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Nampt is required for long-term depression and the function of GluN2B subunit-containing NMDA receptors

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Abstract

Nicotinamide adenine dinucleotide (NAD^+) is an essential coenzyme/cosubstrate for many biological processes in cellular metabolism. The rate-limiting step in the major pathway of mammalian NAD^+ biosynthesis is mediated by nicotinamide phosphoribosyltransferase (Nampt). Previously, we showed that mice lacking Nampt in forebrain excitatory neurons (*CamKIIαNampt*$^{-/-}$ mice) exhibited hyperactivity, impaired learning and memory, and reduced anxiety-like behaviors. However, it remained unclear if these functional effects were accompanied by synaptic changes. Here, we show that *CamKIIαNampt*$^{-/-}$ mice have impaired induction of long-term depression (LTD) in the Schaffer collateral pathway, but normal induction of long-term potentiation (LTP), at postnatal day 30. Pharmacological assessments demonstrated that *CamKIIαNampt*$^{-/-}$ mice also display dysfunction of synaptic GluN2B (NR2B)-containing N-methyl-D-aspartate receptors (NMDARs) prior to changes in NMDAR subunit expression. These results support a novel, important role for Nampt-mediated NAD^+ biosynthesis in LTD and in the function of GluN2B–containing NMDARs.

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Conflict of interest:

S.I. is a co-founder of Metro Midwest Biotech. C.Z. serves on the scientific advisory board of Sage Therapeutics. L.R.S. and Y.I. declare no competing financial interests.

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Keywords

NAD+; Nampt; Long-term depression; NR2B; GluN2B; N-methyl-D-aspartate receptor

1. Introduction

Nicotinamide adenine dinucleotide (NAD^+) is an essential coenzyme/cosubstrate in multiple metabolic reactions, including glycolysis and oxidative phosphorylation [30, 49, 61, 78]. While there are several pathways of NAD^+ biosynthesis, most mammalian cells generate NAD^+ from nicotinamide [30, 78]. The rate-limiting step of this pathway is performed by nicotinamide phosphoribosyltransferase (Nampt) [65]. Recently, we found that mice lacking Nampt in forebrain excitatory neurons of the hippocampal CA1 subregion and cortical layers II/III (*CamKIIαNampt*$^{-/-}$ mice) exhibited hyperactivity, impaired learning and memory, and reduced anxiety-like behaviors at 2–3 months of age [79]. And yet, at postnatal day (P) 60, *CamKIIαNampt*$^{-/-}$ mice had intact tetanic long-term potentiation (LTP), a form of synaptic plasticity considered to be an electrophysiological correlate of learning and memory [20]. Both spatial memory deficits and decreased expression of the immediate early genes *Egr1* and *Arc*, which were exhibited by *CamKIIαNampt*$^{-/-}$ mice, have been strongly linked to LTP [15, 40, 46, 83]. Thus, it was surprising that the spatial memory performance and loss of immediate early gene expression in *CamKIIαNampt*$^{-/-}$ mice were not intrinsically linked with LTP. Our findings raised an important question: does loss of Nampt in forebrain excitatory neurons have any synaptic manifestations?

Little is known regarding the importance of NAD^+ for synaptic transmission. Besides LTP, another form of synaptic plasticity is long-term depression (LTD), or a persistent decrease in synaptic strength [20, 43, 94]. Consolidation of hippocampal-dependent memory has been linked to N-methyl-D-aspartate receptor (NMDAR) dependent LTD in CA1 [7, 25, 91]. NMDAR activation modulates induction of LTP and LTD [94] and occurs during conditions of energy deprivation, such as hypoxia and hypoglycemia [13, 14, 26, 31, 81]. NMDARs are tetrameric receptors consisting of two obligatory NR1 (GluN1) subunits and two regulatory subunits, usually a combination of GluN2A (NR2A) and GluN2B (NR2B) [94]. Previous work found that mice lacking GluN2B function behave similarly to *CamKIIαNampt*$^{-/-}$ mice [79], with hyperactivity [1, 29, 85], memory impairments [7, 25], and reduced levels of anxiety-like behaviors [2, 7, 85].

Here, we show that *CamKIIαNampt*$^{-/-}$ mice exhibit a specific defect in induction of LTD in the Schaffer collateral pathway. Moreover, *CamKIIαNampt*$^{-/-}$ mice display insensitivity to pharmacological inhibition of synaptic GluN2B–containing NMDARs. These functional changes occur prior to decreased expression of hippocampal NMDARs and in the absence of changes in basal transmission or presynaptic mechanisms. Together, the constellation of phenotypes that we report in *CamKIIαNampt*$^{-/-}$ mice informs our understanding of the effects of NAD^+ depletion, a form of energy deprivation, on synaptic plasticity and function of NMDARs.

2. Materials and Methods

2.1 Animals

Mice were bred and maintained as described [79]. Briefly, *Nampt*$^{flox/flox}$ mice [71] were crossed to *CamKIIαCre* mice [The Jackson Laboratory, Stock #005359, T29-1 [84]] to generate *CamKIIαNampt*$^{-/-}$ mice. In all experiments, control mice were age-matched littermates. Since no sex differences were observed, both male and female mice were used. All animal procedures were approved by the Washington University Animal Studies Committee, Division of Comparative Medicine, Washington University School of Medicine, St. Louis, MO, and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised 1996. All efforts were made to minimize the number of animals used and their suffering.

2.2 Reagents

Chemicals were purchased from Sigma or Tocris (St. Louis, MO). Drugs were freshly dissolved in artificial cerebrospinal fluid (ACSF) at the time of experiment and administered by bath perfusion as noted in the text. The concentrations and durations of drug administration were based on prior studies indicating that the agents are effective at altering synaptic transmission or synaptic plasticity as administered.

2.3 Western Blotting

Protein was isolated and analyzed as previously described [79]. Briefly, protein extracts (15–50 μg) were prepared from acutely isolated mouse hippocampi flash frozen in liquid nitrogen, and stored at −80C until use. Membranes were incubated with primary antibodies in Tris-buffered saline containing 0.1% Tween 20 (TBST) overnight at 4°C. Primary antibodies used: Gapdh (1:1000; Millipore, CB1001, 6C5), Nampt (1:3000; Alexis Biochemicals, ALX-804-717-C100, mouse), GluN2B (1:1000; NeuroMab, N59/36, mouse).

2.4 Quantitative real-time RT-PCR

RNA was isolated and analyzed as previously described [79]. Briefly, mouse hippocampi were flash frozen in liquid nitrogen and stored at −80 C. Total RNA was isolated from the hippocampus using the RNeasy kit (Qiagen) and reverse-transcribed into cDNA with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative real-time RT-PCR was conducted with the TaqMan Fast Universal PCR Master mix and appropriate TaqMan primers in the GeneAmp 7500 fast sequence detection system (Applied Biosystems). Relative expression levels were calculated for each gene by normalizing to levels of Gapdh and then to a control.

2.5 Immunohistochemistry

Mice were anesthetized by intraperitoneal injection of ketamine and xylazine, and perfused transcardially through the left ventricle with cold phosphate buffer (0.1 M, pH 7.4) followed by a phosphate-buffered solution of 4% paraformaldehyde (PFA). Brains were postfixed with 4% PFA overnight, equilibrated in 15% sucrose overnight, equilibrated in 30% sucrose overnight, frozen, and stored at −80°C until sectioning. 30 μm coronal sections in a 1 in 8

series were made by cryostat and stored at −30°C in cryoprotectant until use. Every eighth section was processed. Tissue sections were incubated in 50% formamide in 2x saline/sodium citrate (SSC) at 65°C for 2 h and incubated with 3% H2O2 for 15 min to remove endogenous peroxidase activity. Tissue sections were incubated in blocking/permeabilization solution containing 10% normal goat serum, 1% bovine serum albumin (BSA), and 0.3% Triton-X in PBS for 45 to 60 min prior to 24 or 48 h of incubation with primary antibodies in 5% normal goat serum and 0.1% Triton-X in PBS at 4°C at the following concentrations: Iba1 (1:500; Wako, #019–19741, rabbit), Gfap (1:1000; Millipore, MAB360, mouse), Nampt (1:1000; Alexis Biochemicals ALX-804-717-C100, mouse), GluN2B (1:10; NeuroMab, N59/36, mouse). Antibody specificity was determined by lack of staining after omission of primary or secondary antibodies. Alexa647 (1:200), Alexa488 (1:200), or Cy3 (1:400) conjugated-secondary antibodies (Jackson ImmunoResearch) diluted in 2% normal goat serum, 1% BSA, and 0.1% Triton-X in PBS were added for 2 h at room temperature. Detection of Nampt and GluN2B was performed using the TSA-Plus kit (PerkinElmer, Boston, MA). Nuclei were stained with 4,6-diamidino-2-phenylindole (Sigma) for 10 min at room temperature. High-magnification (20x, 0.8DICII or 40x oil 1.3DICII) microscopic imaging was performed using a Zeiss Axioimager.Z1 or an Olympus NanoZoomer 2.0-HT. Images were taken in z-stacks of 1 μm steps through the range of tissue section immunoreactivity. ImageJ was used to 3D render z-stacks.

2.6 Hippocampal slice physiology

Hippocampal slices were prepared from postnatal day 30 (P30) mice using standard methods [79]. Mice were anesthetized with isoflurane and decapitated. Right hippocampi were rapidly dissected and placed in ACSF containing (in mM): 124 NaCl, 5 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 22 $NaHCO_3$, 10 glucose, gassed with 95% O_2–5% CO_2 at 4–6°C, and sectioned transversely into 400 μm slices using a rotary slicer. Slices were incubated in gassed ACSF for at least 2 h at 30°C. Experiments were performed in a submersion-recording chamber at 30°C with continuous perfusion of ACSF (2 ml/min). Extracellular recordings were obtained from the CA1 apical dendritic region (*stratum radiatum*) for analysis of field excitatory postsynaptic potentials (fEPSPs) using 2 M NaCl glass electrodes (5–10 MΩ). Responses were elicited with 0.1 ms constant current pulses through a bipolar electrode in the Schaffer collateral pathway. fEPSPs were measured by the maximal slope of their rising phase. A baseline (control) input-output curve was obtained to determine stimulus intensities for subsequent analyses. Input-output curves were generated using stimuli of 6 different intensities to allow determination of half maximal responses. The smallest stimulus was set to evoke a response less than half maximal while the largest stimulus was designed to evoke a fully saturated response. During an experiment, evoked fEPSPs were monitored by applying single stimuli to the Schaffer collateral pathway every 60 s at intensity sufficient to elicit half maximal responses. After establishing a stable baseline, LTD was induced by applying 1 Hz low frequency stimulation (LFS) to the Schaffer collateral pathway for 15 min (900 pulses), as previously described [38]. LTP was induced by a single 100 Hz × 1 s high frequency stimulus (HFS) using a stimulus of the same intensity, as previously described [8, 82]. Input-output curves were repeated 60 min after delivery of either LFS or HFS to determine the magnitude of LTD and LTP based on changes in half maximal responses. Isolated NMDAR synaptic responses were studied in an

extracellular solution containing 2 mM calcium and 0.1 mM magnesium. 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 30 μM, Sigma) was added to this solution to inhibit AMPA receptor-mediated fEPSPs, and responses were evoked once per min [32, 36]. fEPSPs were analyzed by measuring their rising slopes.

2.7 Statistical Analyses

Numerical data are presented as mean ± standard error of the mean (S.E.M.). Statistical significance was determined by unpaired Student's *t*-tests, Mann-Whitney U-tests in cases of irregular distribution, or Analysis of variance (ANOVA) tests in cases of more than two groups. Statistical analyses and plots of electrophysiology data were performed using Rstudio, SigmaPlot 5.01, SigmaPlot 9.0, and SigmaStat 3.1 (Systat Software Inc., Richmond, CA). *P*-values of less than 0.050 were considered significant.

3. Results

3.1 CamKIIαNampt$^{-/-}$ mice show impaired induction of LTD but not LTP

As previously shown [17, 38], low-frequency stimulation (LFS) of the Schaffer collateral pathway in P30 control mice resulted in induction of LTD (Fig. 1A, 64.2 ± 9.5% of baseline 60 min after LFS, n=5). However, the same treatment could not induce LTD in *CamKIIαNampt*$^{-/-}$ mice (103.0 ± 4.1%, n=5, p=0.006, Student's *t*-test). On the other hand, P30 control and *CamKIIαNampt*$^{-/-}$ mice exhibited normal induction of LTP in response to high-frequency stimulation (HFS, Fig. 1B), as we had previously shown for P60 control and *CamKIIαNampt*$^{-/-}$ mice [79].

A deficit in LTD induced by 1 Hz in the absence of a deficit in LTP induced by 100 Hz may be caused by several mechanisms. For example, synaptic strength could be depressed in *CamKIIαNampt−/−* mice. Alternatively, the optimal frequency for LTD induction may be altered in *CamKIIαNampt−/−* mice. To distinguish between these possibilities, we obtained full frequency/response curves in control and *CamKIIαNampt*$^{-/-}$ mice using 900 pulses administered at various frequencies (Fig. 1C; 1, 3, 10, 30, and 100 Hz; n=5). As expected, there was a significant effect of stimulation strength [Hz, $F(4,45)=7.85$, $p=0.00009$, ANOVA] on the percentages of evoked LTP and LTD. However, besides the loss of LTD at 1 Hz in *CamKIIαNampt*$^{-/-}$ mice, there was no significant difference between genotypes [$F(1,48)=0.61$, $p=0.438$, ANOVA]. Thus, the major defect in synaptic plasticity in *CamKIIαNampt*$^{-/-}$ mice involves a loss in the ability of low frequency Schaffer collateral pathway stimulation to induce LTD.

Importantly, at P30, *CamKIIαNampt*$^{-/-}$ mice lacked the weight loss (Fig. 2A, n=4–5, p=0.92, Student's *t* -test), astrogliosis (Fig. 2B,C, *Gfap*, n=4–5, p=0.23, Student's *t*-test), or microgliosis (Fig. 2B,C, *Iba1*, n=4–5, p=0.60, Student's *t*-test) that they displayed at 2–3 months of age [79]. Hematoxylin and eosin (H&E) staining (Fig. 2D,E) and immunofluorescence for Dapi (Fig. 2C) confirmed the lack of the overt cell loss in the pyramidal cell layer that we had observed at 2–3 months of age [79].

3.2 CamKIIαNampt$^{-/-}$ mice show impaired GluN2B–containing NMDAR-mediated fEPSPs

LTD of the Schaffer collateral-CA1 synapse induced by 1 Hz LFS depends on NMDARs [43]. Moreover, energy deprivation often damages the central nervous system by altering NMDAR activity [23, 33, 37, 62, 68]. Thus, we tested if the NAD+ deficiency present in *CamKIIαNampt*$^{-/-}$ mice altered NMDAR function and expression. Because GluN2B–containing NMDARs are known to contribute to LTD in CA1 [7, 32, 48, 94], we hypothesized that GluN2B–containing NMDAR activity was altered in *CamKIIαNampt*$^{-/-}$ mice. To test this, we treated hippocampal slices with ifenprodil, a selective antagonist of GluN2B–containing NMDARs [90], alone and in combination with low concentrations of D-2-amino-5-phosphonovalerate (D-APV), a broad-spectrum NMDAR antagonist. Although there are no reliable, selective antagonists against ifenprodil-insensitive NMDAR subtypes [4, 32, 89], low concentrations of D-APV (5 μM or less) have a limited preference for, and thus behave as an antagonist of, ifenprodil-insensitive NMDARs [9, 32, 36]. In all control mice tested, ifenprodil (10 μM) partially depressed NMDAR-mediated fEPSPs (Fig. 3A, 54.6 ± 7.6% compared to baseline, n=5). Subsequent co-administration of D-APV (2.5 μM) almost completely suppressed the remaining NMDAR-mediated fEPSPs (Fig. 3A, 4.7 ± 3.1%), as previously reported [32]. These observations indicate that GluN2B contributes to approximately 45% of synaptic NMDAR activity in P30 control slices, whereas the remainder is mediated by ifenprodil-insensitive NMDARs. In contrast, the depression induced by ifenprodil in *CamKIIαNampt*$^{-/-}$ mice was minimal (Fig. 3B, 95.4 ± 10.7% of baseline, n=5, p=0.006, Student's *t*-test). And yet, coadministration of D-APV (2.5 μM) successfully suppressed NMDAR-mediated fEPSPs (10.8 ± 2.4% of baseline), with the residual fEPSPs likely due to the fact that a minority of CA1 neurons remained positive for Nampt in P30 *CamKIIαNampt*$^{-/-}$ mice (Fig. 2C). Thus, it appears that fEPSPs mediated by NMDARs are present in *CamKIIαNampt*$^{-/-}$ mice, but these NMDARs do not display functional GluN2B subunits in response to synaptic stimulation. In support of this notion, in control mice, 2.5 μM APV treatment alone partially depressed NMDAR-mediated fEPSPs (Fig. 3C, 48.8 ± 3.7%, n=5). Since low concentrations of D-APV behave as an antagonist of ifenprodil-insensitive NMDARs [9, 32, 36], the partial depression of NMDAR-mediated fEPSPs induced by 2.5 ^M APV treatment is consistent with an intact GluN2B–containing NMDAR component. On the other hand, in *CamKIIαNampt*$^{-/-}$ mice, D-APV treatment alone suppressed NMDAR-mediated fEPSPs to a much greater extent than we observed in control mice (16.6 ± 4.2%, p<0.001, Student's *t*-test), suggesting a reduced functional GluN2B component. To verify these findings, we repeated this experiment with TCN 213 in place of ifenprodil. TCN 213 [N-(cyclohexylmethyl)-2-[{5 [(phenylmethyl)amino]-1,3,4-thiadiazol-2-yl}thio]acetamide] was initially thought to be a selective antagonist of GluN2A–containing NMDARs [5]. However, we recently showed that inhibition of NMDAR EPSPs by ifenprodil and TCN 213 largely overlapped in the CA1 region of hippocampal slices from 30-day-old rats [39]. We speculated that ifenprodil and TCN 213 may act on the same subset of GluN2A/GluN2B triheteromers [39]. Like ifenprodil, TCN 213 (10 μM) partially depressed NMDAR-mediated fEPSPs in all control mice tested (Fig. 3D, 50.9 ± 6.5% compared to baseline, n=5). Subsequent co-administration of D-APV (2.5 μM) suppressed the remaining NMDAR-mediated fEPSPs (Fig. 3D, 14.4 ± *7.3%*). These observations suggest that GluN2B contributes to approximately 48% of synaptic NMDAR activity in P30 control slices, which is similar to the 45% we noted with ifenprodil (Fig. 3A).

On the other hand, TCN 213, like ifenprodil, did not depress NMDAR fEPSPs in slices from *CamKIIαNampt*$^{-/-}$ mice (Fig. 3D, 101.6 ± 6.5% of baseline, n=5, p=0.0002, Student's *t*-test). And yet, co-administration of D-APV (2.5 μM) successfully suppressed NMDAR-mediated fEPSPs (13.3 ± 2.0% of baseline), further supporting our notion that *CamKIIαNampt*$^{-/-}$ mice have altered NMDAR function.

3.3 GluN2B expression is unaltered in P30 CamKIIαNampt$^{-/-}$ mice

Given the lack of responsiveness of *CamKIIαNampt*$^{-/-}$ mice to ifenprodil, we examined the mRNA expression of *GluN2B*. Quantitative RT-PCR analysis of hippocampal RNA samples showed no difference in *GluN2B* mRNA expression between P30 control and *CamKIIαNampt*$^{-/-}$ mice (Fig. 3A). While *GluN2B* mRNA expression was not altered at P30, it decreased with age, showing a 20% reduction by 6 months of age (n=3–4, p=0.076, Student's *t*-test) and a 27% reduction by 10 months of age (Fig. 4A, n=4–5, p=0.0004, Student's *t* -test). To determine if this change in *GluN2B* mRNA expression was specific, we also assessed age-related changes in other NMDAR subunits, *GluN2A* and *GluN1*, as well as two immediate early genes shown to have altered expression by 2–3 months of age [79], *Arc* and *Egr1*. Like *GluN2B*, *GluN2A* expression was not altered at P30, but decreased with age (Fig. 4B), showing 26% and 32% reductions by 6 (n=3–4, p=0.084, Student's *t*-test) and 10 months of age (n=4–5, p=0.039, Student's *t*-test), respectively. *GluN1* expression also significantly decreased with age (Fig. 4C), but at a lesser extent, showing 16% and 20% reductions by 6 (n=3–4, p=0.288, Student's *t*-test) and 10 months of age (n=4–5, p=0.009, Student's *t*-test), respectively. Expression of the immediate early genes *Arc* and *Egr1* were even more affected (Fig. 4D,E), showing an approximately 60% decrease at 6 (n=3–4, *Arc*: p=0.0006, *Egr1:* p=0.008, Student's *t*-test) and 10 months of age (n=4–5, *Arc*: p=0.0013, *Egr1:* p=0.083, Student's *t*-test). These data suggest that the change in GluN2B function at P30 is unlikely to result from transcriptional modifications and that the change in *GluN2B* mRNA expression with age is representative of broader hippocampal defects.

To determine whether GluN2B protein levels were altered in *CamKIIαNampt*$^{-/-}$ mice, we immunoblotted hippocampal extracts for GluN2B. However, control and *CamKIIαNampt*$^{-/-}$ mice showed similar protein levels of GluN2B (Fig. 4F,G). To examine whether the distribution of GluN2B was changed in *CamKIIαNampt*$^{-/-}$ mice, we performed immunohistochemistry for GluN2B on P30 brain sections. Control and *CamKIIαNampt*$^{-/-}$ mice appeared to show similar patterns of GluN2B protein distribution on hippocampal sections (Fig. 4H). Thus, loss of Nampt affects the function of GluN2B–containing NMDARs through a mechanism independent of global GluN2B expression profiles.

To assess if changes in GluN2B sensitivity involved alterations in presynaptic mechanisms, we assessed the paired pulse ratio by delivering two identical stimuli at an interval of 21 msec. We then used a baseline stimulus-response curve to calculate changes in the second fEPSP slope at a stimulus intensity that evoked a half maximal response for the first response (Fig. 5A). In this paradigm, the second fEPSP evoked by the second stimulus with the same intensity are usually bigger (>100%) than the half maximal fEPSP evoked by the initial stimulus. Such an occurrence is termed paired pulse facilitation. Similar paired pulse facilitation was seen in slices from control and *CamKIIαNampt*$^{-/-}$ mice (Fig. 5B, p=0.151,

Mann-Whitney Rank Sum Test). Thus, presynaptic mechanisms do not appear to be significantly affected in *CamKIIαNampt*$^{-/-}$ mice and cannot explain their insensitivity to LTD or GluN2B antagonists.

Having found that synaptic responses from *CamKIIαNampt*$^{-/-}$ mice lacked sensitivity to GluN2B antagonists despite normal expression of GluN2B and normal presynaptic function, we next investigated if GluN2B was properly targeted to synapses. To address this possibility, we administered DL-threo-β-benzyloxyaspartate (DL-TBOA), a potent, selective nontransportable inhibitor of excitatory amino acid transporters [16, 75], at 10 μM for 30 min. DL-TBOA blocks recycling of presynaptically-released glutamate and causes accumulation of glutamate in the synaptic cleft, thus enhancing 'spillover' and increasing the likelihood of extrasynaptic GluN2B activation [52, 92]. DL-TBOA did not depress basal response in *CamKIIαNampt*$^{-/-}$ mice (Fig. 5C, 111.8±6.5, n=5). However, in the presence of DL-TBOA, 1 Hz LFS induced LTD in *CamKIIαNampt*$^{-/-}$ mice (Fig. 5C, 65.9±7.5%, n=5). These data suggest that blocking glutamate uptake results in the induction of LTD by allowing glutamate released by LFS to activate receptors not normally activated during LFS.

4. Discussion

Here we report a novel assessment of Nampt, the rate-limiting enzyme in the major pathway of mammalian NAD^+ biosynthesis [65], in LTD and NMDAR-mediated synaptic transmission. We show that *CamKIIαNampt*$^{-/-}$ mice have impaired induction of LTD and function of GluN2B–containing NMDARs which manifest prior to changes in NMDAR subunit expression.

Why do *CamKIIαNampt*$^{-/-}$ mice exhibit intact induction of LTP but abolished induction of LTD? NMDARs can drive both LTP and LTD, but potentially in a subunit dependent manner [94]. The idea that subunit identity differentially affects synaptic plasticity is reasonable as GluN2A–containing NMDARs have faster rise and decay times than GluN2B–containing NMDARs [11, 18]. Several studies have shown that LTP requires GluN2A–containing NMDARs, but not GluN2B–containing NMDARs [48, 73, 77, 85]. However, we and others have found that NMDAR-mediated LTP is not subunit specific [4, 32, 89]. In contrast, many studies have shown that LTD involves GluN2B–containing NMDARs [7, 32, 36, 48, 94]. While not all studies support this functional dichotomy [3, 27, 45], our results are consistent with a link between LTD and GluN2B–mediated synaptic transmission. We show that *CamKIIαNampt*$^{-/-}$ mice have markedly dampened responsiveness to GluN2B antagonism at synapses, suggesting significantly reduced function of synaptic GluN2B–containing NMDARs. Moreover, a specific impairment of GluN2B synaptic transmission is consistent with the behavioral phenotypes of *CamKIIαNampt*$^{-/-}$ mice, including hyperactivity, impaired spatial memory and fear conditioning, and diminished anxiety-like behaviors [79]. Similar phenotypes are also seen in mice lacking functional GluN2B [1, 2, 7, 29, 85].

A selective impairment in LTD accompanied by intact LTP has been observed before [19, 32, 35, 43, 54]. However, the finding that *CamKIIαNampt*$^{-/-}$ mice have impaired induction of LTD but intact LTP was surprising since we previously showed that *CamKIIαNampt*$^{-/-}$

mice exhibit impaired spatial memory [79]. Traditionally, LTP has been considered a major contributor to hippocampal spatial memory [40, 43, 83]. In contrast, LTD has been given an auxiliary role in signal-to-noise regulation or in forgetting [43]. However, LTD may also contribute to hippocampal-dependent memory as mice with impaired LTD displayed impaired spatial memory [7, 19, 25, 43, 54, 55]. LTD may also be relevant to novelty detection, acquisition [42, 50], and habituation [19]. Thus, impaired LTD is consistent with the hyperactivity and lack of habituation displayed by *CamKIIαNampt*$^{-/-}$ mice [79].

Importantly, these phenotypes are not typical effects of energy deprivation. While P30 *CamKIIαNampt*$^{-/-}$ mice exhibit relatively normal CA1 population spikes and fEPSPs, other conditions involving energy deprivation, such as hypoglycemia [72, 81], anoxia [31, 33], or ischemia [60, 87] are associated with reduced or abolished population spikes and fEPSPs. Similarly, *CamKIIαNampt*$^{-/-}$ mice exhibit normal LTP, whereas hypoglycemia [37], anoxia [33], ischemia [13, 87], or pharmacological glycolytic blockade [34] can abolish LTP. LTD has been less studied in the context of energy deprivation. However, hypoxia and/or hypoglycemia can cause non-tetanic LTD [76] and depress synaptic transmission in CA1 neurons [23]. Similarly, reduced Na,K-ATPase activity, which occurs upon energy deprivation [53], induces, rather than abolishes, LTD [64]. Energy deprivation also occurs with NMDAR over-activation [13, 14, 26, 31, 33, 81]. In fact, GluN2B–containing NMDARs appear to play a key role in this over-activation as antagonism of GluN2B improved fEPSP recovery following hypoxia [68] and blocked non-tetanic LTP post-ischemia [62]. Metabolic inhibition also increased the expression of GluN2B in CA1 [10]. Thus, our analysis of *CamKIIαNampt*$^{-/-}$ mice suggests that NAD^+ depletion generates specific effects that differ from other forms of energy deprivation and/or that energy deprivation is not the primary cause of *CamKIIαNampt*$^{-/-}$ phenotypes.

What could be the potential cause of *CamKIIαNampt*$^{-/-}$ phenotypes? One possibility would be the accumulation of Nampt's substrate, nicotinamide. However, nicotinamide appears to have unrelated effects from those we find upon deletion of Nampt. In particular, bath applied nicotinamide blocks LTP [44, 74] and presynaptic injection of nicotinamide reduces basal transmission without affecting LTP [59]. Moreover, in preliminary studies, we found that application of nicotinamide (200 μM, 15 min) prior to LFS did not inhibit LTD (58.0 ± 8.1%, n=3). Thus, accumulation of nicotinamide is unlikely to play a causal role.

Another possibility is the functional defect in key mediators produced from NAD^+ or requiring NAD^+. One potential mediator is cyclic ADP (cADP)-ribose. cADP-ribose is a second messenger hydrolyzed from NAD^+ by cADPR-ribose synthases (CD38 and CD157) [78], and cADPR-mediated signaling pathways play a key role in LTD induction [66, 67]. Induction of LTD begins with calcium entry via NMDARs and this calcium influx activates CD38, which produces cADP-ribose from NAD^+. cADP-ribose binds ryanodine receptors, thus modulating calcium release from intracellular stores that is necessary for induction of LTD [66, 67, 69]. In addition to cADP-ribose synthases, NAD^+ is a substrate for poly(ADP-ribosyl) polymerases (ie. Parp1) and sirtuins (ie. Sirt1) [30, 78]. However, to our knowledge, neither factor has been assessed in LTD. Therefore, further investigation will be necessary.

The age-dependent decline in the expression of the immediate early gene *Arc* could be a possible cause, as genetic reduction of *Arc* reduced LTD [63]. While *Egr1* mRNA expression also declined with age, *Egr1* appears to be more important for LTP than LTD [12, 88]. Both *Egr1* [41, 58] and *Arc* [70] are upregulated after ischemia and/or hypoxia, suggesting that their downregulation in *CamKIIαNampt*$^{-/-}$ mice is a specific change.

Currently, the possibilities outlined above do not provide a direct connection to the impaired GluN2B–containing NMDAR function exhibited by *CamKIIαNampt*$^{-/-}$ mice. Although we show that NMDAR subunit mRNA expression significantly decreases with age in *CamKIIαNampt*$^{-/-}$ mice, GluN2B mRNA and protein levels were not altered at P30. Moreover, the immunostaining profile of GluN2B did not reveal an overt difference. Thus, the reduced activity of GluN2B in P30 *CamKIIαNampt*$^{-/-}$ mice likely resulted from something besides GluN2B expression levels. Nevertheless, it is important to acknowledge that immunohistochemical analysis was performed on slices from mice anesthetized with ketamine. Some have found that ketamine down-regulated GluN2B in rat forebrain culture [21] and upon subcutaneous injection in neonatal mice [47]. However, these effects were shown with ketamine exposure for 12 hours [21] and upon reaching adulthood [47], respectively. We sacrificed our mice within 15 minutes of intraperitoneal ketamine administration, which is scant time for ketamine to reach the hippocampus and alter GluN2B expression. Thus, we perceive it to be unlikely that use of ketamine affected our results.

One potential explanation is that the trafficking of GluN2B is altered in *CamKIIαNampt*$^{-/-}$ mice. It is still possible that GluN2B subunits were present, but intracellular or extrasynaptic [93]. This notion is supported by our finding that in the presence of the glutamate uptake inhibitor, DL-TBOA, 1 Hz stimulation induced LTD in *CamKIIαNampt*$^{-/-}$ mice. Indeed, other studies have found that spillover of glutamate released by LFS results in the induction of GluN2B–dependent LTD [51]. While it is currently difficult to distinguish between synaptic and extrasynaptic NMDARs, others have defined extrasynaptic receptors as those that are not activated by 1 Hz LFS unless a glutamate uptake blocker is applied [51]. This definition implies that the GluN2B–containing NMDARs in *CamKIIαNampt*$^{-/-}$ mice are extrasynaptic. As such, future work with surface biotinylation, measurements of intra- and extra-synaptic receptors, and electron microscopy are prime areas for future pursuit. Another potential explanation is that GluN2B is correctly localized, but inactive. NMDAR function is dependent upon intracellular oxidation-reduction state [6], which could change upon loss of NAD^+ biosynthesis. Thus, maybe oxidation-reduction state can inactivate GluN2B subunits. It should be noted that GluN2B–containing NMDARs are vulnerable to a variety of insults [22, 35]. Thus, an alterative explanation is that rather than a specific effect on GluN2B, GluN2B function is a particularly sensitive synaptic component.

In any genetic model, effects could result from compensatory changes during development. However, with the *CamKIIα*Cre driver, recombination begins in CA1 at P14–18 and plateaus at P28 [56, 84, 86]. Thus, the *CamKIIα*Cre driver avoids any compensatory changes during embryonic and early postnatal development and is therefore a preferred Cre driver [24]. Other models for inducible genetic deletion also have caveats. For example, the tetracycline regulatory system requires long-term exposure to doxycycline or tetracycline just as the Cre lines CrePR, CreERT, CreERT2, CreER™ lines require tamoxifen or RU486.

The inducible models not only have lower efficiency in the brain, but can have other serious drug-induced side effects [24, 28, 57, 80]. Nevertheless, recapitulation of these effects in an adult knockdown model is a prime area for future work and a study that we are currently undertaking.

In conclusion, in a model that minimizes compensatory changes during development by postnatal activation of Cre recombinase [56, 84, 86], loss of Nampt selectively impairs LTD and the function of GluN2B–containing NMDARs. These findings support an intricate link between neuronal metabolic status and synaptic plasticity, but are not typical effects of energy deprivation. Thus, loss of neuronal NAD^+ and/or the manner in which the brain compensates for loss of neuronal NAD^+ appears to generate significant and novel effects. Further analysis of *CamKIIαNampt*$^{-/-}$ mice should provide meaningful insight into the involvement of GluN2B in synaptic transmission.

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Abbreviations

CA1	Cornu ammonis region 1
fEPSP	Field excitatory postsynaptic potential
HFS	High frequency stimulation
LTD	Long-term depression
LTP	Long-term potentiation
LFS	Low frequency stimulation
NAD^+	Nicotinamide adenine dinucleotide
Nampt	Nicotinamide phosphoribosyltransferase
NMDAR	N-methyl-D-aspartate receptor
P	Postnatal day

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Highlights

1. *CamKIIαNampt*$^{-/-}$ mice lack the NAD^+ biosynthetic enzyme Nampt in forebrain neurons.
2. *CamKIIαNampt*$^{-/-}$ mice have impaired induction of long-term depression (LTD).
3. *CamKIIαNampt*$^{-/-}$ mice have normal induction of long-term potentiation (LTP).
4. *CamKIIαNampt*$^{-/-}$ mice have dysfunctional GluN2B–containing NMDARs.
5. Nampt plays a critical, novel role in LTD and GluN2B–containing NMDAR function.

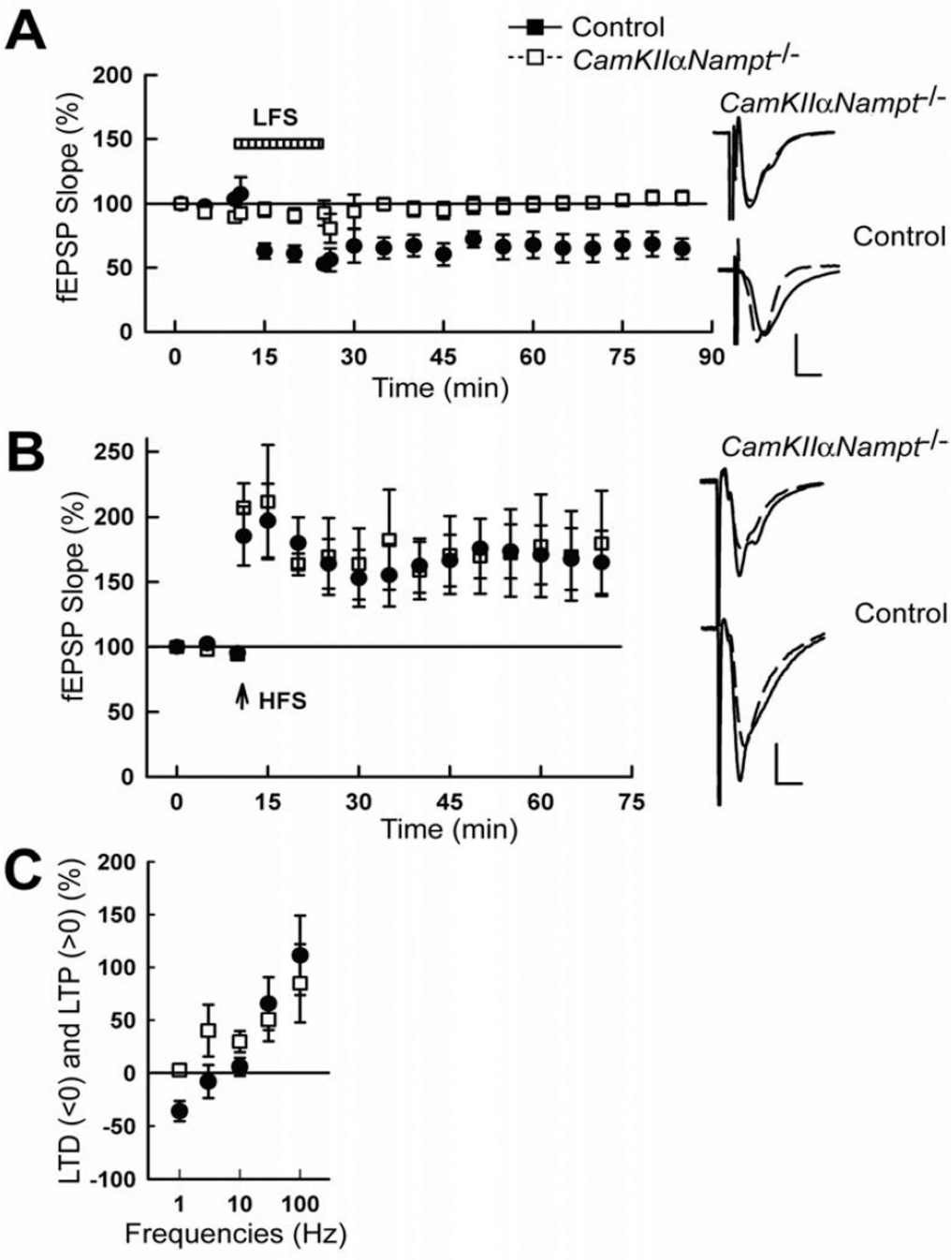

Figure 1.

Postnatal day 30 *CamKIIαNampt*$^{-/-}$ mice showed impaired induction of long-term depression (LTD) but not long-term potentiation (LTP). **A**, LTD was not induced by low frequency stimulation (LFS) in *CamKIIαNampt*$^{-/-}$ mice (white squares), but was induced in control mice (black circles; n=5 each). **B**, LTP was induced by high frequency stimulation (HFS, arrow) in all *CamKIIαNampt*$^{-/-}$ and control mice tested (n=5 each). **A,B**, Traces to the right depict representative field excitatory postsynaptic potentials (fEPSPs) before (dotted lines) and 60 min after HFS or LFS (solid lines). Calibration: 1 mV, 5 ms. **C**, A

frequency response curve for LTD and LTP evoked by 900 pulses delivered at 1, 3, 10, 30, and 100 Hz (n=5). Data are presented as mean ± S.E.M.

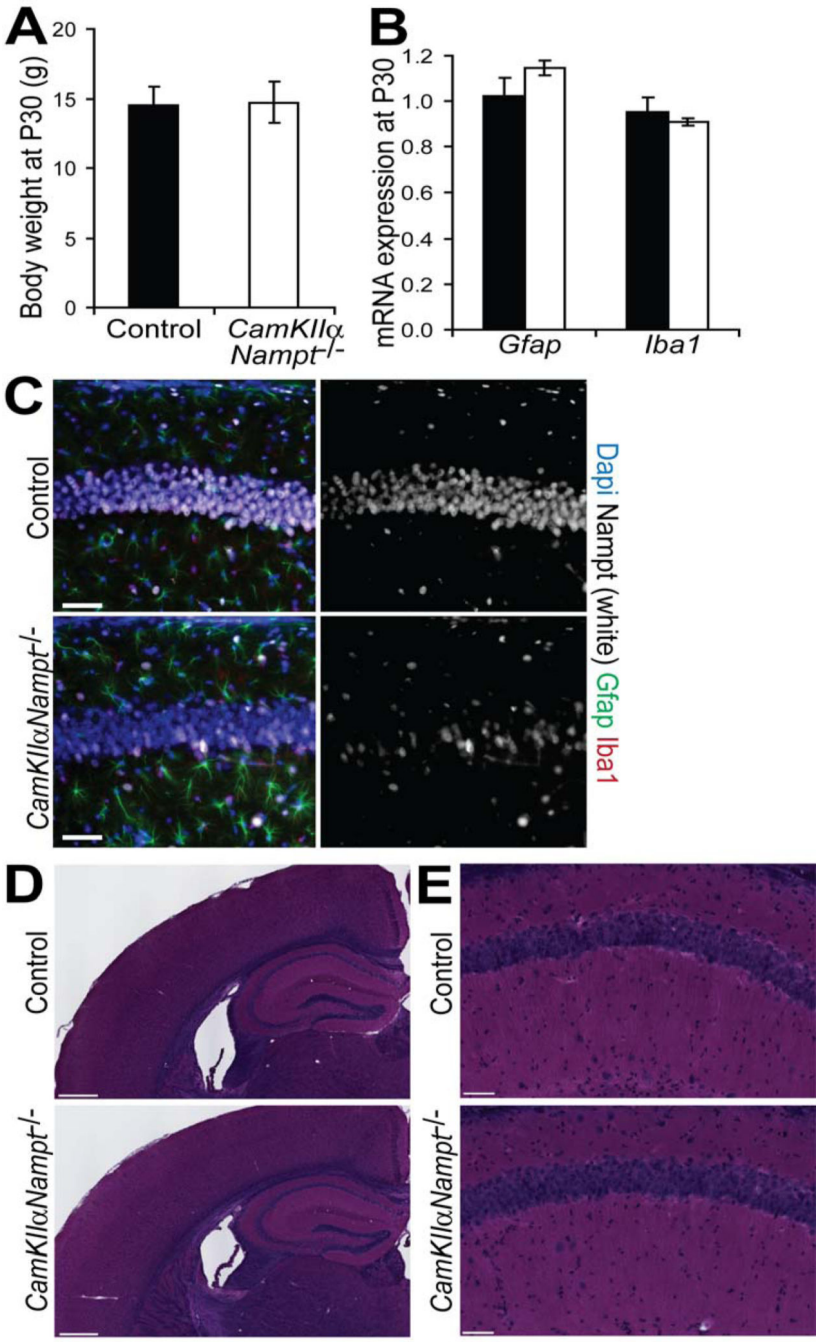

Figure 2.

Postnatal day 30 *CamKIIαNampt*$^{-/-}$ mice were overtly normal. **A**, The body weights of *CamKIIαNampt*$^{-/-}$ (n=4) and control (n=5) mice were similar. **B**, Quantitative PCR analysis of RNA isolated from the hippocampi of *CamKIIαNampt*$^{-/-}$ and control mice (n = 3–5) showed no astrogliosis or microgliosis. **C**, Representative images of immunofluorescence for Dapi (blue), Nampt (white), Gfap (green), Iba1 (red) in coronal sections (n = 4–6). Scale bars represent 50 μm. **D,E**, Hematoxylin and eosin (H&E) staining of coronal brain sections.

D, **S**cale bars represent 500 μm. **E**, Magnification of the CA1 hippocampal subregion. Scale bars represent 50 μm. Data are presented as mean ± S.E.M.

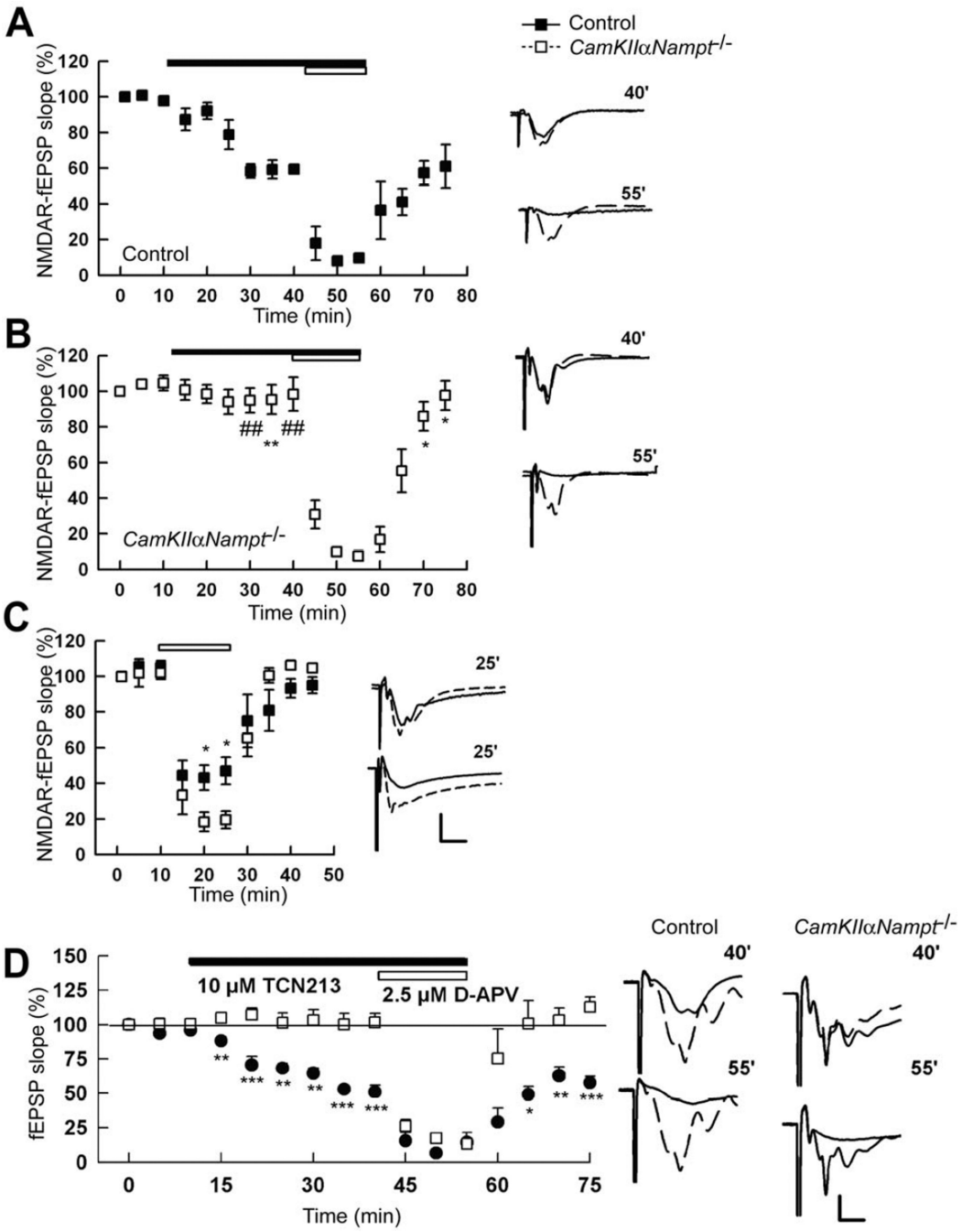

Figure 3.

Postnatal day 30 *CamKIIαNampt*$^{-/-}$ mice showed impaired GluN2B–containing N-methyl-D-aspartate receptor (NMDAR)-mediated field excitatory postsynaptic potentials (fEPSPs). **A**, In control mice, ifenprodil (10 μM, filled bar) partially depressed NMDAR-mediated fEPSPs. Co-administration of D-APV (2.5 μM, open bar) thereafter almost completely suppressed the remaining NMDAR-mediated fEPSPs (n=5). **B**, In *CamKIIαNampt*$^{-/-}$ mice, ifenprodil only minimally depressed NMDAR-mediated fEPSPs. Co-administration of D-APV thereafter successfully suppressed NMDAR-mediated fEPSPs (n=5). **C**, D-APV (2.5

μM, open bar) alone suppressed NMDAR-mediated fEPSPs to a greater extent in *CamKIIαNampt*$^{-/-}$ mice than in control mice. **D**, In control mice, TCN 213 (10 μM, filled bar) partially depressed NMDAR-mediated fEPSPs. Co-administration of D-APV (2.5 μM, open bar) almost completely suppressed the remaining NMDAR-mediated fEPSPs (n=5). In *CamKIIαNampt*$^{-/-}$ mice, TCN 213 did not depress NMDAR-mediated fEPSPs. Co-administration of D-APV successfully suppressed NMDAR-mediated fEPSPs in both genotypes (n=5). **A-D**, Traces to the right depict representative NMDAR-mediated fEPSPs at the times denoted. **A-C**, Calibration: 1 mV, 5 ms. **D**, Calibration: 1 mV, 10 ms. Data are presented as mean ± S.E.M. *, # represents significance generated from Student *t*-tests and Mann-Whitney U-tests, respectively, in comparisons between control and *CamKIIαNampt*$^{-/-}$ mice. *,$^{\#}$P < 0.05. **,$^{\#\#}$P < 0.01. ***,$^{\#\#\#}$P < 0.001.

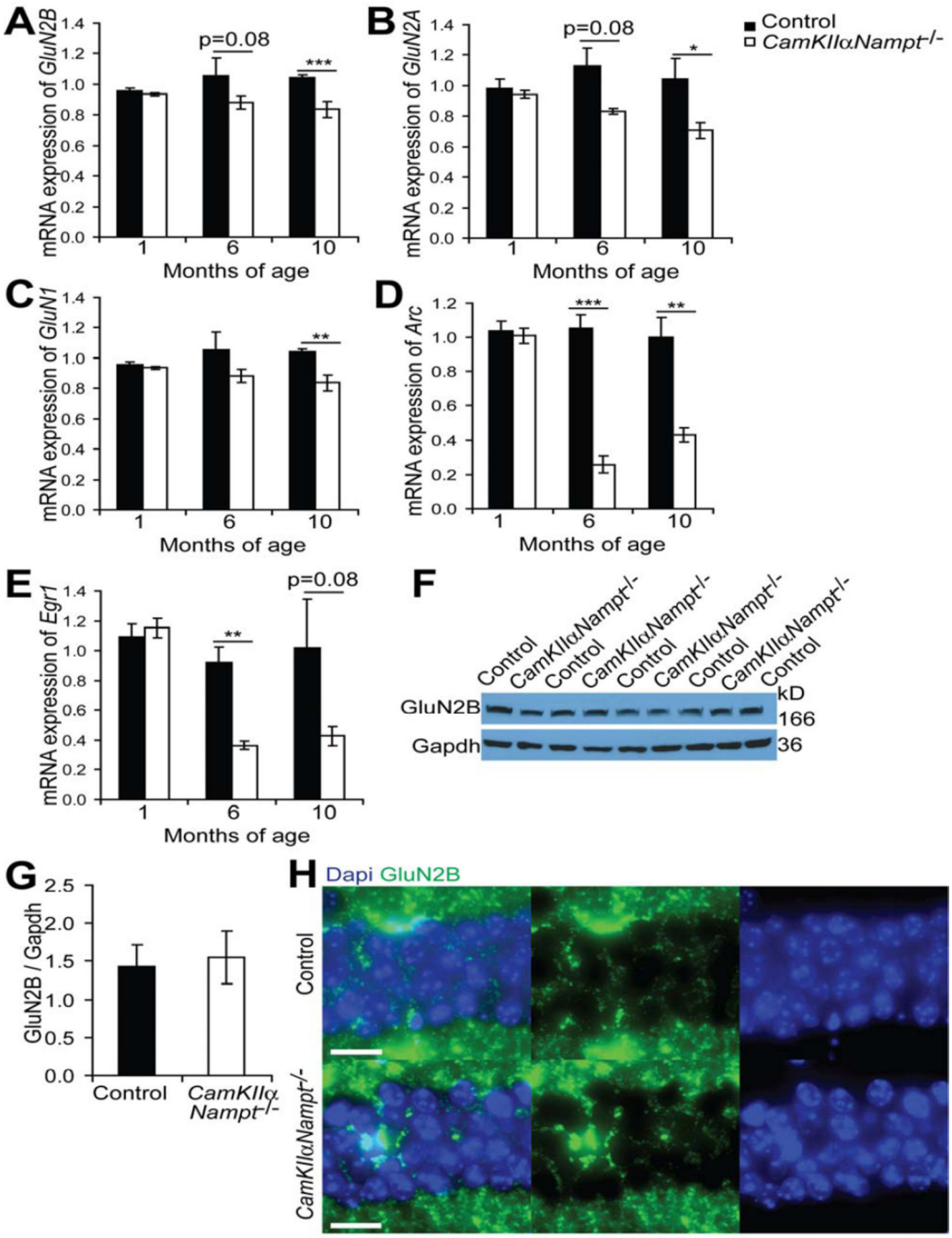

Figure 4.

CamKIIαNampt$^{-/-}$ mice showed an age-related decline in N-methyl-D-aspartate receptor (NMDAR) subunits and immediate early genes. **A-E**, Quantitative PCR analysis in RNA isolated from the hippocampi of *CamKIIαNampt*$^{-/-}$ and control mice at the ages indicated (n = 3–5). **F**, Representative immunoblot of hippocampal extracts of postnatal day 30 *CamKIIαNampt*$^{-/-}$ and control mice for GluN2B normalized by Gapdh. **G**, Quantification of F. **H**, Representative images of immunofluorescence in postnatal day 30 *CamKIIαNampt*$^{-/-}$ and control mice for Dapi (blue) and GluN2B (green) in CA1 (n = 4–6).

Scale bars represent 20 μM Data are presented as mean ± S.E.M. *P < 0.05. **P < 0.01. ***P < 0.001.

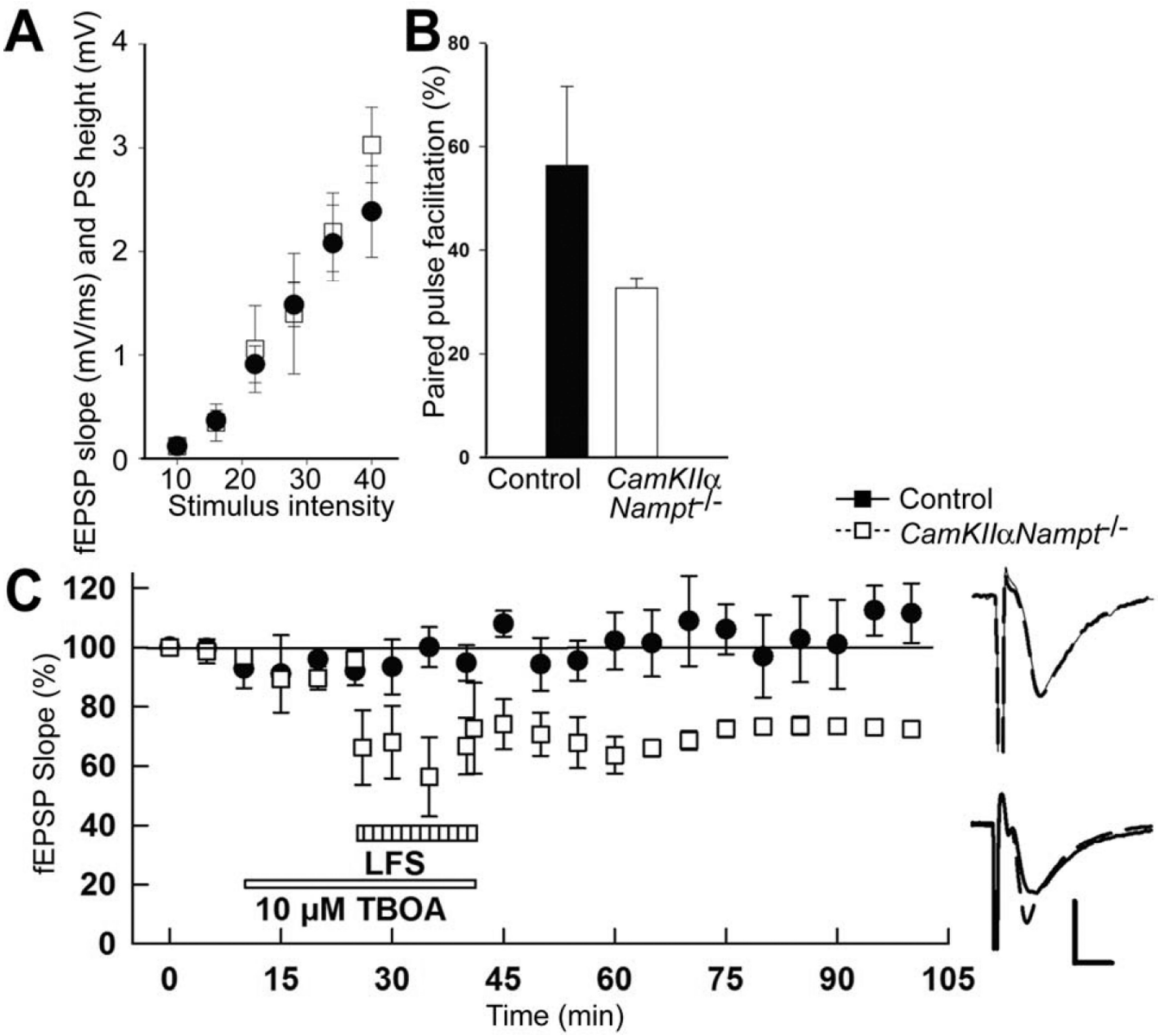

Figure 5.

Postnatal day 30 *CamKIIαNampt*$^{-/-}$ mice showed normal baseline synaptic responses as well as presynaptic mechanisms and their impaired induction of LTD was rescued by inhibition of glutamate uptake. **A**, Base line response was evaluated by obtaining baseline stimulus response curves with the same set of 6 stimuli (10, 16, 22, 28, 34, and 40 V; n=5). **B**, Paired pulse facilitation was evaluated by delivering two identical stimuli at an interval of 21 msec and calculated changes in the second EPSP slopes a stimulus intensity that evoked a half maximal response for the first response based on a baseline stimulus-response curve. Compared to the half maximal fEPSP evoked by the initial stimulus the second fEPSP evoked by the second stimulus with the same intensity are usually bigger (>100%). Similar paired pulse facilitation was seen in slices from control and *CamKIIαNampt*$^{-/-}$ mice (n=5). **C**, The presence of the glutamate uptake inhibitor DL-threo-β-benzyloxyaspartate (DL-

TBOA) at 10 μM for 30 min allowed 1 Hz LFS to induce LTD in slices from *CamKIIαNampt*$^{-/-}$ mice (65.9±7.5%, white squares, n=5). The failure of LFS to induce LTD in the absence of DL-TBOA is shown in black circles. Calibration: 1 mV, 5 ms.