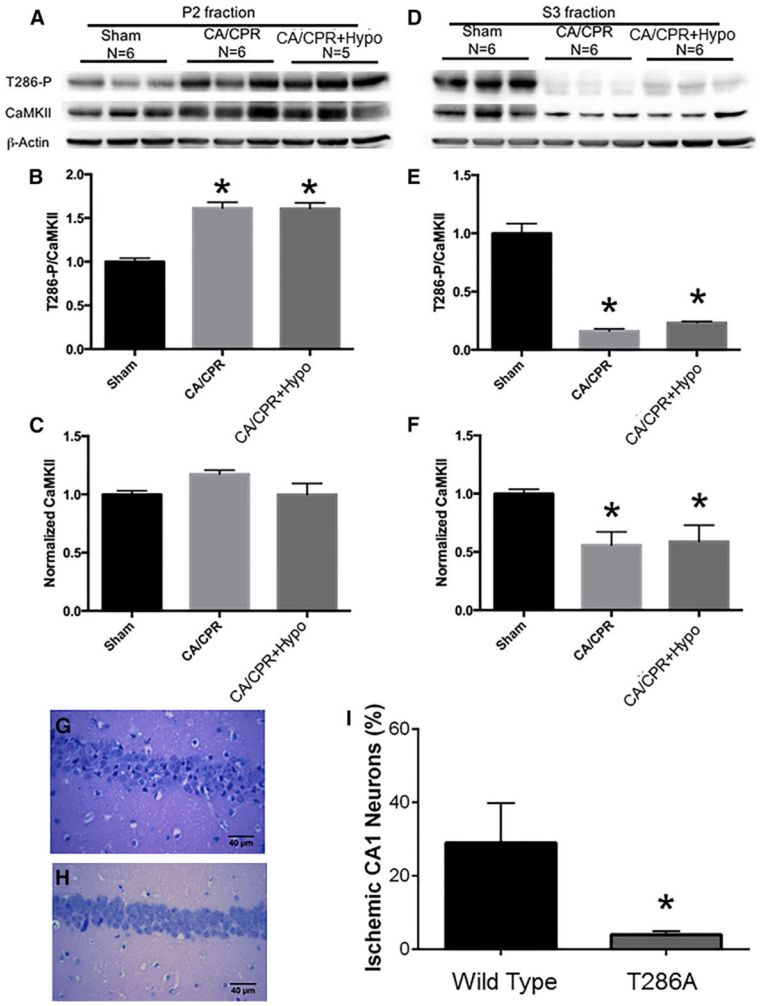

Figure 2. Autonomous CaMKII Activity Contributes to Ischemic Injury

(A–F) CaMKII autophosphorylation at T286, total CaMKII were assessed in synaptosome (P2) membrane fraction (A) and cytosolic (S3) fraction (D) 3 hr following resuscitation by western analysis. Quantification of the ratio of phosphorylated CaMKII to total CaMKII in P2 fraction (B); sham, 1.0 ± 0.04 (n = 5) and CA/CPR, 1.6 ± 0.07 (n = 5). Analysis of S3 fraction (E); sham, 1.0 ± 0.08 and CA/CPR, 0.2 ± 0.02. Total CaMKII expression showed no differences in P2 fractions (C), but a significant decrease after CA/CPR in S3 fraction (F). *$p < 0.05$ compared to sham.

(G and H) Representative photomicrographs of hippocampal CA1 neurons from WT control mice (G) and T286A mutant mice (H).

(I) Quantification of ischemic neurons in CA1 region of hippocampus 3 days after CA/CPR (n = 8). *$p < 0.05$ compared to WT control mice.

Error bars indicate SEM.

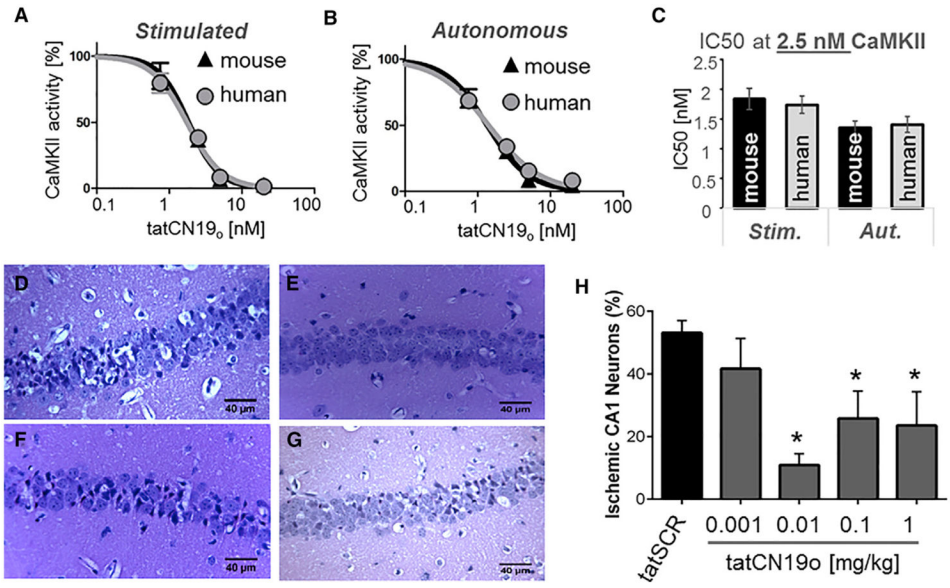

Figure 3. Optimized CaMKII Inhibitory Peptide Is Neuroprotective

(A and B) The optimized CaMKII inhibitor CN19o, fused with tat sequence (tatCN19o) efficiently blocked both stimulated (A) and autonomous (B) activity of both mouse and human CaMKII (n = 3).

(C) Quantification of tatCN19o IC_{50} in assay performed in presence of 2.5 nM CaMKII.

(D–G) Representative photomicrographs of hippocampal CA1 neurons from mice injected with 0.001 mg/kg tatCN19o (D), 0.01 mg/kg tatCN19o (E), 0.1 mg/kg tatCN19o (F), or 1 mg/kg tatCN19o (G) i.v. 30 min after resuscitation and stained with H&E 3 days later.

(H) Quantification of ischemic CA1 neurons 3 days after resuscitation; tatSCR (n = 11), 1 mg/kg = 53.2% ± 3.9% (n = 11); tatCN19o, 0.01 mg/kg = 10.9% ± 3.7% (n = 8); 0.1 mg/kg = 25.8% ± 8.8% (n = 8); and 1 mg/kg = 23.6% ± 10.8% (n = 7). *p < 0.05 compared to tatSCR control.

Error bars indicate SEM.

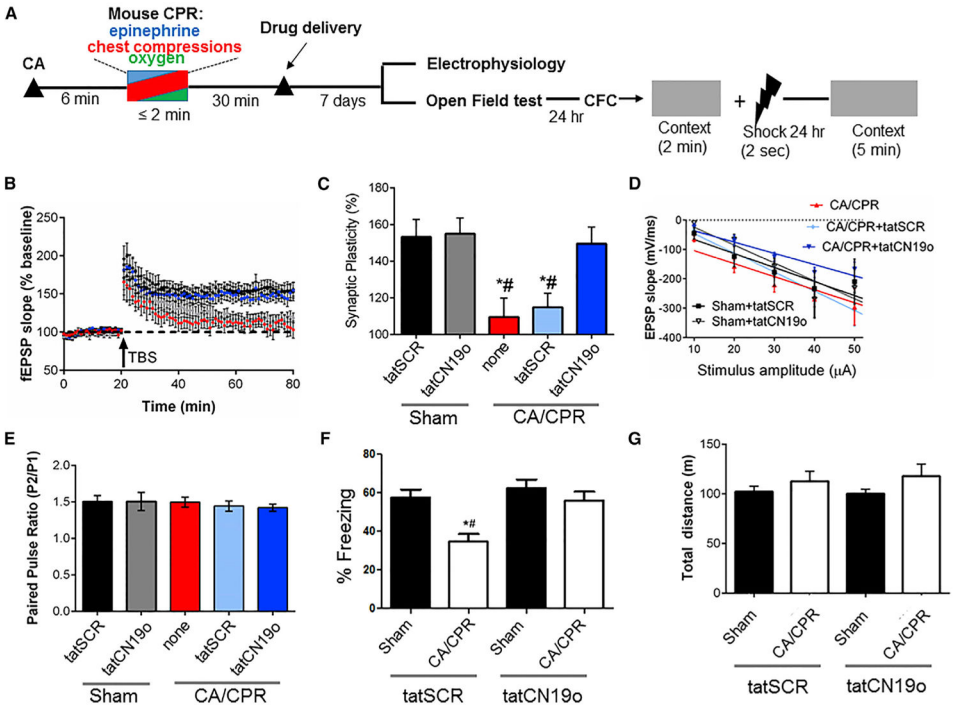

Figure 4. Acute Inhibition of CaMKII Reduces Synaptic Deficits and Improves Memory Function

(A) Experimental timeline.

(B) Time course of fEPSP slope from sham mice (black), mice after CA/CPR only (red), CA/CPR + tatCN19o (blue), and CA/CPR + tatSCR (gray).

(C) Quantification of change in fEPSP slope after 60 min following TBS (40 pulses) normalized to baseline. Sham LTP, 153.3% ± 9.6% of baseline (n = 8); sham + tatCN19o LTP 155.0% ± 8.6% of baseline (n = 6); CA/CPR, 109.5% ± 10.3% (n = 7); CA/CPR + tatSCR, 114.8% ± 7.7% of baseline (n = 7); CA/CPR + 0.01 tatCN19o, 149.6% ± 9.1% (n = 7). *p < 0.05 compared to sham; #p < 0.05 compared to tatCN19o-treated.

(D) Input-output curve showing fEPSP slope plotted against stimulus intensity.

(E) Quantification of paired-pulse ratio, 50-ms inter-stimulus interval.

(F) Quantification of freezing behavior 24 hr after contextual fear conditioning in a novel environment (n = 5–8/group). *p < 0.05 compared to sham; #p < 0.05 compared to tatCN19o-treated.

(G) Quantification of distance traveled in the open field task (n = 5–8/group).

Error bars indicate SEM.