HHS Public Access

Author manuscript

Brain Res. Author manuscript; available in PMC 2022 December 15.

Published in final edited form as:

Brain Res. 2021 December 15; 1773: 147699. doi:10.1016/j.brainres.2021.147699.

CaMKIIα knockout protects from ischemic neuronal cell death after resuscitation from cardiac arrest

Nicole L. Rumian[1,2,‡], **Nicholas E. Chalmers**[3,‡], **Jonathan E. Tullis**[1], **Paco S. Herson**[1,2,3,4,*], **K. Ulrich Bayer**[1,2,*]

[1]Department of Pharmacology, University of Colorado Anschutz Medical Campus, Aurora, CO 80045

[2]Program in Neuroscience, University of Colorado Anschutz Medical Campus, Aurora, CO 80045

[3]Department of Anesthesiology, University of Colorado Anschutz Medical Campus, Aurora, CO 80045

[4]Present address: Department of Neurosurgery, Ohio State University College of Medicine, Columbus, OH 43210

Abstract

CaMKIIα plays a dual role in synaptic plasticity, as it can mediate synaptic changes in opposing directions. We hypothesized that CaMKIIα plays a similar dual role also in neuronal cell death and survival. Indeed, the CaMKII inhibitor tatCN21 is neuroprotective when added during or after excitotoxic/ischemic insults, but was described to cause sensitization when applied long-term prior to such insult. However, when comparing long-term CaMKII inhibition by several different inhibitors in neuronal cultures, we did not detect any sensitization. Likewise, in a mouse *in vivo* model of global cerebral ischemia (cardiac arrest followed by cardiopulmonary resuscitation), complete knockout of the neuronal CaMKIIα isoform did not cause sensitization but instead significant neuroprotection.

Graphical Abstract

*Corresponding author at: Department of Pharmacology, University of Colorado Anschutz Medical Campus, Mail Stop 8303, RC1-North, 12800 East 19th Ave, Aurora, CO 80045, USA. Fax: +1 (303) 724 3663, ulli.bayer@cuanschutz.edu ; paco.herson@osumc.edu.

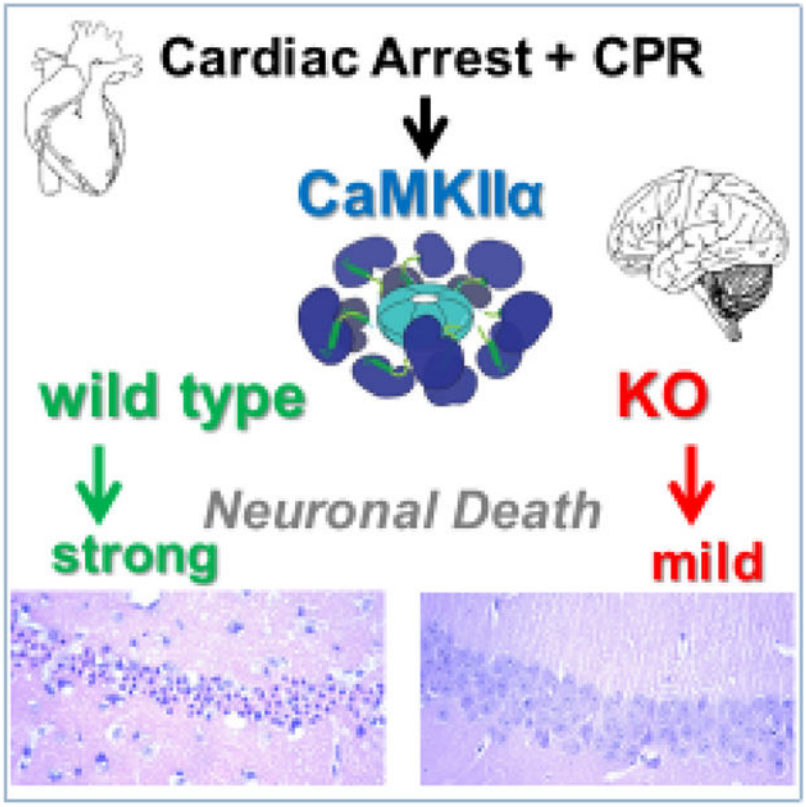
‡These authors contributed equally.

Declaration of Competing Interests

K.U.B. is co-founder and board member of Neurexis Therapeutics, a company that seeks to develop the tatCN19o inhibitor into a therapeutic drug.

Credit author statement:

Conceptualization, N.L.R., N.E.C., J.E.T., P.S.H. and K.U.B.; Investigation, N.L.R., N.E.C., J.E.T.; Writing – Original Draft, K.U.B.; Writing – Review & Editing, N.L.R., N.E.C., J.E.T., P.S.H. and K.U.B.; Visualization, N.L.R., J.E.T., P.S.H., and K.U.B.; Supervision, P.S.H. and K.U.B.; Funding Acquisition, P.S.H., and K.U.B.



Keywords

CaMKII; ischemia; excitotoxicity; cardiac arrest; cardiopulmonary resuscitation; neuronal cell death

1. Introduction

The Ca^{2+}/calmodulin(CaM)-dependent protein kinase II (CaMKII) is a major mediator of higher brain functions such as learning and memory, as well as of the underlying forms of synaptic plasticity, including long-term potentiation and long-term depression (LTP and LTD; for review see Bayer and Schulman, 2019; Coultrap and Bayer, 2012; Hell, 2014; Lisman et al., 2012). However, CaMKII signaling also mediates excitotoxic neuronal cell death after cerebral ischemia (for review see Coultrap et al., 2011). LTP, LTD, and ischemic neuronal cell death all involve CaMKII regulation by autophosphorylation at T286 (which generates Ca^{2+}-independent "autonomous" CaMKII activity; Coultrap et al., 2010; Coultrap et al., 2012; Miller and Kennedy, 1986): T286A mutant mice are impaired for both LTP (Giese et al., 1998) and LTD (Coultrap et al., 2014), and are protected from ischemic neuronal injury (Deng et al., 2017). CaMKII binding to the NMDA-type glutamate receptor subunit GluN2B is required for normal LTP and ischemic cell death, but not for LTD: Mice with a GluN2B mutant that prevents CaMKII binding showed impaired LTP (Halt et al., 2012) and reduced ischemic cell death (Buonarati et al., 2020), but normal LTD (Halt et al., 2012). Importantly, CaMKII inhibition was neuroprotective even when done at

time points significantly after excitotoxic insults in culture (Ashpole and Hudmon, 2011; Vest et al., 2010) or cerebral ischemia *in vivo* (Deng et al., 2017; Vest et al., 2010), thus providing a potential avenue for therapeutic intervention. However, long-term inhibition of CaMKII activity by tatCN21 in cortical cultures or by CaMKIIα knockout *in vivo* prior to ischemic/excitotoxic insults have been described to instead increase neuronal cell death (Ashpole and Hudmon, 2011; Waxham et al., 1996), an effect that we here refer to as sensitization to neuronal cell death. Thus, similar to the dual function of CaMKII in LTP and LTD (for review see Bayer and Schulman, 2019), CaMKII appeared to have a dual role in both neuronal cell death and survival (for review see Coultrap et al., 2011). Indeed, several known CaMKII downstream signaling events could promote cell death, whereas others could promote survival (Coultrap et al., 2011). As this proposed dual role is an interesting concept with potential implications also for therapeutic strategies involving CaMKII inhibition, we decided to investigate this further. Surprisingly, after long-term CaMKII inhibition, we detected only protective but no sensitizing effects: (i) constitutive CaMKIIα knockout protected from the neuronal cell death after global cerebral ischemia (GCI) that was induced by cardiac arrest followed by cardio-pulmonary resuscitation (CA/CPR) in mice, and (ii) in our hands, long-term CaMKII inhibition with tatCN21 or other CaMKII inhibitors had no sensitizing effect in cortical cultures.

2. Results

2.1 CaMKIIα knockout protects from neuronal cell death after CA/CPR

CaMKIIα is the main CaMKII isoform in the mammalian brain (Bayer et al., 1999; Bayer and Schulman, 2019; Cook et al., 2018). In order to determine the effect of CaMKIIα knockout on ischemic neuronal cell death, we used our *in vivo* mouse model that closely mimics the most prevalent GCI condition in humans: cardiac arrest followed by CPR (CA/CPR) (Buonarati et al., 2020; Deng et al., 2017). In this model, mice are treated very similarly as human patients would be treated after cardiac arrest, with CPR consisting of an epinephrin shot, chest compressions, and oxygen supply (Fig. 1A). The resulting ischemic neuronal cell death is most excessive in the hippocampal CA1 region, and was assessed here by H&E staining three days after CA/CPR (Fig. 1B). Compared to their wild type littermates, the CaMKIIα knockout mice showed dramatically reduced cell death (Fig. 1B,C). In fact, cell death in the CaMKIIα knockout mice was so minimal that it was statistically undistinguishable from sham treated knockout mice that did not receive any CA/CPR (Fig. 1C). Thus, constitutive knockout of the CaMKIIα isoform had a strong neuroprotective effect; sensitization to neuronal cell death was not observed.

2.2 Various CaMKII inhibitors fail to sensitize cortical cultures to excitotoxic death

As CaMKIIα knockout was found here to be neuroprotective rather than sensitizing, we hypothesized that the previously described sensitization to excitotoxic neuronal cell death by an 8h pre-treatment with the CaMKII inhibitor tatCN21 may have been due not to CaMKII inhibition but to an off-target effect of tatCN21. Thus, we decided to compare the effect of several different CaMKII inhibitors: tatCN21, tatCN19o, and KN93. The cell penetrating tatCN21 peptide is an active-kinase inhibitor that binds to the T-site of CaMKII (Vest et al., 2007), tatCN19o is an optimized peptide with higher potency and selectivity (Coultrap and

Bayer, 2011; Deng et al., 2017), and KN93 is a small molecule with a different inhibitory mechanism that is competitive with the activation by Ca^{2+}/calmodulin (Sumi et al., 1991; Tokumitsu et al., 1990; Vest et al., 2010). Excitotoxic cell death was induced in cortical cultures (DIV 14) by a 5 min treatment with 20 μM glutamate, and assessed 24 h later by the release of lactate dehygrogenase (LDH) into the culture medium from the dying cells. This treatment was chosen as it causes submaximal cell death (Fig. 2A), thus allowing to detect increases in cell death that would result from sensitization. This stimulation was expected to suffice for CaMKII activation, as increased T286 autophosphorylation has been observed also in response to much weaker chemical LTD stimuli, at least in cultures from hippocampus (Cook et al., 2021). Indeed, an increase in T286 phosphorylation was observed also after our submaximal excitotoxic stimulus in cortical cultures (Fig. 2B,C). As expected, the 8h pre-treatment with either tatCN19o or KN93 did not cause any sensitization (Fig. 3A). If any, there appeared to be a slight reduction of cell death (especially with the higher dose of tatCN19o), although this did not reach statistical significance by ANOVA with Newman-Keuls posthoc analysis (Fig. 3A). However, in contrast to our expectation, tatCN21 did not cause any sensitization either (Fig. 3A).

2.3 No sensitization by the tatCN21 inhibitor at various time point prior to the insult

As our finding of no sensitization by 8h pre-treatment with tatCN21 appeared to be in conflict with a previous study, we decided to test a time course of tatCN21 pre-treatment (Fig. 3B). While we were using similar culture conditions as the previous study, we argued that potential small differences could lead to different timing of the sensitization effect. However, no sensitization was found for any of the timepoints of tatCN21 pre-treatment tested, which ranged 1h to 24 h (Fig. 3B). Thus, our results did not reveal any evidence for any sensitization of neuronal cultures by long-term treatment with any of the CaMKII inhibitors tested, consistent with the lack of sensitization found here also *in vivo* by constitutive knockout of CaMKIIα.

3. Discussion

The results of this study clearly support a role for CaMKII in ischemic neuronal cell death, which enables neuroprotection by CaMKII inhibition. However, they do not support the proposed dual effect also in the opposite function. A dual role of CaMKII in neuronal cell death has been appealing conceptually, and a dual role of CaMKII in opposing functions has been described for LTP versus LTD (Bayer and Schulman, 2019; Cook et al., 2021; Coultrap et al., 2014; Giese et al., 1998) and for LTP versus Aβ-mediated LTP impairment (Cook et al., 2019; Opazo et al., 2018). By contrast, for neuronal cell death versus survival, this study did not find evidence for a role of CaMKII that would cause sensitization to ischemic neuronal cell death by CaMKII inhibition. Lack of sensitization to neuronal cell death may help alleviate a potential hurdle for the use of CaMKII inhibitors in human therapy. However, chronic CaMKII inhibition is still contraindicated by the strong adverse effect on learning and memory that is seen in CaMKIIα knockout mice (Silva et al., 1992a) and even after acute CaMKII inhibition (Buard et al., 2010). Thus, for chronic use, CaMKII inhibitors would still have to be prevented from penetrating the blood brain barrier. Obviously, this would restrict any chronic use of CaMKII inhibitors to therapeutic

indications in the periphery, such as the proposed treatment of heart conditions by inhibition of the CaMKIIγ and δ isoforms that are not restricted to the brain (Bayer et al., 1999; Pellicena and Schulman, 2014; Schulman and Anderson, 2010; Zhang et al., 2005). In the central nervous system, therapeutic CaMKII inhibition would have to be restricted to acute therapy. This likely precludes CaMKII inhibition from therapeutic use in Alzheimers disease: While CaMKII inhibition can prevent some of the mechanisms by which Aβ impairs LTP (Cook et al., 2019; Opazo et al., 2018), continued CaMKII inhibition would instead itself directly impair LTP and thereby learning (Buard et al., 2010; Malinow et al., 1989). However, as the learning and memory deficits that are caused by CaMKII inhibition are quickly reversible (within 24h or less) (Buard et al., 2010), it could still be used for the acute therapy that was found to be effective in mouse models of global cerebral ischemia and stroke (Deng et al., 2017; Vest et al., 2010).

While our studies did not indicate any sensitization to ischemic cell death by CaMKII inhibition, they cannot completely rule out a role of CaMKII also in neuronal cell survival. For instance, it is possible that under our conditions, the protective effects of CaMKII inhibition outweighed any opposing effects. Indeed, while CaMKIIα knockout was found here to protect from ischemic damage (consistent with prior findings after more subtle mutations of CaMKII signaling)(Buonarati et al., 2020; Deng et al., 2017), a combined double knockout that additionally included CaMKIIβ (the second most prevalent isoform in the brain)(Bayer et al., 1999; Cook et al., 2018) directly results in mortality during early postnatal development, even without any ischemic insults (Kool et al., 2019). Thus, CaMKII indeed appears to have a dual role in death and survival, even if this additional role did not manifest itself here in any detectable sensitization to ischemia. However, while the effect has likely neuronal origins (as at least CaMKIIα is exclusively expressed in neurons), it is not clear if this neuronal origin of mortality is neuronal cell death or another critical functional impairment. Notably, individual CaMKIIα knockout has been described previously to sensitize to ischemic damage in a mouse model of stroke (Waxham et al., 1996). This apparent discrepancy to the findings of this study could be due to major mechanistic differences in the cell death after stroke versus GCI. However, acute CaMKII inhibition was neuroprotective in the same stroke and GCI models (Deng et al., 2017; Vest et al., 2010). Thus, a more likely alternative explanation could be differences between the different lines of CaMKIIα knockout mice that were used in the studies. For instance, our CaMKIIα knockout mouse line showed slightly increased expression of the CaMKIIβ isoform (Coultrap et al., 2014), a potentially compensatory effect that was not observed for the original CaMKIIα knockout mice (Silva et al., 1992b).

In summary, while our study cannot rule out a role for CaMKII in neuronal survival, it confirms that such a function is not selectively linked to the α isoform and indicates that it does not pose a concern for acute treatment of ischemic damage with CaMKII inhibitors.

4. Materials and Methods

4.1 Experimental Animals

All animal treatments in this study were approved by the Institutional Animal Care and Use Committee of the University of Colorado Anschutz Medical Campus (CU AMC; Aurora,

CO), in accordance with NIH guidelines. Animals were housed in ventilated cages on a 12 h light/ 12 h dark cycle and were provided ad libitum access to food and water. The strains of CaMKIIα knockout mice (Coultrap et al., 2014) and T286A mutant mice (Giese et al., 1998) used here have been described previously. Mouse breeding was done in heterozygous pairs on a C57BL/6 background for use of littermates in CA/CPR experiments. For neuronal cultures from pups, pregnant Sprague-Dawley rats were supplied by Charles River Labs.

4.2 GCI induced by CA/CPR in mice

GCI induction in mice and assessment of the resulting neuronal cell death three days later was done essentially as we have described previously (Buonarati et al., 2020; Deng et al., 2017; Orfila et al., 2014). Briefly, asystolic cardiac arrest was induced by KCl injection via jugular catheter. After 6 mins, CPR was initiated by injecting 0.5-1 mL epinephrine (16 mg/mL in saline solution), chest compressions at 300/min, and ventilation with 100% O2. For animals that did not regain spontaneous circulation within 2 min of CPR, resuscitation was stopped and the animal was excluded from the study. Mortality was 2 of 5 mice in the CaMKIIα knockouts, and 1 of 6 animals in their wild type littermates. Brain tissue was collected 72 h after CA/CPR for use in hematoxylin and eosin (H&E) staining for cell death assessment. The tissue was then post-fixed with paraformaldehyde and embedded in paraffin. Serially cut coronal sections 6 uM thick were stained with H&E and three levels of the hippocampal CA1 region were analyzed (100 uM apart) starting at 1.5 mm bregma. Neurons with hypereosiniophilic cytoplasm and pyknotic nuclei were deemed nonviable. The percentage of nonviable neurons was calculated for hippocampus in both hemispheres (average of three levels for each animal). Littermates from heterozygous breeding were used, with the experimenter blinded of the genotype.

4.3 Primary cortical cultures

Primary cortical cultures were prepared from P0-P1 neonatal Sprague-Dawley rat pups of both sexes, similarly as described previously (Ashpole and Hudmon, 2011; Buonarati et al., 2020). After decapitation and dissection, cortical tissue was incubated in dissociation solution (7 mL HBSS buffered saline, 150 μL 100 mM $CaCl_2$, 10 μL 1 M NaOH, 10 μL 500 mM EDTA, 200 units Papain [Worthington]) at 25°C for 1 h. The tissue was washed 5X in plating media (DMEM, 1-% FBS, 50 units/mL pen/strep, 2 mM L-glutamine, filter sterilized). After dissociation by trituration, the cells were counted using a hemocytometer. Neurons were plated on poly-D-lysine (0.1 mg/mL in 1 M Borate Buffer: 3.1 g boric acid, 4.75 g Borax, in 1 L deionized H2O)-coated 96 well plates (for LDH assays) or 6 well plates (for biochemistry) at a density of 75,000-100,000 or 350,000-400,000 cells per well, respectively, in plating media and maintained at 37°C with 5% CO2. After 1 day *in vitro* (DIV), media was switched to 100% feeding media (Neurobasal-A, B27 supplements, and 2 mM L-gluta-mine, filter sterilized). At DIV 4, neurons were treated with a FDU (70 μM 5-fluoro-2'-deoxyuridine/140 μM uridine) to halt mitosis and suppress glial growth.

4.5 Excitotoxic cell death in neuronal cultures

DIV 14 cortical neurons were pre-treated with tatCN21 (5 μM), tatCN19o (5 or 0.2 μM), or KN93 (5 μM), or water control; pre-treatment occurred 24, 8, 5, 2.5, or 1 h before the glutamate insult (20 or 500 μM glutamate for 5 min, or 200 μM glutamate for 60

min), as indicated. Prior to the insult, half of the media was removed (and saved for later use as conditioned media) from each well and replenished with fresh feeding media. After the insult, media was exchanged with a 1:1 mix of fresh and conditioned media and neurons were returned to 37°C and 5% CO2 for 20-24 h. Cell death was then assessed as described previously (Vest et al., 2010) by measuring the lactate dehydrogenase (LDH) that leaked from cells into the media using a CyQUANT LDH Cytotoxicity Assay Kit (Thermo Scientific). The kit was used as directed by the manuracturer and cytotoxicity results were calculated as a percentage of control values.

4.6 Western-analysis of CaMKII phosphorylation at T286

For Western-analysis, DIV 14 cortical neurons were switched from media to artificial cerebral spinal fluid (ACSF) for 10 mins before wells were treated with a water control or glutamate (20 μM or 500 μM glutamate for 1 min). Cells were then harvested in buffer containing 1% SDS, 1 mM EDTA, and 10 mM Tris pH 8 and boiled for 10 min. Protein content was determined using the Pierce BCA protein assay (Thermo-Fisher). Western-analysis was performed essentially as described previously (Cook et al., 2021). Briefly, 4 to 10 μg of total protein was separated on SDS-PAGE on 10% polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membrane for 1–2 h at 4°C. Membranes were blocked in 5% milk for 1 h and first incubated with anti-CaMKIIα 1:5000 (CBα2, available at Invitrogen, but made in house) and later, after stripping and re-blocking, anti-pT286 1:1000 (PhosphoSolutions). Primary incubation was followed by Amersham ECL anti-mouse or anti-rabbit IgG, horseradish peroxidase-linked secondary 1:5000 (GE Healthcare). Blots were developed using chemiluminescence (Super Signal West Femto, Thermo-Fisher), imaged using the Chemi-Imager 4400 system (Alpha-Innotech), and analyzed by densitometry (ImageJ).

Acknowledgements

This work was supported by National Institutes of Health grants F31AG069458, T32AG000279 (supporting N.L.R.), T32GM007635 (supporting J.E.T.), R01NS081248 (to K.U.B.) and R01NS118786 (to K.U.B. and P.S.H.).

References

Ashpole NM, Hudmon A, 2011. Excitotoxic neuroprotection and vulnerability with CaMKII inhibition. Mol Cell Neurosci. 46, 720–30. [PubMed: 21316454]

Bayer KU, et al. , 1999. Developmental expression of the CaM kinase II isoforms: ubiquitous gamma- and delta-CaM kinase II are the early isoforms and most abundant in the developing nervous system. Brain Res Mol Brain Res. 70, 147–54. [PubMed: 10381553]

Bayer KU, Schulman H, 2019. CaM kinase: Still intriguing at 40. Neuron. 103, 380–394. [PubMed: 31394063]

Buard I, et al. , 2010. CaMKII “autonomy” is required for initiating but not for maintaining neuronal long-term information storage. J Neurosci. 30, 8214–20. [PubMed: 20554872]

Buonarati OR, et al. , 2020. CaMKII versus DAPK1 binding to GluN2B in ischemic neuronal cell death after resuscitation from cardiac arrest. Cell Rep. 30, 1–8. [PubMed: 31914378]

Cook SG, et al. , 2018. Analysis of the CaMKIIalpha and beta splice-variant distribution among brain regions reveals isoform-specific differences in holoenzyme formation. Sci Rep. 8, 5448. [PubMed: 29615706]

Cook SG, et al. , 2019. Simultaneous Live Imaging of Multiple Endogenous Proteins Reveals a Mechanism for Alzheimer's-Related Plasticity Impairment. Cell Rep. 27, 658–665 e4 (as cover article). [PubMed: 30995464]

Cook SG, et al. , 2021. CaMII holoenzyme mechanisms that govern the LTP versus LTD decision. Science Advances. 7, eabe2300. [PubMed: 33853773]

Coultrap SJ, et al. , 2010. CaMKII autonomy is substrate-dependent and further stimulated by Ca2+/calmodulin. J Biol Chem. 285, 17930–7. [PubMed: 20353941]

Coultrap SJ, Bayer KU, 2011. Improving a natural CaMKII inhibitor by random and rational design. PLoS One. 6, e25245. [PubMed: 21984908]

Coultrap SJ, et al. , 2011. CaMKII in cerebral ischemia. Acta Pharmacol Sin. 32, 861–72. [PubMed: 21685929]

Coultrap SJ, Barcomb K, Bayer KU, 2012. A significant but rather mild contribution of T286 autophosphorylation to Ca2+/CaM-stimulated CaMKII activity. PLoS One. 7, e37176. [PubMed: 22615928]

Coultrap SJ, Bayer KU, 2012. CaMKII regulation in information processing and storage. Trends Neurosci. 35, 607–618. [PubMed: 22717267]

Coultrap SJ, et al. , 2014. Autonomous CaMKII mediates both LTP and LTD using a mechanism for differential substrate site selection. Cell Reports. 6, 431–437. [PubMed: 24485660]

Deng G, et al. , 2017. Autonomous CaMKII Activity as a Drug Target for Histological and Functional Neuroprotection after Resuscitation from Cardiac Arrest. Cell Rep. 18, 1109–1117. [PubMed: 28147268]

Giese KP, et al. , 1998. Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. Science. 279, 870–3. [PubMed: 9452388]

Halt AR, et al. , 2012. CaMKII binding to GluN2B is critical during memory consolidation. EMBO J. 31, 1203–16. [PubMed: 22234183]

Hell JW, 2014. CaMKII: claiming center stage in postsynaptic function and organization. Neuron. 81,249–65. [PubMed: 24462093]

Kool MJ, et al. , 2019. CAMK2-Dependent Signaling in Neurons Is Essential for Survival. J Neurosci. 39, 5424–5439. [PubMed: 31064859]

Lisman J, Yasuda R, Raghavachari S, 2012. Mechanisms of CaMKII action in longterm potentiation. Nat Rev Neurosci. 13, 169–82. [PubMed: 22334212]

Malinow R, Schulman H, Tsien RW, 1989. Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. Science. 245, 862–6. [PubMed: 2549638]

Miller SG, Kennedy MB, 1986. Regulation of brain type II Ca2+/calmodulin-dependent protein kinase by autophosphorylation: a Ca2+-triggered molecular switch. Cell. 44, 861–70. [PubMed: 3006921]

Opazo P, et al. , 2018. CaMKII Metaplasticity Drives Abeta Oligomer-Mediated Synaptotoxicity. Cell Rep. 23, 3137–3145. [PubMed: 29898386]

Orfila JE, et al. , 2014. Increasing small conductance Ca2+-activated potassium channel activity reverses ischemia-induced impairment of long-term potentiation. Eur J Neurosci. 40, 3179–88. [PubMed: 25080203]

Pellicena P, Schulman H, 2014. CaMKII inhibitors: from research tools to therapeutic agents. Front Pharmacol. 5, 21. [PubMed: 24600394]

Schulman H, Anderson ME, 2010. Ca/Calmodulin-dependent Protein Kinase II in Heart Failure. Drug Discov Today Dis Mech. 7, e117–e122. [PubMed: 21503275]

Silva AJ, et al. , 1992a. Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. Science. 257, 206–11. [PubMed: 1321493]

Silva AJ, et al. , 1992b. Deficient hippocampal long-term potentiation in a-calcium-calmodulin kinase II mutant mice. Science. 257, 201–6. [PubMed: 1378648]

Sumi M, et al. , 1991. The newly synthesized selective Ca2+/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. Biochem Biophys Res Commun. 181, 968–75. [PubMed: 1662507]

Tokumitsu H, et al. , 1990. KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazi ne, a specific inhibitor of Ca2+/calmodulin-dependent protein kinase II. J Biol Chem. 265, 4315–20. [PubMed: 2155222]

Vest RS, et al. , 2007. Dual Mechanism of a Natural CaMKII Inhibitor. Mol Biol Cell. 18, 5024–33. [PubMed: 17942605]

Vest RS, et al. , 2010. Effective post-insult neuroprotection by a novel Ca(2+)/calmodulin-dependent protein kinase II (CaMKII) inhibitor. J Biol Chem. 285, 20675–82. [PubMed: 20424167]

Waxham MN, et al. , 1996. Ischemia-induced neuronal damage: a role for calcium/calmodulin-dependent protein kinase II. J Cereb Blood Flow Metab. 16, 1–6. [PubMed: 8530541]

Zhang R, et al. , 2005. Calmodulin kinase II inhibition protects against structural heart disease. Nat Med. 11, 409–17. [PubMed: 15793582]

Highlights:

- CaMKIIα knockout protected from ischemic neuronal death after cardiac arrest
- 5 min 20 μM glutamate induced submaximal excitotoxic death in cortical cultures
- The submaximal stimulus increased CaMKII activation in cortical cultures
- No sensitization to cell death was observed even by long-term CaMKII inhibition

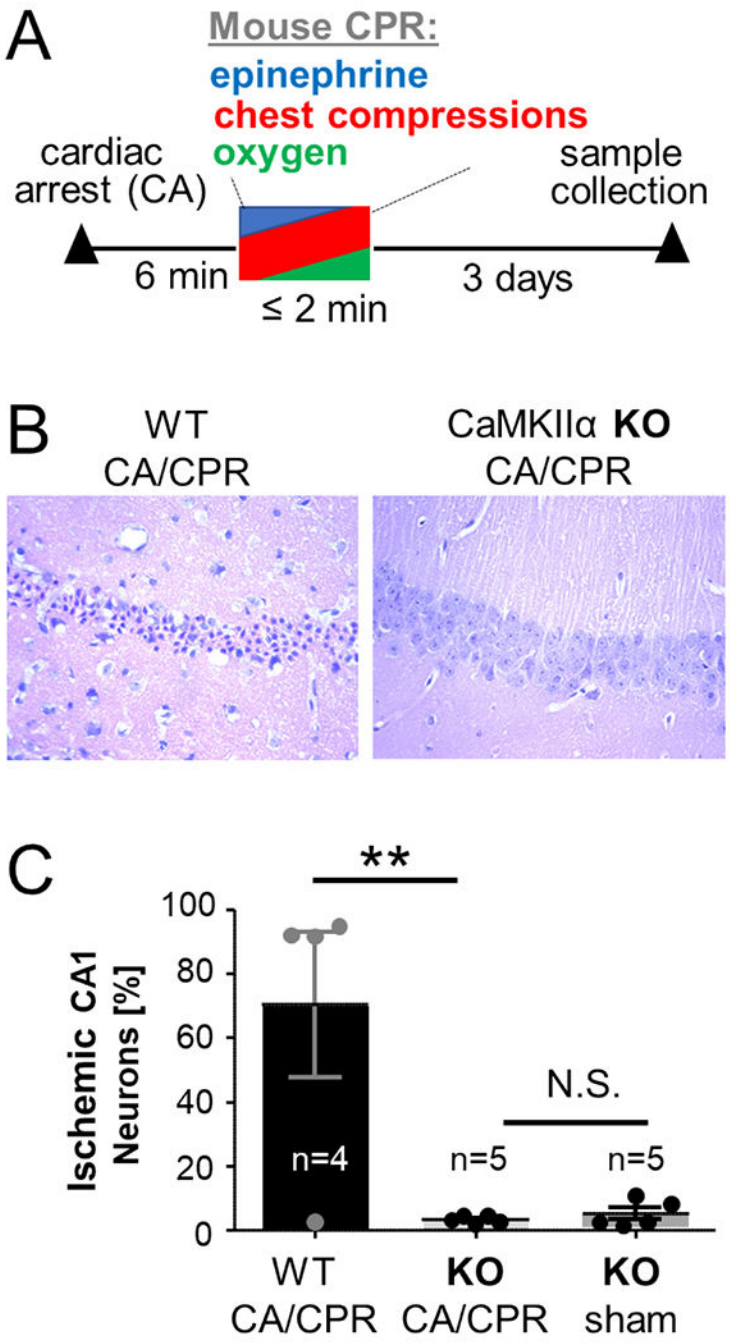


Fig. 1. CaMKIIα knockout protects from neuronal cell death after CA/CPR *in vivo*.

(A) Experimental timeline for the CA/CPR model of global cerebral ischemia.

(B) Representative images of H&E stained hippocampal CA1 region, to visualize cell death.

(C) Quantification of neuronal cell death in the CA1 region after CA/CPR. CaMKIIα KO mice showed significantly less cell death as compared to WT (**: $p<0.01$, ANOVA with Newman-Keuls posthoc analysis; N.S.: not significant).

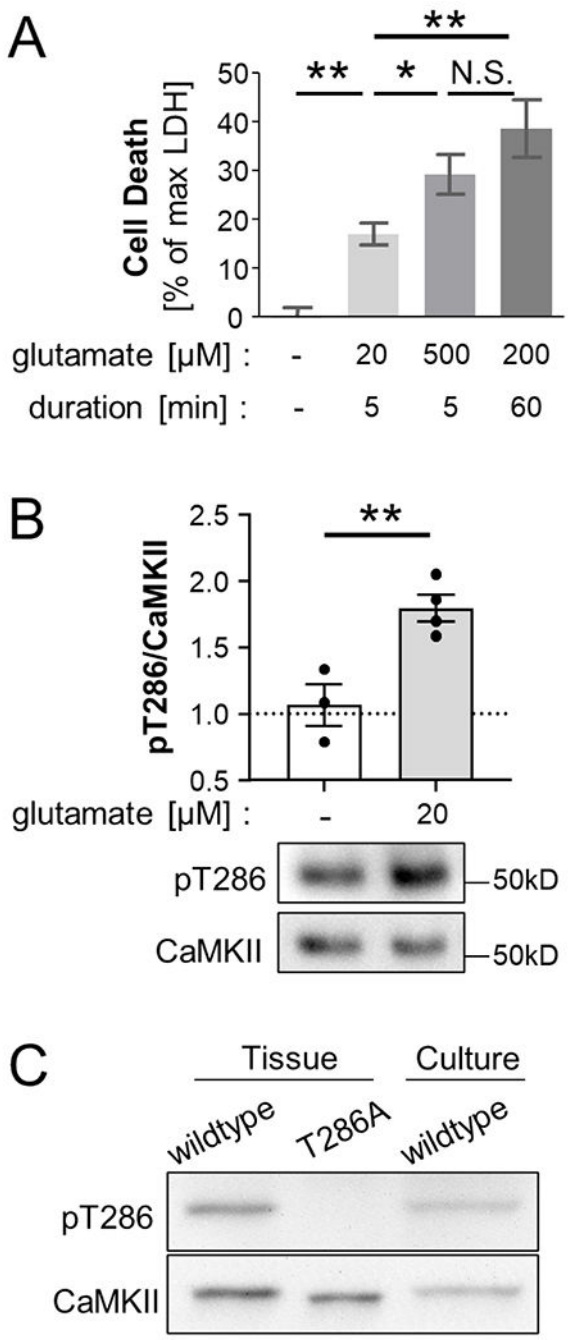


Fig. 2. Treating cortical cultures for 5 min with 20 μM glutamate causes submaximal cell death and is sufficient to activate CaMKII.

Cortical neurons were treated with glutamate as indicated.

(A) A 5 min treatment with 20 μM glutamate induces significant but submaximal neuronal cell death (n=20), as cell death was significantly further increased by increasing the dose to 500 μM glutamate (n=20) and by a 60 min treatment with 200 μM glutamate (n=8), a condition previously shown to elicit maximal cell death in cortical cultures (Ashpole and Hudmon, 2011). Cell death was measured by LDH release into the culture medium. N.S.: $p>0.05$; *: $p<0.05$; **: $p<0.01$ in ANOVA with Newman-Keuls posthoc analysis.

(B) A 5 min treatment with 20 μM glutamate is sufficient to stimulate CaMKII activity in cortical neurons, as indicated by an increase in T286 autophosphorylation (pT286) detected by Western-analysis; **: $p<0.01$ in unpaired two-tailed t-test.
(C) Basal T286 phosphorylation was detected in both cortical tissue and cultures; this detection was abolished in tissue from T286A mutant mice.

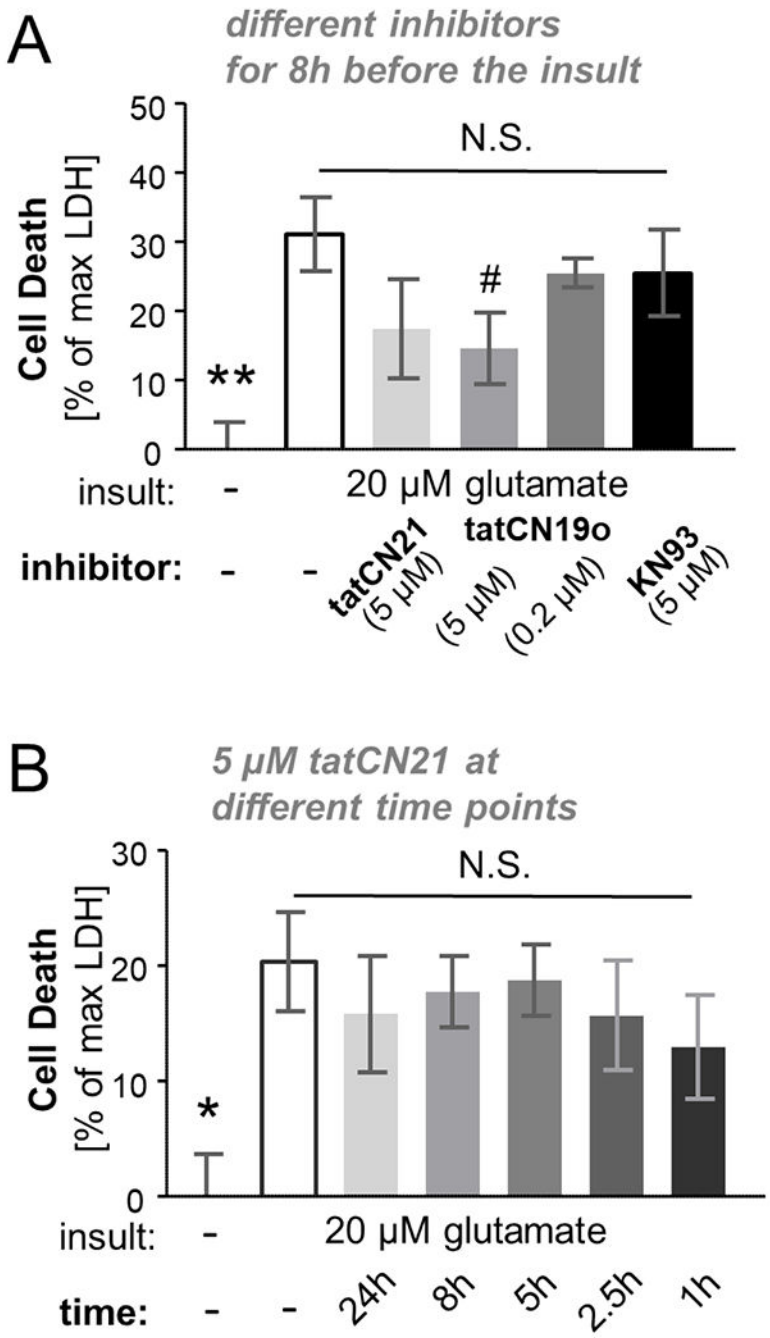


Fig. 3. No sensitization of cortical cultures detected after CaMKII inhibition.

Cortical neurons were pre-treated with tatCN21, tatCN19o, or KN93 at various timepoints prior to glutamate insult. N.S.: $p>0.05$; *: $p<0.05$; **: $p<0.01$ compared to a 5 min 20 μM glutamate treatment without inhibitor in ANOVA with Newman-Keuls posthoc analysis (n=9).

(A) Pre-treatment with the various CaMKII inhibitors 8 h prior to glutamate insult did not lead to sensitization to cell death. If any, pre-treatment with tatCN19o (5 μM) appeared to reduce glutamate-induced cell death (#: $p<0.05$ for posthoc analysis by Bonferroni single

pair of columns comparison instead of the Newman-Keuls posthoc analysis that did not indicate significance).

(B) No sensitization to cell death by tatCN21 was observed also when a wider range of pre-treatment times was tested (1 h to 24 h prior to glutamate).