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CaMKII inhibitor 1 (CaMK2N1) mRNA is upregulated following LTP induction in hippocampal slices

Daniela Astudillo[a], **Daniel Karmelic**[a], **Barbara S. Casas**[b], **Nikolai Otmakhov**[c], **Veronica Palma**[b], **Magdalena Sanhueza**[a]

[a]Cell Physiology Center, Department of Biology, Faculty of Sciences, Universidad de Chile, Santiago, Chile.

[b]Laboratory of Stem Cells and Developmental Biology, Department of Biology, Faculty of Sciences, Universidad de Chile, Santiago, Chile.

[c]Brandeis University, Waltham, MA, USA

Abstract

CaMK2N1 and CaMK2N2 (also known as CaMKIINα and β) are endogenous inhibitors of calcium/calmodulin-dependent kinase II (CaMKII), an enzyme critical for memory and long-term potentiation (LTP), a form of synaptic plasticity thought to underlie learning. CaMK2N1/2 mRNAs are rapidly and differentially upregulated in the hippocampus and amygdala after acquisition or r*et*rieval of fear memory. Moreover, CaMK2N2 protein levels increase after contextual fear conditioning. Therefore, it was proposed that CaMK2N1/2 genes (*Camk2n1/2)* could be immediate-early genes (IEG) transcribed promptly (30–60 min) after training. As a first approach to explore a role in synaptic plasticity, we assessed a possible regulation of *Camk2n1/2* during the expression phase of LTP in hippocampal CA3-CA1 connections in rat brain slices. Quantitative PCR revealed that *Camk2n1,* but not *Camk2n2*, is upregulated 60 min after LTP induction by Schaffer collaterals high-frequency stimulation. We observed a graded, significant positive correlation between the magnitude of LTP and *Camk2n1* change in individual slices, suggesting a coordinated regulation of these properties. If mRNA increment actually resulted in the protein upregulation in plasticity-relevant subcellular locations, CaMK2N1 may be involved in CaMKII fine-tuning during LTP maintenance or in the regulation of subsequent plasticity events (metaplasticity).

Keywords

CaMKII; CaMK2N1; CaMK2N2; LTP; gene expression

Corresponding author: Dr. Magdalena Sanhueza, Department of Biology, Faculty of Sciences. Universidad de Chile, Las Palmeras 3425, Nunoa, 7800003 Santiago, Chile, Phone: 56-2-2978-7344, masanhue@uchile.cl.

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Data associated with the manuscript will be available upon request

LTP is a form of synaptic plasticity characterized by a long-lasting increase in synaptic strength after a brief, high-frequency stimulation (HFS) of afferent pathways. The mechanisms of LTP are proposed to be similar, at least in part, to those underlying short and long-term memory (Whitlock *et al.*, 2006; Nabavi *et al.*, 2014). The role of CaMKII in LTP has been extensively studied in CA3-CA1 synapses of the rodent hippocampus (reviewed in Lucchesi *et al.*, 2011; Lisman *et al.*, 2012; Hell, 2014; Herring and Nicoll, 2016), where a N-methyl-D-aspartate glutamate receptor (NMDAR)-dependent LTP is expressed. LTP induction is triggered by calcium entry through postsynaptic NMDARs, leading to CaMKII activation in dendritic spines (Lee *et al.*, 2009). The activated enzyme can undergo autophosphorylation at T286, switching the kinase to a partially calcium-independent or "autonomous" state of activity (Hanson *et al.*, 1989; Lisman & Goldring, 1988; Miller & Kennedy, 1986; Coultrap and Bayer, 2012). Upon activation, the kinase can translocate to the stimulated synapses (Shen and Meyer, 1999; Otmakhov *et al.*, 2004), where it binds to NMDAR and other postsynaptic density (PSD) proteins (Coultrap and Bayer, 2012; Hell, 2014), enhancing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptor (AMPAR)-mediated transmission (Barria and Malinow, 2005; Herring and Nicoll, 2016; Incontro *et al.*, 2018) and spine size (Nakahata and Yasuda, 2018). Notably, CaMKII binding to the GluN2B subunit of the NMDAR also locks the kinase in an active state (Bayer *et al.*, 2001). Pharmacological or genetic manipulations that prevent CaMKII activation or autophosphorylation cause a deficit in LTP induction and severe learning defects (reviewed in Lucchesi *et al.*, 2011; Lisman *et al.*, 2012; Herring and Nicoll, 2016). Moreover, inhibition of CaMKII-GluN2B binding also causes LTP and memory impairment (Barria and Malinow, 2005; Halt *et al.*, 2012; Incontro *et al.*, 2018). CaMKII is one of the most abundant proteins in neurons, specifically in synapses, where it regulates numerous processes (Coultrap and Bayer, 2012; Hell, 2014). Thus, it is expected that the kinase activity and localization should be tightly regulated. This regulation could involve changes in the expression of two endogenous inhibitors: CaMK2N1 and CaMK2N2. According to *in vitro* studies, these proteins inhibit both calcium-dependent and -independent CaMKII activity over exogenous substrates but are not efficient at inhibiting the kinase's autophosphorylation of T286, which is critical for maintaining its autonomous activity and synaptic targeting (Chang *et al.*, 2001; Vest *et al.*, 2007; Hell, 2014). Early studies on cultured pyramidal hippocampal neurons showed that, at basal conditions, these inhibitors are only present in the cell bodies and dendrites of (Chang *et al.*, 2001). Later work, however, detected CaMK2N1 in hippocampal PSD and synaptosome fractions (Saha *et al.*, 2007). Moreover, shorter CaMK2N1 derivative peptides inhibit CaMKII-GluN2B binding both *in vitro* and in slices (Vest *et al.*, 2007; Sanhueza *et al.*, 2011; Barcomb *et al.*, 2016), which could influence CaMKII synaptic targeting.

Behavioral reports indicated that *Camk2n1,* but not *Camk2n2,* is transiently upregulated in the mouse hippocampus and amygdala 30 min after animals were trained in an auditory fear-conditioning paradigm (Lepicard *et al.*, 2006). Moreover, a later study showed that CaMK2N2 protein is increased in the hippocampus and amygdala for a few hours after contextual fear conditioning in mice (Radwańska et al., 2010), with a similar kinetics to that observed for *Camk2n1* (Lepicard *et al.*, 2006). Additionally, a recent study showed that 30 min after retrieval of a contextual fear memory, *Camk2n1* is upregulated and CaMK2N1

knockdown causes an impairment in contextual fear memory maintenance after retrieval (Vigil *et al.*, 2017). Therefore, the question arises whether changes in gene expression of the inhibitors also take place following LTP induction. To assess this possibility, we performed quantitative PCR (qPCR) to study *Camk2n1/2* expression after inducing LTP in the Schaffer collaterals-CA1 pyramidal cells synapses in single, transversal hippocampal slices of rats. For this, P18–22 male Sprague-Dawley rats were used; all animal procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethical Committee of the Faculty of Sciences of the University of Chile. Slices were prepared and maintained as in Sanhueza et al. (2011), but CA3 region was not removed. Slices were transferred to a recording chamber with oxygenated artificial cerebrospinal fluid containing (in mM): 125 NaCl, 26 $NaHCO_3$, 2.6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1 NaH_2PO_4*H2O, 10 D-glucose. A 15 min stable field excitatory postsynaptic potentials (fEPSP) baseline was recorded before LTP induction by HFS (Fig. 1A) and synaptic transmission was further monitored for 60 min (percent LTP: 58% ± 4.6%, mean ± SEM; relative to baseline, calculated for the last 20 min). For each slice in which LTP was induced, an adjacent slice or the contralateral slice at the same longitudinal position was used as a control. In control slices, basal transmission was recorded but HFS was not applied.

60 min after LTP induction or control recording, the entire hippocampal slices were processed for qPCR experiments to examine CaMK2N1 and CaMK2N2 gene expression. Specific primers pairs that span at least one exon-exon junction were designed (Primer-BLAST) to determine the expression levels of *Camk2n1* (forward: 5'-GCAAGCGCGTTGTTATTGAAG-3', reverse: 5'-TTCCAAAGTGCTTTCTCCTCCT-3'), *Camk2n2* (forward: 5'-CGCCATGTCCGAGATCCTAC-3', reverse: 5'-TCGATCACCACTCTCTTGGC-3') and *Gapdh* (forward: 5'-CTCTCTGCTCCTCCCTGTTC-3', reverse: 5'-CGATACGGCCAAATCCGTTC-3') for a control. qPCR was performed on the Stratagene Mx3000P System (Agilent Technologies) using 2X Brilliant II SYBR® Green Master mix according to the manufacturer instruction. For each determination, samples were run in duplicates and the appropriate final primer concentration was determined empirically.

The experiments revealed an upregulation of *Camk2n1* expression after LTP induction, in all potentiated slices analyzed (Fig. 1B). Percent *Camk2n1* expression in LTP slices relative to control slices revealed a 2.03 ± 0.28-fold increase in gene expression. In contrast, *Camk2n2* expression did not change (Fig. 1C). These results are consistent with those previously reported by Lepicard *et al.* (2006) and Vigil *et al.* (2017), who observed an increase in hippocampal *Camk2n1*, but not *Camk2n2*, 30 and 60 min after fear-conditioned learning or memory retrieval, respectively.

Next, we quantified the relationship between the increase of *Camk2n1* levels and the magnitude of the LTP induced in each slice (Fig. 2). Spearman's coefficient analysis between the two variables revealed a significant positive correlation (r=0.943, P=0.018). This result indicates that changes in *Camk2n1* expression induced by synaptic activity are triggered in a graded manner, following a monotonic direct relationship with the percent fEPSP increase induced by the HFS.

It is worth noting that the increment in CaMK2N1 mRNA that we measured after LTP induction does not necessarily imply an upregulation of the inhibitor protein. Therefore, from our results it is not possible to assert that CaMK2N1 protein is being upregulated after LTP induction. This possibility, however, is consistent with the observation that CaMK2N2 protein levels rapidly raised after a different fear conditioning protocol (Radswanska *et al.,* 2010) and this increase was specifically observed in the brain regions involved in this type of memory (hippocampus and amygdala). Furthermore, for several LTP-related genes, a correlation between changes in mRNA and protein levels has been reported (for an early work, see Abraham 1991). In the case of *Camk2n1,* however, this remains to be investigated. However, a critical point for the inhibitor to be effective in regulating CaMKII-dependent plasticity, is that its expression should co-localize with plasticity-relevant pools of active CaMKII. Considering the evidence on learning-related CaMK2N mRNA or protein changes and additional *in vitro* and slices data, we can speculate about possible mechanisms by which eventual increases in CaMK2N1 during LTP might participate in synaptic memory. CaMKII is a ubiquitous kinase in the brain and is abundantly expressed at synapses, where it binds to and phosphorylates a wide variety of substrates (Hanson et al., 1998; Lucchesi *et al.*, 2011; Lisman *et al.*, 2012; Hell, 2014; Herring and Nicoll, 2016). Several CaMKII synaptic targets (as GluN2B, densin-180 and SAP97) bind to a specific site in the kinase catalytic domain called the ‘T-site’ (Bayer *et al.*, 2001; Nikandrova *et al.*, 2010; Jiao *et al.*, 2011), which is also a binding site for CaMK2N (Vest *et al.*, 2007). Therefore, these are not only specific inhibitors of CaMKII enzymatic activity but may also interfere with kinase synaptic targeting (Vest *et al.*, 2007; Sanhueza *et al.*, 2011). It is, however, clear that although both inhibitors have similar potency for CaMKII (Chang *et al.*, 2001), they could play different roles in LTP and memory, because of their different regulation after LTP and learning (our data and Lepicard *et al.*, 2006; Radwańska *et al.*, 2010; Vigil *et al.*, 2017).

We and others have shown earlier that CaMK2N1-derived short peptides applied to the bath before induction reduce or abolish LTP in slices (Sanhueza *et al.*, 2007; Buard *et al.*, 2010), as expected for CaMKII inhibitors. In agreement with those experiments in slices, overexpression of CaMK2N2 protein before fear conditioning impaired learning (Vigil *et al.*, 2017). These findings suggest that basal CaMK2N may negatively regulate LTP induction and memory formation. In the same line, if upregulation of *Camk2n1* after LTP or memory formation results in higher CAMK2N1 levels, the inhibitor may interfere with similar plasticity events that follow, which could be considered a form of metaplasticity or LTP saturation.

Interestingly, some data suggest that after activation during LTP induction, CaMKII may need to be inhibited to maintain the potentiation. For example, translocation of active CaMKII to spines activates proteasomes, which leads to degradation of some synaptic proteins and a decrease in synaptic response (Bingol *et al.,* 2010). In line with this, it can be speculated that an emergent role for CaMKII activity in memory destabilization may be prevented by CaMK2N1 (Vigil et al., 2017). These authors claim that the upregulation of *Camk2n1* and the related decrease in CaMKII autophosphorylation on T286 in the PSD fraction are required for the maintenance of fear memory after recall. It is not clear if there is a causal relationship between the upregulation of *Camk2n1* and CaMKII dephosphorylation in that study, because *in vitro* data indicate that CaMK2N1 is not a strong inhibitor of the

autophosphorylation reaction (Vest *et al.*, 2007). To further interpreting these results, it would be beneficial to determine if T286 dephosphorylation occurred in synapses involved in the memory storage or in neighboring synapses. For example, the early gene (*Arc*), which is known to be strongly upregulated after LTP induction, was found to be expressed mostly in inactive synapses, leading to their depression (Okuno *et al.*, 2018).

Previous studies demonstrated that short derivatives of CaMK2N1 depressed both potentiated and non-potentiated synaptic transmission (Sanhueza *et al.*, 2007, 2011; Incontro *et al.*, 2018). It was concluded that the suppression was a result of the interference of the inhibitor with CaMKII-GluN2B binding, because larger concentrations of the inhibitor than those needed to inhibit enzymatic CaMKII activity were required (Buard *et al.*, 2010; Sanhueza *et al.*, 2011). Therefore, it would be interesting to quantify the level of CaMK2N1 upregulation after LTP induction and determine how it correlates with CaMKII-GluN2B binding and synaptic strength. It is likely that, as for CaMK2N2, if CaMK2N1 is indeed increased after LTP induction (following the upregulation of its gene, *Camk2n1*), the concentration of the inhibitor at the synapse may not reach the level needed to interfere with CaMKII-NR2B binding.

It is important to note that as CaMK2N1 mRNA levels were evaluated using the whole hippocampal slice, it remains to be determined if Schaffer collaterals HFS upregulates gene expression specifically in CA1 or if it could increase expression in a widespread manner in other directly or indirectly stimulated hippocampal subregions, such as CA3. Future experiments, such as measuring mRNA in punch-out CA1 sections or *in situ* hybridization are needed to address this issue.

A correlation between LTP magnitude and local gene expression indicates a coordinated modulation by activity and has been reported for several IEGs or “effector genes” expressed later (Meberg *et al.*, 1993; Brackmann *et al.*, 2004), suggesting shared signaling pathways.

Motivated by previous behavioral experiments, in this work we explored a possible modulation of the CaMKII inhibitor proteins mRNA during synaptic plasticity. We demonstrated that the *Camk2n1* gene is upregulated in a graded manner after LTP induction, opening the possibility of a role of CaMK2N1 protein in synaptic plasticity, by contributing to LTP consolidation or modulating further LTP induction as a form of metaplasticity.

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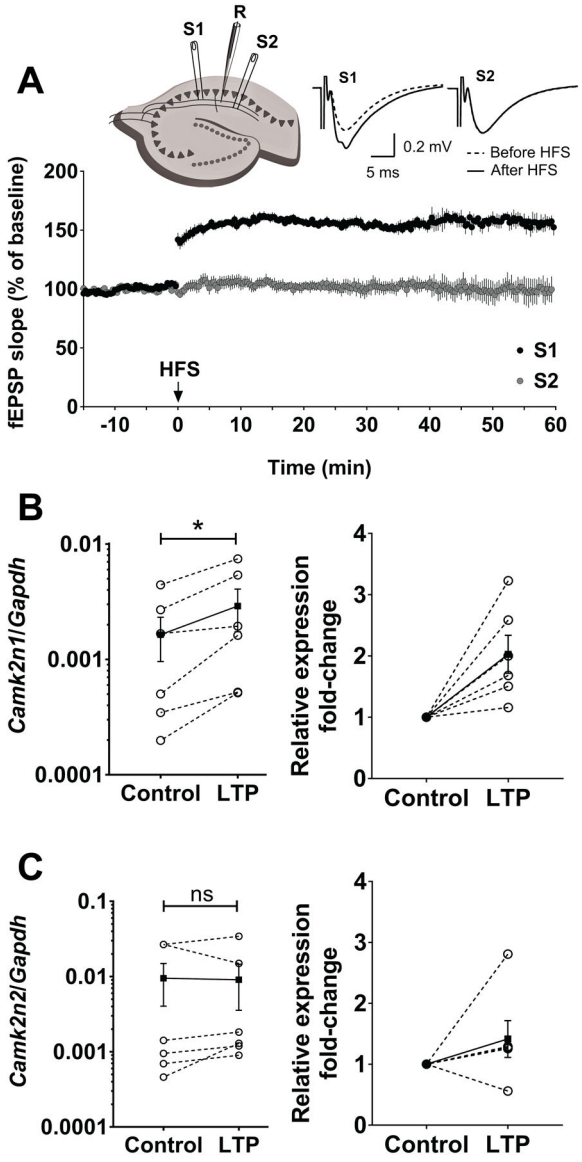

Figure 1. *Camk2n1* expression levels increase after induction of LTP in the CA1 region.

(A) LTP induction in CA3-CA1 synapses. Top left, sketch representing the experimental setup: S1, S2 and R, stimulation and recording electrodes, placed in *stratum radiatum.* S1, S2 were located at 100–200 μm from R. LTP was induced by HFS (four tetani at 100 Hz, 1 s each, separated by 20 s). Top right, sample average traces evoked by basal stimulation at 0.05 Hz, before (−15–0 min) and after (50–60 min) induction. Bottom, summary plot of fEPSP slope relative to baseline for all experiments (N=6). **(B)** qPCR revealed an increase in *Camk2n1* expression 60 min following LTP induction. Left, *Camk2n1* expression relative to *Gapdh* in individual slices for each condition (LTP and Control). Right, relative expression fold-change of *Camk2n2* in LTP slices compared to their control slices. **(C)** *Camk2n2* expression did not change after LTP induction. Left, Right: same as B for *Camk2n2*. Experiments were conducted at 31°C ± 1 °C. Wilcoxon signed-rank test, **P*=0.031; n.s.= not significant.

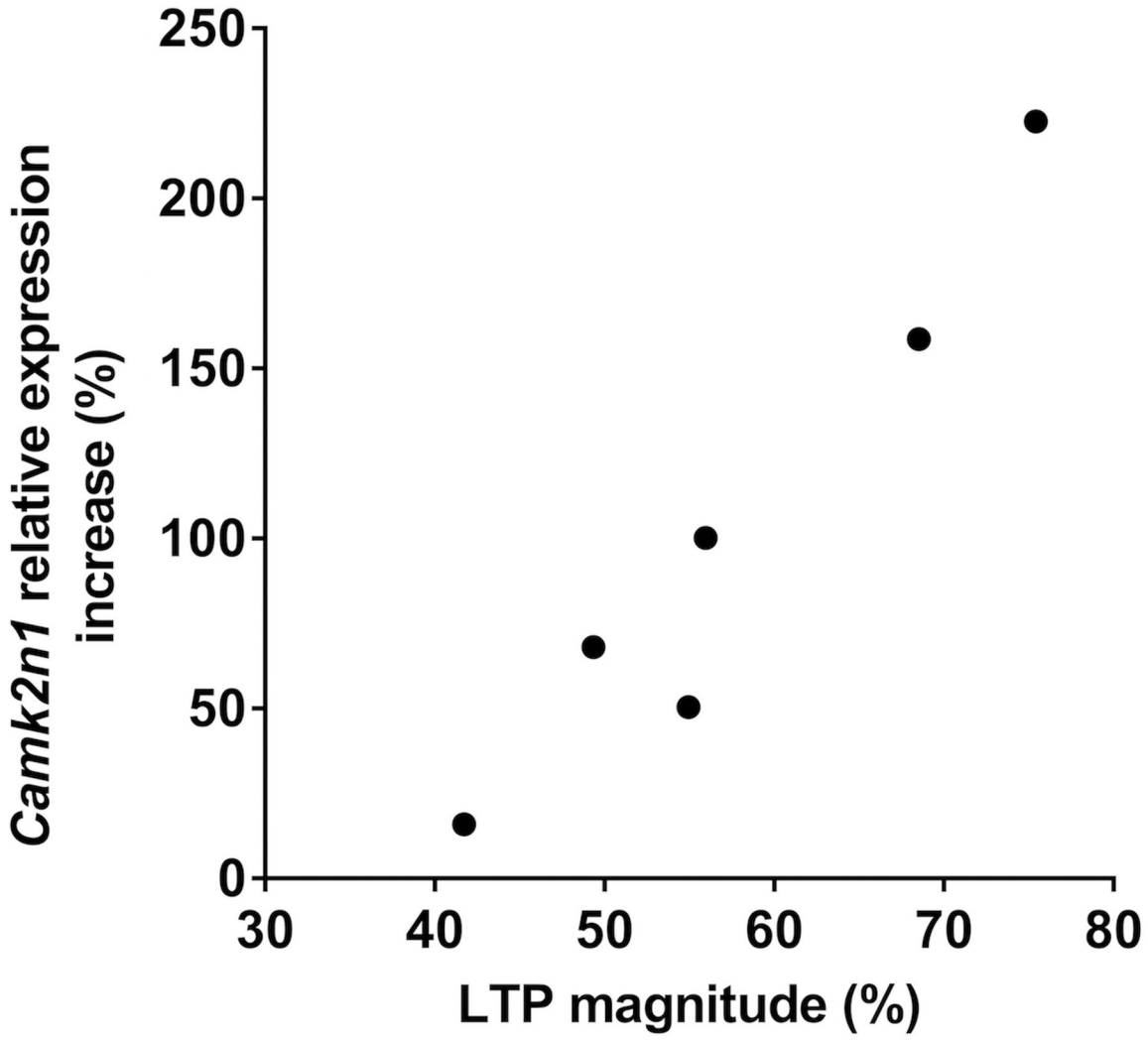

Figure 2. *Camk2n1* expression increase correlates with LTP magnitude in each slice.

Camk2n1 percent expression change in each LTP slice was calculated relative to the corresponding control slice, for each animal (N=6). LTP magnitude (last 20 min) relative to baseline. Spearman's rank correlation coefficient indicates a statistically significant positive correlation between *Camk2n1* expression increase and LTP magnitude (r_s=0.943, *P*=0.018).