

Derangements of Hippocampal Calcium/Calmodulin-Dependent Protein Kinase II in a Mouse Model for Angelman Mental Retardation Syndrome

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Angelman syndrome (AS) is a disorder of human cognition characterized by severe mental retardation and epilepsy. Recently, a mouse model for AS (*Ube3a* maternal null mutation) was developed that displays deficits in both context-dependent learning and hippocampal long-term potentiation (LTP). In the present studies, we examined the molecular basis for these LTP and learning deficits. Mutant animals exhibited a significant increase in hippocampal phospho-calcium/calmodulin-dependent protein kinase II (CaMKII), specifically at sites Thr²⁸⁶ and Thr³⁰⁵, with no corresponding change in the levels of total CaMKII. In addition, mutants show a reduction in CaMKII activity, autophosphorylation capability, and total CaMKII associated with postsynaptic density. These findings are the first to implicate misregulation of CaMKII as a molecular cause for the neurobehavioral deficits in a human learning disorder.

Key words: Angelman syndrome; calcium/calmodulin-dependent protein kinase II; long-term potentiation; postsynaptic density; protein phosphatase; autophosphorylation

Introduction

Angelman syndrome (AS) is a neurological disorder characterized by severe mental retardation, propensity for seizure (associated with a characteristic electroencephalogram), and an ataxic gait (Williams et al., 1995; Buoni et al., 1999; Laan et al., 1999). AS is estimated to occur in 1 of every 15,000–20,000 births and is most often caused by maternal chromosomal deletion of 15q11-q13; however, maternal uniparental disomy and imprinting center mutations also can cause AS (Clayton-Smith, 1993). In 1997, the *Ube3a* gene within the 15q11–13 chromosomal region was identified as the genetic locus for AS (Kishino et al., 1997; Matsuura et al., 1997; Sutcliffe et al., 1997). *Ube3a* codes for an E6-AP ubiquitin ligase, an enzyme involved in protein degradation through the ubiquitin-associated proteasome-mediated pathway. The E6-AP ubiquitin ligase is known to be involved in the degradation of four proteins, including the p53 tumor suppressor protein (Huibregtse et al., 1991), a human homolog to the yeast DNA repair protein Rad23 (Kumar et al., 1999), the multicopy maintenance protein 7 subunit involved in the initiation of DNA replication (Kuhne and Banks, 1998), and E6-AP, which is a target for itself (Nuber et al., 1998). Unfortunately, identification of these target proteins has yet to shed light on any possible mech-

anism underlying the etiology and learning and memory deficits of AS.

Since the identification of the AS locus, some progress has been made in understanding the genetic and biochemical mechanisms underlying this disorder. One important step forward in this endeavor was the development of a mouse model for AS (Jiang et al., 1998). This mouse model, generated by a maternal null mutation in the *Ube3a* gene, revealed the characteristic subregion-specific paternal silencing of the *Ube3a* gene in the brain. Because of imprinting, the *Ube3a* gene product in the hippocampus and Purkinje cell layer of the cerebellum is derived primarily from the maternal copy of the gene. Importantly, the mouse model recapitulates the brain-specific imprinting of *Ube3a* in humans, and also the seizure, ataxic gait, and hippocampus-dependent abnormalities observed in human AS.

In undertaking these studies, we reasoned that understanding the deficits in hippocampal long-term potentiation (LTP) that we had observed previously in the AS mouse model should facilitate identifying the putative molecular mechanisms underlying the behavioral learning deficits in AS. Thus, in the present studies, we examined the hippocampal LTP deficits observed in the *Ube3a* maternal-deficient (m^{-}/p^{+}) mice and investigated the biochemical basis for these deficits. *Ube3a* maternal-deficient mice exhibited deficits in both NMDA receptor (NMDAR)-dependent and -independent LTP, suggesting that the source of the observed LTP deficits resides downstream of calcium influx. These results led us to examine calcium-dependent signal transduction pathways activated during LTP induction. We found aberrant autophosphorylation and diminished activity of hippocampal calcium/calmodulin-dependent protein (CaM) kinase II (CaMKII), without a change in total CaMKII concentrations. The change in the phosphorylation

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state of CaMKII was coupled to a significant reduction in CaMKII associated with postsynaptic density (PSD). In addition, we found that activity for the major phosphatases for CaMKII, protein phosphatase 1 (PP1) and PP2A, is significantly reduced in m^{-}/p^{+} mice. Together, these results suggest that changes in CaMKII underlie the hippocampal LTP deficits. More importantly, these data indicate that alterations in calcium-dependent hippocampal CaMKII activation may play a role in the learning and memory deficits in the m^{-}/p^{+} mutant mice and in humans with AS as well.

Materials and Methods

Hippocampal slice preparation and electrophysiology. Mutants with the *Ube3a* null mutation were developed as described previously (Jiang et al., 1998). Hippocampal slice preparation and electrophysiology were performed as described previously (Roberson and Sweatt, 1996). Briefly, hippocampal slices (400 μ M) were maintained on an interface chamber and bathed in oxygenated artificial CSF (in mM: 125 NaCl, 2.5 KCl, 1.24 NaH_2PO_4 , 25 NaHCO_3 , 10 D-glucose, 2 CaCl_2 , and 1 MgCl_2) (1 ml/min) in an interface chamber maintained at either 25 or 32°C. Extracellular field recordings were made in area CA1 stratum radiatum by stimulation of the Schaffer collateral synapse. Stable baseline synaptic transmission was established for 20 min at an intensity of 40–50% of the maximum population EPSP (pEPSP) before LTP-inducing high-frequency stimulation (HFS). HFS consisted of one or three sets, each set consisting of two trains of 100 Hz stimulation for 1 sec, separated by 20 sec (NMDA-dependent LTP induction) or three trains of 200 Hz stimulation for 0.5 sec, separated by 2 min (NMDA-independent LTP induction). Stimulus intensity of the HFS was matched to the intensity used in the baseline recordings. Responses were monitored for 20 min before high-frequency stimulation was given to ensure a stable baseline. Measurements are shown as the average slope of the pEPSP from six individual traces and are standardized to baseline recordings.

Tissue preparation. Mouse brains were immediately removed and perfused in ice-cold saline (in mM: 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 25 NaHCO_3 , 25 D-glucose, 2 CaCl_2 , and 1 MgCl_2 , saturated with 95% O_2 –5% CO_2 , pH 7.4). Hippocampi were dissected and then homogenized in 2–3 ml of buffer (20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 25 μ g/ml aprotinin, 25 μ g/ml leupeptin, 1 mM $\text{Na}_4\text{P}_2\text{O}_7$, 500 μ M phenylmethylsulfonyl fluoride, 4 mM *para*-nitrophenylphosphate, and 1 mM sodium orthovanadate). Protein concentrations were determined using the BCA protein determination assay (BCA protein assay reagent; Pierce, Rockford, IL).

Western blot analysis. Hippocampal tissue homogenates were resolved on 10% SDS-polyacrylamide gels and transferred to Immobilon-P membranes. The membranes were blocked in 5% dry-milk solution for 1 hr. The primary antibody was diluted 1:3000 in the same 5% dry-milk solution. The membranes were incubated in the primary antibody for 1 hr at room temperature and then washed in Tween-TBS (TTBS) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20). The secondary antibody was diluted 1:30,000 in 5% dry-milk solution, and the membranes were incubated in the secondary antibody for 1 hr at room temperature. The membranes were again washed in TTBS before being developed using the enhanced chemiluminescence method (Amersham Pharmacia Biotech, Arlington Heights, IL). The bands of each Western blot were quantified with densitometry using a StudioScan desktop scanner and NIH Image software to determine the amount of each of the tested proteins.

Kinetic assays. CaMKII enzyme activity was measured by quantifying incorporation of [^{32}P]PO $_4$ into a synthetic peptide substrate as described by Roberson et al. (1996). Briefly, *in vitro* assays consisted of 5–10 μ g of hippocampal homogenate protein resuspended in reaction buffer (in mM: 200 Tris, 5 EGTA, 10 EDTA, and 20 Na_2PO_4), 100 μ M autocamide peptide substrate, [γ - ^{32}P]ATP, 0.5 mM ATP, and H_2O in a final assay volume of 50 μ l. The reaction samples were incubated at 37°C for 20 min and terminated by addition of 25 μ l of stop solution (in mM: 225 H_3PO_4 and 1 ATP). Two aliquots from each sample were transferred onto Whatman P-51 paper. After drying at room temperature, the filter papers were washed three times for 10 min in 0.25 M H_3PO_4 and once for 2 min in

95% (v/v) ethanol with gentle agitation. Chromatography papers were air dried before quantitation by liquid scintillation counting.

Autophosphorylation. CaMKII autophosphorylation assays were performed from whole hippocampal homogenates. Homogenates were thawed before use, and 15 μ g of protein was used for each assay. Each 50 μ l assay reaction was incubated on ice for 15 min with the addition of 10 μ M ATP to allow the CaMKII enzyme to reach a state of autophosphorylation equilibrium. Reactions were initiated with the addition of 0.5 μ Ci of [γ - ^{32}P]ATP and 0.5 mM ATP with or without the 10 nM calmodulin and 1 mM CaCl_2 . Reactions were incubated at 32°C for 3 min and stopped with the addition of SDS-containing sample buffer. Samples were heated at 90°C for 1 min, and 25 μ l was loaded onto a 7.5% SDS-polyacrylamide gel. After electrophoresis, gels were dried and placed on Biomax film overnight at –70°C in the presence of an intensifying screen. Band intensities were analyzed and quantified by densitometry using a StudioScan desktop scanner and NIH Image software.

Thr 305 serum specificity. The synthetic peptides CRRRLKLGAIL-pT-TMLATRN containing the phosphorylated threonine (corresponding to α CaMKII-Thr 305) were coupled to keyhole limpet hemocyanin and injected into rabbits (Elgersma et al., 2002). The antisera were screened for recognition to activated CaMKII using purified CaMKII (Sigma, St. Louis, MO) subjected to a kinase reaction in the presence or absence of Ca^{2+} and calmodulin (see Fig. 4A). No immunoreactivity was seen when blots were probed with P-Thr 305 CaMKII serum to purified CaMKII samples incubated with PP1 (Sigma) after the autophosphorylation reaction (data not shown). Specificity to the P-Thr 305 CaMKII site was tested by preincubation of the serum with either the synthetic peptide phosphorylated at Thr 305 or a synthetic peptide lacking the Thr 305 phosphate. Precleared serum was used to probe blots from hippocampal homogenates that were subjected to a kinase phosphorylation reaction in the presence or absence of Ca^{2+} and calmodulin (see Fig. 4A). Because of the apparent high specificity of the serum exclusively to the P-Thr 305 CaMKII site, the serum was not affinity purified and was directly used to probe blots of hippocampal homogenates from wild-type (WT) and m^{-}/p^{+} animals.

Postsynaptic density isolation. PSD was isolated as described previously (Carlin et al., 1980). After decapitation, brains were removed and hippocampi were dissected and frozen on dry ice within 2 min. The speed of hippocampus isolation and flash freezing were optimized to reduce ischemia-induced CaMKII translocation to the PSD, as described previously by Suzuki et al. (1994). Hippocampi were homogenized by hand using a homogenization buffer without detergents (0.32 M sucrose, 1 mM NaHCO_3 , 1 mM MgCl_2 , and 0.5 mM CaCl_2). The homogenates are then centrifuged at 1400 \times g for 10 min. The resulting pellet is washed in the homogenization buffer and centrifuged at 710 \times g for 10 min. The supernatant from this spin is combined with the one from the first spin, and this sample is centrifuged at 13,800 \times g for 10 min. The pellet from this spin is then resuspended in a second homogenization buffer (0.32 M sucrose and 1 mM NaHCO_3). This sample is then placed on top of a trilevel sucrose gradient (0.85, 1.0, and 1.4 M sucrose). The sample is centrifuged through the gradient at 82,500 \times g for 1 hr. The protein fraction between the 1.0 and 1.4 M sucrose layers is removed. A Triton solution (1% v/v Triton X-100 in 0.32 M sucrose and 12 mM Tris-HCl, pH 8.1) and the second homogenization buffer are added to this fraction in equal amounts. This suspension is stirred gently and then centrifuged at 30,800 \times g for 20 min. The resulting pellet is resuspended in the second homogenization buffer and used in subsequent Western blotting experiments.

Immunohistochemistry/microscopy. Mice were perfused, and brains were harvested as described previously (Varga et al., 2000). Forty micrometer coronal hemisections were cut on a cryostat and placed in cryoprotectant until time of experiment. Sections were first washed three times for 10 min in TBS and then blocked for 2 hr (blocking buffer: 1% BSA, 5% NGS, and 0.3% Triton X-100 in TBS). Sections were then incubated in primary antibody for 24 hr (antibody buffer: 0.25% BSA, 5% NGS, and 0.3% Triton X-100 in TBS). Phospho-Thr 286 CaMKII primary antibody (Promega, Madison, WI) was used at 1:500. Sections were again rinsed three times for 10 min with TBS and then incubated in fluorescent-labeled secondary antibody for 2 hr in the dark. Alexa Fluor

568 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) was diluted to 1:500 in TBS. Sections were again rinsed three times for 10 min, mounted on Plus slides, and coverslipped with Supermount aqueous mounting medium (Biogenex). Confocal imaging was accomplished using a FluoView FV300 confocal laser scanning microscope on a BX50WI fixed-stage upright microscope equipped with a FV5-ZM stepper motor and FluoView software (Olympus Optical, Melville, NY). Fluorescent images were acquired via excitation with a krypton laser (568 nm line), a 510–550 nm bandpass emission filter set, and a 10× or 20× objective (Olympus Optical) using the confocal aperture suggested by the manufacturer. Kalman accumulation averaging of three or four was used. Maximum projection images were generated with the FluoView software. All figures were ultimately prepared using Photoshop software (Adobe Systems, San Jose, CA).

PP1/PP2A phosphatase assay. Phosphatase activity in whole hippocampal homogenates was measured using a serine–threonine phosphatase assay kit with a PP1/PP2A-specific phosphopeptide (K-R-pT-I-R-R) (Upstate Biotechnology, Lake Placid, NY). Free phosphate was detected after a 20 min incubation at 32°C using a colorimetric assay and compared with a freshly prepared phosphate standard. Specific activity was determined as the amount of picomoles of phosphate released per minute per microgram of protein. Data represent mean \pm SEM. An analysis with a value of $p < 0.05$ was considered to be statistically significant.

Data collection and statistical analysis. Hippocampal area CA1 pEPSPs were recorded using Axon 1320 Digidata data acquisition hardware operated by Axon pClamp 8.2 software. One-way ANOVA was used for multiple group data, and the paired Student's t test was used to evaluate differences in experimental control and mutant values. GraphPad Prism data analysis software was used for graph production, curve fitting, and statistical analysis. Data represent mean \pm SEM. An analysis with a value of $p < 0.05$ was considered to be statistically significant.

Results

The AS mouse model is a powerful tool that can be effectively used to better understand the biochemical mechanisms involved in human maternal E6-AP deficiency. However, relating the genetic and biochemical abnormalities observed in a mouse model for AS to humans with AS has certain limitations. For instance, we have gained substantial insight into the brain region-specific maternal imprinting of *Ube3a* from investigation of the AS mouse model. This is consistent with our current knowledge that the human *Ube3a* gene undergoes brain-specific imprinting (Rougeulle et al., 1997; Vu and Hoffman, 1997), but the exact brain regions for *Ube3a* imprinting in the human can only be inferred. However, there are many phenotypic and morphological abnormalities that are quite similar in the AS mouse model. For example, human newborns appear to be physically well formed, but by 12 months of age show signs of cranial growth decline and decreased brain size. Although not as prominent, the m^{-}/p^{+} mice show similar changes in head size, with reduced brain weight at 18 d of age that persists into adulthood (Jiang et al., 1998). In addition, many other phenotypic characteristics of human AS are also seen in the AS mouse model, such as an ataxic gait, seizure, an abnormal electroencephalogram, and learning deficiencies. We reasoned that, by understanding the deficits in hippocampal synaptic function and molecular and biochemical changes in the AS mouse model, we could identify putative molecular mechanisms contributing to the cognitive deficits in human AS.

Recovery of *Ube3a* mutant LTP

Numerous examples exist of differential responses of hippocampal CA1 LTP induction with variations in stimulus intensities, duration of stimulation, or changes in incubation temperatures (Krelstein et al., 1990; Muller and Lynch, 1990). A standard LTP-

inducing stimulation consisting of a 1 sec, 100 Hz stimulation elicits a long-lasting increase in synaptic potentiation when slices are maintained at room temperature (25°C). Alternatively, maintaining slices at a higher temperature (30–32°C) and/or increasing the number of high-frequency trains of stimulation can produce a potentiation that is substantially higher in magnitude and longer lasting. Manipulating variables such as stimulus intensity, temperature, and number of stimulations is often used to determine the efficacy or potency of an applied drug or, as in this case, to evaluate the severity or penetrance of a mutant genotype.

Initial studies showing hippocampal CA1 LTP deficits in the *Ube3a* m^{-}/p^{+} mutants (Jiang et al., 1998) were performed in an interface chamber maintained at 25°C and coupled with a modest LTP-inducing stimulus (consisting of two 1 sec trains of 100 Hz stimulation separated by 20 sec). As with any LTP deficit determined with a single LTP-induction protocol, it is unknown whether the LTP impairment in the m^{-}/p^{+} mutants is caused by the inability to reach an LTP induction threshold for a specific type of LTP, or alternatively, whether it reflects a more severe perturbation in a signal transduction cascade absolutely required for the induction of LTP. Therefore, we varied temperature and LTP-induction protocols to determine the nature of the LTP deficit in the *Ube3a* knock-out.

To test the possibility that the LTP deficits in m^{-}/p^{+} mice were caused by an increase in the threshold of LTP induction, LTP was induced while maintaining hippocampal slices at increased temperatures and increasing the number of stimulating trains. In the first experiment, we maintained the interface chamber temperature at 32°C. As observed previously for LTP induced at 25°C, LTP induced at 32°C was essentially eliminated in AS mice (Fig. 1A). In the next experiment, we combined the increase in temperature (32°C) with an increase in the number of stimulus trains delivered to the slice. Surprisingly, when the slice was maintained at 32°C and 100 Hz stimulation was increased from one to three sets of HFS, LTP induction was rescued in the *Ube3a* mutant (Fig. 1B). These results suggest that the LTP impairment in *Ube3a*-deficient mice is primarily attributable to an alteration in the threshold of synaptic stimulation necessary for LTP induction to occur.

The induction of LTP is highly dependent on an influx of postsynaptic Ca^{2+} . The ability to rescue LTP in m^{-}/p^{+} mice with saturating HFS raises the question of whether the derangement of LTP induction was upstream (NMDA receptor activation) or downstream of Ca^{2+} influx. To help determine whether the increase in LTP threshold observed in m^{-}/p^{+} *Ube3a* mutants is caused by insufficient postsynaptic Ca^{2+} influx, slices were stimulated in a manner that allows calcium-dependent but NMDA receptor-independent LTP. Thus, LTP was induced with repeated very-high-frequency stimulation (200 Hz for 1 sec at 32°C) in the presence of the NMDA receptor antagonist AP-5. Using this protocol, a robust, long-lasting potentiation was induced in m^{+}/p^{+} controls, but *Ube3a* m^{-}/p^{+} mutants exhibit a loss of potentiation by 30 min after the last stimulus (Fig. 1C). The inability of m^{-}/p^{+} mutants to achieve NMDA receptor-independent LTP induction suggests that at least one locus of the observed LTP deficit resides downstream of calcium influx.

Potential protein kinase targets

The absence of E6-AP ubiquitin ligase causes the accumulation of E6-AP targeted proteins (such as p53) in areas for which maternal gene imprinting is occurring, but no known LTP-associated protein has been identified as a target of E6-AP. Therefore, potential candidates for the observed physiologic deficits downstream of

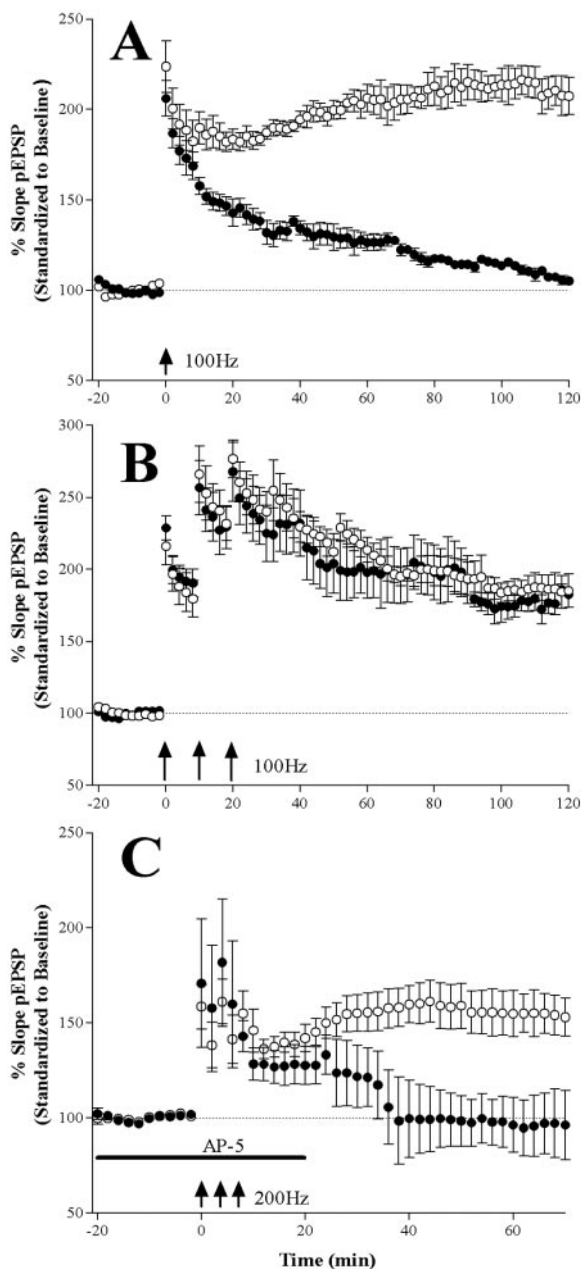


Figure 1. The LTP deficit in m^{-}/p^{+} mice is NMDA receptor dependent. Baseline synaptic responses of wild-type (m^{+}/p^{+}) (○) and maternal-deficient mutant (m^{-}/p^{+}) (●) animals were measured before application of LTP-inducing HFS (indicated by arrows) to hippocampal area CA1. *A*, Slices were maintained at 32°C, and LTP was induced with a single set of HFS consisting of two trains of 100 Hz stimulation for 1 sec separated by 20 sec. *B*, Slices were maintained at 32°C, and NMDA receptor-dependent LTP was induced with three sets of HFS, with each set separated by 10 min. *C*, NMDA receptor-independent LTP was induced with three sets of HFS consisting of 200 Hz stimulation for 1 sec separated by 4 min. The black bar represents the application of the NMDA receptor antagonist AP-5. All results are graphed as the percentage of potentiation standardized to the baseline recording. Dashed lines represent the 100% mark of baseline synaptic responses. Data represent mean \pm SEM.

calcium influx could include any calcium-dependent, or alternatively, calcium-activated enzyme necessary for LTP induction (Fig. 2*A*). Thus, in the next phase of our experiments, we focused on four calcium-regulated protein kinase families that have gained attention for playing prominent roles in LTP and learning and memory in the behaving animal: protein kinase C (PKC), cAMP-dependent protein kinase A (PKA), extracellular signal-

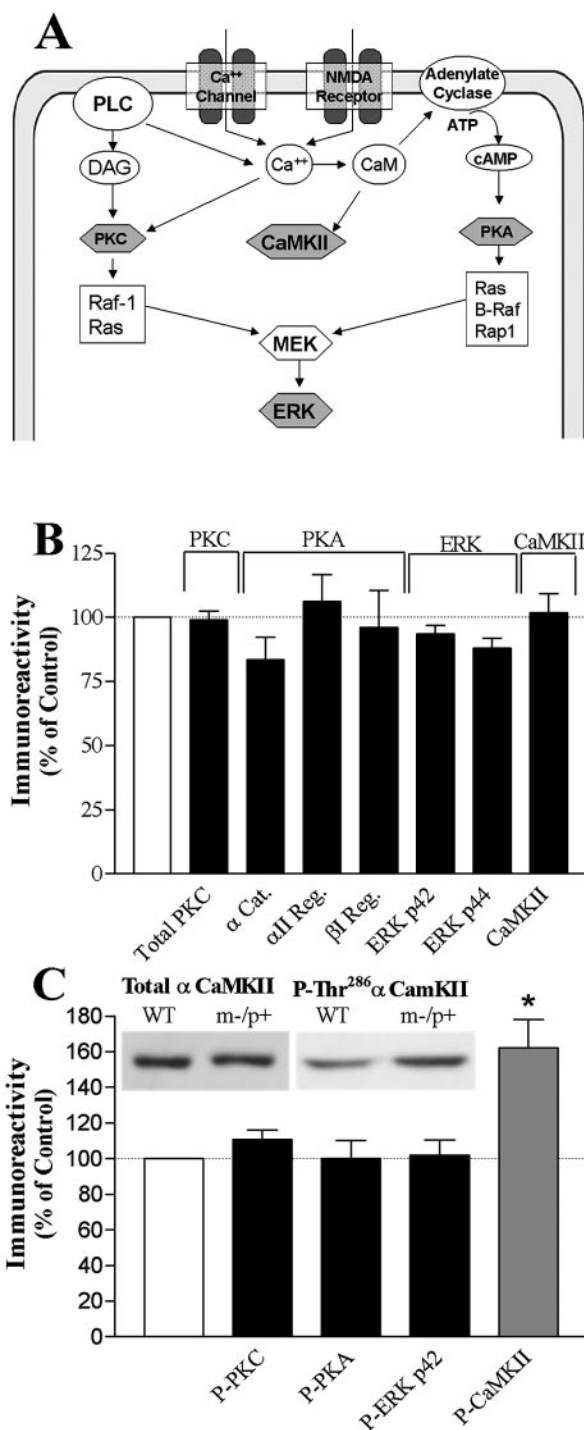


Figure 2. Kinase concentrations in m^{-}/p^{+} mutant hippocampus. *A*, A schematic diagram of a postsynaptic neuron showing potential candidates (shaded hexagons) for LTP and learning disruption observed in E6-AP-deficient mice. *B*, Quantitative Western blot analysis on hippocampal homogenates from wild-type and m^{-}/p^{+} mutants probed for total PKC, PKA [α catalytic (α Cat.), α II regulatory (α II Reg.), and β I regulatory (β I Reg.) subunits], ERK (p42 and p44 MAP kinase), and CaMKII. *C*, Top, Representative Western blot showing no change in total CaMKII but an increase in P-Thr²⁸⁶ α CaMKII in m^{-}/p^{+} mouse hippocampal homogenates. Bottom, Quantitative Western blot analysis of hippocampal homogenates for protein levels of phospho-PKC, phospho-PKA, phospho-p42 ERK, and phospho- α CaMKII. A significant increase was detected in m^{-}/p^{+} mice only for total P-Thr²⁸⁶ α CaMKII (WT, 100 \pm 7.1%, n = 10; m^{-}/p^{+} , 162 \pm 16.2%, n = 10; p = 0.0024). Results shown are the percentage difference of m^{-}/p^{+} protein levels compared with that of wild type. Dashed line represents control level of 100%. * p < 0.05. Data represent mean \pm SEM. PLC, Phospholipase C; DAG, diacylglycerol; MEK, mitogen and extracellular signal regulated kinase.

regulated kinase (ERK), and CaMKII. Common characteristics make these attractive candidates for possible involvement in the m^{-}/p^{+} mutant LTP and behavioral phenotype. For example, all four kinases are (1) activated by HFS in the hippocampus, (2) directly or indirectly affected by calcium influx into the postsynaptic neuron, (3) involved in the induction of hippocampal LTP, and (4) known to be necessary in mammalian learning. Because the m^{-}/p^{+} mutants are deficient for E6-AP ubiquitin ligase in the hippocampus, we predicted that there would be a significant increase in the relevant protein target of E6-AP in the mouse. Therefore, an examination was performed of the levels of a wide variety of specific members of these protein kinase families in the hippocampus of our m^{-}/p^{+} mutant mice.

PKC/PKA/ERK/CaMKII protein levels in m^{-}/p^{+} mutants

Knock-out mouse models for PKC- β and PKC- γ make it evident that these specific isoforms likely play important roles in mammalian synaptic plasticity and memory (Abeliovich et al., 1993a,b; Weeber et al., 2000). Therefore, any ubiquitin ligase-dependent alterations in PKC isoform expression could have profound effects on LTP induction and learning. Levels of seven different PKC isoforms were unchanged in the hippocampus of *Ube3a* mutants (data not shown). To test whether slight changes in each isoform could result in a significant change in the total PKC complement, a broad-spectrum antibody for total PKC detection was used. We determined that the total PKC protein level in m^{-}/p^{+} homogenates was no different from that of controls (Fig. 2B).

We were particularly interested in the levels of PKA, because ubiquitin-proteasome-dependent reductions in the regulatory subunit concentrations of PKA are required for long-term facilitation in *Aplysia* as part of a mechanism by which autonomously active PKA is generated (Chain et al., 1999). In addition, PKA is known to be important in triggering the late phase of LTP and in long-term memory in mice (Chetkovich et al., 1991; Roberson and Sweatt, 1996; Abel et al., 1997). However, we found no difference in the α (catalytic) and αI or βII (regulatory) subunits of PKA in the hippocampus of AS mice (Fig. 2B). This suggests that E6-AP is not involved in PKA regulatory or catalytic subunit degradation, and that this site is not the locus of derangement in m^{-}/p^{+} mice.

It has only recently become clear that the ERK cascade is essential for the induction of NMDA receptor-dependent and -independent LTP and plays a critical role in mammalian learning (Adams and Sweatt, 2002). This prompted us to examine the protein levels of hippocampal p42 and p44 ERK levels in the m^{-}/p^{+} *Ube3a* mutant. Hippocampal homogenates from *Ube3a* mutant animals probed for p42 and p44 ERK MAP (mitogen-activated protein) kinase immunoreactivity showed no change in protein levels compared with wild-type controls (Fig. 2B).

CaMKII is particularly enriched in the brain and exhibits multifunctional roles in calcium-mediated signal transduction processes. CaMKII is known to be involved in synaptic plasticity and learning. Neuronal CaMKII is predominately composed of the α and β subunits, with sizes of 52 and 60 kDa, respectively. The regulation of CaMKII is complex, and is dependent on many factors, including calcium influx, subunit composition, binding to the postsynaptic density, calmodulin availability, and the autophosphorylation state of the holoenzyme. These characteristics make this enzyme especially susceptible to changes within the cellular milieu. However, no changes in total CaMKII concentrations were seen in AS mice (Fig. 2B). Thus, the results on levels of specific PKC, PKA, ERK, and CaMKII isoforms strongly

suggest that protein levels of several kinases known to be involved in synaptic plasticity and learning are normal in the AS hippocampus.

Hippocampal protein phosphorylation

Although an absence of significant changes in protein concentration were observed for PKC, PKA, ERK, and CaMKII, changes in the phosphorylated form of these kinases may not be evident by total protein determinations. The important regulatory actions of phosphorylation are well established, especially regarding the regulation of protein kinase activity. Such is the case in each of the kinases we investigated. Alterations in levels of phosphorylated protein kinases may underlie the disruption in LTP and learning in the AS mouse. Moreover, there are several examples of protein phosphorylation acting as a molecular switch to target a protein for ubiquitination and degradation through the proteasome pathway (Basu and Haldar, 2002; Zheng et al., 2002). Thus, phosphorylated kinases involved in LTP may be a target of E6-AP. To test this hypothesis, we examined the level of phosphorylated PKC, PKA, ERK, and CaMKII. No changes were seen in the phosphorylation levels of PKC, PKA, or ERK (P42) isolated from hippocampal homogenates of m^{-}/p^{+} mice. However, a significant increase was detected in phospho- α CaMKII at the autophosphorylation site threonine 286 (Thr²⁸⁶) (Fig. 2C).

These data suggest that alterations in CaMKII activity, secondary to altered autophosphorylation, could contribute to the LTP and learning phenotype of the AS mouse. This observation becomes increasingly important as it has been widely demonstrated, using genetic and pharmacological manipulations, that normal CaMKII function is necessary for learning and memory. Moreover, the alteration in CaMKII autophosphorylation appears to be a specific and restricted phenomenon. Importantly, absence of E6-AP in the hippocampus of m^{-}/p^{+} mice does not affect the overall phosphorylation state of any of the other proteins we tested. These results highlight the specific nature of the hippocampal derangement associated with the *Ube3a* maternal-deficient phenotype.

Autophosphorylation assays

To confirm the changes in α CaMKII autophosphorylation, we performed another experiment using a back-phosphorylation approach. Our rationale for this experiment is as follows: the result showing an increase in hippocampal P-Thr²⁸⁶ α CaMKII in our *Ube3a* maternal-deficient mice suggested that sites for additional autophosphorylation would no longer be available. Therefore, we performed an autophosphorylation assay in which CaMKII-activating concentrations of Ca^{2+} and CaM were added to hippocampal homogenates, and the amount of subsequent additional autophosphorylation was quantified by assessing ³²P incorporation from [³²P]ATP. Figure 3A shows that the amount of Ca^{2+} /CaM-induced CaMKII autophosphorylation was significantly reduced in hippocampal homogenates from our *Ube3a*-deficient mice. An important caveat to keep in mind when assessing these results is that this assay does not give an indication of which sites are autophosphorylated [i.e., the assay does not distinguish between autophosphorylation at threonine 286 versus threonine 305 or Ser³¹⁴ (Patton et al., 1990) of the α catalytic subunit]. Nevertheless, this observation supports the Western blot analysis results showing that increased autophosphorylated α CaMKII is present in the *Ube3a* maternal-deficient mouse hippocampus.

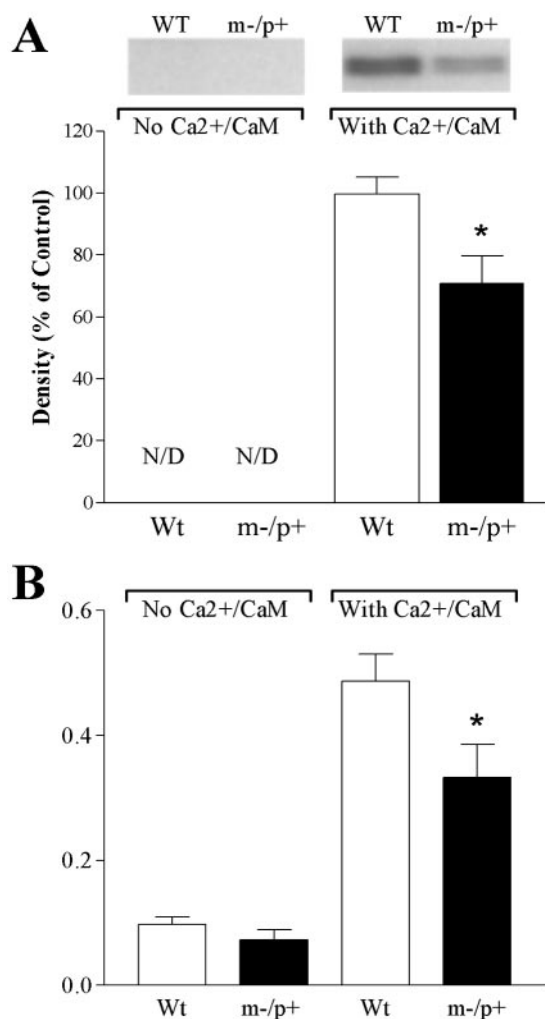


Figure 3. Increased phosphorylated Thr²⁸⁶- α CaMKII corresponds to reduced *post hoc* autophosphorylation and kinase activity. *A, Top*, Representative autoradiograph from hippocampal homogenates corresponding to α and β catalytic subunits of CaMKII in the presence of [γ -³²P]ATP before and after Ca²⁺/calmodulin stimulation. *Bottom*, Quantitative analysis of the total Ca²⁺/CaM-dependent CaMKII autophosphorylation in hippocampal homogenates of wild-type animals (\square) or m⁻/p⁺ mutants (\blacksquare) shows a significant reduction in total CaMKII autophosphorylation after activation with calcium and calmodulin (wild type, 100 \pm 5.5%, n = 6; m⁻/p⁺, 71 \pm 8.9, n = 7; p = 0.002). Bars represent the percentage of quantified signal compared with that of wild types. *B*, Hippocampal homogenates from wild-type animals (\square) or m⁻/p⁺ mutants (\blacksquare) were assayed for basal CaMKII activity and CaMKII activity after activation with Ca²⁺ and CaM. The m⁻/p⁺ mutants (n = 6) show no significant difference in basal phosphotransferase activity compared with wild-type controls (n = 7). Lower total activity after CaMKII activation in the presence of Ca²⁺ and CaM was seen in m⁻/p⁺ mutants (wild type, 0.33 \pm 0.05, n = 6; m⁻/p⁺, 0.487 \pm 0.04, n = 7; p = 0.048). Data represent mean \pm SEM. N/D, Not detected; Wt, wild type. * p < 0.05.

In vitro CaMKII assay

Although the activity of CaMKII is highly sensitive to calcium influx, the activity of the enzyme and its sensitivity to successive increases in calcium concentrations are altered after autophosphorylation. Changes in the phosphorylation state of the α subunit of CaMKII can alter the kinetic properties of the holoenzyme, control translocation, and modify enzyme function, all without a detectable change in total CaMKII concentrations. Our observation of an altered phosphorylation state of CaMKII suggests a resulting change in CaMKII enzymatic activity. Specifically, a greater ratio of P-Thr²⁸⁶- α CaMKII to nonautophosphorylated α CaMKII enzyme would suggest an increased basal

calcium/calmodulin-independent activity. The increase in autonomously active CaMKII complement should also translate into a reduced fold-increase in activity in response to activation with Ca²⁺ and CaM. We tested this hypothesis by performing *in vitro* CaMKII activity assays using hippocampal homogenates and comparing the amount of basal activity and Ca²⁺/CaM-stimulated activity between wild-type and m⁻/p⁺ animals. The maternal-deficient mice showed a reduced, but not quite significant, basal CaMKII phosphotransferase activity (Fig. 3*B*). Moreover, assays performed in the presence of calcium and calmodulin revealed a significant reduction in the phosphotransferase activity of the maternal-deficient mutants (Fig. 3*B*). No differences are seen when the percentage of activation is determined (percentage change of basal vs activated phosphotransferase activity) (data not shown). These data demonstrate that the increases in the autophosphorylation state of CaMKII associated with E6-AP deficiency cause an overall reduction in CaMKII activity, most noticeably as a significant reduction in response to Ca²⁺ and CaM.

Thr³⁰⁵- α CaMKII autophosphorylation

Our *in vitro* CaMKII activity results were unexpected. We hypothesized that a greater P-Thr²⁸⁶- α CaMKII complement would translate into a greater overall basal CaMKII activity in the absence of Ca²⁺ and CaM. One explanation for these results is that a subset of CaMKII in the assay is in an inactive state. Previous *in vitro* experiments indicate that autophosphorylation of Thr³⁰⁵ and Thr³⁰⁶ sites on α and β CaMKII, respectively, occurs after Thr²⁸⁶- α CaMKII phosphorylation under some circumstances (Colbran and Soderling, 1990; Patton et al., 1990; Mukherji and Soderling, 1994). This can affect both the Ca²⁺/CaM-independent activity (Lou and Schulman, 1989) as well as the Ca²⁺/CaM-dependent activity (Kuret and Schulman, 1985; Hashimoto et al., 1987; Lickteig et al., 1988; Lou and Schulman, 1989; Mukherji and Soderling, 1994).

Therefore, we hypothesized that increased phosphorylation of Thr³⁰⁵- α CaMKII (P-Thr³⁰⁵- α CaMKII) was occurring in the AS mice as an explanation for the decreased basal enzyme activity despite the increase in Thr²⁸⁶- α CaMKII autophosphorylation. We tested this hypothesis using an antiserum raised against a peptide duplicate of the phosphorylated Thr³⁰⁵, Thr³⁰⁶ CaMKII site for the α and β CaMKII subunits, respectively (CRRKLGAIL-pT-TMLATRN). It should be noted that, because of the high homology between the α and β subunits of CaMKII, the peptide used resulted in an antiserum that was able to recognize both the Thr³⁰⁵- α CaMKII and Thr³⁰⁶- β CaMKII subunits (Fig. 4). Figure 4*A* shows the specificity of the serum to the phosphorylated Thr^{305/306} CaMKII site. Purified CaMKII (Sigma) was placed in a kinase reaction mixture in the presence or absence of Ca²⁺ and CaM (described in Materials and Methods). Western blot analysis using the Thr^{305/306} CaMKII serum showed immunoreactivity specifically to the activated CaMKII. We then tested for specificity to the P-Thr^{305/306} CaMKII site using hippocampal homogenates from control wild-type mice. Again, homogenates were placed in a kinase reaction with or without Ca²⁺ and CaM, and Western blot analysis was performed using the Thr^{305/306} CaMKII serum. Both the α and β subunits of CaMKII were detected using the P-Thr^{305/306} CaMKII serum to probe hippocampal homogenates from wild-type animals (Fig. 4*A*). The wild-type control samples (no peptide preincubation) show immunoreactivity corresponding to the α and β subunits of CaMKII, which is increased in the homogenate after activation with Ca²⁺ and CaM. Immunoreactivity is nearly absent if the

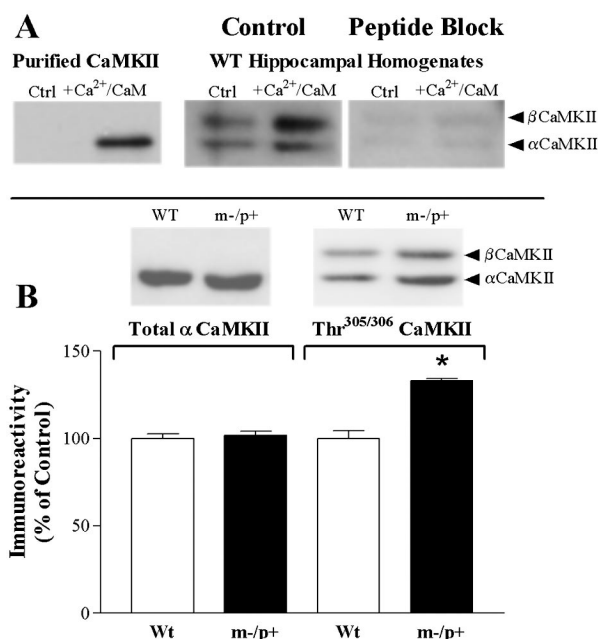


Figure 4. Increased phosphorylated Thr³⁰⁵ CaMKII in m^{-}/p^{+} mutants. *A, Left blot*, Western blot analysis showing that P-Thr^{305/306} CaMKII serum recognizes purified CaMKII activated with Ca^{2+}/CaM , but not nonactivated CaMKII. *Right blot*, Specificity for the Thr³⁰⁵ autophosphorylation site was tested with autophosphorylation reactions with hippocampal homogenates from wild-type mice in the absence [control (*Ctrl*)] or presence ($+Ca^{2+}/CaM$) of calcium and calmodulin. Control Western blots show an increase in immunoreactivity for activated CaMKII corresponding to the α and β subunits of CaMKII. Addition of antigen completely blocked recognition of phospho-Thr^{305/306} CaMKII in control and activated homogenates (peptide block). *B, Top*, Representative Western blot analysis of hippocampal homogenates probed for P-Thr^{305/306} CaMKII (*right blot*). The same blot was then stripped and reprobed for total α CaMKII (*left blot*). *Bottom*, Quantitative Western blot analysis shows no change in total α CaMKII but an increased Thr³⁰⁵ α CaMKII phosphorylation in m^{-}/p^{+} mutants in whole hippocampal homogenates [wild type (*Wt*), 100 ± 12 , $n = 7$; m^{-}/p^{+} , 133.2 ± 3.3 , $n = 7$; $p = 0.02$]. Data represent mean \pm SEM. * $p < 0.05$.

Thr^{305/306} CaMKII serum is preincubated with a phosphorylated Thr^{305/306} peptide (Fig. 4A), and no change is seen in immunoreactivity if serum was preincubated with the nonphosphorylated synthetic peptide (data not shown). These results suggest that the Thr^{305/306} CaMKII serum is highly sensitive to the autophosphorylated species of CaMKII, is specific to the Thr^{305/306} CaMKII autophosphorylation site, and is reliable to test for changes in P-Thr³⁰⁵ α CaMKII in hippocampal extracts.

Using the Thr^{305/306} CaMKII serum, we found that mice maternally deficient for *Ube3a* showed a significant increase in Thr³⁰⁵ α CaMKII autophosphorylation in extracts of hippocampal homogenates (Fig. 4B). Thus, the m^{-}/p^{+} mutants exhibit altered phosphorylation of α CaMKII at both sites of autophosphorylation, Thr²⁸⁶ (Fig. 2C) and Thr³⁰⁵ (Fig. 4B). The increase in P-Thr³⁰⁵ α CaMKII is consistent with the observed reduction in basal CaMKII activity in our *in vitro* assays and also with the loss of Ca^{2+}/CaM -induced CaMKII activation, and indeed appears to be sufficient to explain the observation.

Postsynaptic density isolation

Recently, a T305D CaMKII-transgenic mouse was generated that mimics persistent inhibitory phosphorylation. This mutation is capable of decreasing the association of CaMKII with the PSD and can detrimentally affect LTP and learning (Elgersma et al., 2002). In contrast, blocking inhibitory phosphorylation increases CaMKII in the PSD. These remarkable results raise the interesting

question of whether increased Thr³⁰⁵-phosphorylated α CaMKII in our m^{-}/p^{+} mice could result in decreased PSD-associated CaMKII. To test this hypothesis, we examined levels of total and autophosphorylated CaMKII from PSDs isolated from the hippocampus of wild-type and m^{-}/p^{+} mice. We found that, in our m^{-}/p^{+} mutants, the total PSD-associated CaMKII was significantly reduced, to nearly one-half of that found in wild types (Fig. 5A). These results suggest that in the AS mice, there are sufficient amounts of phosphorylated Thr³⁰⁵ α CaMKII to cause a considerable translocation of the CaMKII complement away from the PSD. To test further the phosphorylated state of the CaMKII present in the PSD, we probed for P-Thr²⁸⁶ and P-Thr³⁰⁵ CaMKII. Surprisingly, we found a 2.5-fold increase in P-Thr²⁸⁶ CaMKII but no change in P-Thr³⁰⁵ CaMKII (Fig. 5B). These results support recent data showing that α CaMKII autophosphorylated at Thr^{305/306} has lower affinity for the PSD than nonphosphorylated α CaMKII (Strack et al., 1997a; Shen et al., 2000).

A caveat to these results arises because of the ability of CaMKII to rapidly translocate to the PSD after animals have been killed (Suzuki et al., 1994). Suzuki et al. (1994) have reported that, in rat frontal cortex, the amount of α CaMKII is 2.7, 4.0, 7.8, 8.6, or 9.4% of the total PSD protein at 0, 2, 5, 30, or 60 min after decapitation. To minimize the potential of postkilling CaMKII translocation, we rapidly removed and flash froze hippocampi within 2 min of decapitation. It is believed that an ischemia-induced chemical modification renders the enzyme insoluble and causes the association of CaMKII with the PSD; however, the exact mechanism underlying the translocation is unknown. Thus, it is difficult to link changes in PSD-associated CaMKII in mice deficient for hippocampal E6-AP with this phenomenon. Despite this caveat, our observations of an increased concentration of P-Thr²⁸⁶ α CaMKII in PSDs from maternal-deficient mice support findings by Elgersma et al. (2002) of the subsequent Thr³⁰⁵ phosphorylation step promoting PSD dissociation. These observations also indicate that not all Thr²⁸⁶-phosphorylated CaMKII enzyme is also phosphorylated at Thr³⁰⁵, suggesting that the Thr²⁸⁶-autophosphorylated kinase does not automatically undergo subsequent phosphorylation at Thr³⁰⁵. Overall, these results are in remarkable agreement with the observations reported by Elgersma et al. (2002) and are consistent with our hypothesis of altered Thr³⁰⁵ autophosphorylation in the AS mouse model.

Immunohistochemistry

Immunohistochemical techniques were used to localize the changes seen in immunoreactivity to phosphorylated CaMKII at Thr²⁸⁶. Because we had seen a significant increase in Thr²⁸⁶-autophosphorylated CaMKII in our Western blot analysis of whole hippocampal homogenates (Fig. 2C) and in an isolated PSD fraction (Fig. 5B), we used immunohistochemistry to determine whether there might be qualitative differences in the cellular distribution of hippocampal CaMKII in AS mice. As shown in Figure 6, phosphorylated Thr²⁸⁶ CaMKII is localized in the wild-type mouse under basal conditions most strongly in the pyramidal cell layer of area CA1 as well as the granule cell layer of the dentate gyrus. The greatest increase in immunoreactivity is seen in the pyramidal cells in area CA3 (Fig. 6E,F). Weaker immunoreactivity is seen in the dendritic layers of these cells, including the stratum oriens and stratum radiatum of areas CA1 and CA3 and the molecular layer of the dentate gyrus. The distribution of phosphorylated CaMKII at Thr²⁸⁶ in an Angelman mouse hippocampus appears qualitatively the same as that of a wild-type mouse, yet the total immunoreactivity is increased across all

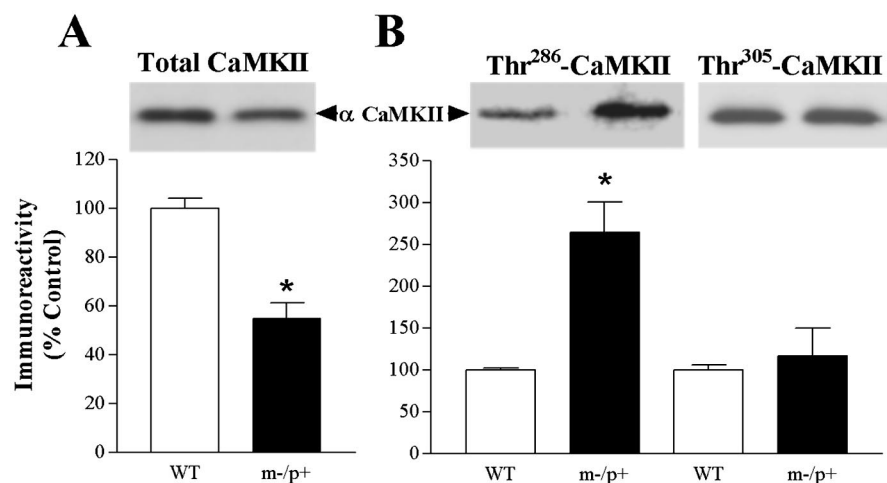


Figure 5. PSD-associated CaMKII and autophosphorylated CaMKII. *A, B, Top*, Representative Western blot for PSD-associated total CaMKII (*A*) and P-Thr²⁸⁶ and P-Thr³⁰⁵ CaMKII (*B*). *A, Bottom*, PSD fractions isolated from the hippocampus of WT (□) or m⁻/p⁺ mutants (■) show a reduction of ~50% in total PSD-associated CaMKII (WT, 100.0 ± 4.2, *n* = 3; m⁻/p⁺, 54.6 ± 6.7, *n* = 4; *p* = 0.045). *B, Bottom*, P-Thr²⁸⁶ CaMKII shows an ~2.5-fold increase in the PSD fraction (WT, 100.0 ± 1.9, *n* = 3; m⁻/p⁺, 264.3 ± 36.4, *n* = 4; *p* = 0.012). P-Thr³⁰⁵ CaMKII levels in the PSD were detectable but showed no significant differences between wild-type and m⁻/p⁺ mice. Data represent mean ± SEM. **p* < 0.05.

labeled areas. Overall, these data suggest that the increase in autophosphorylated CaMKII in AS mice is not specifically localized to one cellular subregion.

Protein phosphatases PP1/PP2A

The observed increases in autophosphorylated CaMKII suggest that a causative candidate for this disruption would be a disruption of a CaMKII phosphatase. Previous investigation has revealed the necessity for phosphatase activity in the regulation of neuronal plasticity and memory formation (for review, see Winder and Sweatt, 2001). A group of these phosphatases, such as PP1 and PP2A, exhibit high CaMKII-specific phosphatase activity and are associated with LTP and learning (Strack et al., 1997b; Blitzer et al., 1998; Kasahara et al., 1999; Brown et al., 2000; Benecib et al., 2001; Winder and Sweatt, 2001). Therefore, we sought to determine whether alterations in the levels of the major CaMKII phosphatases, PP1 and PP2A, were responsible for the increase in the P-Thr²⁸⁶ αCaMKII species. We found no significant difference in PP1 or PP2A total protein levels in hippocampal homogenates from m⁻/p⁺ mice (Fig. 7*A*). These results indicate that *Ube3a* deficiency does not affect protein levels of two known phosphatases associated in P-Thr²⁸⁶ αCaMKII dephosphorylation.

In a final series of experiments, PP1/PP2A phosphatase activity was assayed to determine whether the increase in CaMKII autophosphorylation was a result of alterations in phosphatase capacity. Phosphatase activity was measured using a PP1/PP2A-specific phosphopeptide (K-R-pT-I-R-R). Phosphatase activity using this assay system was found to be significantly reduced in the m⁻/p⁺ mutants (0.659 ± 0.08 pmol · min⁻¹ · μg⁻¹ protein; *n* = 5) compared with controls (1.758 ± 0.27 pmol · min⁻¹ · μg⁻¹ protein; *n* = 8; *p* = 0.009) (Fig. 7*B*). The prodigious 2.5-fold decrease in PP1/PP2A phosphorylase activity in the m⁻/p⁺ mutants strongly suggests that the aberrant state of P-Thr²⁸⁶ and P-Thr³⁰⁵ αCaMKII phosphorylation is attributable to changes in the activity of one or both of these important phosphatases. This observation also suggests that the increase in Thr³⁰⁵ αCaMKII is attributable to both the increase in P-Thr²⁸⁶ αCaMKII, because phosphorylation at Thr²⁸⁶ precedes phosphorylation at Thr³⁰⁵ (Colbran and Soderling, 1990;

Hanson and Schulman, 1992), and reduced PP1 and/or PP2A activity, which are known to dephosphorylate P-Thr³⁰⁵ αCaMKII *in vitro* (Patton et al., 1990).

Discussion

Consistent with the hypothesis that altered LTP parallels a learning deficit in mice and humans, we show that a mouse model for a human mental retardation syndrome displays pronounced hippocampal LTP deficits. Furthermore, the finding that CaMKII is perturbed, without changes in other learning-related kinases, suggests CaMKII as the site for the decreased plasticity and learning and memory defects. These results are consistent with the current literature implicating a role for CaMKII in learning, but extend this to include human learning as well. We reason that our mouse model reflects the mechanisms that are in place in children afflicted with AS. This is supported by our knowledge that the mater-

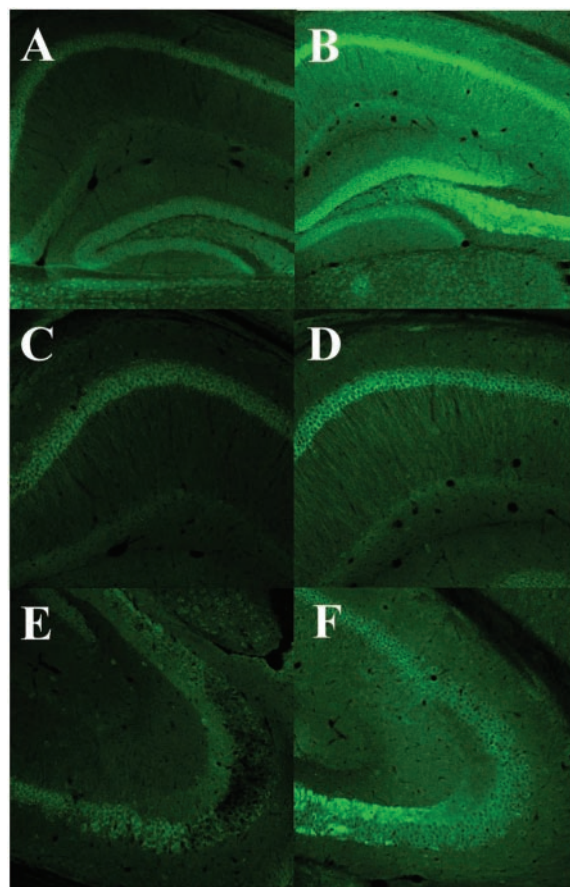


Figure 6. Increased immunoreactivity to phosphorylated CaMKII at Thr²⁸⁶ in Angelman mouse hippocampus. Phosphorylated CaMKII at Thr²⁸⁶ was detected immunohistochemically in the hippocampi of wild-type (*A, C, E*) and Angelman (*B, D, F*) mice. Increases in immunoreactivity are seen in the stratum pyramidale, stratum oriens, and stratum radiatum of CA1 and CA3 as well as the molecular layer and granule cell layer of the dentate gyrus. *A, B*, 100× magnification of the hippocampus. *C, D*, 200× magnification of area CA1. *E, F*, 200× magnification of area CA3.

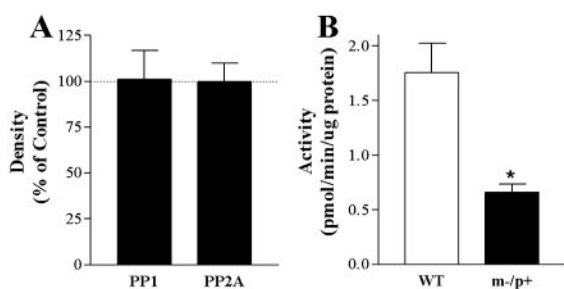


Figure 7. Decreased PP1/PP2A activity in m^{-}/p^{+} mutants. *A*, Quantitative Western blot analysis showing no change in total protein levels of PP1 and PP2A from hippocampal homogenates of m^{-}/p^{+} mutant animals compared with WT controls. The dashed line indicates wild-type protein levels. *B*, Phosphatase activity was measured for PP1/PP2A using a PP1/PP2A-specific phosphopeptide (K-R-pT-I-R-R). Phosphatase activity was significantly reduced in the m^{-}/p^{+} mutants (■) (0.659 ± 0.08 pmol \cdot min $^{-1}$ \cdot μ g $^{-1}$ protein; $n = 5$) compared with wild-type controls (□) (1.758 ± 0.27 pmol \cdot min $^{-1}$ \cdot μ g $^{-1}$ protein; $n = 8$; $p = 0.009$). Data represent mean \pm SEM. * $p < 0.05$.

nal imprinting of *Ube3a* occurs in both systems, and that this imprinting is restricted to the brain in both humans (Rougeulle et al., 1997; Vu and Hoffman, 1997) and mice (Jiang et al., 1998). Finally, the CaMKII-dependent LTP deficit appears to be caused by altered CaMKII regulation in the hippocampus, specifically because of alterations in the autophosphorylation state of the enzyme. These data thereby implicate CaMKII as being required for normal learning in humans.

Insights into the LTP defects in *Ube3a* maternal-deficient mice

The necessity of CaMKII for synaptic plasticity is well established. The induction of NMDA receptor-dependent LTP requires CaMKII activation in the postsynaptic neuron (Nicoll and Malenka, 1995; Lisman et al., 1997), and mice deficient for α CaMKII show deficits in hippocampal LTP (Silva et al., 1992; Hinds et al., 1998). The sites of CaMKII autophosphorylation are also important in LTP induction. Mutations of the Thr²⁸⁶ site to prevent autophosphorylation or, conversely, to produce a calcium-independent form of α CaMKII, result in LTP deficits for some types of LTP inducing stimulation (Giese et al., 1998). With the recent production of a knock-in mouse with mutations that either mimic or prevent inhibitory phosphorylation comes the first insight into the physiologic role of inhibitory autophosphorylation of CaMKII in synaptic plasticity and learning (Elgersma et al., 2002). These studies reveal that T305D mutants show a significant LTP deficit in studies using theta burst stimulation and 100 Hz, 1 sec HFS protocols. These results are especially exciting because we are investigating effects of both phospho-Thr²⁸⁶ and Thr³⁰⁵ CaMKII increases that, because of maternal imprinting of the *Ube3a* gene, are nearly exclusive to the hippocampus. We conclude that the derangements in CaMKII autophosphorylation at Thr²⁸⁶ and Thr³⁰⁵ contribute to the overall AS phenotype. Indeed, the results of Elgersma et al. (2002) suggest that the specific molecular change in phospho-Thr³⁰⁵ α CaMKII is sufficient to explain the LTP and learning deficits seen in the AS mouse model.

Regulatory consequences of altered CaMKII phosphorylation

What are the potential effects associated with an increased Thr²⁸⁶ and Thr³⁰⁵ phosphorylation in CaMKII in m^{-}/p^{+} mutants? In a population of CaMKII molecules in which Thr²⁸⁶ and Thr³⁰⁵ are chronically phosphorylated, one would predict that the overall

effect would be reduced basal activity, because of Thr³⁰⁵ phosphorylation, and insensitivity to increased Ca²⁺/CaM concentrations, because of the combination of Thr²⁸⁶ and Thr³⁰⁵ autophosphorylation. This scenario is in agreement with our *in vitro* assay results, which do not show an increase in basal activity in the absence of Ca²⁺/CaM, despite the presence of increased P-Thr²⁸⁶ α CaMKII. Also in agreement is the reduced activity in the presence of Ca²⁺/CaM, which could reflect both the Ca²⁺/CaM-insensitive Thr²⁸⁶ and inhibited Thr³⁰⁵ CaMKII complement. Moreover, this may also explain the hippocampal LTP deficit when using a modest LTP-inducing HFS protocol, but in which there is normal LTP with saturating HFS. A compromised, but not absent, CaMKII signal in CA1 may result in an inability of LTP induction with the application of a modest stimulus input. However, if an LTP-inducing protocol is robust, the result is sufficient CaMKII activation needed to trigger LTP induction.

Altered PSD-associated CaMKII

The potential regulatory problems involved with the aberrant autophosphorylated CaMKII are compounded in light of recent studies focusing on CaMKII autophosphorylation and enzyme translocation. In a series of elegant studies by Shen and colleagues (Shen and Meyer, 1999; Shen et al., 2002), it was found that the activation of NMDA receptors results in translocation of CaMKII from the cytosol to the postsynaptic density regions (Shen and Meyer, 1999). Also, HFS triggers a transient translocation of CaMKII from the cytosol to the PSD that is primarily dependent on the autophosphorylation state of the CaMKII at Thr²⁸⁶ (Shen et al., 2000). Results by Elgersma et al. (2002) reveal that a T305D CaMKII mouse, which mimics persistent inhibitory phosphorylation, shows decreased association of CaMKII with the PSD. Conversely, a TT305/6VA CaMKII mutation results in greater PSD-associated CaMKII. Together, these results are consistent with published reports that P-Thr²⁸⁶ α CaMKII is localized to the PSD, whereas P-Thr³⁰⁵ α CaMKII causes disassociation from the PSD. Our results suggest a derangement of this regulatory mechanism in our AS mouse model.

Our results show that the total pool of CaMKII in AS mice has more Thr²⁸⁶ and Thr³⁰⁵ autophosphorylation. However, the PSD-associated fraction of CaMKII shows increased Thr²⁸⁶ phosphorylation but not increased Thr³⁰⁵ phosphorylation. This supports the model that Thr²⁸⁶ phosphorylation promotes PSD association by binding to the NMDAR and is in agreement with the observation that association with the NMDAR effectively blocks inhibitory phosphorylation (Bayer et al., 2001) and that Thr³⁰⁵ phosphorylation promotes PSD dissociation (Elgersma et al., 2002). This situation does not preclude there being a population of unaffected CaMKII available for normal signaling and activation, supported by our saturating HFS protocol LTP results. The *in vitro* activity results showing CaMKII activation in the m^{-}/p^{+} mutants, albeit reduced total activation, is also consistent with this idea. Regardless, the alterations seen for both CaMKII autophosphorylated regulatory sites (Thr²⁸⁶ and Thr³⁰⁵) may represent a cumulative disruption in CaMKII signaling capability in the hippocampus. This may explain why the behavioral and LTP deficits in the E6-AP maternal-deficient mice are more pronounced than those in the transgenic mice described by Elgersma et al. (2002).

Protein phosphatase activity and altered autophosphorylated CaMKII

Our data support the hypothesis that CaMKII is not a target of E6-AP-dependent degradation but is indirectly affected by E6-AP

deficiency, as observed by the significant increase in the P-Thr²⁸⁶ and P-Thr³⁰⁵ α CaMKII without an increase in total CaMKII complement. The considerable reduction in PP1/PP2A phosphatase activity suggests that the changes in CaMKII phosphorylation are a direct result of reduced protein phosphatase activity, specifically PP1 and/or PP2A. What may be the underlying mechanism of reduced PP1/PP2A activity? The observed alterations in P-Thr²⁸⁶ and P-Thr³⁰⁵ CaMKII may be caused by the endogenous alterations associated with hippocampal E6-AP deficiency, specifically an increase in proteins usually targeted by a now-absent ubiquitin ligase. For example, p53 is a known target of E6-AP and grossly accumulates in the hippocampus of m^{-}/p^{+} mice, as well as in humans with AS. An interesting possibility arises from the identification of a p53-associated protein, designated p53 binding protein 2 (p53BP2), which can bind p53 and PP1 in a mutually exclusive manner (Helps et al., 1995). Very little is known about p53BP2, and its physiological importance in the CNS is unclear. However, it is known that p53BP2 can inhibit the phosphatase activity of PP1 at nanomolar concentrations. It is hypothesized that p53BP2 may be involved in the dephosphorylation and regulation of p53, but its effect on CaMKII phosphorylation is completely unknown at present. This raises the interesting possibility that the chronic presence and considerable increase in p53 in the imprinted areas of AS mice and humans can directly affect PP1 activity through p53BP2. The effect of p53BP2 may occur either through an upregulation of p53BP2 in response to p53 levels or through a change in the localization of p53, and subsequently p53BP2, throughout the neuron. An alternative hypothesis is that phosphorylation of copious p53 protein can cause phosphatase inhibition. This hypothesis is supported by recent research showing that inhibition of PP1 can occur after p53 phosphorylation in rat cardiomyocytes (Long et al., 2002). The actions of p53BP2 or p53 phosphorylation in Thr²⁸⁶ CaMKII dephosphorylation represent an exciting area of future research.

Conclusions

The association of the observed neurobehavioral features of AS is consistent with our current knowledge of the role of CaMKII in mammalian neural processes. The most important implication of our results relates to learning in humans. Various experimental data support the presupposition that the AS mouse model accurately reflects the condition *in vivo* in AS children. Our data support the hypothesis that the loss of hippocampal LTP underlies the learning deficit in humans. Finally, our observations support the hypothesis that the *Ube3a* maternal deficient-associated increase in the P-Thr²⁸⁶ and Thr³⁰⁵ CaMKII may underlie the deficits in synaptic plasticity and learning in our AS mouse model, and potentially underlie the etiology of human AS. This study is significant because it represents the first report that an alteration in CaMKII has the potential to cause a human disorder associated with a severe learning deficiency.

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