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The Implications of CaMK2A And MeCP2 Signalings In The Cognitive Ability of Adolescents

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Research Article

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Abstract

The glutamatergic signaling pathway is involved in molecular learning and human cognitive ability. Specific single nucleotide polymorphisms (SNPs) in the genes encoding NMDA receptor subunits have been associated with neuropsychiatric disorders by altering glutamate transmission. But how these polymorphisms associated with cognition and brain psychological activities were rarely been explored in healthy adolescents. In this study, we screened SNPs of the glutamatergic signaling pathway to identify genetic variants associated with cognitive ability. We found that single nucleotide polymorphisms (SNPs) in subunits of ionotropic glutamate receptors, including *GRIA1*, *GRINN1*, *GRIN2B*, *GRIN2C*, *GRIN3A*, *GRIN3B*, and Calcium/ Calmodulin-dependent protein kinase II α (*CaMK2A*) associated with the cognitive function of students. Importantly, the plasma *CaMK2A* levels correlated positively with the cognitive ability of senior high school students in Taiwan. We demonstrated that the elevated CaMK2A increased its autophosphorylation at T286 and increased the expression of its downstream targets, including GRIA1 and phosphor GRIA1 *in vivo*. Additionally, the Methyl CpG binding protein 2 (MeCP2), a downstream target of CaMK2A, can activate the expression of CaMK2A, suggesting that MeCP2 and CaMK2A could form a positive feedback loop. In summary, we concluded that members of the glutamatergic signaling, CaMK2A, and MeCP2 were implicated in the cognitive ability of adolescents, and alternating in the CaMK2A expressing may have collective effects on the cognitive ability of youths.

Introduction

N-methyl-D-aspartate receptors (NMDARs) are ionotropic glutamate receptors and are crucial for neuronal communication, which plays a central role in learning, memory, and synaptic development. NMDARs form tetrameric complexes that consist of two GRIN1 subunits and two GRIN2 or GRIN3 subunits. Although NMDARs are widely expressed throughout the central nervous system, their number, localization, and subunit composition are strictly regulated and differ in a cell and synapse-specific manner. All NMDAR subunits contain modular domains that are responsible for controlling distinct functions. All of the ionotropic glutamate receptor subunits, including the seven GRINs, share a common membrane topology, however, there are developmental and regional variations depending on the type of GRIN1 isoform [1, 2]. The GRIN2 subunits have expression patterns that differ strikingly in both times (during development) and space (different brain regions) [3–5]. The GRINA3 and GRIN3B subunits were the last NMDA receptor subunits to be cloned around two-decade ago [6, 7]. GRIN3A expression is low before birth, peaks during early postnatal life, and decreases to low levels in adulthood. In contrast, the GRIN3B subunit expression level is low in early life but increases progressively until adulthood.

Mutations in synaptic proteins playing crucial roles in synaptic activity and dendritic spine morphogenesis lead to cognitive deficits. Because cognitive abilities highly affect intellectual capacity, these proteins are associated with the academic achievements of youths. Of all the identified genes involved in cognitive function, members of the glutamatergic signaling pathway are of specific interest [8]. Glutamate potentially is an excitatory neurotransmitter involved in learning and memory, long-term potentiation, and synaptic plasticity [9]. In neurons, glutamate binds to and activates ionotropic receptors (iGluRs; e.g., N-methyl-D-aspartate [NMDA]), and a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and mediates Ca^{2+} transport, thus activating intracellular signaling cascades to alter synaptic efficacy and trigger long-term potentiation (LTP) induction [10].

One important signaling pathway activated by iGluR-mediated Ca^{2+} influx is that involving Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII), a serine/threonine kinase enriched at excitatory synapses and postsynaptic densities (PSDs) [11]. Upon the binding of the Ca^{2+}/CaM, CaMKII phosphorylates numerous substrates responsible for LTP, including voltage- and ligand-gated Ca^{2+} channels, cAMP-response element-binding protein (CREB), ERK, and voltage-gated sodium channels [12]. One important aspect of CaMKII regulation is autophosphorylation. After initial activation by Ca^{2+}-bound CaM, CaMKII autophosphorylates at Thr286/287, thus enhancing its binding affinity to Ca^{2+}/CaM and inducing Ca^{2+}/CaM-independent autonomous phosphorylation [13]. This autonomous kinase activity of CaMKII has been hypothesized to provide biochemical-based memory storage for LTP [11, 14]. Blockade of the autophosphorylation of CaMK2A (Thr 286), a forebrain-enriched CaMKII isoform, impaired LTP, sLTP, and spatial learning and memory in knock-in mice [15].

Recently several studies demonstrated that *de novo* mutations in CaMK2A disrupt the function of synaptic proteins, which provide novel insights into the synaptic mechanisms contributing to disease pathological pathway[16]. For instance, Glu831 to Val (CaMK2A^{E831V}) mutation in the CaMK2A catalytic domain, decreases both CaMK2A substrate phosphorylation and regulatory auto-phosphorylation. Additionally, CaMK2A^{E831V} could inhibit the phosphorylation of CaMK2A in a dominant-negative manner [17]. Loss of function of CaMK2A mutation (CaMK2A^{H477Y}) mutation causes growth delay, and seizures [18]. Association studies from two *de novo* variants in CaMK2A and CaMK2B demonstrated that Thr286/Thr287, pay a pivotal role in neuronal plasticity in the brain [16, 19]. Activated CaMK2A is targeted to dendritic spines and postsynaptic density via interactions with various CaMKII associated proteins (CaMKAPs) including GluN2B NMDA receptor subunits [20].

CaMKII also functions in the nucleus through nuclear Ca^{2+} signaling. One important substrate of CaMKII is the Methyl-CpG-binding protein 2 (MeCP2). Upon neuronal activity and subsequent Ca^{2+} influx, CaMKII phosphorylates MeCP2 on S421 [21, 22]. This neuronal activity-induced phosphorylation (NAIP) of MeCP2 is essential for numerous neuronal functions and neurodevelopment [23]. MeCP2 phosphorylation at various sites regulates numerous target genes including brain-derived neurotrophic factor (*Bdnf*), *Rab3d*, *Vamp3*, and *Igsf4b*. The NAIP of MeCP2 might function as a molecular switch regulating the dynamic expression of neuronal genes [24].

Methyl CpG binding protein 2 (MeCP2) is an X-linked global transcription regulator that binds methylated sites in DNA, and its dysfunction implicates in Rett syndrome and MeCP2 duplication syndrome. Well-defined mouse models for both syndromes show impairments in learning and memory [25–27]. MeCP2 is an excellent epigenetic biomarker in learning and memory, brain circuits, and behaviors [28–30]. Apart from the neuropathological lesions of MeCP2-related disorders, the mechanism through which MeCP2 affects learning in human cognitive ability (such as intelligence quotient, IQ) remains

unknown. The research aimed at the underlying pathophysiological mechanisms of RTT and MDS has significantly advanced our understanding of MeCP2 functions in the nervous system [31]. In screening for genetic and epigenetic factors associated with the cognitive ability of adolescents, we previously reported that multiple epigenetic biomarkers regulating MeCP2 homeostasis are associated with students' academic performance [32]. Because components of glutamatergic signaling, including, CaMK2A, NMDAR, and AMPAR, are essential for appropriate synaptic development and plasticity, and their disruption leads to cognitive deficits, we speculated that subtle and chronic alterations in these genes may collectively affect the cognitive ability of students.

In our continuous efforts to search for genetic and epigenetic factors associated with the cognitive ability of adolescents, we previously demonstrated that multiple epigenetic biomarkers regulated MeCP2 homeostasis were associated with students' academic performance. Because components of the glutamatergic signaling pathway, including MeCP2, CaMK2, NMDAR, and AMPAR, are essential for proper synapse development and plasticity whose disruption leads to cognitive deficits, we speculated that subtle and chronic alterations in these genes may collectively affect the academic performance of students. In this study, we found that single nucleotide polymorphisms (SNPs) in Calcium/Calmodulin-dependent protein kinase IIα (*CaMK2A*) and subunits of ionotropic glutamate receptors, including *GRIN1*, *GRIN2B*, *GRIN2C*, *GRIN3A*, *GRIN3B*, *GRIA1*, and *GRID1*, associated with the cognitive function of students. Importantly, the protein levels of CaMK2A were elevated in the peripheral blood samples of senior high school students with higher reasoning skills. Using cultured cell systems, we showed that the elevated CaMK2A increased the expression of pCaMK2A and GRIA1. Moreover, a downstream target of CaMK2A, MeCP2 could increase the expression of CaMK2A, suggesting the CaMK2A and MeCP2 could form an autoregulatory positive feedback signal transduction loop. We believed that alteration in the expression of CaMK2A might affect the cognitive ability of adolescents.

Material And Methods

Participants and human blood samples

Three public senior high schools were selected (one each in southern, central, and northern Taiwan). In total, 832 tenth-grade volunteers (269 male; median age, 16.3 ± 0.5 [range, 16–17] years) were recruited. This study was approved by the Institutional Review Board of the National Taiwan University Hospital (Research Ethics identifier: NCT00713570). Volunteers and their parents were explicitly informed about the plan, protocol, study procedure, publication of the data, and any accompanying images or information, and written consent was obtained before the study commenced.

Gene screening, variation analysis, and bioinformatics

DNA samples from 20 healthy participants were genotyped in a pilot study. SNPs with a minor allele frequency of > 5% of samples were considered the most promising candidates, having representative common variants. From the pilot study, SNPs were identified as the most promising candidates. The SNP variants selected were genotyped in all participants. Genotyping was conducted through DNA sequencing of the relevant PCR products using ABI Prism_ BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kits and an ABI Prism_ 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) following manufacturer instructions.

Cognitive ability assessments

Cognitive ability assessment: Cognitive abilities were assessed through the Multiple Aptitude Test Battery (MAT)[33, 34], which is commonly used, thoroughly standardized, and suitable for assessing different cognitive abilities in adolescents. The Chinese version of the MAT was revised from the Differential Aptitude Tests (DAT) [34] for an integrated, scientific, and well-standardized procedure for measuring the cognitive abilities of Taiwanese adolescents [35]. Theoretically, aptitude testing can provide more accurate predictions for learning and working performance than traditional intelligence tests. The MAT comprises eight subtests: verbal reasoning, numerical ability, mechanical reasoning, perceptual speed and accuracy, space relations, abstract reasoning, verbal comprehension, and grammar and language usage. This test contains 496 items and takes approximately 80 min. Mao and Lu reported internal consistency reliability of the MAT ranging from 0.5 to 0.9.

ELISA

Volunteer plasma samples were collected on ice using EDTA as an anticoagulant. Samples were then centrifuged at 4°C for 10 min at 3000 ·g. Plasma was stored at − 80°C until use. Platelets were eliminated via centrifugation for 10 min at 10 000 ·*g*. CaMK2A/CaMKII Alpha levels were measured using this platelet-poor plasma supernatant for assaying. CaMK2A levels were measured using a high-sensitivity (< 0.094 ng/mL) quantitative ELISA (LifeSpan BioSciences, Inc.).

CaMK2A cDNA constructs and cloning

CaMK2A mRNA (cDNA clone MGC: 95) was purchased from the Bioresource Collection and Research Center in Taiwan. CaMK2A cDNA was excised with *NotI* and *ApaI* restriction endonucleases and subcloned with EGFP (fragment from EGFP-N1) at the N-terminal or with DsR (fragment from pDsR-Monomer-C1) at the C-terminal into the pcDNA/Flp Recombination Target (FRT)/TO cloning vector between *HindIII* and *ApaI* sites.

Cell culture and transfection

The Flp-In™ T-REx system (Invitrogen, Carlsbad, CA, USA) was used to generate stably induced HEK293 and SH-SY5Y cell lines exhibiting tetracycline-inducible CaMK2A and MeCP2 expression. First, HEK293-derived Flp-In host cells were purchased from a commercial retailer (Flp-In™ T-REx™ Cell Line, Invitrogen). SH-SY5Y-derived Flp-In host cells were generated via the independent integration of the plasmids pcDNA6/TR (a plasmid expressing the Tet repressor; selected with 5 mg/mL of blasticidin) and pFRT/lacZeo (a plasmid containing the FRT site; selected with 100 mg/mL of Zeocin selected and

transferred to the SH-SY5Y cell genome [ATCC No. CRL-2266]. Thereafter, among the SHSY5Y host cells, which were established by Lee et al. in 2012[36], two cell lines were cotransfected with the pOG44 plasmid (constitutively expressing the Flp recombinase) and the pcDNA5/FRT/TO-CaMK2A or pcDNA5/FRT/TO-MeCP2 plasmid following the supplier instructions. These stable cell lines were cultured in a medium containing 5 mg/mL of blasticidin and 100 mg/mL of hygromycin. Doxycycline (Dox, 1 mg/mL) was added to induce CaMK2A and MeCP2 expression for 2–6 d. Proteins were prepared for Western blot analysis using anti-CaMK2A (1:1000, GeneTex, Irvine, CA, USA), anti-MeCP2 (1:2000, GeneTex), anti-phospho-CaMK2A, anti-phospho-MeCP2, anti-GRIA1, anti-BDNF, and anti-H3.3B antibodies (details provided in the following section).

Western blotting

Total soluble protein samples from HEK293-derived and SH-SY5Y-derived cells with inducible CaMK2A and MeCP2 expression for 0, 2, 4, and 6 d were treated with 3 μM calmodulin (Bovine brain Millipore Sigma-Aldrich) 2 h before harvesting and prepared using a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol-bis (β-aminoethyl ether)-N, N, NN-tetraacetic acid, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, and a protease inhibitor cocktail (Life Technologies [Thermo Fisher Scientific], Carlsbad, CA, USA). After sonication and centrifugation at 15 000 ×g for 10 min at 4°C, the protein concentration was determined (Bio-Rad Protein Assay, Hercules, CA, USA) using bovine serum albumin as the standard. Soluble proteins (25 μg) were separated through SDS-PAGE (12% gel) and electro-blotted onto nitrocellulose membranes. Thereafter, the membrane was blocked with 10% nonfat milk and probed with anti-MeCP2 (1:2000; GeneTex), anti-pMeCP2 (phospho-S80) (1:1000; GeneTex), anti-CaMK2A (1:1000; GeneTex), anti-pCaMK2A (phospho-T286) (1:1000; GeneTex), anti-BDNF (1:2000; GeneTex), anti-GRIA1 (1:2000; Abcam, Cambridge, UK), anti-GRIA1 (phospho-S831) (1:1000; Abcam), and anti-H3.3B (1:2000; GeneTex) antibodies. Immune complexes were detected using horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit (Jackson Immuno Research, West Grove, PA, USA) IgG (1:10000), and a chemiluminescent substrate (Millipore, Burlington, MA, USA).

Chromatin immunoprecipitation

SH-SY5Y-derived cells with inducible MeCP2 expression for 0, 2, 4, and 6 d were treated with Cross-link chromatin with formaldehyde (1% final concentration) for 10 min at 37 ℃ and then quenched with 0.125 M glycine for 5 min. After being washed with PBS twice, samples were homogenized in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris; pH 8.1) containing protease inhibitor (Thermo Fisher Scientific). Chromatin was sheared using a Bioruptor sonicator for 35 30-s-ON/30-s-OFF cycles in a 4℃ water bath, yielding 300–400-bp fragments, as confirmed by agarose gel electrophoresis. For immunoprecipitation, 150 μL of chromatin was diluted at a ratio of 1:10 in chromatin immunoprecipitation (ChIP) dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 167 mM NaCl; pH 8.1) and approximately 7% of the diluted sample was used as input control. Before sample sonication, magnetic protein A/G beads (Merck Millipore, MA, USA) were incubated with anti-MeCP2 antibodies (GeneTex) or rabbit IgG overnight at 4 ℃ under constant rotation in a blocking solution (0.5% BSA in PBS). After washing and resuspension of the antibody-bead conjugates, chromatin samples were added in antibody-beads and incubated for 16 h under constant rotation at 4 ℃. After immunoprecipitation, the beads were washed at least six times (5 min each) with wash buffer (50 mM HEPES-KOH, 500 mM LiCl, 1 mM EDTA, 1% NP-40, 0.7% Na-deoxycholate; pH 7.6), once with TE buffer (10 mM, 1 mM EDTA; pH 8.1), and eluted with 200 μL elution buffer (50 mM Tris-HCl, 1 mM EDTA, 1% SDS; pH 8.1). The eluate was incubated at 65 ℃ for 4 h to reverse the cross-linkage. The input DNA was diluted in elution buffer (up to 200 μL) and processed for cross-link reversal. Samples were then digested sequentially with 0.2 μg/mL RNase A for 2 h at 37 ℃ and with 20 μg of proteinase K for 30 min at 55 ℃. DNA was extracted using the phenol/chloroform/isoamyl alcohol method and subjected to quantitative PCR using the ViiA 7 real-time PCR system (Thermo Fisher Scientific) with primer pairs for *GRIN*, *GRIA1*, and *GRID1*. Relative gene expression levels were determined using the comparative Ct method.

Statistical analysis

The effects of genotypes on cognition (MAT) in participants were assessed through ANOVA tests using genotypes as the dependent variable. Univariate ANOVA was performed to assess differences in the scores of the MAT subtests among the three genotype groups for each SNP. Significance was set at $p < 0.05$. Bonferroni's correction was used for multigroup comparisons. Post hoc testing was performed using Scheffe's F test owing to its high statistical power [37]. (For each SNP, the participants were assigned to one of three groups based on their genotype, and deviation from the Hardy–Weinberg equilibrium was tested using a chi-squared test. Gene-gene interactions were assessed using ANCOVA. The assumptions used for ANOVA and inferential statistical analyses were applied using SPSS version 23.0. Haplotype analyses were performed using Haploview 4.1 software.

Results

Gene screening, variation analysis, and bioinformatics

Our previous studies found that genetic variants in glutamatergic signaling were associated with emotions and social behaviors of adolescents [38]. In this study, we found that specific SNPs in genes encoding NMDA receptor subunits were associated with academic performance and cognitive abilities (Table 1). The observed genotype distribution for each SNP appeared consistent with that expected for the Hardy–Weinberg Equilibrium. Furthermore, the genotype frequencies for each SNP in the study population were consistent with the HapMap-HCB population studies reported by the International HapMap project (http://www.hapmap.org). The participants' background (age, years of education, academic performance, and eight subtests of MAT; Table 1) and the scores obtained for each MAT subtask (verbal reasoning, numerical ability, mechanical reasoning, perceptual speed and accuracy, space relations, abstract reasoning, verbal comprehension, and grammar and language usage) for different genotype groups for each SNP were analyzed through ANOVA (Table 2 and Table 3).

N-Methyl-D-aspartate receptors (NMDARs) are glutamate-gated cation channels that are expressed throughout the brain and play an essential role in physiological and pathological processes in the central nervous systems. Diversity of the subunits and their Spatio-temporal expression imparts distinct functional properties, such as channel kinetics, permeation, or block by divalent cations or sensitivity to endogenous modulators. Students with *GRIN1* (rs4880213) and *CaMK2A* (rs2241694) variants were significantly associated with perceptual speed and accuracy (p = 0.018 and 0.041). *GRIN2B* (rs1805502) and *GRIN2C* (rs3744215) were significantly associated with abstract reasoning, grammar and language usage and mechanical reasoning (p = 0.022, p = 0.053 and p = 0.013). Two SNPs for *GRIN3A* (rs10989589 and rs3739722) were significantly associated with verbal comprehension and abstract reasoning (p = 0.017 and p = 0.033). The other six SNPs for *GRIN3B* have significantly associated eight subtests of MAT as follows. *GRIN3B* (rs2240154) was significantly associated with abstract reasoning (p = 0.012). *GRIN3B* (rs4807399) was significantly associated with abstract reasoning and grammar and language usage (p = 0.035 and p = 0.024). *GRIN3B* (rs2240157) was significantly associated with abstract reasoning (p = 0.025). *GRIN3B* (rs2285906) was significantly associated with mechanical reasoning, abstract reasoning, and perceptual speed accuracy (p = 0.018, 0.03, and 0.018. *GRIN3B* (rs10417824) was significantly associated with abstract reasoning and perceptual speed and accuracy (p = 0.012 and p = 0.033). *GRIN3B* (rs10401454) was significantly associated with five of eight subtests of MAT; such as verbal reasoning, space relations, abstract reasoning, verbal comprehension, and grammar and language usage (p = 0.005, 0.043, 0.008, 0.006, and 0.003, respectively) as listed in Table 3.

Table 1
Demographic characteristics

	Male	Female	*p*
Age (yrs)	16.8 ± 0.32*	16.8 ± 0.30	
Academic performance			
BCT	245.9 ± 19.69	235.8 ± 20.61	< 0.01
Cognitive abilities			
Verbal reasoning	21.5 ± 5.31	21.9 ± 5.16	
Numerical ability	11.1 ± 3.56	10.90 ± 3.37	
Mechanical reasoning	14.3 ± 3.91	13.3 ± 3.46	< 0.01
Space relations	17.1 ± 4.49	15.4 ± 4.49	< 0.01
Abstract reasoning	20.2 ± 4.81	19.1 ± 4.95	< 0.01
Verbal comprehension	21.5 ± 6.14	22.2 ± 5.91	< 0.05
Grammar and language usage	17.9 ± 5.04	18.5 ± 5.00	< 0.05
Perceptual speed and accuracy	67.2 ± 21.33	68.1 ± 20.19	

Table 2
Genotype distributions and chromosome locations of the SNPs

Gene	SNP ID	Allele/genotype	Subjects	Chromosome region	Genotype frequency
GRIN1				9q34.3	
	rs4880213	CC/CT/TT	124/368/340		0.15/0.44/0.41
	rs11146020	CC/CG/GG	565/239/28		0.68/0.29/0.03
GRIN2B				12p12	
	rs3764028	CC/CG/GG	250/396/186		0.30/0.48/0.22
	rs1806201	AA/AG/GG	223/438/171		0.27/0.53/0.20
	rs1805247	AA/AG/GG	608/204/20		0.73/0.25/0.02
	rs1805502	AA/AG/GG	608/203/20		0.73/0.24/0.03
	rs7301328	AA/AC/CC	148/397/287		0.18/0.48/0.34
GRIN2C				17q25	
	rs3744215	AA/AC/CC	143/423/266		0.17/0.51/0.32
GRIN3A				9q34.1	
	rs10989591	CC/CT/TT	738/90/4		0.89/0.11/0.01
	rs10989589	CC/CT/TT	527/265/40		0.63/0.32/0.05
	rs3739722	CC/CT/TT	508/287/37		0.61/0.35/0.04
	rs62000403	TT/AT/AA	708/115/9		0.85/0.14/0.01
GRIN3B				19p13.3	
	rs2240154	CC/CT/TT	254/405/173		0.31/0.49/0.21
	rs35592366	CC/AC/AA	627/191/14		0.75/0.23/0.02
	rs55646937	GG/AG/AA	562/231/39		0.58/0.28/0.05
	rs12978900	TT/CT/TT	697/133/2		0.84/0.16/0.01
	rs4807399	CC/CT/TT	707/119/6		0.86/0.14/0.01
	rs2240157	CC/CT/TT	207/413/212		0.25/0.50/0.26
	rs10666583	MM/Mm/mm	730/98/4		0.88/0.12/0.01
	rs2240158	CC/CT/TT	597/214/21		0.72/0.26/0.03
	rs2285906	GG/AG/AA	626/183/23		0.75/0.22/0.03
	rs10417824	TT/AT/AA	362/355/115		0.23/0.22/0.08
	rs10401454	CC/CG/GG	622/163/47		0.75/0.23/0.02
GRIA1				5q33.2	
	rs548294	CC/CT/TT	374/372/86		0.45/0.45/0.10
GRID1				10q23.1-2	
	rs3814614	GG/AG/AA	522/266/42		0.63/0.32/0.05
CaMK2A				5q32	
	rs2241694	GG/AG/AA	580/235/16		0.70/0.28/0.02

Table 3
SNPs associated with the cognitive abilities of the students and the synaptic plasticity of glutamatergic neurons

Gene	SNP	Cognitive abilities	Verbal reasoning	Numerical ability	Mechanical reasoning	Space relations	Abstract reasoning	Verbal Comprehension	Grammar and language	Perceptual speed and accuracy
GRIN1	rs4880213									F = 4.090 P = 0.018
GRIN2B	rs1805502						F = 3.827 P = 0.022		F = 2.948 P = 0.053	
GRIN2C	rs3744215				F = 4.340 P = 0.013					
GRIN3A	rs10989589							F = 4.081 P = 0.017		
	rs3739722						F = 3.420 P = 0.033			
GRIN3B	rs2240154						F = 4.423 P = 0.012			
	rs4807399								F = 3.755 P = 0.024	
	rs2240157				F = 3.697 P = 0.025		F = 3.360 P = 0.035			
	rs2285906		F = 4.103 P = 0.017		F = 4.034 P = 0.018		F = 3.543 P = 0.030			
	rs10417824			F = 3.064 P = 0.047			F = 4.454 P = 0.012			F = 4.056 P = 0.018
	rs10401454		F = 5.412 P = 0.005			F = 3.158 P = 0.043	F = 4.883 P = 0.008	F = 5.123 P = 0.006	F = 5.832 P = 0.003	F = 3.437 P = 0.033
GRIA1	rs548294				F = 4.000 P = 0.019					
CaMK2A	rs2241694									F = 3.248 P = 0.041

Plasma CaMK2A levels were associated with cognitive abilities

To assess cognitive ability using the MAT, students without physical or psychiatric disorders were included in our studies. As shown above, SNPs of GRIA1 (rs548294) and CaMK2A were highly associated with cognition ability, prompted us to study how the SNPs affect the functions of these genes and how these genes affect the cognitive ability of students. To address this, 120 senior high school students were selected for the study based on their MAT scores. Sixty students with MAT score higher than 80% were assigned as high cognitive ability group, whereas the other sixty students with MAT lower than 12% were assigned as low cognitive ability group. The levels of plasma CaMK2A were measured using the enzyme-linked immunosorbent assay (ELISA). Plasma CaMK2A levels of students with high and low cognitive ability were 529 pg/ml and 271pg/mL respectively. Quantification analysis revealed that plasma CaMK2A levels correlated positively with the cognitive ability of senior high school students in Taiwan (Table 4.).

Table 4
Plasma CaMK2A is positively correlated with students' cognitive abilities.

	HMAT group (n = 60)	LMAT group (n = 60)	T	p
CaMK2A Plasma level (pg/ml)	523.3 (189.5) (pg/ml)	272.1 (214.3) (pg/ml)	6.80	< 0.001
*Mean values (standard deviation) HMAT: Higher Multiple Aptitude Test; LMAT: Lower Multiple Aptitude Test				
***P < 0.001; n = 120, Student's t-test.				

CaMK2A upregulation triggers an autophosphorylation signaling cascade

To assess the effect of CaMK2A upregulation in cells, quantitative immunoblotting was performed. CaMK2A autophosphorylation increased proportionately with its expression, plateauing 2 d after CaMK2A induction. CaMK2A expression persisted on days 2–6, whereas pCaMK2A was slightly downregulated on day 6 (Fig. 1B). Additionally, GRIAl, a component of the AMPA receptor, is phosphorylated by CaMK2 at S831. GRIA1 was concomitantly upregulated with pCaMK2A upregulation on days 2–4 and was downregulated on day 6. CaMK2 and GRIA1 phosphorylation were both increased, suggesting that CaMK2A upregulation might initiate CaMK2 phosphorylation and phosphorylated its downstream targets such as GRIA1 (Fig. 1C).

MeCP2 is involved in CaMKII-mediated phosphorylation regulation

We previously reported a homeostatic regulatory control mechanism of MeCP2, similar to that of CaMKII, in both Rett syndrome progression and synaptic plasticity [32]. Because CaMKII autophosphorylation is essential for LTP induction and memory consolidation, and MeCP2 is a direct target of CaMKII, we investigated whether MeCP2 is involved in an autoregulatory loop to activate CaMKII and increase CaMKII autophosphorylation. Quantitative immunoblotting revealed that CaMK2A and phospho-MeCP2 (S80) were significantly upregulated 2 d after MeCP2 induction in stably transfected SH-SY5Y cells. The expression was persisted through days 2–6 after MeCP2 induction. This indicates that CaMK2A and MeCP2 could form a positive feedback loop. Furthermore, BDNF, a downstream target of MeCP2, was significantly upregulated upon MeCP2 overexpression in SH-SY5Y cells, indicating that phospho-MeCP2 was released from the BDNF promoter, thereby facilitating BDNF expression.

GRIA1 and GRID1 are direct targets of MeCP2

To determine the correlation between the identified SNPs and their potential regulation of synaptic plasticity, we performed ChIP to investigate whether MeCP2 binds to the promoter regions encompassed by these SNPs (e.g., *GRIA1*_Promoter_548294, *GRID1*_Promoter_3814614, and *GRIN1*_Promoter_4880213). We found that an anti-MeCP2 antibody could pull down the promoters of *GRIA1* and *GRID1* in SH-SY5Y neurons, indicating that MeCP2 directly regulates *GRIA1* and *GRID1* (Fig. 3). By contrast, MeCP2 could not bind to the *GRIN1* promoter (Fig. 3). Furthermore, the binding of MeCP2 to the promoters of *GRIA1* and *GRID1* was time-dependent, indicating that *GRIA1* and *GRID1* are direct downstream targets of MeCP2 (Fig. 3).

Discussion

In this study, we correlated the genetic variations, the expression of key genes involved in synaptic plasticity with the academic performance of juvenile students. To identify genetic variants that implicated in the cognitive ability of adolescents, we have chosen to study members of the NMDA-dependent AMPA receptor trafficking cascade, including *GRIN1*, *GRIN2B*, *GRIN2C*, *GRIN3A*, *GRIN3B*, *GRIA1*, and *CaMK2A* because functions of the glutamate receptors and CaMKII in neural plasticity and memory has been well-demonstrated [39]. The repeated action potential firing and subsequent Calcium influx induce autonomous activation of CaMKII is key for memory formation. The activation of CaMKII is also tightly linked to NMDAR, whose main function is to reshare the synaptic structure and formation of long-term memory (LTM) [40, 41]. Importantly, we found that the plasma CaMK2A levels were positively associated with the cognitive abilities of students. Using cultured cell systems we demonstrated that the expression levels of the above-mentioned proteins were altered. We believed that the study in a simpler cultured cell system would pave a way for uncovering the potential role of these elementary building blocks of learning and memory in more complex organisms.

The participated students in our study were physically healthy from homogeneous socioeconomic areas, which presented a more difficult task for us to identify SNPs from psychiatric disorders patients with more heterogeneous genetic backgrounds. Additionally, despite the effective sample size of the study was narrow, we were able to detect the associations between SNPs in glutamatergic system genes and the cognitive ability of senior high students of Taiwan. For instance, polymorphisms in *CaMK2A* (rs2241694) and several glutamatergic system genes including *GRIA1*, *GRIN1*, *GRIN2*, *GRIN3*, and *GRIN3B*, were also associated with cognitive ability in adolescents. Consistent with our findings, several SNPs of GRIN3B have shown to be associated significantly with cognitive function. *GRIN3*B, an unconventional member of the NMDA receptor family, complexes with NR1 and NR2 subunits, and modulated Ca2 + permeability and membrane trafficking. It has been shown that stimulating the unconventional receptors, such as *GRIN3B*, may compete against conventional NMDA receptors (GRIN1/GRIN2) to favor synaptic depotentiation in response to subsequent synaptic stimulations [42]. This

metaplasticity is thought to be essential for LTP, LTD, and memory storage. It would be of our future interest to dissect how the variations of *GRIN3B* affect the function of glutamatergic receptors and the cognitive ability of students.

Using ELISA, we found that students who performed well in MAT have higher plasma CaMK2A expression, whereas students who scored poorly in MAT have lower plasma CaMK2A. The concentration of plasma CaMK2A quantified by mass spectrometry and by ELISA was compatible because both were in pg/L ranges (https://www.proteinatlas.org/ENSG00000070808-CAMK2A/blood). CaMK2A is expressed mostly in CNS, adrenal gland, stomach, kidney, liver, and bone marrow (https://www.proteinatlas.org/ENSG00000070808-CAMK2A/tissue). It can also be detected in neutrophil and memory B-cells of the circulatory system. Although we do not know the likely source of the plasma CaMK2A and how it is secreted into serum, the detection sensitivity of ELISA is good enough for us to quantify its concentration in the blood samples. Because blood plasma contains proteins/peptides exported from the brain and the blood sample is relatively easy for us to collect, the blood plasma would be ideal for the discovery of biomarkers that affect academic performance in youths.

To gain insights into the effect of the elevated CaMK2A *in vivo*, we found that the expressions of pCaMK2A (Thr286), GRIA1, and pGRIA1 (Ser831) were increased in the stably transfected HEK293 cells-expressing CaMK2A, suggesting that CaMK2A could initiate autophosphorylation cascade thereby activating downstream target gene (Fig. 1). Additionally, MeCP2, a downstream target of CaMK2A, and BDNF can be activated by MeCP2 in SH-SY5Y cells (Fig. 2). Despite we did not know whether MeCP2, a transcription regulator that binds methylated DNA, activates CaMK2A directly, this result demonstrates that CaMK2A and MeCP2 could form an autoregulatory loop. Interestingly, a 1.5-fold increase in CaMK2A, phospho-CaMK2A was not increased in SHSY5Y cells expressing MeCP2 (Fig. 2). It is possible that the amount of the increased CaMK2A is not sufficient to initiate its autophosphorylation. It is equally possible that a Ca^{2+} influx is required initially to trigger CaMK2A phosphorylation, and cytosolic Ca^{2+} was not sufficient to initiate CaMK2A autophosphorylation in MeCP2-expressing cells. We believe that a sufficient amount of the upregulated CaMK2A would be autophosphorylated when a Ca^{2+} influx is triggered by neuronal activity.

In summary, we found that single nucleotide polymorphisms (SNPs) in Calcium/Calmodulin-dependent protein kinase IIα (*CaMK2A*) and subunits of ionotropic glutamate receptors, including *GRIA1*, *GRIN1*, *GRIN2*, and *GRIN3*, associated with the cognitive function of students (Table 1,2,3). The protein levels of CaMK2A were elevated in the peripheral blood samples of senior high school students with higher reasoning skills. These results indicate that the primary function of the CaMK2A phosphorylation signaling pathway plays a critical role in synaptogenesis and molecular learning mechanism (Fig. 4). Physiological and pathological roles of CaMK2A, which have attracted substantial attention due to its function in synaptic plasticity. Our SH-SY5Y-derived CaMK2A expression cell model will be a good system to study the associations of synaptogenesis synapses, long-term potentiation (LTP) and long-term depression (LTD), and cognitive function of students.

Abbreviations

ANOVA: One-way analysis of variance; AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BDNF: Brain-derived neurotrophic factor; CaMK2A: Calcium/ Calmodulin-dependent protein kinase II α; ELISA: Enzyme-linked immunosorbent assay; GRINs: Glutamate ionotropic receptors NMDA type subunits; GRIA1: Glutamate ionotropic receptor AMPA type subunit 1; GRID1: Glutamate ionotropic receptor delta type subunit 1; GRINN1: Glutamate ionotropic receptor NMDA type subunit 1; GRIN2B: Glutamate ionotropic receptor NMDA type subunit 2B; GRIN2C: Glutamate ionotropic receptor NMDA type subunit 2C; GRIN3A: Glutamate ionotropic receptor NMDA type subunit 3A; GRIN3B: Glutamate ionotropic receptor NMDA type subunit 3B; LTP: long-term potentiation; LTD: long-term depression; LTM: long-term memory; MAT: Multiple Aptitude Test Battery; MeCP2: Methyl CpG binding protein 2; NMDAR: N-methyl-D-aspartate receptor; SNPs: Single nucleotide polymorphisms.

Declarations

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The authors are grateful to all those who participated in the study.

Authors' contributions

LCL contributed to the study's conception and design, data analysis, and drafted the article. MTS contributed to the study's conception and design and revised the paper. HYH contributed to the study's operation and data acquisition. YCC was involved in data analysis. CYC was involved in the study's conception design and revised manuscript. TKY contributed to the study's conception design, data analysis, revised the paper. All authors read and approved the final manuscript.

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Availability of data and materials

The data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All participants provided written informed consent before participation. The study was approved by the National Taiwan University Hospital Research Ethics Committee in Taiwan (Research Ethics identifier: NCT00713570) and all study procedures followed the Ministry of Science and Technology in Taiwan.

Consent for publication

Not applicable

Competing of interests

The authors declare that they have no conflict of interest.

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Figures

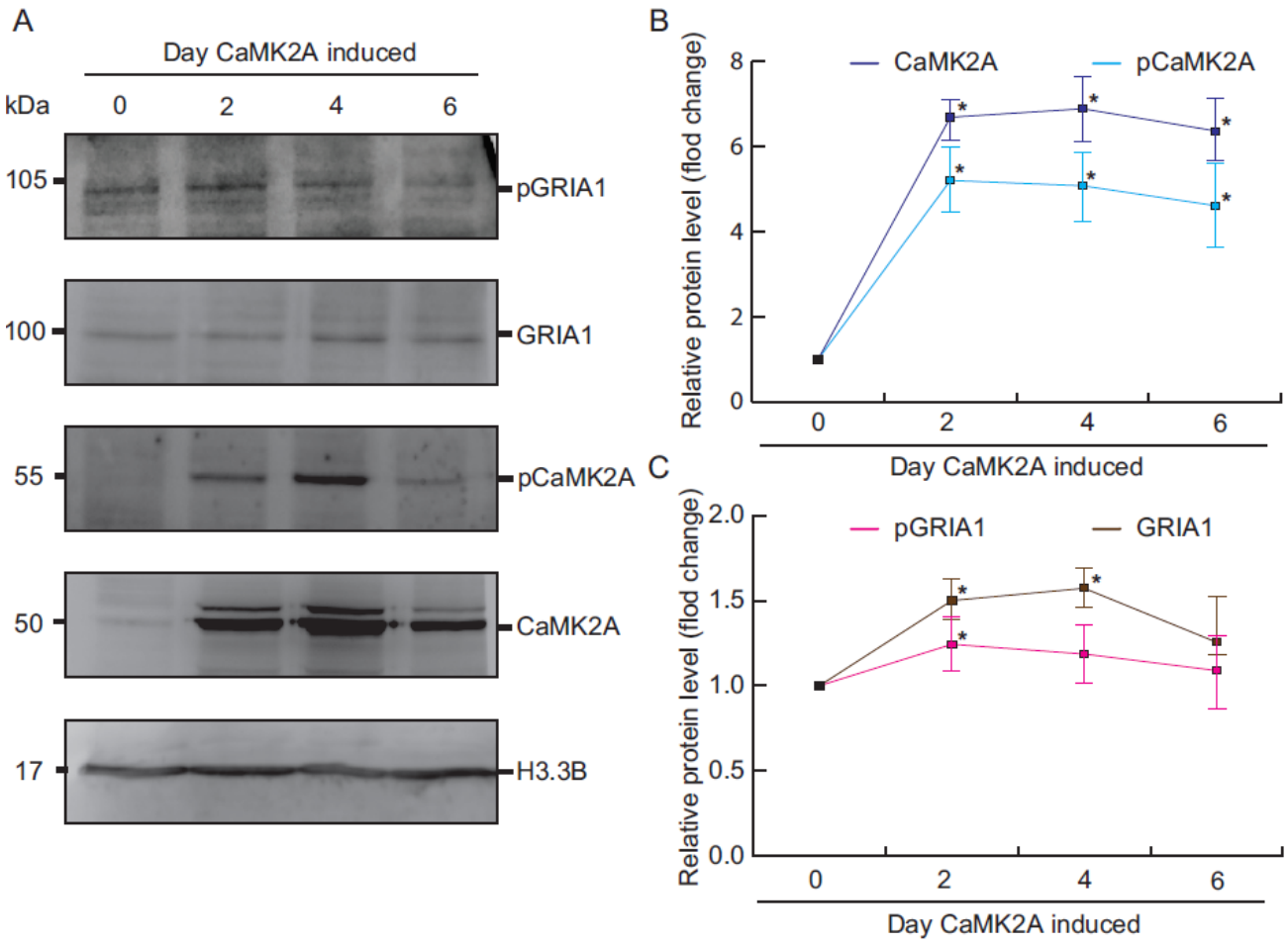

Figure 1

Increased CaMK2A activated autophosphorylation. Total soluble protein from HEK293-derived cells were harvested at 2, 4, and 6 d after the induction of CaMK2A expression with Dox. (A) Representative immunoblots displaying CaMK2A, phospho-CaMK2A (T286), and GRIA1, phospho-GRIA1 (S831), expression. H3.3B was considered loading control. (B, C) Autophosphorylation of CaMK2A and phosphorylation of GRIA1 were increased after CaMK2A induction. Quantification of relative protein expression levels. Data are presented as mean ± SD values. *P < 0.05, n = 5, Student's t-test.

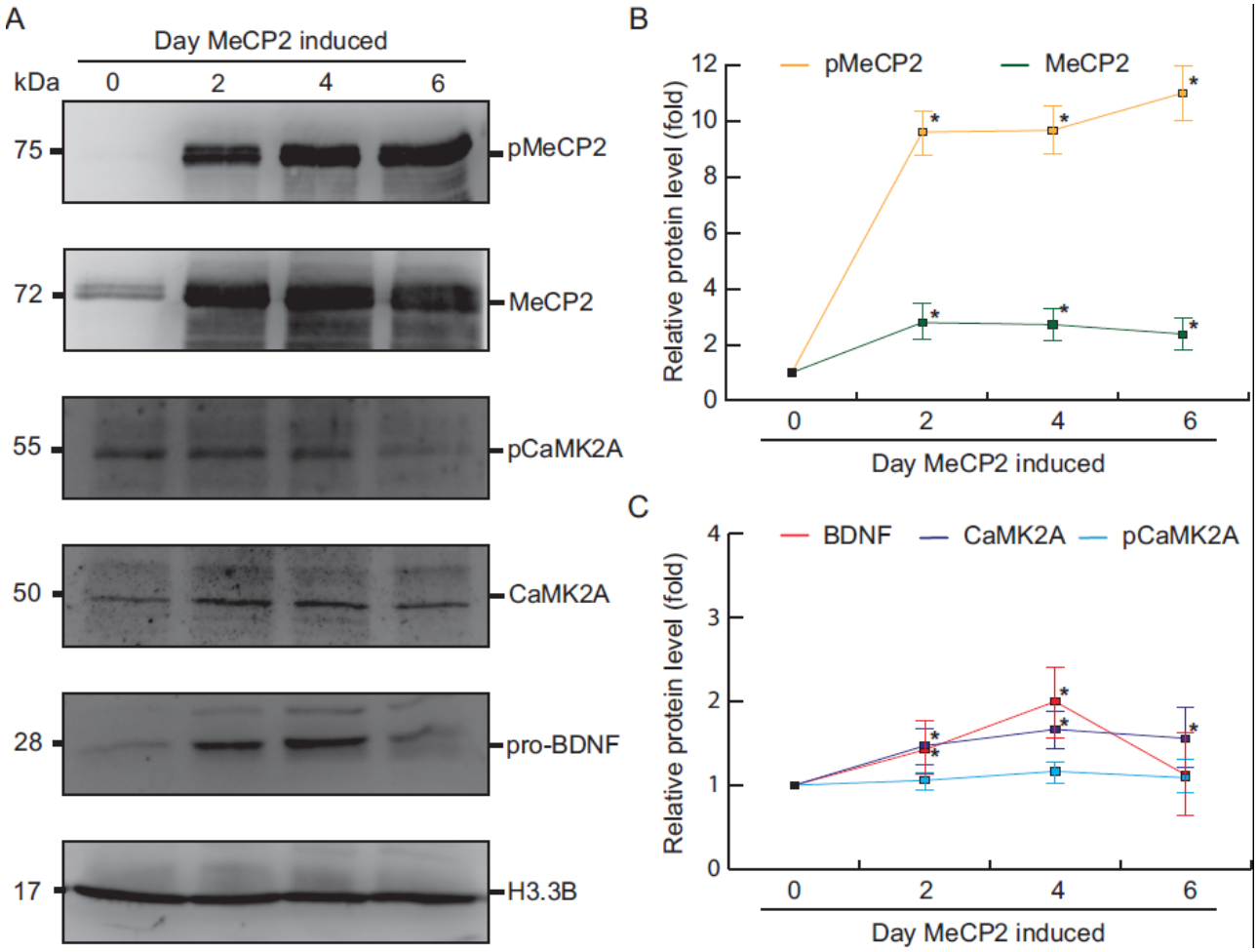

Figure 2

MeCP2 is involved in CaMK2A-mediated phosphorylation. Total soluble protein from stably transfected SH-SY5Y cells were harvested at 2, 4, and 6 d after initiation of MeCP2 expression with Dox. (A) Representative immunoblots displaying phospho-MeCP2 (S80), MeCP2, phospho-CaMK2A (T286), CaMK2A, and pro-BDNF expression. H3.3B was considered loading control. (B, C) Quantification of relative protein expression levels. Data are presented as mean ± SD values. *P < 0.05, n = 5, Student's t-test.

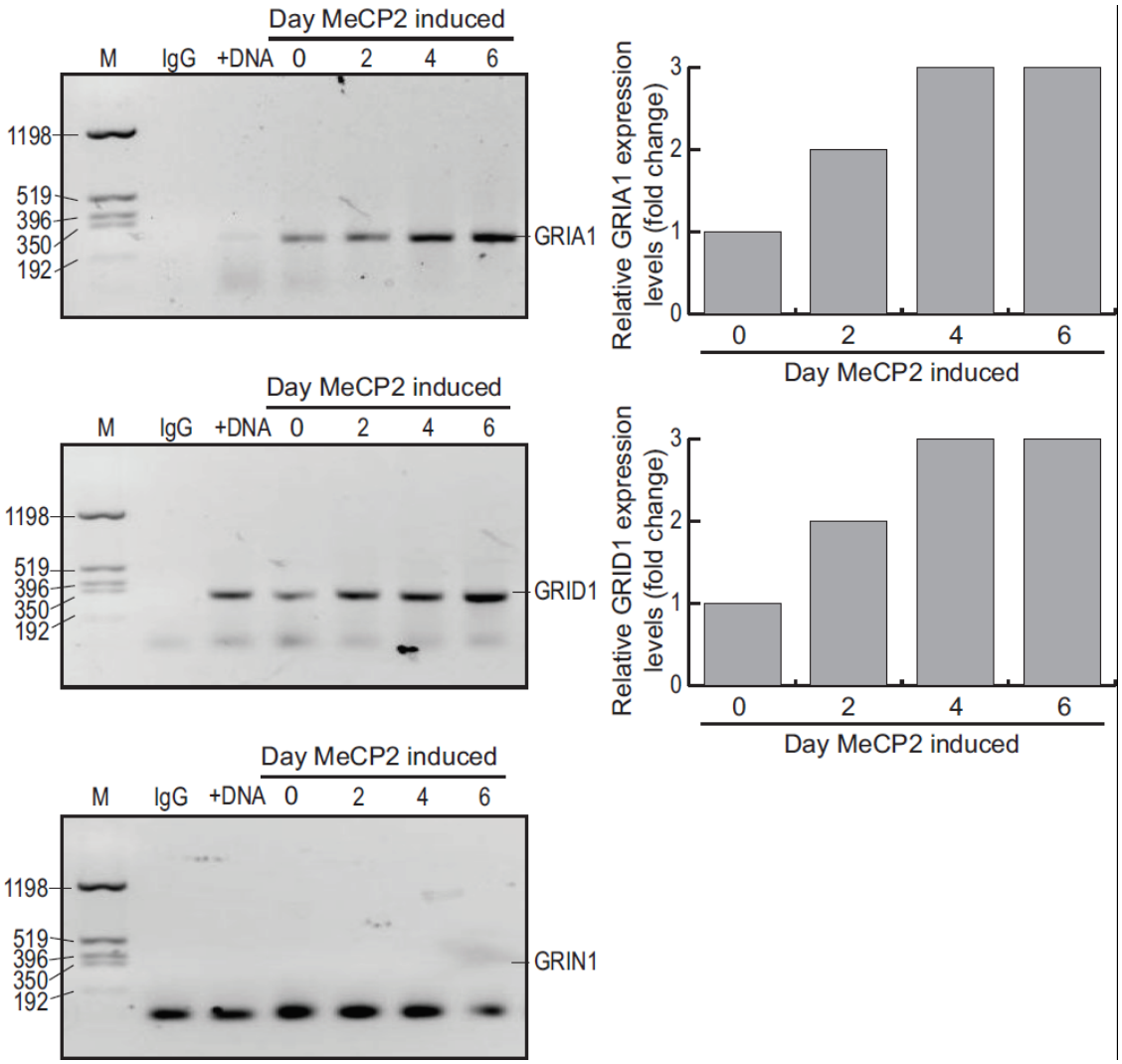

Figure 3

MeCP2 binds to the promoters of GRIA1 and GRID1. ChIP was performed using anti-MeCP2 antibodies on sheared chromatin from SH-SY5Y neuroblastoma cells expressing MeCP2 on days 0, 2, 4, and 6. Purified DNAs from immunoprecipitated chromatin were amplified using optimized primers for the promoters (GRIA1, GRID1, and GRIN1). M: Molecular weight markers are on the left in bases. IgG: Normal rabbit IgG was used as a negative IP control. +DNA: Purified chromosomal DNA was used as a positive control.

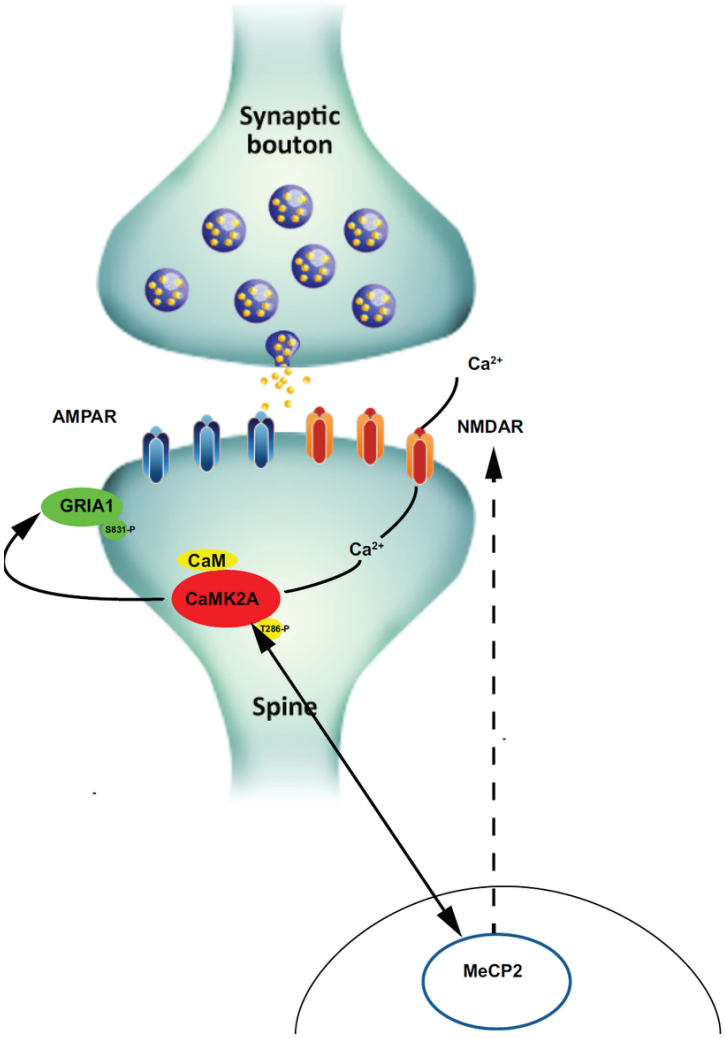

Figure 4

MeCP2 interacted with CaMK2A and CaMK2A phosphorylation signaling pathway plays a critical role in synaptogenesis and molecular learning mechanism (see text for detail).