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Loss of Thr286 phosphorylation disrupts synaptic CaMKII α targeting, NMDAR activity and behavior in pre-adolescent mice

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Abstract

In order to provide insight into *in vivo* roles of CaMKII α autophosphorylation at Thr286 during postnatal development, behavioral, biochemical, and electrophysiological phenotypes of pre-adolescent Thr286 to Ala CaMKII α knock-in (T286A-KI) and WT mice were examined. T286A-KI mice displayed cognitive deficits in a novel object recognition test and an anxiolytic phenotype in the elevated plus maze, suggesting disruption of normal developmental processes. At the molecular level, the ratio of total CaMKII α to CaMKII β in hippocampal lysates was significantly decreased $\approx$ 2-fold in T286A-KI mice, and levels of both isoforms in synaptic subcellular fractions were decreased by $\approx$ 80%. Total levels of GluA1 AMPA-glutamate receptor subunits and phosphorylation of GluA1 at the CaMKII site (Ser831) in synaptic fractions were unaltered, as were the frequency and amplitude of AMPAR-mediated spontaneous excitatory postsynaptic currents at hippocampal CA3-CA1 synapses. Synaptic levels of NMDA-glutamate receptor GluN1, GluN2A and GluN2B subunits also were unaltered. However, the reduced ratio of CaMKII to NMDAR subunits in synaptic fractions was linked to increased synaptic NMDAR-mediated currents in T286A-KI mice, apparently due to increased functional contributions by GluN2B NMDARs (assessed by Ro 25-6981 sensitivity). Thus, disruption of CaMKII synaptic targeting caused by elimination of Thr286 autophosphorylation leads to synaptic and behavioral deficits during pre-adolescence.

INTRODUCTION

Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) integrates transient, localized changes of Ca²⁺ to induce diverse downstream responses (reviewed by (Colbran and Brown, 2004; Hudmon and Schulman, 2002; Lisman et al., 2002). Autophosphorylation can modulate CaMKII activity, interactions with CaMKII-associated proteins and subcellular targeting. For example, Thr286 autophosphorylation confers autonomous activity and stabilizes binding of CaMKII α to GluN2B subunits of NMDA-type glutamate receptors

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(NMDARs) and to intact postsynaptic densities (reviewed in (Colbran, 2004; Griffith, 2004). Thr286 autophosphorylation is also required for CaMKII α to enhance the desensitization of GluN2B-containing NMDARs, but not those containing GluN2A (Sessoms-Sikes et al., 2005).

CaMKII α expression is strongly induced beginning at postnatal day (P) 5 in rodents (Brocke et al., 1995; Sugiura and Yamauchi, 1992). This coincides with the onset of crucial sensitive periods in the activity-dependent establishment of connectivity, synapses and behavior. Studies in neuronal cultures have shown that CaMKII α is important for development and refinement of synaptic connectivity (Thiagarajan et al., 2002; Wu and Cline, 1998). Indeed, CaMKII binding to NMDAR GluN2B subunits is required for LTP in organotypic hippocampal slice cultures (Barria and Malinow, 2005). However, the role of autophosphorylation-dependent synaptic targeting of CaMKII during development *in vivo* is poorly understood. Here we show that Thr286 to Ala knock-in (T286A-KI) mutation of CaMKII α in mice produces cognitive and anxiety phenotypes during pre-adolescence, disrupts synaptic targeting of CaMKII, and enhances the activity of GluN2B-NMDARs at hippocampal CA3-CA1 synapses. Our findings identify important roles for CaMKII autophosphorylation during synaptic and behavioral development *in vivo*.

MATERIALS AND METHODS

Mice

T286A-KI mice (Giese et al., 1998) on a C57BL/6J background were housed on a 7:00 am-7:00 pm light-dark cycle. All experiments were performed at postnatal days 25–28. At P25, weights of WT and T286A-KI mice were 10.9 ± 0.3 g (N=17) and 9.3 ± 0.5 g (N=18) ($p < 0.02$).

Behavior

Behavioral testing was performed under normal lighting (250–300 lux) from 10 am–1 pm using procedures modified for pre-adolescent mice. *Novel object recognition*: Weanlings were housed in cages containing a 1/2" PVC pipe extender (familiar object) from P21 until testing. One day prior to testing, mice explored testing chambers (standard mouse cage) for 20 min. On testing day, mice were placed in the chamber with 2 copies of the familiar object for 5 min. Mice were then placed back in their home cage for 3 min while one familiar object was replaced with a novel object (50 ml conical tube weighted with sand); locations of novel and familiar objects within the arena were counterbalanced between subjects. Mice were then placed back in the chamber and allowed to explore for an additional 5 min. Time spent exploring each object was assessed by video analysis (ANY-maze, Stoelting). No preference between objects corresponds to 50% time. *Y-maze*: The Y-maze had visual cues on the floor and at the end of each arm. Mice were placed at the end of one arm, facing the center, and allowed to explore for 6 min. Consecutive entries into each of the three arms were scored as spontaneous alternations from a video record and expressed as a percentage of total arm entries. *Elevated-plus maze (EPM)*: Mice were placed on open-arms (10 x 10 x 30 cm) facing the center of the maze and were allowed to explore for 5 min. Time spent in open-arms was recorded and expressed as a percentage of total time.

Whole-cell recordings

Hippocampal slices were prepared for whole cell recordings from WT or T286A-KI mice and spontaneous excitatory postsynaptic currents (sEPSCs) were recorded from CA1 pyramidal neurons in the presence of picrotoxin (25 μ M) at a -70 mV holding potential, as described previously (Kash et al., 2008a; Kash et al., 2008b). Recording electrodes were filled with (in mM) 135 Cs⁺-gluconate, 5 NaCl, 10 HEPES, 0.6 EGTA, 4 ATP, 0.4 GTP, pH

7.2, 290–295 mOsmol. AMPAR-dependent EPSCs were also evoked at 0.05 Hz and a -70 mV holding potential using a glass, ACSF-filled pipette placed in the Schaffer collateral pathway ≈ 100 μ m from the cell soma. NMDAR-dependent EPSCs were evoked after the addition of NBQX (10 μ M) to inhibit AMPAR components and adjusting to $+40$ mV. Averages of 10 responses under each condition were expressed as the NMDA/AMPA ratio. Slices were then perfused with Ro 25-6981 (2 μ M) for 15 min before again recording evoked NMDAR-EPSCs. Current decays for baseline and Ro 25-6981-insensitive evoked NMDAR-EPSCs were fitted to double-exponential functions (Clampfit 10.0) to estimate weighted decay tau values (τ_w) (Kash et al., 2008b).

Subcellular fractionation

S1 (soluble proteins), S2 (Triton-soluble proteins), S3 (Triton/ deoxycholate-soluble proteins) and P3 (Triton/deoxycholate-insoluble proteins) fractions were generated from rapidly dissected whole hippocampus. S3 and P3 fractions are highly enriched in a postsynaptic marker (PSD95), and are deficient in cytosolic (GAPDH), presynaptic (syntaxin), and internal membrane (IP₃R) markers (Gustin et al., 2010). In some studies the S3 and P3 fractions were not separated by centrifugation. Synaptic S3/P3 fractions isolated from P25 WT mouse hippocampus are highly enriched in NMDAR subunits, CaMKII when normalized to protein loading, but these proteins are also present in S2 and/or S1 (Supplementary Fig. 1). GluA1 subunits of the AMPA type glutamate receptor are most abundant in S2, but are enriched to a similar extent in the synaptic S3/P3 fraction.

Immunoprecipitation

S3/P3 synaptic fractions were immunoprecipitated (Brown et al., 2008) using an affinity-purified goat polyclonal CaMKII antibody (McNeill and Colbran, 1995).

Immunoblot Analysis

Immunoblots developed using enhanced chemi-luminescence were quantified and normalized for protein loading (based on Ponceau-S stained membranes) (Brown et al., 2005; Gustin et al., 2010). Mouse primary antibodies: CaMKII α (ABR); CaMKII β (Zymed); GluN1 (BD Pharmingen); GluN2B (Transduction Laboratories). Rabbit primary: GluN2A (Millipore). Pre-absorbed secondary antibodies were from Santa Cruz.

Statistical analysis

Unpaired Student's *t*-test or two-way repeated measures ANOVA with Bonferroni post-tests were used, as appropriate. Sample numbers and statistical significances are indicated on the figures (*, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.002$).

RESULTS

Altered behavior in pre-adolescent T286A-KI mice

Since CaMKII α is initially expressed at about postnatal day (P) 5 (Bayer et al., 1999), we hypothesized that T286A-KI mutation would disrupt subsequent development of normal molecular and synaptic processes *in vivo*. In order to determine whether such changes result in early behavioral phenotypes, we initially modified simple tests of baseline behavior that do not demand extensive training sessions for pre-adolescent mice (P25–28) (Materials and Methods). Exploratory behavior in novel object arenas was not significantly different between WT and T286A-KI mice when using two copies of a familiar object. T286A-KI mice had a significant novel object recognition deficit, in that they showed no preference for exploration of a novel object, in marked contrast to WT mice (Fig. 1A). The T286A-KI mice appeared hyperactive in the Y-maze, based on the total number of arm entries, but spatial

working memory was normal because the fraction of spontaneous alternations was unaffected (Fig. 1B). However, the hyperactive phenotype appeared to be context-dependent, because T286A-KI and WT mice displayed a similar total number of entries into the open and closed arms in the EPM (Fig. 1C, left). Notably, the EPM revealed a robust anxiolytic phenotype in the T286A-KI mice, based on a significant increase in the fraction of time spent in the open-arms (Fig. 1C right). The anxiolytic phenotype of T286A-KI mice was also evident based on a significant increase in the fraction of total entries into the open arms in the EPM (WT: $31 \pm 3\%$, N=13. T286A-KI: $54 \pm 5\%$, N=8. $P=0.0004$).

CaMKII expression in pre-adolescent T286A-KI mice

Immunoblotting of whole hippocampal homogenates revealed a significant decrease of total CaMKII α levels in T286A-KI mice, but a significant increase of total CaMKII β levels. Thus, the ratio of CaMKII α to CaMKII β was significantly decreased ≈ 2 -fold (Fig. 2).

CaMKII subcellular localization in pre-adolescent T286A-KI mice

CaMKII isoforms are present in all subcellular fractions in P25 WT mice, but are enriched in synaptic S3 and P3 fractions, co-distributing with PSD95 and NMDAR subunits (Fig. S1) (Gustin et al., 2010). In T286A-KI mice, CaMKII α levels were significantly decreased in both cytosolic S1 ($\approx 35\%$) and postsynaptic (S3/P3) ($>90\%$) fractions (Fig. 3A). Interestingly, even though the CaMKII β gene was unaltered, CaMKII β levels were significantly increased ≈ 2 -fold in S1 and S2 fractions, but significantly decreased $\approx 70\%$ in postsynaptic S3/P3 fractions (Fig. 3A). In order to ascertain whether altered CaMKII β phosphorylation contributes to changes in CaMKII β distribution in T286A-KI mice, immunoprecipitated CaMKII holoenzymes were immunoblotted using phospho-Thr286/7 specific antibodies. The stoichiometry of CaMKII β phosphorylation at Thr287 (normalized to total levels of CaMKII β) was unaltered in T286A-KI mice (Fig. 3B).

Hippocampal AMPARs in pre-adolescent T286A-KI mice

The best-characterized synaptic target of CaMKII is likely the AMPAR GluA1 subunit (Derkach et al., 2007; Kessels and Malinow, 2009). In order to test the hypothesis that disruption of CaMKII targeting in T286A-KI mice affects synaptic trafficking and/or phosphorylation of GluA1, synaptic S3/P3 fractions were immunoblotted for GluA1, as well as for Ser831 phosphorylation. However, total GluA1 protein levels and Ser831 phosphorylation were no different between WT and T286A-KI mice (Fig. 4A). Moreover, the amplitude and frequency of AMPAR-mediated spontaneous excitatory postsynaptic currents (sEPSCs) at hippocampal CA3-CA1 synapses were unaffected in T286A-KI mice (Fig. 4B).

Evoked synaptic transmission in pre-adolescent T286A-KI mice

In order to screen more broadly for synaptic phenotypes in T286A-KI mice, we recorded evoked synaptic currents at hippocampal CA3-CA1 synapses. Inward currents evoked at -70 mV are predominantly AMPAR mediated, but slower outward currents recorded at $+40$ mV are mediated by a combination of AMPARs and NMDARs. Contributions of NMDARs to synaptic currents at $+40$ mV were estimated in the presence of AMPAR antagonist. Notably, there was a significant ≈ 2 -fold increase in the ratio of evoked NMDAR-synaptic currents to evoked AMPAR-synaptic currents in T286A-KI mice compared to WT (Fig. 5A). Since sEPSC data suggest that synaptic AMPARs are unaltered (Fig. 4), the difference in NMDA:AMPA ratios presumably arises from an increase in synaptic NMDAR currents in T286A-KI mice.

Altered synaptic NMDAR kinetics in pre-adolescent T286A-KI mice

In order to better understand differences in synaptic NMDARs between WT and T286A-KI mice, we compared decay kinetics of NMDAR-mediated synaptic currents in the presence of an AMPAR antagonist. Interestingly, decay kinetics were significantly ≈ 2 -fold slower in T286A-KI than in WT cells (Fig. 5B). Ro 25-6981, a GluN2B-selective NMDAR antagonist, had no effect on decay kinetics in WT cells (Fig. 5B) and only modestly reduced total NMDAR-EPSCs by $13 \pm 10\%$ (area under the curve; $N=5$, NS). However, Ro 25-6981 significantly accelerated decay kinetics in T286A-KI cells to baseline WT values (Fig. 5B), and significantly reduced total NMDAR-EPSCs by $25 \pm 10\%$ ($p < 0.05$, $N=6$). Thus, GluN2B-NMDARs make a more substantial contribution to total synaptic NMDAR currents in T286A-KI than in WT cells. The slower synaptic NMDAR kinetics in T286A-KI mice do not appear to be due to changes of NMDAR subunit levels, because amounts of GluN1, GluN2B and GluN2A in synaptic S3/P3 fractions are not significantly different between T286A-KI and WT mice (Fig. 5C). However, since CaMKII α levels in synaptic S3/P3 fractions are reduced by $>90\%$ (Fig. 3), the ratio of CaMKII to NMDAR subunits in synaptic fractions is decreased in T286A-KI mice.

CaMKII-NMDAR interactions in pre-adolescent T286A-KI mice

Interactions of synaptic NMDAR subunits with CaMKII were directly assessed by co-immunoprecipitation from S3/P3 fractions. Synaptic CaMKII complexes isolated from T286A-KI mice contained significantly reduced levels of both CaMKII isoforms and all NMDAR subunits tested: decreases of CaMKII β , GluN1 and GluN2B levels appeared somewhat less robust than the decrease of CaMKII α or GluN2A levels (Fig. 6A). In fact, the ratio of CaMKII β , GluN1 and GluN2B to CaMKII α in synaptic complexes isolated from T286A-KI mice was significantly increased >2 -fold, but the ratio of GluN2A to CaMKII α was not significantly increased (Fig. 6B). In contrast, the ratio of GluN1 and GluN2B to CaMKII β in the immune complexes was not significantly different between T286A-KI and WT mice (not shown).

DISCUSSION

It is well established that genetic alterations of the expression or function of developmentally important molecules can result in complex age-dependent phenotypes and neuroadaptive changes (see for example, (Adusei et al., 2010; Burrows et al., 2000); reviewed in (Thompson and Stanwood, 2009)). Changes of CaMKII isoform expression levels or CaMKII interactions with NMDAR GluN2B subunits disrupt synaptic development and plasticity in neuronal cultures (Barria and Malinow, 2005; Thiagarajan et al., 2002; Wu and Cline, 1998). However, prior studies of T286A-KI mice focused mostly on adults, typically >3 months of age. These studies established that Thr286 autophosphorylation of CaMKII α is critical for normal synaptic plasticity, learning and memory, although the specific mechanistic explanation of these phenotypes is poorly understood (Giese et al., 1998; Glazewski et al., 2000). In order to provide insights into the *in vivo* impact of autophosphorylation at Thr286 on developmental mechanisms, we focused the present studies on pre-adolescent mice. We found that the T286A-KI mutation reduces CaMKII α to CaMKII β expression ratios in hippocampus (Fig. 2) and profoundly disrupts synaptic CaMKII targeting (Fig. 3A). These findings represent the first molecular signaling deficits to be described in T286A-KI mice. We therefore hypothesize that the T286A-KI mutation disrupts synaptic signaling to reduce or delay normal increases in the CaMKII α to CaMKII β expression ratio during development (Bayer et al., 1999; Brocke et al., 1995).

While adult T286A-KI mice display robust deficits in hippocampus-dependent spatial learning (Giese et al., 1998), the hippocampus is important for many other behaviors

(Barkus et al., 2010; Engin and Treit, 2007; Packard, 2009). In addition, the T286A-KI mutation may affect behaviors that involve other brain regions that express CaMKII α . Our testing of pre-adolescent mice used simple methods that do not require substantial training, permitting tighter correlations with observed baseline synaptic and biochemical changes. The pronounced novel object recognition deficit (Fig. 1A) establishes that cognitive changes arise relatively early in development in T286A-KI mice, coincident with a disruption of synaptic CaMKII α and CaMKII β targeting (Fig. 3A). The robust anxiolytic phenotype in T286A-KI mice (Fig. 1C) may relate to prior studies showing that changes in CaMKII α expression levels affect fear responses, anxiety and aggression (Chen et al., 1994; Hasegawa et al., 2009). Interestingly, transient receptors from P13 to P34 increases hippocampal pharmacological blockade of serotonin 5HT_{1A} CaMKII α autophosphorylation at Thr286, and increases basal anxiety levels in adulthood (Lo receptor Iacono and Gross, 2008). Moreover, the anxiogenic phenotype of global 5HT_{1A} knockout mice is attenuated by crossing them onto a heterozygous T286A-KI CaMKII α background, thereby limiting the extent of Thr286 autophosphorylation throughout development (Lo Iacono and Gross, 2008). Our study focused on the midpoint of this sensitive period. Thus, CaMKII α autophosphorylation at Thr286 during development appears to be important for anxiety programming, apparently downstream of serotonin signaling.

The disruption of synaptic CaMKII α and CaMKII β targeting in T286A-KI mice (Fig. 3A) is selective, because synaptic levels of other proteins are unaltered (e.g., GluA1, NMDAR subunits: Figs. 4A/5C). This observation is consistent with studies showing that autophosphorylation at Thr286 stabilizes CaMKII α binding to NMDAR-GluN2B subunits (but not to GluN2A), as well as CaMKII targeting to dendritic spines in cultured neurons (Bayer et al., 2006; Shen et al., 2000; Strack and Colbran, 1998). GluN2B is the predominant GluN2 subunit during early postnatal periods, with GluN2A expression increasing during development (Hsia et al., 1998; Philpot et al., 2007; Quinlan et al., 1999a; Steigerwald et al., 2000). Taken together, these data suggest that decreased association of CaMKII α with GluN2B may explain the disruption of CaMKII synaptic targeting in pre-adolescent T286A-KI mice (Fig. 3). Although CaMKII α and CaMKII β bind similarly to GluN2B *in vitro* (Robison et al., 2005) and CaMKII β autophosphorylation at Thr287 is unaltered in T286A-KI mice (Fig. 3B), T286A-KI mutation may dominantly disrupt targeting of CaMKII β because the two isoforms co-assemble in the dodecameric holoenzyme. Immunoprecipitation studies confirmed that the proportion of synaptic GluN1, GluN2A and GluN2B subunits associated with CaMKII was indeed decreased $\approx$ 2-fold in T286A-KI mice (Fig. 6A), but these immune complexes actually contained $\approx$ 4-fold reduced levels of CaMKII α (Fig. 6A). Thus, ratios of GluN1 and GluN2B to CaMKII α in the synaptic immune complexes were significantly increased $\approx$ 2-fold in T286A-KI mice (Fig. 6B), suggesting tighter association of a sub-population of NMDAR subunits with residual synaptic CaMKII holoenzymes in T286A-KI mice. This may reflect partial compensation for the lack of Thr286 autophosphorylation by an independent mechanism for recruiting CaMKII to NMDAR-GluN2B subunits. For example, α -actinin can interact independently with both GluN1 and GluN2B, and with CaMKII (Robison et al., 2005; Wyszynski et al., 1997). Alternatively, it is interesting to note that there was no difference in the ratios of GluN1 and GluN2B to CaMKII β in synaptic CaMKII complexes isolated from WT and T286A-KI mice, suggesting that CaMKII β may play an important role in controlling CaMKII holoenzyme association with synaptic NMDARs. Thus, it is difficult to predict mechanistic effects of specific molecular manipulations *in vivo*, even after thorough *in vitro* characterization, emphasizing the importance of careful evaluation of multi-protein complexes involved in postsynaptic signaling *in vivo*.

CaMKII has pleiotropic actions in dendritic spines (Colbran and Brown, 2004). CaMKII-dependent LTP is generally accepted to involve enhanced synaptic trafficking of GluA1

subunits, as well as phosphorylation of GluA1 at Ser831 to enhance AMPAR conductance (Derkach et al., 2007; Kessels and Malinow, 2009). Therefore, we tested the hypothesis that the T286A-KI mutation would disrupt normal developmental regulation of synaptic AMPARs *in vivo*. Notably, there was no significant difference in levels of GluA1 subunits or Ser831 phosphorylation in synaptic S3/P3 fractions (Fig. 4A). Only a small fraction of total hippocampal GluA1 subunits is present in the S3/P3 fraction and most GluA1 is extracted into S2 using Triton X-100 alone (Fig. S1). In fact, the distribution of GluA1 resembles that of IP3 receptors (Gustin et al., 2010), a marker for intracellular membrane proteins. We interpret this to indicate that S2 contains a combination of intracellular and extra-/peri-synaptic GluA1 subunits, with S3 being enriched in synaptic GluA1 subunits. Although interpretation of our biochemical data may be confounded by non-synaptic AMPAR GluA1 subunits in the S3/P3 fraction, the apparent lack of effect of T286A-KI mutation on synaptic AMPARs is further supported by the similarity of spontaneous synaptic AMPAR currents in WT and T286A-KI neurons (Fig. 4B). Presumably, other pathways have compensated for possible deficiencies in CaMKII regulation of GluA1-AMPA receptors in T286A-KI mice during development. For example, Ser831 can also be targeted by PKC to regulate GluA1 AMPARs (Brooks and Tavalin, 2011; Tavalin, 2008). Thus, it may be interesting to compare synaptic PKC signaling in WT and T286A-KI mice in future studies.

We also found that evoked NMDA:AMPA current ratios were significantly increased in pre-adolescent T286A-KI mice relative to WT (Fig. 5A). Our data indicating that baseline synaptic AMPAR function is normal in T286A-KI mice (see above) are in agreement with a previous study showing no differences in input-output relationships of field recordings between (older) WT and T286A-KI mice (Giese et al., 1998). Thus, although we cannot formally exclude a contribution from decreased evoked synaptic AMPAR currents, the combined evidence suggests that increased NMDA:AMPA current ratios in T286A-KI mice at P25 largely arise from an increase in synaptic NMDAR currents.

Enhanced synaptic NMDAR currents in T286A-KI mice appear to be explained by slower NMDAR decay kinetics (Fig. 5B), but the molecular basis for the altered kinetics is unclear. Decay kinetics of di-heteromeric NMDARs containing 2 GluN2B subunits and 2 GluN1 subunits (GluN2B-NMDARs) are slower than decay kinetics of di-heteromeric GluN2A-NMDARs (Monyer et al., 1994), suggesting that there is a larger functional contribution of GluN2B-NMDARs in T286A-KI mice. Previous studies have shown that acceleration of NMDAR decay kinetics correlates with a decreased GluN2B:GluN2A expression ratio during normal postnatal development and can be driven by synaptic plasticity (Philpot et al., 2007; Quinlan et al., 1999a; Quinlan et al., 1999b; Steigerwald et al., 2000). However, biochemical data showing that synaptic levels of GluN2A and GluN2B are not significantly different in WT and T286A-KI mice (Fig. 5C) indicate that changes in NMDAR subunit ratios likely do not explain slower baseline NMDAR decay kinetics.

Studies using Ro 25-6981, a selective antagonist of GluN2B-NMDARs, provide another perspective on possible mechanisms underlying the slower NMDAR kinetics in T286A-KI mice. Acceleration of NMDAR decay kinetics during normal development is associated with decreased sensitivity to GluN2B-selective antagonists; for example, CP-101,606 accelerates NMDAR decay kinetics in CA1 pyramidal neurons at P15, but not at P30 (Steigerwald et al., 2000). Similarly, we failed to detect a significant effect of Ro 25-6981 on baseline NMDAR decay kinetics in WT CA1 pyramidal neurons at P25 (Fig. 5B). These data indicate that any GluN2B-NMDARs present in WT neurons are unavailable for activation in preadolescent WT mice, perhaps because they are extrasynaptic, and that evoked synaptic NMDAR currents are largely carried by GluN2A-NMDARs or tri-heteromeric NMDARs (containing 1 GluN2B, 1 GluN2A and 2 GluN1 subunits). However,

slower baseline decay kinetics in T286A-KI neurons were sensitive to Ro 25-6981, which reduced the decay time constants to WT values (Fig. 5B). These findings suggest a larger functional contribution of GluN2B-NMDARs in T286A-KI neurons.

At first glance, the slower NMDAR decay kinetics in T286A-KI neurons without changes in NMDAR subunit expression appear incongruent. However, these findings are consistent with a working model in which pre-adolescent WT neurons contain synaptic GluN2B-NMDARs, but that CaMKII has accelerated their baseline decay kinetics by a mechanism that requires autophosphorylation at Thr286. Thus, GluN2B-NMDAR antagonists would have no significant effect on baseline decay kinetics in WT neurons. Modulation of synaptic GluN2B-NMDARs by CaMKII is prevented in T286A-KI neurons, thereby slowing baseline synaptic NMDAR decay kinetics and conferring sensitivity to Ro 25-6981. Although the precise role of CaMKII in modulating synaptic GluN2B-NMDARs is poorly understood, there is a significant decrease in CaMKII binding to synaptic NMDARs in T286A-KI mice (see above). Moreover, CaMKII α can accelerate the desensitization kinetics of GluN2B-NMDARs in heterologous cells by a mechanism that requires autophosphorylation at Thr286 (Sessoms-Sikes et al., 2005). These findings indicate that it will be important to develop a better understanding of the relationship between NMDAR desensitization in heterologous cells and synaptic NMDAR decay kinetics as well as the role of CaMKII in NMDAR regulation.

In summary, the T286A-KI mutation of CaMKII α reduces postsynaptic CaMKII targeting to NMDAR subunits. Despite the uncertain molecular mechanisms, our findings clearly indicate that lack of Thr286 autophosphorylation also increases functional synaptic contributions of GluN2B-NMDARs, and is associated with cognitive/anxiolytic phenotypes in pre-adolescent mice. These findings highlight specific mechanisms underlying an important role for CaMKII autophosphorylation during synaptic development *in vivo*. Understanding the impact of genetic and environmental factors on CaMKII regulation during development will shed light on the pathophysiology of complex brain disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AMPA	α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
EPM	elevated-plus maze
EPSC	excitatory postsynaptic current
NMDAR	N-methyl-D-aspartate receptor

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Fig. 1. Behavioral phenotypes in pre-adolescent T286A-KI mice

A. WT and T286A-KI mice display no preference between 2 familiar objects (*left*). However, WT mice preferentially explore a novel object over a familiar object, whereas T286A-KI mice cannot differentiate novel and familiar objects (*right*). **B.** In the Y-maze, T286A-KI mice appear more active than WT, based on total number of arm entries (*left*), but exhibit the same fractional number of spontaneous alternations (*right*), indicating spatial working memory processes are normal. **C.** In the EPM, T286A-KI mice have normal activity, based on the total number of entries into both the open and closed arms, but display an anxiolytic phenotype, spending significantly more time in the open arms compared to their WT littermates (*right*).

Fig. 2. Altered total CaMKII expression levels in pre-adolescent T286A-KI mice
Whole hippocampal extracts from WT and T286A-KI mice were immunoblotted as indicated in the representative images (A) and relative levels of CaMKII α and CaMKII β were quantified (B).

Fig. 3. Disrupted subcellular targeting of CaMKII α and CaMKII β in pre-adolescent T286A-KI mice

A. Cytosolic (S1), Triton-soluble membrane (S2) and postsynaptic (S3/P3) hippocampal subcellular fractions isolated from WT and T286A-KI mice were immunoblotted for CaMKII α and CaMKII β (*upper panels*) and relative levels of each isoform were quantified (*lower panels*). Note that different film exposures are used for each subcellular fraction so band intensities should only be compared within each subcellular fraction, not between fractions. **B.** CaMKII holoenzymes were immunoprecipitated from synaptic S3/P3 fractions isolated from WT and T286A-KI mice. After immunoblotting for total levels and Thr287 phosphorylation of CaMKII β (*left*), the ratio of these signals was quantified (*right*).

Fig. 4. Synaptic AMPARs are unaltered in pre-adolescent T286A-KI mice

A. Immunoblots of combined S3/P3 hippocampal fractions from WT and T286A-KI mice for levels of total GluA1 (*left*) and phospho-Ser831 (*right*) were quantified (*lower panels*). **B.** Representative sEPSC traces (*left*) recorded from CA1 pyramidal neurons from WT and T286A-KI mice, with adjacent average sEPSC traces (*center*), and bar graphs (*right*) reporting average sEPSC amplitudes and frequencies.

A. NMDAR/AMPA ratios

B. Synaptic NMDAR currents

C. In synaptic fraction

Fig. 5. Altered hippocampal synaptic transmission in pre-adolescent T286A-KI mice

A. *Left:* Evoked EPSC traces were scaled to normalize AMPAR components (-70 mV: downward currents) to illustrate proportionately larger NMDAR components (+40 mV: upward currents) in T286A-KI cells compared to WT. *Right:* Plot of average NMDA:AMPA current ratios. **B.** Scaled representative evoked NMDAR-EPSCs (see Methods) from WT (*left*) and T286A-KI (*right*) cells before (black) and after (gray) perfusion with 2 μ M Ro 25-6981. Average weighted tau (τ_w) values for each condition are plotted below. **C.** Immunoblots of combined S3/P3 fractions from hippocampus of WT and T286A-KI mice and quantification of total levels of NMDAR subunits.

Fig. 6. Altered synaptic CaMKII complexes in pre-adolescent T286A-KI mice
A. Immunoblots of CaMKII immune complexes isolated from combined S3/P3 fractions of WT and T286A-KI mice and quantification of total levels of CaMKII isoforms and NMDAR subunits in the complexes. **B.** Plot of normalized ratios of CaMKIIβ and GluN subunits to CaMKIIα in synaptic CaMKII complexes.