Role of the CaMKII/NMDAR complex in the maintenance of the basal and potentiated Synaptic transmission.

(Rol del complejo CaMKII/NMDAR en la mantención de la transmisión sináptica basal y potenciada.)

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INFORME DE APROBACION

TESIS DE MAGÍSTER

Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Magíster presentada por el candidato

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A mi familia, que siempre ha estado ahí.

BIOGRAFÍA

Nací un 25 de Septiembre de 1981, a la hora de las teleseries según mi mamá, a un costado del Zanjón de la Guada, en el hospital Félix Bulnes. Cuando llegue estaba toda mi familia esperándome y desde ahí que no se han despegado más de mí.

Tengo varios recuerdos de pequeño, muchos de ellos son cosas inconexas y tienen que ver con varias cosas que pasaban a mi alrededor, desde las marchas y protestas que pasaban en la esquina, hasta lo yogures que dejaba todas las mañanas mi abuelo Juan en la ventana antes de irse al trabajo.

Lamentablemente no tuve la oportunidad de conocer tanto a mis abuelos, el materno nos dejó muy pronto, el paterno por distintas razones no lo puede conocer lo suficiente, pero creo que fueron hombres con historias bien entretenidas que de a poco le he ido sacando a mis abuelas (ojala pueda seguir con eso), con ellas me crié durante gran parte de mi infancia y adolescencia.

Hice mi básica en un colegio cerca de mi casa, un colegio "de monjitas" donde recibí gran parte de lo que sé de religión católica (el otro tanto me lo enseñaron mi abuela y mi mamá quienes son evangélicas). La secundaria la realicé en el Liceo de Aplicación, un lugar al que le debo harto, no tanto por la educación formal que recibí, si no por darme la oportunidad de salir y conocer otras realidades, mejores y peores que la mía, por enseñarme acerca de la sociedad y el rol que uno desempeña en ella.

Luego de una estadía de dos años en Coquimbo, me volví a Santiago, a la Universidad de Chile, específicamente a la facultad de ciencias, a estudiar biología, carrera que elegí porque me daba la oportunidad de mirar y aprender mas acerca de la naturaleza, algo que me ha cautivado desde pequeño. A pesar de que mi incursión en la fisiología y la neurociencia resultaron de un hecho meramente accidental a finales de mi carrera, es un área que he aprendido a querer, ya que implica un área donde se plantean preguntas muy interesante acerca de como funcionamos y como nos relacionamos con el mundo.

Lo que tiene en sus manos es un resumen de los primero pasos formales que doy en la ciencia, y es el fruto de mi trabajo durante los últimos años. Es un trabajo sincero y que implicó mucho esfuerzo y dedicación, características que pretendo conservar en el futuro. Espero que esta tesis sirva de puerta de entrada a muchas otras investigaciones que pretendo realizar, en las cuales me encuentro trabajando ahora, mientras usted lee esta línea.

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ÍNDICE DE MATERIAS

INDICE DE FIGURAS

LISTA DE SÍMBOLOS, ABREVIATURAS O NOMENCLATURA

CaMKII	quinasa II dependiente de Ca+2/Calmodulina
AMPA	ácido α-amino-3-hydroxy-5-metil-4-isoxazolepropiónico
NMDAR	Receptor de N-metil-D-aspartato
NR2B	Subunidad 2B del receptor de NMDA
Hz	Hertz
min	minuto
s	Segundo
ms	milisegundo
ml	mililitro
µl	microlitro
mM	milimolar
µM	micromolar
V	Volt
mV	milivolt
Ω	Ohm

RESUMEN

La quinasa II dependiente de Ca+2/Calmodulina (CaMKII) es una proteína abundante en varios tipos de tejido. Esta constituye el 1-2% de la proteína total del cerebro (Lisman et al., 2002). CaMKII se encuentra enriquecida en las densidades postsinápticas (PSD), una especialización del citoesqueleto que se encuentra presente en las espinas dendríticas de las sinapsis glutamatérgicas, en ellas se organiza la maquinaria sináptica, que incluye receptores de neurotransmisores proteínas de andamio y enzimas. En las PSD, CaMKII no solo jugaría un rol como quinasa, también puede unirse directamente a una serie de proteínas, incluyendo al receptor de glutamato tipo NMDA (NMDAR). Durante la potenciación a largo plazo (LTP), un modelo de plasticidad y aprendizaje comúnmente utilizado, las sinapsis experimentan cambios estables en la fuerza sináptica luego de un periodo de intensa actividad. En la región de CA1 del hipocampo, una estructura cerebral relacionada con la memoria y ubicación espacial, la inducción y los mecanismos de expresión de LTP han sido ampliamente estudiados, pero los procesos moleculares que mantienen dichos cambios no han sido identificados. Al aumentar la actividad sináptica, CaMKII migra desde el citoplasma hasta las PSD, donde se une con la subunidad NR2B de los NMDAR. Es destacable que si esta interacción se interrumpe, la inducción de LTP y el aprendizaje también.

En esta tesis se prueba la hipotesis de que el complejo formado por CaMKII y NMDAR podría constituir un tipo de memoria sináptica. Para determinar si una molécula es importante o no para el proceso de memoria celular, se debe primero probar que al intervenir con dicha molécula se produce una reversión persistente de la LTP. Por otro lado, si la LTP ocurre naturalmente en la vida del animal, es de esperar que un componente de la intensidad de la sinapsis dependa de la estabilidad de esta molécula.

Como una primera aproximación para probar la importancia del complejo CaMKII/NMDAR para la mantención de la transmisión sináptica, se utilizaron distintas secuencias peptídicas construidas en base a las regiones de interacción entre CaMKII y NMDAR. Estos agentes fueron aplicados en rebanadas de hipocampo de rata, al mismo tempo que se realizaban registros electrofisiológicos para medir la transmisión sináptica. Los resultados de estos experimentos fueron difíciles de interpretar, debido a un efecto inespecífico de una región de la secuencia peptídica utilizada para la incorporación a las células.

Recientemente se ha descrito una nueva familia de péptidos (péptidos CN), derivados de una proteína endógena de función desconocida. Estos péptidos han mostrado, in vitro, inhibir la actividad de CaMKII inducida por Ca+2/Calmodulina, tanto a nivel de su actividad como quinasa, como su unión a NR2B. Al acoplar estos péptidos con una secuencia de incorporación derivada de una de las proteínas del virus VIH (tatCN) no se observaron efectos inespecíficos como en los primeros experimentos.

En este trabajo se muestra que la aplicación transitoria de estos péptidos produce una reducción persistente en la transmisión sináptica basal. Lo anterior fue demostrado utilizando dos aproximaciones distintas: i) Registros antes, durante y después de la aplicación de tatCN. ii)

Preincubación y posterior medida de la intensidad sináptica a través de curvas entrada/salida (curvas I/O). El mismo tratamiento puede revertir parcialmente procesos de LTP, esto queda de manifiesto ya que, luego de la aplicación transitoria de la droga, es posible inducir nuevamente LTP en vías previamente saturadas. Interesantemente, un efecto análogo se observó en la transmisión basal (no potenciada experimentalmente), en esta, el tratamiento con tatCN facilitó la inducción de una nueva LTP.

Como complemento a esta tesis, nuestros colaboradores realizaron ensayos de coimmunoprecipitation en las mismas rebanadas utilizadas en los registros electrofisiológicos, esto para cuantificar la cantidad de CaMKII unida a NMDAR. Estos datos muestran que tatCN reduce la intensidad de la sinapsis a concentraciones suficientes para desestabilizar el complejo CaMKII/NMDAR, pero no a concentraciones lo suficientemente pequeñas como para afectar solo la actividad quinasa de la proteína.

Otro resultado interesante obtenido por nuestros colaboradores, fue que la secuencia CN también produjo un efecto en rebanadas en cultivo capaces de expresar CaMKII acoplada a proteína fluorescente verde (GFP). En estas se observó una disminución en la cantidad de CaMKII asociada a la espina.

En resumen, este trabajo muestra evidencia suficiente de que la intervención del complejo CaMKII/NMDAR en las PSD produce una reducción de la intensidad sináptica. Cabe destacar que tanto la reducción del complejo, como la caída en la intensidad de la señal, persistieron luego de la remoción del inhibidor. Más aún, la interrupción del complejo produce un desaturación de la LTP y facilita la inducción en una vía en la que no se ha inducido una LTP experimentalmente, sugiriendo una reversión del proceso. Consistente con estos resultados, el

mismo tratamiento produce una relocalización de la quinasa en la espina dendrítica. Estos resultados apoyan la hipótesis de que el complejo CaMKII/NMDAR posee características de switch las cuales son importantes para la mantención de la intensidad sináptica.

ABSTRACT

The multifunctional Ca^{+2}/Calmodulin-dependent kinase II (CaMKII) is an abundant protein in several tissues. It constitutes the 1-2% of total brain protein (Lisman et al., 2002). CaMKII is enriched in the Post Synaptic Densities (PSD), a cytoskeletal specialization of glutamatergic synapses, where the postsynaptic signaling machinery that includes neurotransmitter receptors, scaffold and regulatory proteins and enzymes, is organized. In the PSD, CaMKII may not only play a role as a kinase, it also can directly and indirectly bind a series of other proteins, including the NMDA-type glutamate receptor (NMDAR). During long-term potentiation (LTP), a broadly used synaptic plasticity model, synapses undergo stable changes in synaptic strength after a period of intense synaptic activity. In hippocampal CA1 region, LTP induction and expression mechanisms have been extensively studied, but the molecular processes that maintain strength have not been identified. During high rate synaptic activity, CaMKII is translocated to the PSD, where it binds to the NR2B subunit of NMDARs. Notably, if this interaction is impaired, LTP induction and learning is disrupted.

In this Thesis, we tested the hypothesis that the complex formed by CaMKII and the NMDAR may constitute a molecular memory at the synapse. To establish a molecule as a molecular memory, it must be shown that interfering with the molecule produces a persistent reversal of LTP. On the other hand, if LTP processes in fact occur during the previous animal

life, it would be expected that a component of synaptic strength would depend on the stability of such molecular entity.

As a first approximation to test the importance of the CaMKII/NMDAR complex for synaptic transmission maintenance, peptides derived from the interacting region between NMDAR and CaMKII were used. These agents were applied to brain slices during simultaneous recording of synaptic transmission. The results of these experiments were difficult to interpret, due to an unspecific effect of the peptidic sequence used to allow drug incorporation to cells.

Recently, a new peptide family (CN peptides), derived from an endogenous protein of unknown function has been described. These peptides have been shown to specifically inhibit both CaMKII activity and its binding to NR2B induced in vitro by Ca^{+2}/Calmodulin treatment. These peptides coupled to VIH protein sequence (tatCn) showed no unespecific effects.

In this thesis work it was shown that the transient application of these peptides produces a persistent reduction in basal transmission. This was demonstrated by two different approaches: i) direct recording of transmission before, during, and after transient application of tatCN to the bath. ii) Preincubation and measurement of synaptic strength by construction of Input-Output curves (I/O Curves). The same treatment can also partially reverse saturated LTP, as transient drug application after induction allowed additional LTP to be induced. Interestingly, an analogous effect was observed for basal transmission (not potentiated during the experiment), as CN treatment facilitated posterior LTP induction.

As a complement to this thesis work, coimmunoprecipitation assays were done by our collaborators in the same slices where electrophysiology experiments of this thesis were conducted, to quantify the binding of CaMKII and NMDAR. Comparisons of the data show that

CN peptides decreases synaptic strength only at concentrations that disrupt the CaMKII/NMDAR complex, but not at lower concentrations, sufficient only to inhibit CaMKII activity.

Also, applications of CN peptide to slice cultures expressing GFP-tagged CaMKII actually affected CaMKII in living cells, causing a reduction in the bound amount of the kinase in the spine.

In summary, we here show compelling evidence that interfering with CaMKII/NMDAR binding at PSD causes synaptic strength depression. Importantly, both the reduction of the complex and the reduction of the synaptic strength persisted after removal of the inhibitor. Moreover, complex disruption brings experimentally induced LTP out of saturation and facilitates subsequent LTP induction in a naïve pathway, suggesting a reversal of LTP processes. Consistent with these results, the same treatment caused delocalization of the kinase from a binding partner in the spine. These results support the hypothesis that the CaMKII/NMDAR complex has switch-like properties that are important in the maintenance of synaptic strength.

INTRODUCTION

CaMKII is a critical protein for the induction of long –term potentiation LTP (Lisman et al., 2002). The kinase is activated by Ca2+ entry through the NMDA receptor (NMDAR) (Hudmon and Schulman, 2002)(Fig 1). If this activation is blocked pharmacologically or by CaMKIIα knockout, LTP induction is prevented (Malinow et al., 1989; Silva et al., 1992). A necessary aspect of this process is the autophosphorylation of T286 (Giese et al., 1998; Buard et al., 2010), which makes the kinase partially independent of Ca2+ (autonomous) (Miller and Kennedy, 1986). Once activated, CaMKII undergoes translocation to synaptic sites (Shen and Meyer, 1999; Shen et al., 2000; Bayer et al., 2001; Otmakhov et al., 2004; Hudmon et al., 2005; Bayer et al., 2006; Zhang et al., 2008; Lee et al., 2009) and becomes bound in the postsynaptic density (PSD)(Strack et al., 1997; Dosemeci et al., 2001; Otmakhov et al., 2004).

An important binding partner in the PSD is the NMDAR (Strack and Colbran, 1998; Leonard et al., 1999; Strack et al., 2000; Bayer et al., 2001; Leonard et al., 2002). The CaMKII/NMDAR complex exists under basal conditions but is increased by strong synaptic stimulation (Leonard et al., 1999). Preventing the formation of this complex by overexpression of mutated NR2B or NR2B ΔC-tail strongly reduces LTP induction (Barria and Malinow, 2005; Zhou et al., 2007). These results suggest that complex formation is necessary for LTP induction. Whether the complex also has a role in LTP maintenance, i.e., acts as a molecular memory, remains unclear.

A critical test of any model of LTP maintenance is to apply an agent that interferes with the function of a putative memory molecule and determine whether potentiation is persistently reduced. Several inhibitors of CaMKII activity do not reverse LTP (Malinow et al., 1989; Otmakhov et al., 1997; Chen et al., 2001; Buard et al., 2010). However, it is possible that the CaMKII/NMDAR complex serves as a synaptic memory through a structural rather than enzymatic process. A new class of CaMKII inhibitors derived from the endogenous protein CaMKIIN (Chang et al., 1998, 2001) interfere with the Ca^{+2}/Calmodulin-induced binding of CaMKII to NR2B in vitro (Vest et al., 2007). In the presents work peptides derived from this inhibitor protein (CN peptides) have been used to test the hypothesis that synaptic strength is maintained by the CaMKII/NMDAR complex. Previous experiments utilizing antCN27 (also known as ant-CaMKIINtide) (Sanhueza et al., 2007) supported this hypothesis. However, it was then shown that the cell permeabilizing sequence, ant, directly binds calmodulin (Buard et al., 2010), opening the possibility of an unspecific effect. Here, improved versions of CN peptides have been used, these contain a tat permeabilizing sequence (tatCN) that does not bind calmodulin (Buard et al., 2010). This work shows that tatCN peptides are able to partially reverse LTP maintenance and produce a persistent reduction in basal transmission. Parallel experiments conducted by our collaborators, directly demonstrated by biochemical methods that the treatments we used actually reduced the CaMKII/NMDAR complex in our slices and also removed CaMKII from spines. Overall, these results suggest that the reduction of both synaptic strength and the CaMKII/NMDAR complex is not an equilibrium effect, but instead had the switch-like properties required of a molecular memory.

MATERIALS AND METHODS

Acute slice preparation and preincubation. Animal care was in accordance with institutional guidelines of the University of Chile. Transverse hippocampal slices (400 µm) were prepared from 18- to 23-day-old Sprague Dawley rats in ice-cold dissection solution containing (in mM): 125 NaCl, 2.6 KCl, 10 $MgCl_2$, 0.5 $CaCl_2$, 26 $NaHCO_3$, 1.23 NaH_2PO_4, and 10 D-glucose (equilibrated with 95% O_2 and 5% CO_2), pH 7.3. CA3 area was removed, and slices were allowed to recover for a minimum of 1 h at room temperature in inverted interface chambers (tissue inserts, 8 µm; EMS, Hatfield, PA) maintained in an atmosphere saturated with 95% O_2 and 5% CO_2. Each insert held two slices completely submerged in a drop of 100 µl of ACSF containing (in mM): 125 NaCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 2.6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 D-glucose. In experiments in which slices were preincubated with peptides, the drop of solution bathing slices was carefully removed and replaced by freshly oxygenated ACSF containing 5 or 20 µM peptides. After 1 or 2 h preincubation, drop solution was replaced by regular oxygenated ACSF four times and maintained for at least 1 h before recordings. Slices used for coimmunoprecipitation analyses received identical treatment and were transferred to 2 ml cryotubes (2 slices per tube) in which the ACSF was gently removed. Cryotubes were quickly transferred to a liquid nitrogen tank, where they were stored until biochemical analyses.

Electrophysiological recordings in acute slices. For recordings, slices were transferred to a submersion-type recording chamber mounted on an upright microscope (Nikon E600FN) and were continuously superfused (2–4 ml/min) with ACSF bubbled with 95% O_2 and 5% CO_2 in a 20 ml syringe before entering the chamber. A total volume of 10 ml of ACSF was recirculated during each experiment using a two-way pump. Experiments were conducted at 30°C–31°C. Extracellular field potential recordings were made with borosilicate glass recording electrodes (0.5–1 MΩ) filled with ACSF and placed in stratum radiatum of the CA1 region. Schaffer collaterals were stimulated with monopolar stimulating electrodes (glass pipettes filled with ACSF; 0.5 MΩ) or bipolar electrodes (FHC, Inc), positioned 100–150 µm to the recording electrode. Stimulus duration was 0.1 ms, and interstimulus interval was 10 s.

During the stimulation 3 clear signals were observed (Appendix Fig 1), the first and most quick correspond to the stimulus artifact. This signal was followed by a more slow signal of about 2-3ms of duration and 0.2mV of amplitude that correspond to the synaptic volley, this response is proportional to the presynaptic discharge. The last, and bigger signal, correspond to the Excitatory Postsynaptic Potential (EPSP) and its proportional to the postsynaptic response.

To obtain input-output (I-O) curves, stimuli of increasing intensities were applied (5–100 mA in 5 or 10 steps; 5 records per amplitude) until signal saturation or population spike generation (discharge of the postsynaptic). Construction of I-O curve started only after stable basal transmission was reached (typically, after 10 min). For LTP induction, two independent synaptic pathways were alternately stimulated. LTP was induced in one pathway by 4 tetani (100 Hz, 1 s each), separated by 20 s. In LTP saturation experiments, 1 additional tetanus was

applied 10 min later to confirm that saturation was reached. Stimulus strength was adjusted to have a field EPSP (fEPSP) amplitude of 50%–60% of the population spike threshold. Stimulus strength was the same for both test and tetanic stimulation. A stable fEPSP slope baseline of 20 min was taken before LTP induction, and transmission was further recorded for 30 min. Data were acquired at 20 kHz by a PC with an ITC-1600 interface (HEKA Inst. Inc., Bellmore, NY), using custom programs procedures (G. Fernandez-Villalobos) in Igor Pro 6.03A (WaveMetrics, Lake Oswego, OR).

Field potential data analysis and statistics. Igor Pro 6.03A or Microsoft Excel were used for analysis. fEPSP initial slope and fiber volley peak amplitude were measured to build an I-O curve for each experiment. I-O curve slope per experiment was determined by linear regression, and a mean slope per animal and drug (2–4 slices for each condition) was calculated and used for paired test analyses (Fig. 4C, F). Mean I-O test and control curves (Fig. 4B, D) were calculated by first averaging curves for each drug per animal and then averaging among animals. Plot error bars correspond to SEM. The specific type of statistical analysis used is indicated in legends.

Drug application in the recording chamber. During the experiments, stock peptide solutions were added to the syringe with ACSF to achieve the desired final concentration. We have observed that, during excessive bubbling, there is foam formation in the syringe after peptide addition that could alter the effective peptide concentration in the chamber. Therefore, bubbling was decreased to a level at which foam formation was avoided but enough slice oxygenation was provided (as indicated by stable fEPSP recording and a fEPSP/fiber volley

ratio > 4-fold). This method ensured highly reproducible effects of peptide application. Drugs were washed out by changing syringe solution: recirculation was stopped, and the syringe was refilled with fresh, oxygenated, ACSF. After changing at least four times the original solution volume, the final 10 ml recirculating volume was re-established.

Peptides. Tat-conjugated CaMKIIN-derived peptide, tatCN21 (Vest et al., 2007), and a control peptide that was a fusion of tat to a scrambled CN21 sequence were obtained from Biomatik (Wilmington, DE).

RESULTS

In the original project, It was proposed to use a series of peptides that presumably could disrupt basal CaMKII/NMDAR binding at the synapse . As possible candidates, synthetic peptides constructed in base to the regions of interaction of NMDA with CaMKII were used. These peptides are based on the interaction sequence (of NMDAR) between CaMKII and NMDAR. An additional sequence of 11 Arginine residues (11R) was attached to promote its cellular incorporation.

The first series of experiments showed a persistent decrease in synaptic transmission (Appendix Fig 1). However control experiments where a peptide with two mutated residues that does not interact with CaMKII was applied; produce a similar effect (Appendix Fig 1). This indicates that our peptides produced an unspecific effect related to the incorporation sequence.

After these preliminary results, we decided to use an alternative pharmacological approach. The CN compounds are based on the sequence of an endogenous protein, CaMKIIN (Chang et al., 1998, 2001). CN compounds have been progressively optimized to enhance CaMKII inhibition (and their capacity to bind to the same CaMKII region as NR2B), and shorter (CN27; CN21; CN19) (Vest et al., 2007 ; Coultrap and Bayer, unpublished observations).

Furthermore, the permeabilizing sequence, ant, used in early versions has been replaced by tat, because tat, unlike ant, does not by itself affect calmodulin (Buard et al., 2010).

To evaluate the effect of these peptides on synaptic transmission, extracellular field recordings were done, and the excitatory synaptic field potential (fEPSP) were measured. Fig. 2 shows experiments in which tatCN21 (20 μM) or the more potent version, tatCN19 (5 μM), was applied and then removed. In both cases, there was a reduction in transmission. As a similar effect on synaptic strength was previously reported for antCN27 (Sanhueza et al., 2007) our observations suggest that this type of synaptic depression is not caused by an unspecific effect of the ant sequence, as suggested by Buard et al (2010). The reduction in transmission during application can be in part due to the reversible effects of CaMKII inhibitor on presynaptic release and excitability (Waxham et al., 1993; Sanhueza et al, 2007) and is also due to postsynaptic effects (Sanhueza et al, 2007; also see additional control below). After removal of peptide there was some recovery, but there was a persistent reduction in the fEPSP. This persistent reduction was not seen with scrambled peptide (tatCN21 SCR) and was not evident in the fiber volley, a measure of axon excitability (Fig. 2A, C). These results indicate that a component of synaptic strength can be persistently reduced by transient treatment with CN peptides.

Previous experiments showed that antCN27 transient application persistently reduced the response to the local application of AMPA, demonstrating a postsynaptic effect. To confirm that this was also true for tatCN peptide, a series of experiments were conducted by our collaborators (Dr. J. Lisman lab), where selected CA1 cells were transfect with GFP-CN19 . After 2 days, the excitatory post synaptic currents (EPSC) evoqued in these cells were measured by

whole-cell recording and compared to nearby untransfected cells (Hayashi et al., 2000). EPSC was reduced in transfected cells compared to nearby control cells (Appendix Fig.2). It confirms that CN compounds act postsynaptically to reduce the maintenance of basal transmission. Additional control experiments and their discussion were included in the Appendix.

Given the ability of CN compounds to reverse maintenance processes, it is important to establish whether they in fact affect the CaMKII/NMDAR complex in living cells, as suggested by work in cell free assays (Vest et al., 2007). CaMKII is enriched in spines (Otmakhov et al., 2004; Zhang et al., 2008; Lee et al., 2009) and in the PSD (Otmakhov et al., 2004) even under basal conditions, and this may partly be due to basal CaMKII/NMDAR complex (Leonard et al., 1999). Thus, if the CaMKII/NMDAR complex is reduced by CN compounds, there should be a reduction in spine-bound CaMKII content. To test this prediction, our collaborators made transfections of GFP-CaMMKII and a Volume marker. Measurements of GFP-CaMKIIα fluorescence showed that the peptide produced a 15-20% reduction in the bound fraction of CaMKII (Appendix Fig. 3C, D) in contrast control peptide had no effect on either the synaptic response or the bound fraction GFP-CaMKIIα. Spine volume did not change significantly (Appendix Fig. 3C, D).

The persistent decrease on basal transmission observed after CN peptide treatment may be due to an effect on LTP that occurred when the animal was alive (Whitlock et al., 2006). If so, CN peptides should also be able to reverse LTP that was induced experimentally. To determine whether this is the case, we conducted two-pathway experiments and induced saturating LTP in one pathway. Subsequently, tatCN21 or scrambled peptide was transiently applied; after drug washout, the previously saturated pathway was again tetanized. If this

procedure reversed saturated LTP, it should then be possible to reinduce LTP (Sanhueza et al., 2007) in the saturated pathway. Fig. 3 shows that this is the case. In the example of Fig. 3A, it can be seen that a large LTP was first induced by 4 tetani and that an additional tetanus had no effect, demonstrating saturation. Transient application of peptide produced a persistent reduction of the fEPSP, and after peptide washout, LTP could then be reinduced. Summary results are presented in Fig. 5A, B. When similar protocols were followed using tat-SCR peptide, LTP could not be reinduced. This suggests that tatCN21 can reverse the LTP maintenance process.

The above results suggest that the CaMKII/NMDAR complex is important for synaptic strength and plasticity stability (LTP), but a more direct measurement of the complex is desired. With this purpose average synaptic strength were done in slices transiently preincubated with test CN or control peptides measures. In these experiments the slope of the curve relating fiber volley (FV) to fEPSP (input-output [I-O] curve) was used to measure synaptic strength. After the experiments the slices were quickly frozen and sent to Dr. J Hell lab (UC Davis), where coimmunoprecipitation assays were done in those slices to quantify the effect of CN on the complex (Leonard et al., 1999). This measure was compared for slices incubated for equal times with either tatCN21 or tatCN21SCR. Following a procedure sufficient to inhibit induction, but not maintenance, of LTP (Buard et al., 2010), 1 h incubation with 5 μM tatCN21 were done. Slices were then washed for 1 h. As shown in Fig. 4A-C, this procedure had no effect on basal transmission (Fig. 4C; $p > 0.1$). As shown in Appendix (Appendix Fig. 2A), the procedure also had no effect on the CaMKII/NMDAR complex ($p > 0.1$). Thus, 5 μM tatCN21 does not affect basal

transmission, consistent with previous findings (Buard et al., 2010), but also does not affect the CaMKII/NMDAR complex.

Then a new series of experiments, using the same procedure but this time with 20 μM tatCN21were done (Fig. 5D-F). This higher concentration produced a persistent reduction of synaptic strength (Fig. 4F; $p < 0.05$). Moreover, there was a persistent reduction in the CaMKII/NMDAR complex, as determined by coimmunoprecipitation of either NR2B or NR1 (Appendix Fig. 2B; $p < 0.001$ for NR2B and $p<0.05$ for NR1). Total CaMKII, total NMDAR, and CaMKII binding to α-actinin were unaltered. Thus, tatCN21 concentrations, sufficient to produce a persistent reduction of the CaMKII/NMDAR complex, produce a persistent reduction of synaptic strength.

With that correlation in mind, additional experiments of LTP induction were done, this time, using slices preincubated as described above (no previous experimentally induced LTP. The effect of a series of 4 tetani was evaluated. These experiments show an enhanced LTP in tatCN21-pretreated slices compared with those treated with SCR peptide (Fig 5). This supports the interpretation that the peptide reduced a LTP maintenance process in which potentiation occurred naturally during the life of the animal. These experiments show a clear correlation between the amount of complex and the capacity to induce LTP.

DISCUSSION

If the CaMKII/NMDAR complex has a role in the maintenance of synaptic strength, reducing this complex should produce a reduction of synaptic strength. This work shows the first experiments in which both synaptic strength and the amount of CaMKII/NMDAR complex in the slice preparation could be directly measured. Together with our collaborators found that the CaMKII inhibitor, tatCN21 (Vest et al., 2007), reduced basal synaptic strength at concentrations that disrupt this complex (20 μM). At lower concentrations (5 μM) sufficient to inhibit CaMKII activity (Buard et al., 2010), but not to reduce the complex, there was no reduction in synaptic strength. Also, found that tatCN21 could reverse saturated LTP, a reversal that then allowed additional LTP to be induced.

The effects of tatCN21 persisted after a 1 h washout of the peptide, a persistence has important implications for the kind of biochemical processes involved. The effectiveness of washout is indicated by the fact that LTP cannot be induced in presence of CN peptide(Sanhueza et al., 2007; Buard et al., 2010)but can be induced after washout. The persistence of the effects of peptide thus argue that the CaMKII/NMDAR complex does not spontaneously reform after removal of the peptide, i.e., that the peptide is not simply interfering with equilibrium binding. These findings suggest that the complex has switch-like

properties in which formation of the storage molecule is driven by synaptic activity rather than equilibrium processes.

The reduction in transmission produced by tatCN21 could be due to a dual effect between the reduction in catalytic activity of CaMKII and its localization. This interpretation comes from our observations of the persistent effect of the peptide in the biochemical and fluorescence experiments of our collaborators. These experiments show that the persistent effect produced by 20μM of tatCN implies not only a reduction in the CaMKII/NMDAR complex, also affect the amount of CaMKII in the spine. This kind of change in the subcellular localization has been related with the a series of effects over the protein, these include, changes in the phosphorylation state (Davies et al., 2007; Skelding et al., 2010) and make the kinase sensitive to the action of phosphatases (Mullasseril et al., 2007). This kind of changes could produce long lasting modifications in the synaptic strength. In our case, this kind of changes are not related with other kind of plastic effect, like Long Term Depression (LTD), recent occlusion experiments made in our laboratory show that the effect of the CN peptide do not share the same mechanism of LTD (Gouet, data not published).

Another related point is the observation that 5 ⍰M tatCN21 is sufficient to block LTP induction (Buard et al., 2010), a process that requires CaMKII activity (Malinow et al., 1989; Silva et al., 1992) but does not affect basal synaptic transmission (Buard et al., 2010) or the CaMKII/NMDAR complex (shown here). Other CaMKII inhibitors that are relatively ineffective blockers of the CaMKII/NMDA receptor complex formation (Bayer et al., 2006) can block LTP induction but also do not affect synaptic strength or LTP maintenance (Malinow et al., 1989; Otmakhov et al., 1997; Chen et al., 2001; Wang et al., 2008). Thus, the fact that 5 ⍰M tatCN21 is

not sufficient to disrupt the complex provides a satisfactory explanation for why we did not find any effect of that concentration on synaptic strength, as reported previously (Buard et al., 2010). It is unlikely that the effect of CN21 was due to inhibition of other CaM-kinases or PKC, because inhibition of these kinases does not affect basal transmission (Huber et al., 1995; Kasten et al., 2007; Redondo et al., 2010). Taken together, these results strongly suggest that CN compounds affect synaptic strength through a structural process, and this modification in the subcellular localization could be related with the long lasting changes in synaptic transmission rather than a direct catalytic process.

What might be the structural processes by which CN affects synaptic strength? There are several known proteins that CaMKII binds in addition to NMDA receptors. These include actin;α-actinin; densin-180; and SAP97, MUPP1, and L-type Ca channels (Walikonis et al., 2001 ; Krapivinsky et al., 2004; Okamoto et al., 2004; Hudmon et al., 2005; Robison et al., 2005; Buard et al., 2010; Nikandrova et al., 2010)(for review, see (Colbran and Brown, 2004; Merrill et al., 2005)).Several of these proteins may bind to the same site on CaMKII as the NR2B. Therefore, we cannot exclude that CN compounds might also interfere with the binding of CaMKII to some of these targets. However, it is likely that the changes in synaptic strength that we observe arise mostly from the demonstrated change in the CaMKII/NMDAR complex because blocking the formation of this complex by other means (overexpression of mutated NR2B or NR2B C-tail) strongly reduces LTP induction(Barria and Malinow, 2005; Zhou et al., 2007).Additionally, expression of NR2B has been described to re-constitute activity-driven translocation of CaMKII in heterologous cells (Strack et al., 2000; Bayer et al., 2006).

The binding of CaMKII to the NMDAR places the kinase in a unique environment where the important T286 site is protected from dephosphorylation (Mullasseril et al., 2007). This may account for why T286 phosphorylation is detected in the PSD under basal conditions (Lengyel et al., 2004; Larsson and Broman, 2005; Dosemeci and Jaffe, 2010). Although phospho-T286 is not required for the initial binding to NR2B (Bayer et al., 2001), it strengthens this binding (Strack and Colbran, 1998; Bayer et al., 2006). In addition, several other proteins (Densin-180, NR1) also preferentially bind to the phosphorylated state of CaMKII though different binding sites on CaMKII (Walikonis et al., 2001; Leonard et al., 2002). A 12 subunit holoenzyme CaMKII can simultaneously bind several different PSD proteins, allowing the formation of macromolecular complexes (Robison et al., 2005). A simple possibility is that one or more of these CaMKII binding proteins serve to anchor AMPARs at the synapse and thereby control synaptic strength though mainly structural mechanism (Lisman and Zhabotinsky, 2001).

Changes in the subcellular localization related with the application of CN protein could be an indicator of its function in the inactivation of the kinase. CN binding kinase loose it´s ability to phosphorylate other substrates and only can be phosphorylate by itself. Another important site of phosphorylation of the protein is the T305/306. The phosphorylation of this site could determine the direction of synaptic modification (Pi et al., 2010)and also it´s related with the inactivation of the kinase. This particular site only can be phosphorylated by the self CaMKII in intra subunit way (Bayer et al., 2006) would not be affected by CN. In theory CN could be affecting the intersubunit phosphorylation of the protein, but not the intrasubunit activity, this could produce an increase in the T305/306 phosphorylation and then could be dephosphorylated by Calcineurin. To confirm this hypothesis more experiments are needed.

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FIGURES

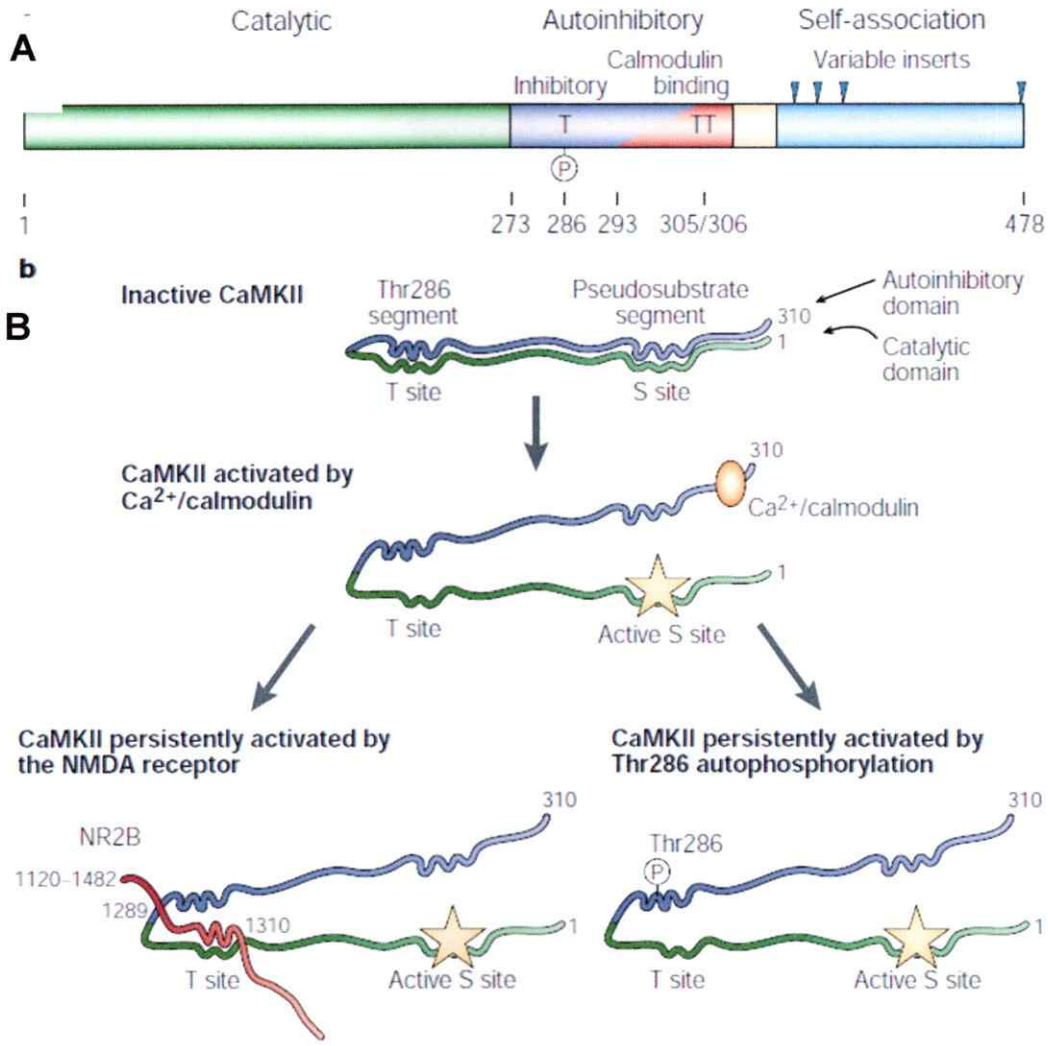


Figure. 1. Structure and phosphorylation cycle of CaMKII. **A,** The different functional domains in the primary structure of calcium/calmodulin-dependent protein kinase II (CaMKII). T represents threonine residues that are crucial phosphorylation sites. **B,** The autoinhibitory and catalytic domains form a gate that regulates activity. The enzyme is inhibited when the gate is closed because the autoinhibitory domain binds to the catalytic domain at the S and T sites (top). The binding of Ca2+/calmodulin opens the gate and the enzyme becomes active (middle). A site on the NMDA (*N*-methyl-D-aspartate) receptor NR2B subunit can bind to the T side, keeping the gate open and the enzyme active even after the dissociation of calmodulin (bottom left). In the presence of Ca2+/calmodulin, the Thr286 site can be phosphorylated by a neighboring subunit. This is also sufficient to keep the gate open and the enzyme active even after dissociation of calmodulin (bottom right). From Lisman, 2002.

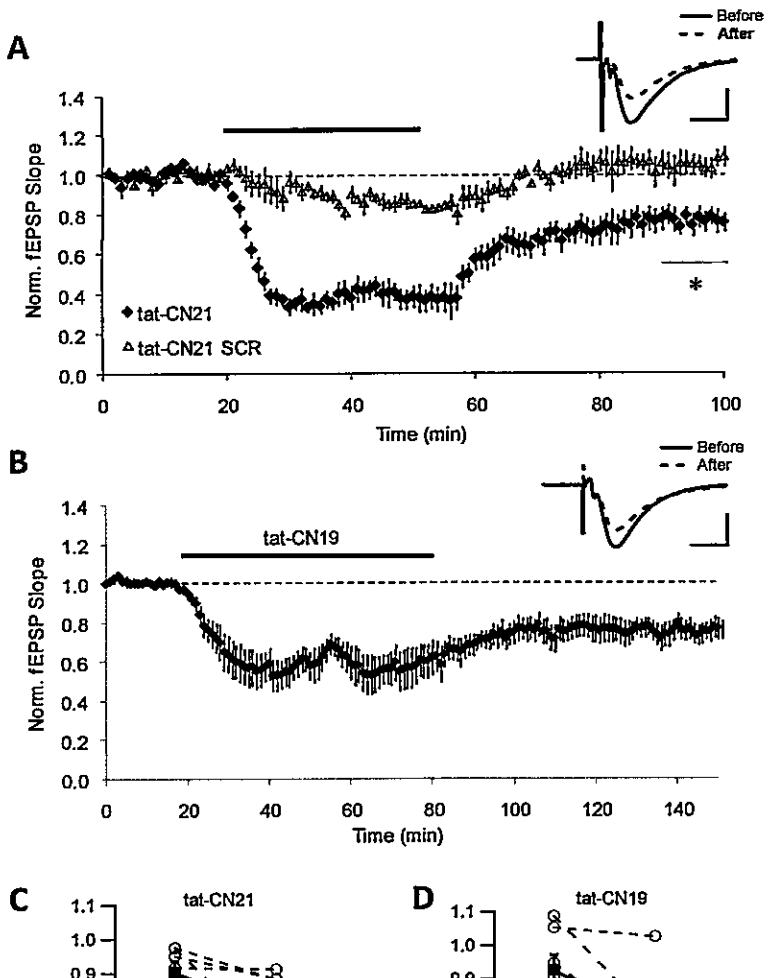


Figure. 2. Persistent depression of basal transmission by bath-applied tat-fused CN peptides. ***A,*** Basal transmission was monitored before, during, and after transient application (30 min) of 20 µM tatCN21 (n = 6) or tatCN21 SCR peptide (n = 4). T test, $p < 0.005$. *Inset*, representative averaged basal fEPSP before and 50 min after drug washout. ***B,*** Same as A for 1 h application of 5 µM tatCN19 (n = 7). *Inset*, averaged fEPSP before and 60 min after drug washout. ***C,*** Graph shows the change of the fiber volley (FV) and EPSP slope after tatCN21was washed out for 1 h, relative to these before drug application. Each pair represents data from a single tatCN21 experiment. Filled symbols: average ± SEM (Wilcoxon test, $p < 0.05$). ***D,*** Same as C, for tat-CN19 ($p < 0.05$). Calibration: 5 ms, 0.4 mV.

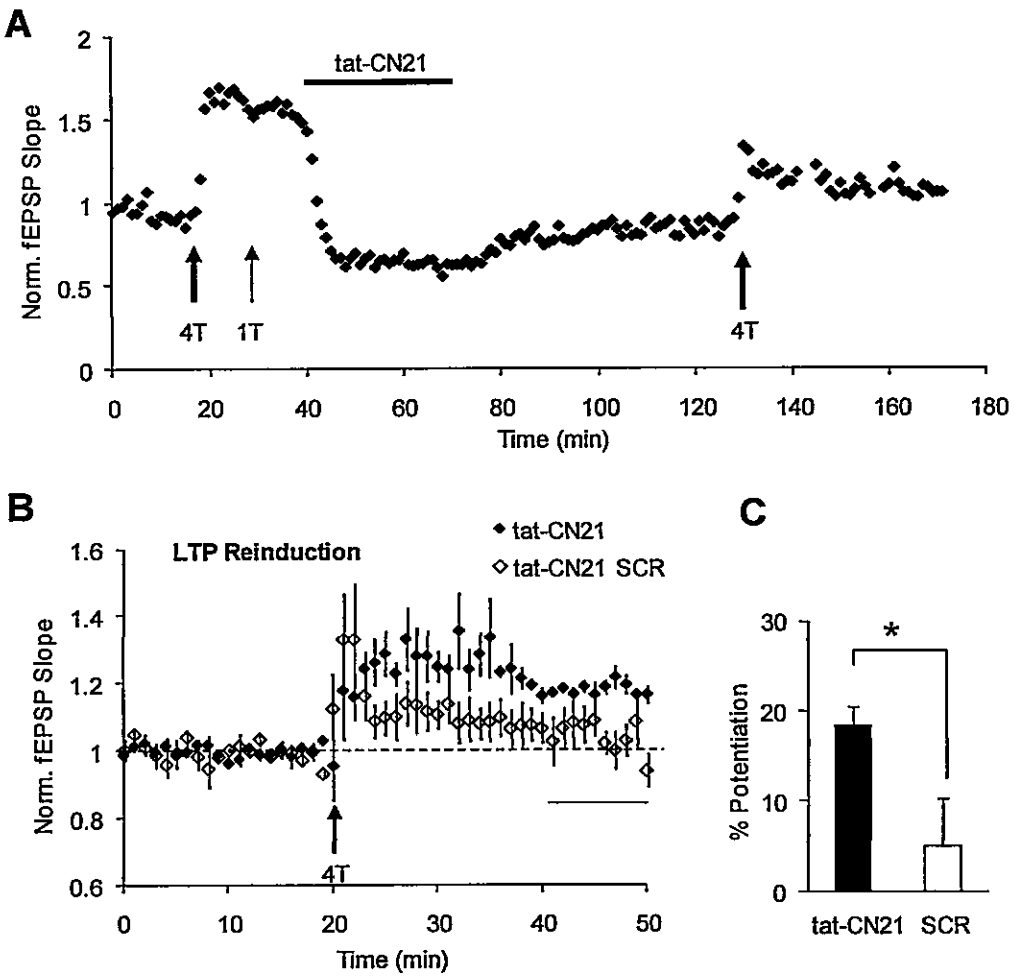


Figure. 3. TatCN21 reverses saturated LTP, allowing subsequent potentiation. ***A,*** Sample experiment in which saturated LTP was induced (by 4 tetani and 1 additional tetanus to show saturation). 20 ⍰M tatCN21 was next applied for 30 min, and drug was washed out for 1 h. Subsequently, LTP reinduction was tested. Similar experiments were conducted with SCR peptide (not shown). ***B,*** Average plot showing the effect of the second series of tetani applied after drug washout (n = 5, for tatCN21; n = 4, for SCR peptide). ***C,*** Summary plot of the magnitude of LTP for the data shown in B. After treatment with SCR peptide, tetanization induced no further potentiation, as fEPSP slope (average ± SEM = 4.9 ± 5.3%, for last 10 min, indicated by continuous line in B), was not statistically different as compared to 20 min baseline (p = 0.156, Wilcoxon rank test), indicating that LTP saturation was unaffected. LTP reinduction after tatCN21 was 18.5 ± 1.9 % (*, p = 0.011; Wilcoxon rank test).

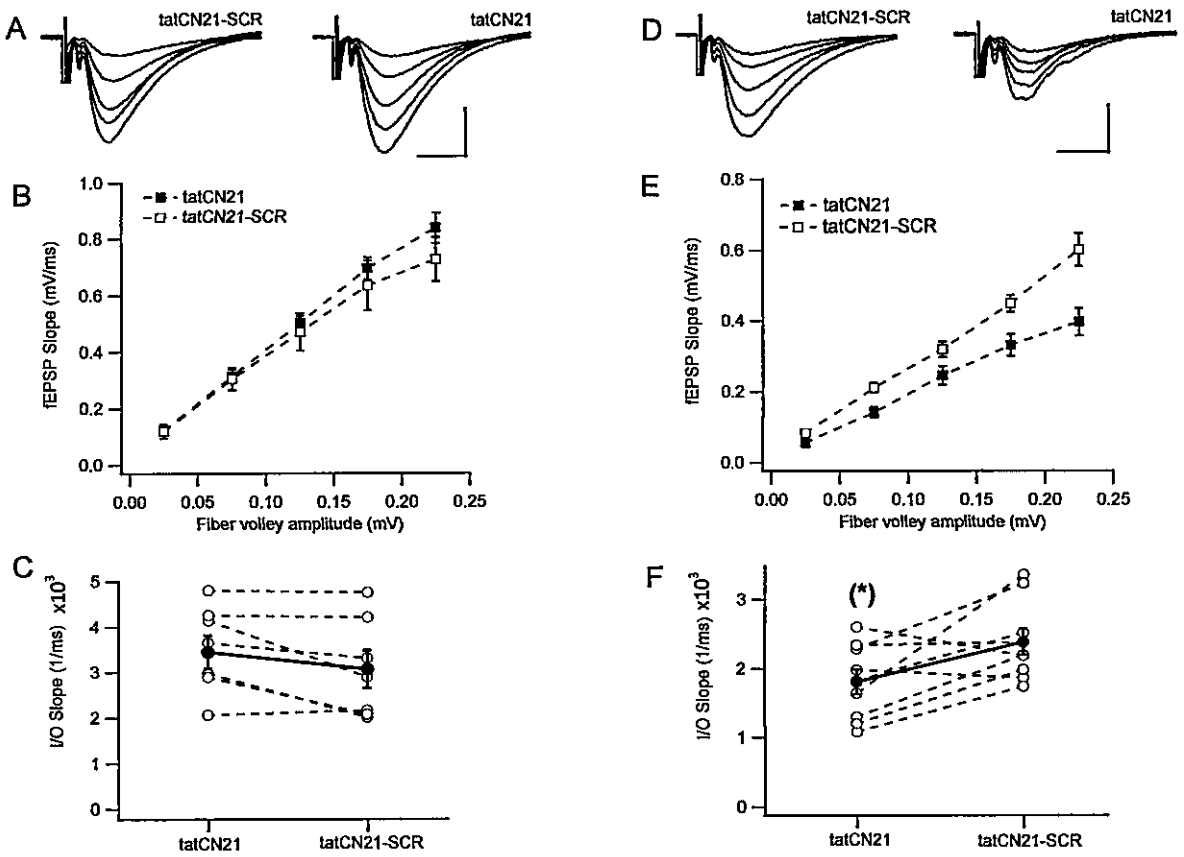


Figure. 4. TatCN21 persistently decreases synaptic strength at 20 □M (but not 5 □M). Acute hippocampal slices were incubated with tatCN21 or tatCN21 SCR peptide and washed with ACSF for 1 h before being transferred to the recording chamber. *(A-C)* TatCN21 (5 □M for 1 h) does not affect synaptic strength. ***A,*** Superimposed fEPSP obtained for increasing stimulation after tatCN21 SCR or tatCN21 treatment. ***B,*** Average input-output (I-O) curves. ***C,*** Each pair represents mean test and control I-O curve slopes from the same animal (filled symbols: average I-O slope ± SEM: 3447.8 ± 968.6 (1/ms), for tatCN21 and 3068.6 ± 1095.8 (1/ms) for tat-CN21 SCR; p = 0.157, paired t test; data from 7 animals, 2-4 slices per treatment). ***D-F,*** Longer incubation with a higher tatCN21concentration (20 µM for 2 h) persistently decreased synaptic strength. ***D, E, F,*** Same as A, B, and C for the present treatment (average I-O slope ± SEM: 1805.8 ± 542.8 (1/ms) for tat-CN21, 2386.0 ± 571.1 (1/ms) for tatCN21 SCR; data from 9 animals, 2-4 slices per treatment). *: $p < 0.05$. Calibration: 0.5 mV, 5 ms.

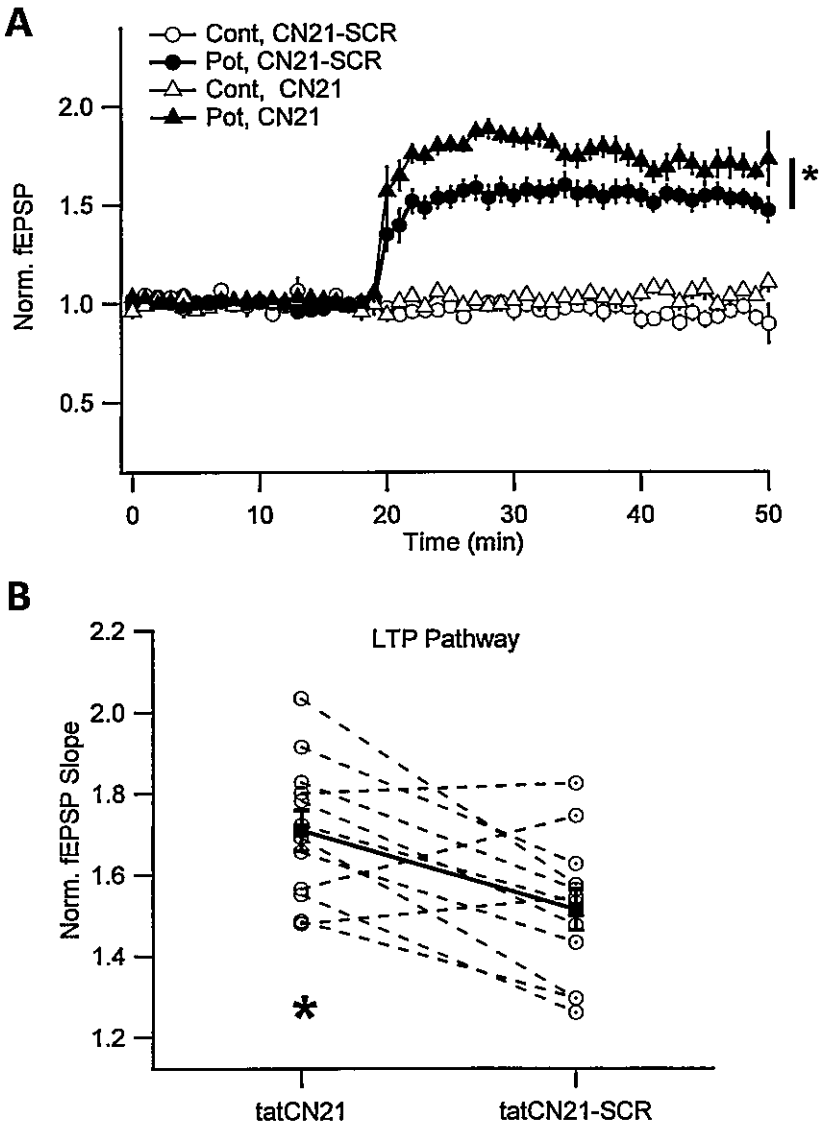


Figure. 5. Transient treatment with tatCN21 allows higher subsequent potentiation of basal transmission. ***A,*** Average plots for LTP induction after preincubation with tatCN21 or SCR peptide (20 μM for 2 h; 1 h drug washout). ***B,*** Each pair represents normalized fEPSP slope in potentiated pathway after tatCN21 or SCR peptide treatment of slices from the same animal (averaging interval: last 10 min). Filled symbols: average ± SEM ($p = 0.0045$, paired t test). Data from 12 animals.

APPENDIX

DISCUSSION OF OUR COLLABORATORS WORK

CaMKII is present both presynaptically and postsynaptically, and both pools have important functions (Lisman, 2003; Hojjati et al., 2007). It has been argued (Sanhueza et al., 2007)that the persistent effects of bath-applied CN are postsynaptic, given that similar effects could be seen in experiments in which the postsynaptic cells were excited by AMPA application. The results here shown by our collaborators strengthen this conclusion by showing that postsynaptic expression of CN19 reduces basal transmission. Similar results (although not in APV) have been reported previously for CN27 (Goold and Nicoll, 2010). In contrast, Zhou et al. (2007) did not observe a significant effect on basal synaptic transmission by NR2B C-tail expression in transgenic mice. However, the variance of the measurements in Zhou et al (2007) is on the same order as the effect he observed (~20%) and so may have been undetected.

Our Collaborators also found that CN compounds produce a reduction of the bound CaMKII content of spines. This is consistent with the hypothesis that CN reduces the CaMKII/NMDAR complex in the PSD. The magnitude of the reduction (10-15%) is roughly consistent with FRAP, as well as photoactivation experiments showing that only ~15% of CaMKII in spines is tightly bound (Otmakhov et al., 2007) and protein measurements indicating

that ~10% of spine CaMKII is in the PSD (Feng, Lisman unpublished observations). A major fraction of spine CaMKII is weakly bound to actin (Shen and Meyer, 1999; Okamoto et al., 2004).

METHODS USED BY OUR COLLABORATORS

Immunoprecipitation and immunoblotting. After treatment, hippocampal slices were shock frozen and later extracted with 1% deoxycholate, cleared by ultracentrifugation, and analyzed by IP and quantitative IB with antibodies against CaMKII□, NR1, NR2B, and □-actinin as described in (Leonard et al., 1999).

Slice culture preparation. Hippocampal slice cultures were prepared from postnatal day 6 (P6) – P7 Sprague Dawley rats in accordance with the animal care protocol of the Brandeis University. In sterile conditions, the rats were decapitated, the brain was removed, and 260 µm hippocampal slices were prepared using a Leica VT 1000S vibratome. Sterile, cold (4°C) modified artificial CSF (ACSF) saturated with 95% O2 and 5% CO2 was used during the preparation. Modified ACSF contained the following (in mM): 248 sucrose, 4 KCl, 1 CaCl2, 5 MgCl2, and 26 NaHCO3, pH 7.3, 320 mOsm. Slices were plated on the membrane of six-well plate inserts (Falcon; 0.4 µm pore size) and incubated in a CO2 incubator at 36°C in culture medium containing MEM (Cellgro 50-019-PB) supplemented with the following: 20% horse serum (Sigma), 1 µg/ml insulin, 1 mM glutamine (or glutaMAX), 30 mM HEPES, 1 mM CaCl2, 2 mM MgSO4, 5 mM NaHCO3, 16.5 mM D-glucose, and 0.5 mM ascorbate. The medium was changed three times per week.

Field Potential recordings in slice cultures. A single cultured slice was cut out from the cell culture insert, the connections between CA1 and CA3 were cut, and the slice was placed in the recording chamber mounted on the microscope stage. Slices were continuously superfused (2.5-3 ml/min) with ACSF containing the following (in mM): 124 NaCl, 2.5 KCl, 2.5 CaCl2, 1.3 MgSO4, 1.25 NaH2PO4, 26 NaHCO3, and 20 D-glucose. In imaging experiments, ACSF was also supplemented by 200 mM Trolox to decrease fluorescence bleaching and related cell damage. In those experiments, the concentration of dextrose was lowered (10 mM) to maintain an osmolarity of 320 mOsm. Before entering the chamber, the ACSF was continuously bubbled with 95% O2 and 5% CO2. The experiments were conducted at room temperature. For field potential recordings, electrodes (glass pipettes filled with ACSF; 300 kΩ resistance) were positioned in the stratum radiatum of the CA1 region. To stimulate two independent inputs, two monopolar stimulating electrodes were positioned to each side of the recording electrode. For imaging experiments with field potential recordings, only one stimulating electrode was used. Single shock stimuli (a square pulse of 50-100 μA for 70-150 μsec duration) were delivered every 30 or 60 seconds. When two stimulating electrodes were used, test stimulation alternated between the two electrodes.

Single cell electroporation. After 7-15 days in vitro, slice cultures were transfected with cDNA containing coding sequences of CaMKIIα fused with that of monomeric green fluorescent protein (GFP-CaMKII) and monomeric red fluorescent protein cherry (RFP). GFP-CN19 plasmid was constructed by inserting CN19 sequence (Coultrap and Bayer, unpublished observations) into EGFP-C1 vector (Clontech, Inc) between BamH1 and XbaII restriction sites. The transfection was performed using single cell electroporation technique (Haas et al., 2001; Pi et

al., 2010). A single cultured slice was cut out from the cell culture insert and placed in electroporation buffer in the chamber (similar to the recording chamber used for imaging) mounted on the microscope stage. The electroporation buffer contained the following (in mM): 160 NaCl, 5.4 KCl, 12 MgCl2, 2 CaCl2, 5 HEPES; pH 7.4; Osmolarity 320 mOsm. Glass pipettes (3-4 MΩ resistance) were filled with DNA diluted in electroporation buffer, and pyramidal cells in the CA1 were transfected by approaching the cell membrane and delivering square pulses of 10 V of 1 ms duration at 200 Hz for 500 ms. Slices were then briefly washed in electroporation buffer and placed back onto the insert membrane and incubated in a CO2 incubator in standard culture medium. Slices were used for imaging 1-3 days after the transfection.

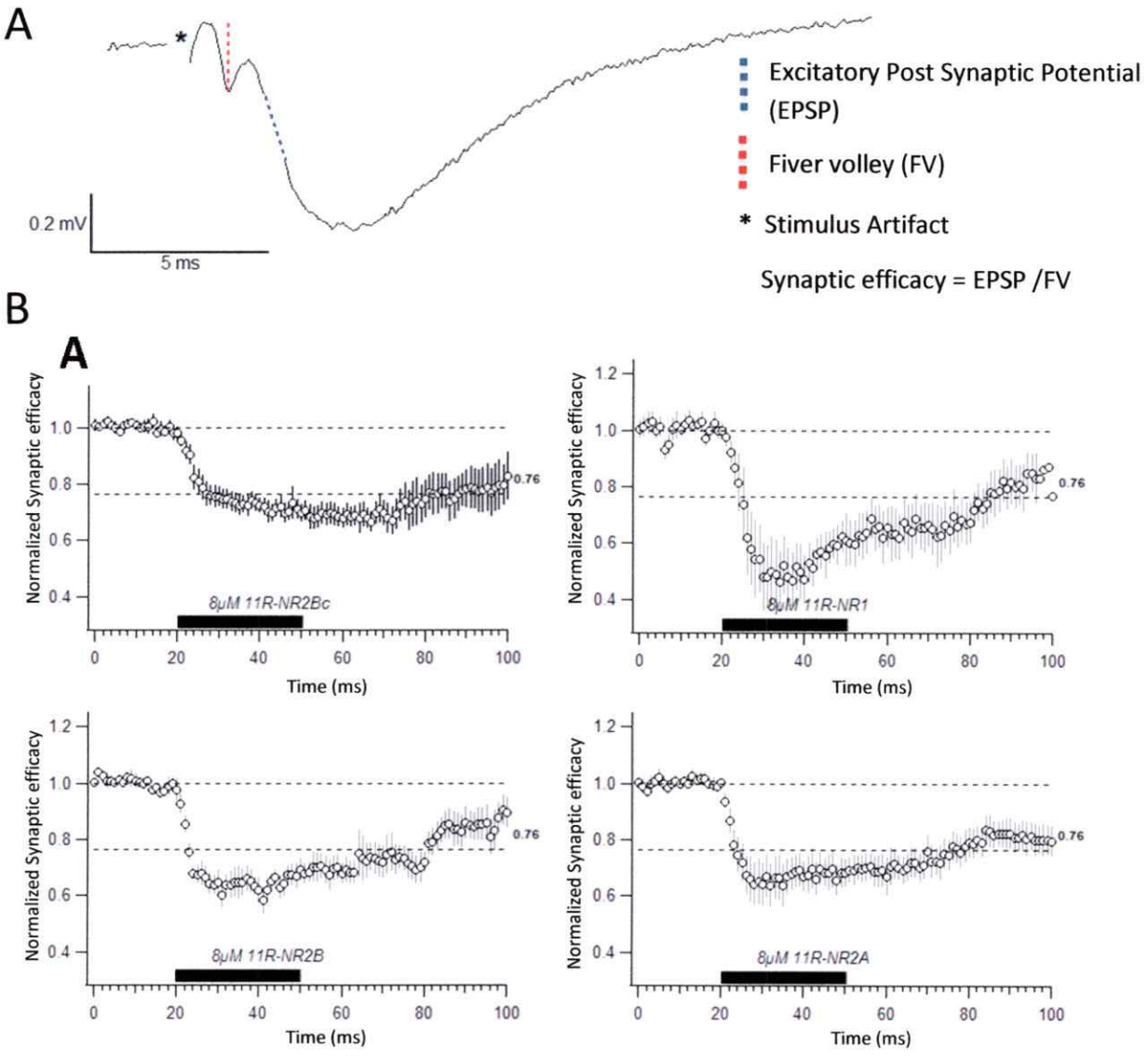
Imaging and Image Analysis. Imaging experiments were performed using a Leica confocal microscope with Ar-Kr lasers. Green and red images were taken consecutively at each focal plane using 488 nm and 543 nm excitation wavelengths and 500-550 and 590-650 emission, respectively. Stacks of 10-15 images (256 x 256, 0.093 nm pixel size, 3 frame averages) with 0.5 μm focal steps were collected. Experiments were analyzed using IGOR Pro custom software. To eliminate background fluorescence, a binary mask was created for each image in a given stack of z-plane images. The mask was constructed to cover all the pixels below an arbitrarily chosen threshold equal to double of the background fluorescence. Regions of interest (ROIs) were drawn around spines and nearby dendrites. For each ROI, the in-focus z-plane was selected from the z-stack and the fluorescence of pixels within the selected region, and not covered by the mask, was measured. All of the data are presented as mean ± standard error. Statistical significance was calculated using two population t-test (independent or paired) or unbalanced two-way ANOVA.

Calculation of bound GFP-CaMKIIα in spines and spine size. The calculation of the bound amount (BA) and bound fraction (BF) was as in (Otmakhov et al., 2004). Briefly, if all the GFP-CaMKIIα in spines is soluble, then the ratio of green fluorescence in spines to green fluorescence in dendrites [(s/d)G] should be equal to the ratio of red fluorescence in spines to red fluorescence in dendrites [(s/d)R]. A ratio of (s/d)G greater than (s/d)R is an indication of a bound pool of GFP-CaMKIIα in the spine. Therefore, the fluorescence of soluble GFP-CaMKIIα in the spines is estimated as: dG • (s/d)R. The amount of GFP-CaMKIIα bound in spines can then be calculated by subtracting the fluorescence of soluble GFP-CaMKIIα in spines from the total green fluorescence in spines: sG - dG • (s/d)R. One limitation in the calculations of bound CaMKII in spines is that it is based on the assumption that all CaMKII in the dendritic region is soluble. Although there are some data supporting this view (Shen and Meyer, 1999), other studies indicate that some CaMKII may also be bound in dendrites. Our unpublished data indicate that the dendritic bound amount of CaMKII is about 10-fold smaller than that in spines. Therefore, the method of calculation of bound CaMKII in spines may have around a 10% error. The fraction of GFP-CaMKIIα bound in the spine is represented by the bound amount of GFP-CaMKIIα divided by the total green fluorescence in the spine: [sG - dG • (s/d)R] /sG or 1 – (d/s)G • (s/d)R. The spine size was measured using the method of (Holtmaat et al., 2005): the integral of red fluorescence in the spine ROI was measured and then adjusted to red fluorescence in the dendrite.

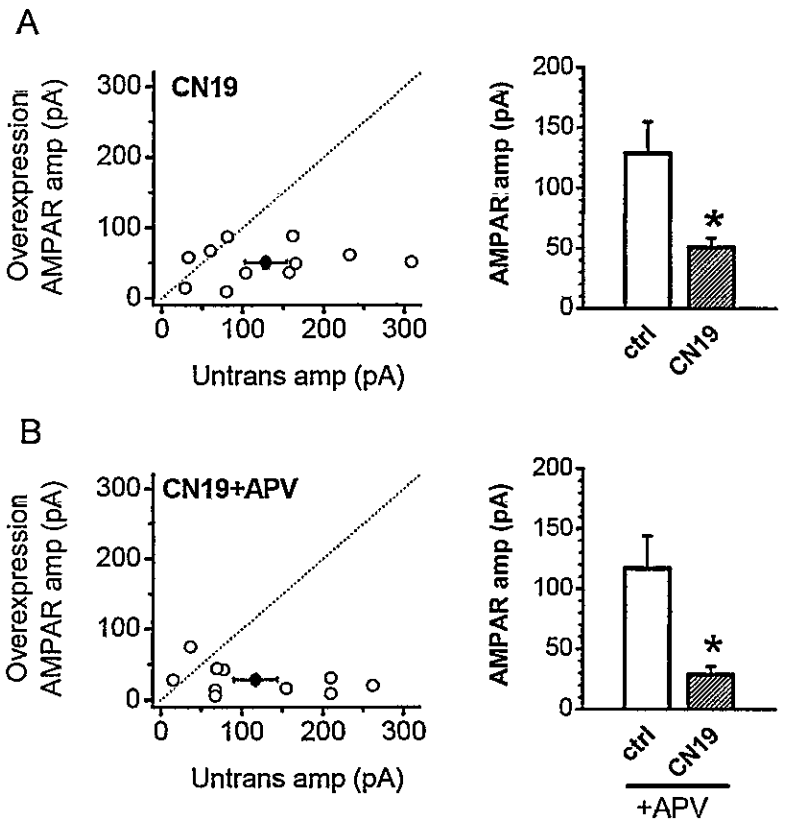
Whole-cell recordings. Whole-cell recordings were performed using standard methods as previously described. Slices were continuously superfused (1.8 ml/min) with artificial CSF (ACSF) containing the following (in mM): 124 NaCl, 2.5 KCl, 4 CaCl2, 4 MgCl2, 1.25, NaH2PO4,

26 NaHCO3, and 0.05 picrotoxin, balanced with 95% O2 and 5% CO2, pH 7.4. Whole-cell recordings were performed at room temperature (22–24°C). The pipette solution contained (in mM) 120 Cs Methane Sulfonate, 20 CsCl, 10 HEPES, 4 MgATP, 0.3 Na3GTP, 0.2 EGTA, and 10 Na2-phosphocreatine, pH 7.3; osmolarity, 320 mOsm. EPSCs were evoked by stimulating the Schaffer-collateral pathway via a stimulation electrode placed at 100-200 μm from cell body layer and measured in voltage-clamp mode at -65 mV. For cell pair recordings, two neurons (one transfected with GFP-CN19 plasmid and one nontransfected) within 30 μm were selected. The test stimulation was delivered every 6 s with 0.2 ms duration, and the current intensity was adjusted to induce 50–100 pA EPSC. After adjusting stimulation strength in one neuron, sequential recording was performed in the other neuron with the same intensity and location of stimulation. EPSC magnitudes were calculated by averaging a 5 msec time window centered at the peak of the EPSC, and 20-50 EPSCs were averaged. Series and input resistance were monitored throughout each experiment, and recordings for which series resistance varied by more than 20% were rejected. In some experiments, 100 μM DL-2-amino-5-phosphono-valeric acid (APV) were added immediately after transfection and were present for the following 2 days; after 2 days, the recordings were performed in ACSF without the APV.

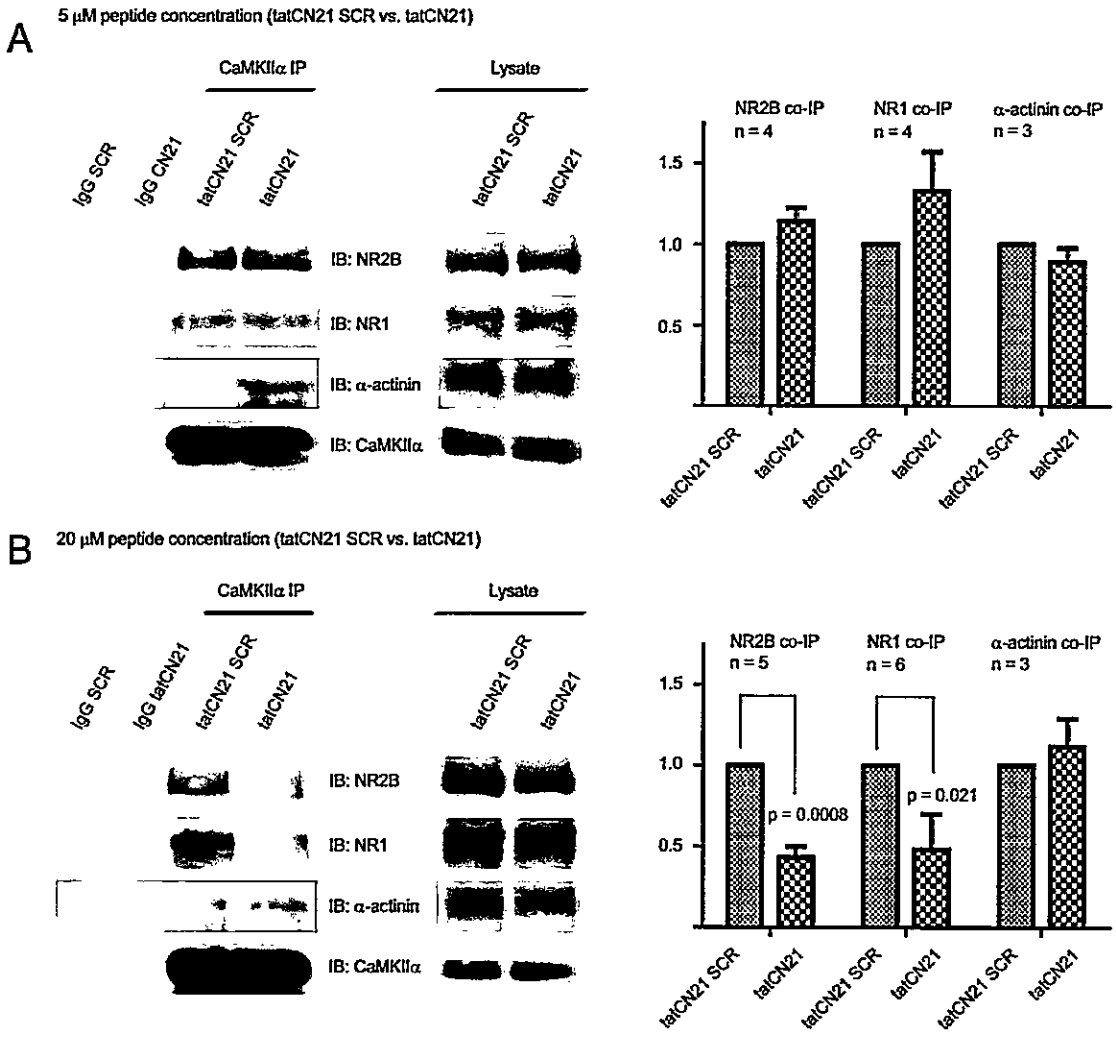
APPENDIX FIGURES



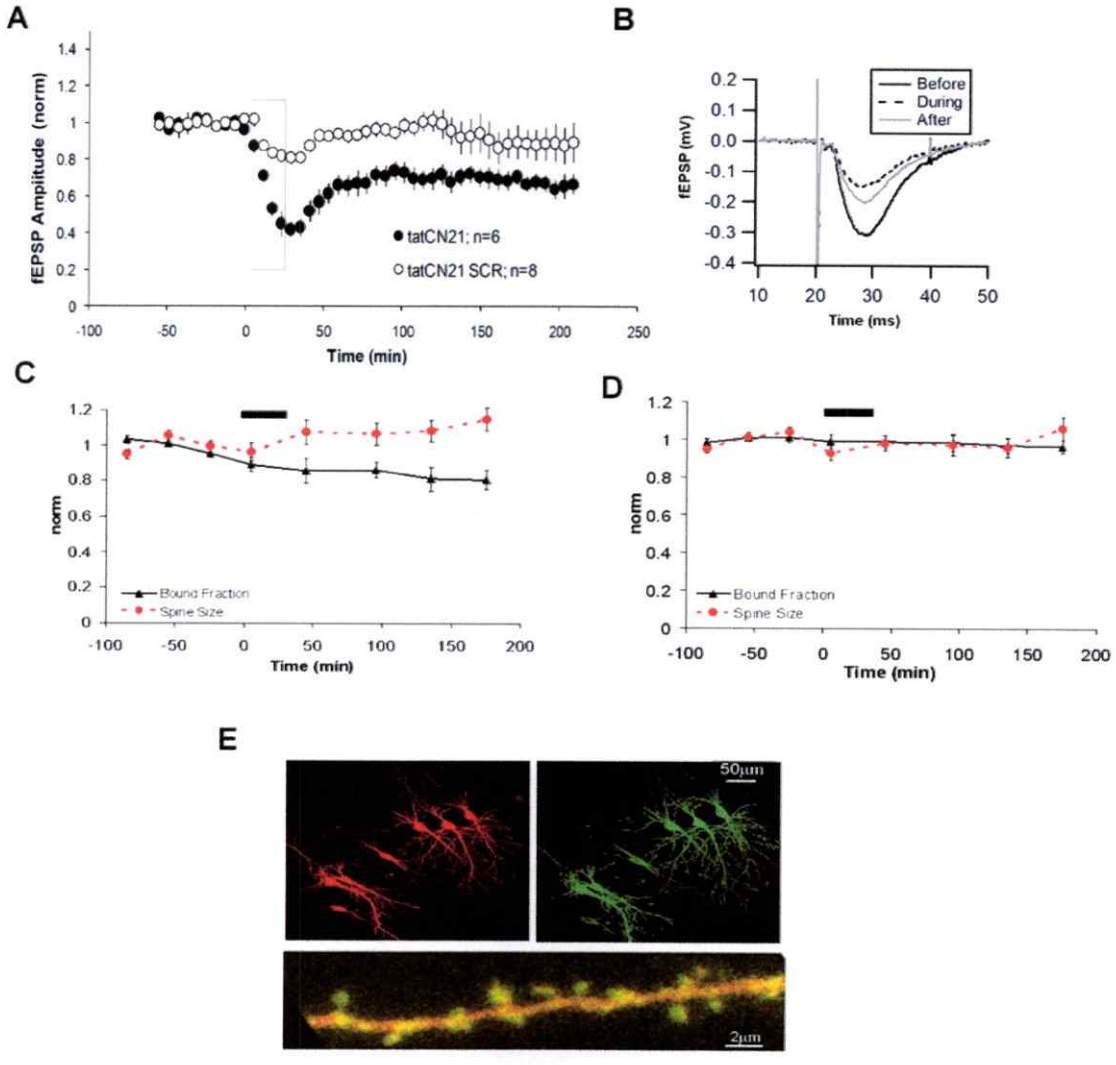
Appendix Fig.1. Effect of 11R coupled peptides over synaptic efficacy. ***A,*** Definition of synaptic efficacy, the fiber volley (FV) is a quick deflection of the recorded potential; this signal is proportional to the discharge of the presynaptic fivers. It was measured as the amplitude of this quick deflection. The excitatory postsynaptic potential (EPSP) correspond to the second deflection of the extracellular potential, this is proportional to the postsynaptic fibers discharge, in this case the initial slope was measured. The synaptic efficacy is defined as proportional presynaptic response to presynaptic stimulus. ***B,*** Synaptic efficacy was monitored before, during, and after transient application (30 min) of 8 μM 11R-NR2Bc (Control, n = 6), 11R-NR1 (n=8), 11R-NR2B (n=6), 11RNR2A (n=7) (Mean every 1min ± SE).



Appendix Fig. 2. CN19 decreases synaptic transmission in an NMDAR-independent way. CA1 neurons from hippocampal slice culture were transfected to overexpress GFP-CN19 for 2 days. Cell-pair recordings were performed from a transfected neuron and a nearby untransfected neuron. ***A,*** AMPAR EPSCs were significantly decreased by the overexpression of GFP-CN19 (average ± SEM: 128.7 ± 26.2 vs. 50.6 ± 7.8 pA for untransfected and transfected cells, respectively; n = 11, p = 0.013 with paired student t test). ***B,*** Slices were incubated in 100 μM APV after transfection for 2 days, and AMPAR EPSCs were recorded with APV removed. CN19 still significantly decreased AMPAR EPSCs after APV treatment (117.0 ± 26.9 vs. 28.7 ± 6.6 pA for untransfected and transfected cells, respectively; n = 10, p = 0.017 with paired student t test).



Appendix Fig. 3. TatCN21 persistently disrupts the CaMKII/NMDAR complex at 20 µM (but not 5 µM). After the same preincubation procedure described in Fig. 5, slices were frozen for biochemical analysis. The NMDA receptor complex was solubilized with 1% deoxycholate before IP with control IgG or CaMKIIα antibody and IB for NR2B, NR1, α-actinin, and CaMKIIα. The immunosignals were quantified by densitometry, NMDAR and α-actinin signals divided by CaMKIIα signals to correct for any variability in CaMKII IP, and tat-CN21 values normalized to SCR values (100%). Bars: averages ± SEM. ***A,*** 5 µM tat-CN21 for 1 h does not affect basal CaMKII interaction with NMDAR (NR2B: CN21/SCR = 1.177 ± 0.076, p = 0.199, n = 4; NR1: CN21/SCR = 1.399 ± 0.135, p = 0.266, n = 4; averages ± SEM, paired t test) or α-actinin (CN21/SCR = 0.892 ± 0.088). ***B,*** 20 µM tatCN21for 2 h produces persistent reduction of basal CaMKII interaction with NMDAR (NR2B: CN21/SCR = 0.386 ± 0.068, p = 0.0008, n = 5; NR1: CN21/SCR = 0.508 ± 0.156, p = 0.021, n = 6), but not α-actinin (CN21/SCR = 1.083 ± 0.189).



Appendix Fig. 3. TatCN21 decreases synaptic transmission and bound fraction of CaMKII in dendritic spines in slice cultures. ***A,*** The graph shows a significant and persistent (for at least 3 h) decrease of fEPSP amplitude after application of tatCN21 (black symbols), while control tatCN21 SCR peptide was ineffective. ***B,*** Examples of average waveforms of fEPSP at different times during the experiment. ***C,*** Graphs showing that bound fraction of GFP-CaMKII in spines (black symbols) but not spine volume (red symbols) significantly and persistently decreases after application of tatCN21. ***D,*** CN21 SCR peptide did not affect either bound fraction (black symbols) or spine volume (red symbols). Concentrations of both peptides were 20 μM. Gray bar indicates the period of drug application. Thin horizontal line shows a period taken for analysis of statistical significance between control and test experiments ($p < 0.001$). ***E,*** Top, A representative example of CA1 neurons transfected with RFP (left) and GFP-CaMKIIα (right). Images are maximum projection of 3D stack. Bottom, A representative image of a dendritic segment used for analysis in these experiments (this is an overlay of green and red fluorescence).