A novel role of CPEB3 in regulating EGFR gene transcription via association with Stat5b in neurons

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Received May 16, 2010; Revised June 23, 2010; Accepted July 1, 2010

ABSTRACT

CPEB3 is a sequence-specific RNA-binding protein and represses translation of its target mRNAs in neurons. Here, we have identified a novel function of CPEB3 as to interact with Stat5b and inhibit its transcription activity in the nucleus without disrupting dimerization, DNA binding and nuclear localization of Stat5b. Moreover, CPEB3 is a nucleocytoplasm-shuttling protein with predominant residence in the cytoplasm; whereas activation of NMDA receptors accumulates CPEB3 in the nucleus. Using the knockdown approach, we have found the receptor tyrosine kinase, EGFR, is a target gene transcriptionally activated by Stat5b and downregulated by CPEB3 in neurons. The increased EGFR expression in CPEB3 knockdown neurons, when stimulated with EGF, alters the kinetics of downstream signaling. Taken together, CPEB3 has a novel function in the nucleus as to suppress Stat5b-dependent EGFR gene transcription. Consequently, EGFR signaling is negatively regulated by CPEB3 in neurons.

INTRODUCTION

Long-term memory requires synthesis of plasticity-related proteins (PRPs) to strengthen synaptic efficacy and consequently consolidate memory. RNA-binding proteins play indispensable roles to control spatial-temporal PRP production by regulating transport, localization, translation and/or degradation of PRP RNAs (1–4). CPEB3 and CPEB4 mRNAs are elevated in the hippocampus after kainate-induced seizure, indicating they are immediate early gene products upon synaptic activation and likely modulate neuronal function (6). Importantly, Orb2 in Drosophila is required for long-term conditioning of male courtship behavior (7), implicating that its mammalian homologs, CPEBs2–4, may also have roles in memory. A recent study has shown that a single nucleotide polymorphism in the CPEB3 gene is associated with human episodic memory (8).

CPEBs2–4 were first identified based on sequence similarity with CPEB (or CPEB1) in the carboxyl terminal RNA-binding domain (9). However, CPEBs2–4 could interact with RNA sequences identified from a SELEX (systematic evolution of ligands by exponential enrichment) screen that are different from the conventional CPEB1-binding site (UUUUA1–2U) (5). Despite CPEB1-controlled translation is characterized at the molecular details and plays important roles in development, cell cycle, neuronal plasticity and cellular senescence (10), much less is known about the functional entities of CPEBs2–4 once they bind to RNAs. A previous study has shown that CPEB3 repressed translation of a reporter RNA and Glu2 RNA (5). Interestingly, a prion-like property has been observed in Orb2 as well as Aplysia CPEB in yeasts (11) and a recent study has shown that multimeric state of CPEB is required for maintaining long-term facilitation in Aplysia (12). Nonetheless, whether any mammalian CPEB possesses prion-like change to modulate its target RNA translation is still in question. To understand how CPEB3 regulates translation, we used a yeast two-hybrid screen to identify its binding partners. Unexpectedly, the screen identified a transcription factor, signal transducer activated transcription (Stat) 5b, interacted with CPEB3. Stat5b is one of the seven Stat family members of which transcriptional

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activity are modulated by Janus tyrosine kinases (JAKs),
that are activated by cytokines and hormones (13,14).
Translocation of dimerized Stat to the nucleus activates
target gene transcription (15). Using promoter assays,
CPEB3 inhibits Stat5b-dependent transcription without
affecting DNA binding, nuclear translocation and dimer-
ization of Stat5b. Moreover, CPEB3 shuttles between the
nucleus and cytoplasm and activation of NMDARs in-
creases nuclear level of CPEB3, suggesting that neuronal
activity regulates CPEB3’s roles in transcription and
translation.

One target gene transcriptionally regulated by Stat5b
and CPEB3 interaction identified from this study is the
receptor tyrosine kinase, epidermal growth factor
receptor (EGFR). Upon ligand binding, the receptors
become phosphorylated on tyrosine residues within their
cytoplasmic kinase domain and activated which then
initiate several downstream signaling pathways, such as
JAK-Stat, mitogen-associated protein kinase (MAPK)
and phosphatidylinositol 3-kinase (PI3K)-Akt. The
elevated EGFR level in CPEB3 knockout neurons,
when stimulated with EGF, results in extended and
amplified downstream signaling measured by phosphoryl-
ation of Stat5b and Akt. Although EGFR has been
studied extensively in cell proliferation (including neuro-
genesis), anti-apoptosis and cancer progression (16–18), its
function in post-mitotic neurons is less characterized. In
the EGFR null mice, abnormal astrocyte development
and neuronal death impede the study of EGFR function
in the adult brain (19,20), but it has been demonstrated
that EGF enhances long-term potentiation in the
hippocampal slices and dentate gyrus of anesthetized
rats after tetanic stimulation (21,22), suggesting its cor-
responding receptor, EGFR, may function as a neuronal
modulator. Using pharmacological approach, activation
or deprivation of EGFR’s kinase activity by infusing
EGF or gefitinib (23), respectively, in the brain, affects
spatial learning and memory performance in mice.
Together, this study first identifies a novel transcriptional
function for the CPEB family members besides their
characterized roles in translation (5,10,24,25). By interact-
ing with Stat5b, CPEB3 downregulates the expression of
EGFR of which kinase activity modulates learning and
memory.

MATERIALS AND METHODS

Antibodies
Antibodies used for the study are, Akt (cat #4691),
pT308-Akt (cat #2965), pY1068-EGFR (cat #2236S) and
pY699-Stat5 (cat #9359) from Cell Signaling; EGFR (cat
#SC-03) and Stat5b (cat #SC1656) from Santa Cruz
Biotechnology; synaptophysin (cat #MAB378) from Chemicon and flag epitope
(cat #F1804) from Sigma-Aldrich.

Yeast two-hybrid screen
Matchmaker Two-Hybrid Library Construction and
Screening Kit (Clontech) was used to construct a
random-primed adult mouse brain cDNA library.

Briefly, mouse brain poly(A) RNA (Ambion) and the
CDS III/6 primer were used to generate double-stranded
cDNA that was co-transformed with linearized pGAD-Rec vector into the yeast strain, AH109.
Approximately 5.5 × 10^5 transformants were harvested,
mixed and stored at −80°C. The N-terminus of hCPEB3
(amo acids 1–427) was cloned into pGBK T7 and trans-
formed into the yeast strain, Y187 (screening bait). Both
yeast strains were mated for 24h at 30°C with gentle
swirling at 40 rpm then plated in nutrient-selective plates
(SD/X-α-gal/-Leu/-Trp/-Ade/-His) to screen for positive
interaction clones.

Plasmid construction
The shRNA sequences, ACAAAACCTGTTCACAATCC
and CAATACTGGGAATAATC targeted against rat
CPEB2 and CPEB4 mRNAs respectively, were cloned
into lentiviral vector pL3.7-Syn (26). The shRNA
clon e, TRCN0000012554 against rat Stat5b (CGGCCA
AAGGATGAAGTATAT), was obtained from the
RNAi Core Facility (Academia Sinica). The various
truncated Stat5b mutants were PCR amplified and
cloned into pGADT7 for two-hybrid interaction.

Cell culture and lentivirus infection
HEK-293T and COS7 cells were cultured in DMEM with
10% FBS. Cultures of rat hippocampal neurons (27) were
grown at a cell density of 8000/cm^2 on coverslips
or 30 000/cm^2 on dishes. Hippocampal neurons of DIV
6 were infected with lentivirus overnight and harvested
on DIV 11 for RNA or protein extraction. On the
day of harvest, the concentrations of reagents used to
treat neurons are: 50 μM NMDA, 15 μM AMPA, 50 μM
DHPG, 30 mM KCl, 30 μM APV, 20 μM NBQX, 5 ng/ml
leptomycin B, 100 ng/ml EGF, 30 μg/ml cycloheximide
and 2 μg/ml actinomycin D.

Co-immunoprecipitation (Co-IP)
For co-IP, 4 μg of DNA mixtures containing equal
amounts of two or three plasmids were co-transfected
in a 6-cm dish of 293T cells using lipofectamine 2000.
The overnight transfected cells were lysed in 120 μl
IP buffer (20 mM Heps, pH 7.4, 100 mM NaCl, 1 mM
MgCl2, 0.1% NP40, 10% glycerol, 0.5 mM DTT,
1× protease inhibitor cocktail and 100 μg/ml RNaseA)
and centrifuged at 10 000 × g for 5 min at 4°C. The super-
natant was incubated with protein G beads bound
with myc or flag antibody (Ab) for 2 h at 4°C. The
beads were washed five times with 300 μl IP buffer
and the precipitated proteins were used for western
blotting. For reciprocal IP of endogenous CPEB3
and Stat5b, ~4 × 10^7 cortical neurons were lysed in
1.6 ml IP buffer. Equal volumes of lysate were incubated
with Stat5b, CPEB3 or control IgG and the immuno-
precipitates were probed with CPEB3 and Stat5b
antibodies.
RNA extraction, cDNA synthesis and quantitative PCR (Q–PCR)

Total RNA was extracted with Trizol (Invitrogen). The cDNA was synthesized using oligo-dT primer and ImPromII Reverse Transcriptase (Promega). Quantitative PCR was conducted using the Universal Probe Library and Lightcycler 480 system (Roche). Data analysis was done using the comparative \( \Delta \Delta C_t \) (threshold cycle value) method with the non CPEB3-targeted RNA, NF-M mRNA as the reference. The PCR primers are: EGFR, 5'-TGCACTCGACACGCTCATAC-3' and 5'-A CTTTTGGGCGGCTATCAG-3'; CPEB3, 5'-TGAGACACTCCATATGGA-3' and 5'-AAGGGACACTTGCTTCTG-3'; Stat5b, 5'-GGAGACACCTACGGA TCAA-3' and 5'-AAGTTTATTCGAGA-3'; EGFR, 5'-CGACTGACCTCTGAATGTCC-3' and 5'-GACCTGGCA-3'; NF-M, 5'-CGTCATTGCGAGATACCA-3' and 5'-TC TCTACCCTTCCAGTTTCT-3'.

Chromatin immunoprecipitation (ChIP)

Approximately 3 × 10^7 cortical neurons of DIV11 were crosslinked with 1.42% formaldehyde for 15 min and quenched with 125 mM glycine for 5 min at room temperature. The fixed cells were washed three times with ice-cold PBS, lysed in 3 ml of ChIP buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP40 and 1% Triton) and sonicated for 10 min with 30 s on/off cycles. The sonicated sample was centrifuged for 5 min at 12 000 g. One-third of beads were eluted with ChIP buffer. The beads were washed three times with ChIP buffer containing 500 mM NaCl followed by another three washes with ChIP buffer. One-third of beads were eluted with Laemmli sample buffer for western blot analysis and the remaining beads along with the saved input sample were incubated at 95°C for 15 min and digested with Proteinase K (1 mg/ml in 50 mM Tris, pH 7.4, 10 mM EDTA, 50 mM NaCl and 1% Triton X-100) at 55°C for 30 min. The supernatants were phenol/chloroform extracted, ethanol precipitated in the presence of 10 μg yeast tRNA as a carrier and used for PCR analysis. Primers used to detect binding to the −3000 to +300 promoter region and a negative control spanning intron 24 and exon 25 of the EGFR gene were: −2790 to −2581: 5'-CGACATACCTCTGAAATGTCC-3' and 5'-TGGTTCGAGCCTAGCAGT-3'; −2299 to −1981: 5'-GAGCTAAGTCTTTATGGAAC-3' and 5'-TACCATGCTGGGAGA-3'; −2001 to −1641: 5'-ATGCCAGGTACCATGGA-3' and 5'-GGAGAGCCTACGGA-3'; −1475 to −1061: 5'-CCACCTTTTATATAAAACCTCG-3' and 5'-AAGACCAACTAATGTCAC-3'; −510 to −101: 5'-GGTACAGTAAACCAGGA-3' and 5'-CTAGCTAGCCTGGTGTC-3'; Intron24 and exon25: 5'-TGGCAGACCTGTGAGACGT-3' and 5'-CGTCTCCTCATGCTCTCTC-3'.

 Luciferase reporter assay

A mixture of ~0.5 μg of plasmid DNA containing 0.2 μg Stat5b, 0.2 μg CPEB3, 0.1 μg firefly luciferase reporter and 5 ng Renilla luciferase were simultaneously transfected into 293T cells with Fugene HD (Roche). Cells were harvested 20–24 h after transfection and analyzed using Dual-Luciferase Reporter Assay System (Promega).

Animal surgery and Morris water maze

All experimental protocols were carried out following guidelines of the Institutional Animal Care & Utilization Committee. For intracerebroventricular drug administration, the 8-week-old male C57BL/6J mouse was anesthetized with ketamine before implanting a cannula at the right lateral ventricle (1.0 mm lateral and 0.5 mm caudal to bregma to a depth of 3.0 mm from the skull) that was attached to an Alzet osmotic Y pump (Model 1004) to infuse drug at 0.11 μl/h for 14 days. Recombinant EGF (Sigma-Aldrich) and gefitinib (first constituted to 5 mM in DMSO, Astra Zeneca) were diluted at the concentration of 50 μg/ml and 50 μM, respectively, in the vehicle (artificial cerebrospinal fluid containing 1 mg/ml BSA). One week after the surgery, mice with complete wound-healing were subject to Morris water maze test (28). Training for the hidden platform version consisted of four trials each day for four consecutive days. The probe trial was administered on the day after training completed. The visible platform task, which consisted of four trials for 1 day with the escape platform marked by a visible flag, was conducted to ensure the intact vision of mice. For all the trials, the maximal swimming duration was 60 s and the inter-trial interval was 60 min. The trajectories of mice were recorded and analyzed with a video tracking system, TrackMot (Singa Technology, Taiwan). Data were represented with mean ± S.E.M. and one-way and two-way ANOVA and post hoc LSD tests were used for statistics.

RESULTS

CPEB3 interacts with Stat5b and inhibits Stat5b-dependent transcription

A yeast two-hybrid screen was performed using the N-terminal 427 amino acids of human CPEB3 as the bait to probe a mouse brain cDNA library. Two positive clones contained the C-terminal regions of Stat5b were identified (Figure 1A). Further analysis showed that the amino acids 639–700 between Src-homology (SH2) and transactivation domain (TAD) of Stat5b were crucial for its association with the N-terminal of CPEB3 (Figure 1A). In this region, tyrosine 699 (Y699) is phosphorylated upon cytokine and growth hormone stimulation that enhances Stat5b’s transactivation ability (13,14). When myc-tagged full length (myc-CPEB3) and C-terminal RNA-binding domain (myc-CPEB3C) of CPEB3 along with flag-tagged Stat5b were expressed in 293T cells, myc-CPEB3 but not myc-CPEB3C was co-precipitated with flag-Stat5b in the presence of RNase A treatment, indicating this interaction is mediated through the CPEB3N-terminus in an RNA-independent manner (Figure 1B). Furthermore, using cortical neuronal lysate to perform reciprocal immunoprecipitation, endogenous CPEB3 was pulled down in the Stat5b immunoprecipitate and vice versa (Figure 1C).
versus WT Stat5b was considered as fold of transcriptional activation caused by phosphorylated Y699 (p-Y699) (Figure 2A). This approach mimics cytokine-induced Stat5b phosphorylation-activated transcription while avoids simultaneously activating other signaling molecules to compromise the promoter assay (31). In the presence of enhanced green fluorescent protein (EGFP, a control), a 3-fold transactivation was observed in the wild-type but not the mutant promoter. Such activation was decreased to 2.2-fold with myc-CPEB3 and further reduced to 1.5-fold with nucleus-localized form of CPEB3 (myc-CPEB3NLS). In contrast, myc-CPEB3C, which was predominantly nuclear, did not affect Stat5b-induced transcription since it did not bind Stat5b (Figure 1B). Because Stat5bWT and Stat5bCA interact similarly with myc-CPEB3 (Figure 2C), the N642H replacement and Y699 phosphorylation have no effect on Stat5b’s association with CPEB3.

CPEB3 suppresses Stat5b’s transcription activity without affecting dimerization and DNA binding of Stat5b

To examine whether CPEB3 could disrupt dimerization of Stat5b and hence inhibit Stat5b’s activity, flag-Stat5b and red fluorescence protein cherry (chRFP)-fused Stat5b along with myc-CPEB3 or EGFP were co-expressed in 293T cells. Figure 3A shows the amount of chRFP-Stat5b dimerized with flag-Stat5b is not influenced by myc-CPEB3. Moreover, growth factor-induced Y699 phosphorylation does not change Stat5b’s self-dimerization and association with CPEB3 (Figure 3B). Because p-Y699 is evidently detected in Stat5bCA (Figure 2B), Stat5b is constantly phosphorylated and dephosphorylated in 293T cells even without growth factor stimulation. To test whether CPEB3 could inhibit the transcription mediated by non-phosphorylated Stat5b, the Y699F mutant was used. The nuclear form of CPEB3 further diminished Stat5b-induced transcription since it did not bind Stat5b phosphorylation-activated transcription while affecting dimerization and DNA binding of Stat5b. CPEB3-mediated transcription regardless of the phosphorylation status of Y699 (Supplementary Figure S1). To confirm that CPEB3-mediated transcription does not require interference with Stat5b’s DNA binding, Stat5b and a control, FoxP2, were fused to Gal4 DNA-binding domain (Gal4DBD) that bound to the Gal4-binding promoter sequences. In the presence of myc-CPEB3, myc-CPEB3NLS or myc-CPEB3C expression, the transcription-activation ability of Gal4DBD-Stat5b but not that of the controls was perceptibly inhibited by nucleus-localized CPEB3 (Figure 3C). Because CPEB3-NLS represses Stat5b’s activity greater than CPEB3, it is unlikely such inhibition is caused by sequestering Stat5b in the cytoplasm. Thus, CPEB3 suppresses Stat5b-dependent transcription without disrupting DNA binding, dimerization and nuclear localization of Stat5b and such a negative regulation is not dependent on Y699 phosphorylation of Stat5b.

Activation of NMDARs influences nucleocytoplasmic distribution of CPEB3

Because CPEB3-repressed Stat5b-dependent transcription was manifest even when CPEB3 was not appended with
NLS (Figure 2A), we questioned if CPEB3 was a nucleocytoplasmic shuttling protein with longer residency in the cytoplasm, and whether neuronal activity could modulate CPEB3 distribution. Hippocampal neurons of day in vitro (DIV) 12 were stimulated with various glutamate receptor agonists, \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA), NMDA and 3,5-dihydroxyphenylglycine (DHPG), for 30 min and immunostained with affinity-purified CPEB3 antibody. Five hundred neurons were scored with the staining signal greater in the cytoplasm than in the nucleus categorized as cytoplasm-localized; while the rest was considered as nucleus-localized. The majority of unstimulated neurons showed a stronger CPEB3 signal in the cytoplasm with <10% of cells displaying nuclear distribution. AMPA and NMDA induced >60% of cells exhibited stronger or similar CPEB3 signals in the nucleus (examples in Figure 4A and quantified results in Figure 4B). To confirm the above observation was not caused by antibody's cross-reactivity or changes in nuclear permeability, neurons expressing myc-CPEB3 and a control flag-FKBP8 were stimulated. Both AMPA and NMDA stimulations shifted the distribution of myc-CPEB3 but not flag-FKBP8 from cytoplasmic to nuclear dominance (Supplementary Figure S2A). KCl depolarization which triggered presynaptic release of glutamate and subsequently activated postsynaptic glutamate receptors, also resulted in nuclear CPEB3 accumulation in a NMDAR-dependent manner since the addition of NMDAR blocker, (2R)-amino-5-phosphonovaleric acid (APV), but not the AMPAR antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzoquinoxaline-2, 3-dione (NBQX), was sufficient to prevent this event (Figure 4B). Interestingly, the AMPA effect on CPEB3 distribution was mediated through NMDAR signaling because it was blocked not only by NBQX but also by APV. Therefore, it appears that AMPAR activation, similar to KCl treatment, results in depolarization of neurons that potentiates synaptic release of glutamate and expels Mg\(^{2+}\) clogs from NMDARs, hence facilitates NMDAR activation. A translation inhibitor, cycloheximide (CHX), did not affect KCl-induced CPEB3 redistribution, so the nuclear accumulated CPEB3 is not newly synthesized (Figure 4B). Despite the NLS and nuclear export sequence (NES) in
CPEB3 have not been identified, nuclear export was mediated by chromosome region maintenance 1 (CRM1) since blocking CRM1 by leptomycin B (LMB) induced nuclear accumulation of CPEB3. Moreover, application of LMB in COS cells expressing GFP-tagged CPEB3 also caused GFP-CPEB3 retained in the nucleus (Figure 4C), suggesting CPEB3 constantly shuttles between nucleo-cytoplasmic compartments and activation of NMDARs modulates its distribution equilibrium. In contrast, Stat5b was present in both nucleus and cytoplasm and the stimulation with AMPA, NMDA and DHPG did not change its distribution (data not shown). Although NMDAR signaling increased nuclear CPEB3, it did not affect Stat5b distribution (see immunostaining in Supplementary Figure S2B and biochemical fractionation in Supplementary Figure S3), indicating the two factors were unlikely co-transported in response to neuronal activity. Moreover, the co-immunoprecipitation assay demonstrated that CPEB3 and Stat5b remained associated in both cytoplasmic and nuclear extracts isolated from neurons treated with NMDA (Supplementary Figure S3).

Figure 3. CPEB3 inhibits Stat5b-dependent transcription without affecting its dimerization and DNA-binding ability. (A) CPEB3 does not affect Stat5b dimerization. The 293T cells expressing flag-Stat5b and red fluorescence protein cherry (chRFP)-tagged Stat5b along with myc-CPEB3 or EGFP, were precipitated with flag Ab and probed with myc and Stat5b Abs. (B) Y699 phosphorylation in Stat5b has no effect on its dimerization as well as association with CPEB3. The 293T cells expressing myc-CPEB3 and red fluorescence protein cherry (chRFP)-tagged Stat5b along with flag-Stat5b or EGFP, were treated with or without EGF for an hour. The extracts were precipitated with flag antibody and probed with myc, Stat5b and pY699-Stat5b antibodies. (C) CPEB3 represses Stat5b-activated transcription without disrupting Stat5b’s DNA binding. The 293T cells were transfected with plasmids encoding (i) myc-CPEB3, myc-CPEB3NLS or myc-CPEB3C, and (ii) Gal4 DNA-binding domain (Gal4DBD) or Gal4DBD fused to Stat5b or FoxP2, and (iii) reporters containing firefly luciferase driven by the promoter containing five Gal4-binding sites and Renilla luciferase. The data from three sets of experiments is expressed as mean ± s.e.m. and the asterisks denote significant difference (Student’s t-test).

Stat5b-activated EGFR transcription is downregulated by CPEB3

To identify a target gene regulated by Stat5b and CPEB3 interaction, we focused on EGFR because hepatic EGFR RNA was reduced in Stat5b knockout mice in the previous microarray study (32) and the isotope-coded affinity tag (ICAT) proteomic analysis displayed a two-fold increase of EGFR protein in CPEB3 knockdown (KD) neurons (unpublished data). Hippocampal neurons of DIV6 were infected overnight with lentivirus containing or lacking a short hairpin sequence for rat CPEB3 or Stat5b. The infected neurons were harvested on DIV11 for RNA and protein analysis. The RNA levels of EGFR, CPEB3 and Stat5b were measured by quantitative PCR following reverse transcription (RT–QPCR). If EGFR transcription is activated by Stat5b whose transactivation ability is offset by CPEB3, a decrease and an increase in EGFR RNA level is expected, respectively, in Stat5bKD and CPEB3KD neurons as seen in Figure 5A. These changes are results of transcription since EGFR RNA stability is similar in the control and knockdown neurons (Figure 5B, normalized curves). To ascertain
that Stat5b binds to the EGFR promoter, we performed chromatin immunoprecipitation (ChIP). Stat5b-binding sites in the proximal 3 kb EGFR promoter (Supplementary Figure S4) were predicted by MATCH analysis (33). Formaldehyde-fixed cortical neurons were lysed and precipitated with Stat5b antibody. In Figure 5C, an extra immunostained band of higher molecular weight likely represents crosslinked dimeric Stat5b as it was not observed when using unfixed neurons (Figure 1C). The promoter regions spanning the predicted Stat5b-binding sites at $\gamma_C0_{2694}$, $\gamma_C0_{2234}$, $\gamma_C0_{2035}$ and $\gamma_C0_{1804}$ were preferentially PCR-amplified from the Stat5b IgG-precipitated substance. In contrast, the areas around $\gamma_C0_{1437}$, $\gamma_C0_{1250}$, $\gamma_C0_{1102}$, $\gamma_C0_{470}$, $\gamma_C0_{467}$, $\gamma_C0_{181}$ as well as a control region spanning intron 24 and exon 25 of EGFR gene were not. The same Stat5b-binding regions in the EGFR promoter were also identified in another ChIP assay (Supplementary Figure S5). Finally, the EGFR protein level was examined using CPEB2, CPEB3, CPEB4 and Stat5b knockdown neurons. As expected, a reduction in CPEB3 leads to an increase in EGFR synthesis whereas a deficiency in Stat5b results in a decrease in EGFR expression (Figure 5D). The elevated EGFR expression in CPEB3 KD neurons could be rescued with the expression of full length human CPEB3 (hCPEB3), to a lesser extent with the N-terminus but not the C-terminus of hCPEB3 (Supplementary Figure S6). Intriguingly, the knockdowns of CPEB2 and CPEB4 have no obvious effect on EGFR expression (5). Since the nuclear history of mRNA could influence its expression pattern later in the cytoplasm (34), we determined if CPEB2 and CPEB4 did not regulate EGFR synthesis because of lacking interaction with Stat5b. In 293T cells that expressing flag-Stat5b along with various myc-tagged CPEBs, only myc-CPEB3 was co-immunoprecipitated with flag-Stat5b (Figure 5E). The inability of CPEB2 and CPEB4 to bind Stat5b is not due to their failure to translocate to the nucleus, as when CRM1-mediated export was blocked, GFP-CPEB2 and GFP-CPEB4 were accumulated in the nucleus (Supplementary Figure S7).

The kinetics of EGFR downstream signaling is altered in CPEB3 knockdown neurons

In cultured hippocampal neurons, the kinase activity of EGFR as assessed by autophosphorylation of Y1068 is relatively inert. Stimulating neurons with DHPG (data not shown), AMPA and NMDA apparently did not release, if any, sufficient amount of endogenous EGFR

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**Figure 4.** NMDAR signaling increases nuclear distribution of CPEB3. (A) Immunostaining of CPEB3 in cultured hippocampal neurons stimulated with or without NMDA for 30 min. MAP2 staining was used to identify neurons. (B) The summary result of CPEB3 localization from analyzing 500 neurons treated with various reagents for 30 min. The staining signal in the cytoplasm greater than that in the nucleus is considered as cytoplasm-localized (light gray bar) and the rest is grouped as nucleus-localized (dark gray bar). (C) The COS7 cells expressing GFP-CPEB3 were treated for 30 min with leptomycin B, fixed and stained for nuclei with Hoechst 33342. Scale: 10 $\mu$m.
ligands to yield detectable p-Y1068 (Figure 6A). To address whether aberrant EGFR expression in CPEB3KD neurons changed downstream signaling, the neurons were stimulated with EGF and monitored for the activation of EGFR (p-Y1068), Stat5b (p-Y699) and Akt (p-T308) (Figure 6B). The increased p-Y1068 signal in KD neurons was caused by elevated EGFR expression; once normalized, the kinetics of EGFR activation (p-Y1068) was similar in control and KD neurons (Figure 6B, top plot). Since ligand-activated EGFR was endocytosed and degraded in proteasome and lysosome-dependent manner (35,36), a decline of EGFR level was observed in EGF-treated samples. In addition, EGF-stimulated CPEB3KD neurons displayed prolonged and augmented activation of Stat5b (p-Y699) and Akt (p-T308), respectively (Figure 6B, bottom plots). As a control, Akt activation showed similar kinetics in NMDA-stimulated control and CPEB3KD neurons, suggesting CPEB3KD effect on Akt signaling is distinctively tailored to specific stimulus like EGF (Figure 6C). In addition, the Y699 phosphorylation of Stat5b is not detectable upon NMDA stimulation, indicating that has implicated in plasticity and memory, perturbation of this receptor tyrosine kinase activity locally in

EGFR is involved in modulating learning and memory

Because activation of EGFR initiates several signaling pathways, such as PI3K-Akt and MAPK, in parallel that have been implicated in plasticity and memory, perturbation of this receptor tyrosine kinase activity locally in
the brain may influence memory performance. To assess whether EGFR is a PRP that functions in learning and memory, pharmacological treatment of mice with EGF ligand and the inhibitor gefitinib was used. EGF-induced EGFR activation in the cultured neurons was effectively suppressed by gefitinib (Figure 7A). Male C57/BL6J mice with indwelling cannulae in the lateral ventricle infused with vehicle, EGF or gefitinib were tested for spatial learning and memory using Morris water maze. The mice received four spatial trials per day with a 60 min inter-trial interval and learned to locate a hidden platform using extra maze visual cues during 4-day spatial acquisition trials. No difference in swimming speed of all drug-treated animals was observed (Figure 7B). Animals infused with EGF trended towards enhanced performance in terms of accuracy and latency of locating the platform (Figure 7C). Such a mild change could be due to the reduced surface level of EGFR since persistent activation by EGF resulted in continuous endocytosis and degradation of the receptors (35,36).

During the probe trial, the hidden platform was removed and the amount of time and distance mice spent in the original platform quadrant was recorded to test their consolidated memory after 4-day trials (Figure 7D). Deprivation of EGFR activity with gefitinib clearly retarded learning and memory performance as demonstrated in decreased accuracy and increased latency in each trial (Figure 7C) as well as reduced retention in the target quadrant in the probe trial (Figure 7D).

**DISCUSSION**

We have identified a novel role for the RNA-binding protein, CPEB3 in transcription. All CPEBs, originally thought to be localized in the cytoplasm, are now proteins shuttling between nucleocytoplasmic compartments. Two recent studies have shown that CPEB1 shuttles to the nucleus where it possibly plays a role in ribosomal biogenesis (37) and alternative splicing (38). One nuclear function of CPEB3 is to downregulate
Stat5b’s transcriptional activity; whereas the roles of nuclear CPEB2 and CPEB4 remain to be determined. Interestingly, NMDAR signaling reorganizes CPEB3 distribution from cytoplasmic to nuclear prevalence, suggesting neuronal activity could regulate and partition CPEB3’s functions between the two compartments to control gene expression. One target gene transcriptionally regulated by CPEB3 is EGFR of which function in modulating learning and memory has been pharmaceutically demonstrated in the present study.

The classical cytokine-Stat-signaling draws a paradigm in which cytokine-induced phosphorylation on a critical tyrosine residue of cytoplasmic Stats is the prerequisite to initiate dimerization and subsequent nuclear translocation of Stats. Thus, this phosphorylation is essential to transduce the extracellular signal to the nucleus and to activate Stat-dependent gene transcription (15,39). However, the finding that non-phosphorylated Stat1 (Y701F mutant) could bind to and activate the low molecular weight polypeptide-2 promoter (40) and accumulating evidences have subsequently demonstrated this phosphorylation event is not necessarily required for Stat’s dimer formation, constitutive transit in and out of nucleus and transcriptional activation (41) that are in accordance with our findings in this study. Silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT), which binds to the N-terminal coiled-coil domain of STAT5 and suppresses the induction of STAT5 target genes (42) is the only identified transcriptional co-repressor of Stat5. Here, we have uncovered CPEB3 as another negative regulator for Stat5b without affecting Stat5b’s dimerization, nuclear localization and DNA binding. The activation domains of transcription factors are known to activate gene transcription via association with a diverse array of coactivators, some of which promote chromatin remodeling, whereas others, such as TFIID and mediator complexes, direct activator recruitment to the transcriptional machinery (43–45). Thus, we hypothesize that the binding of CPEB3 to a region next to the Stat5b’s activation domain likely interferes the association of Stat5b with other coactivators to repress Stat5b-activated transcription. Thus, identification of which coactivator complexes are involved in integrating Stat5b signal and convey it to activate the basal transcription apparatus needs to be first investigated prior to testing our hypothesis.

Although several RNA-binding proteins, such as Pbx-regulating protein 1 (46,47) and FUSE-binding proteins (48), are also able to bind DNAs and function as transcription factors, to our knowledge, nothing is known for a translational repressor in complex with a transcription factor to regulate gene expression. A recent study has identified ribosomal protein S3 (RPS3), a KH domain RNA-binding protein, was in complex with NF-κB and enhanced NF-κB-mediated transcription (49). Although RPS3 is a subunit of 40S ribosome and has a role in general translation, no specific mRNA has been identified to associate with RPS3. Despite CPEB2 and CPEB4 share identical RNA-binding specificity with CPEB3 and constantly shuttle between nucleocytoplasmic compartments, they have no obvious influence on the level of EGFR once knocked down in neurons. Since CPEB2 and CPEB4 do not associate with Stat5b, it appears that the interaction between a translation regulator and a transcription factor may help CPEB3 has regulatory roles in a spectrum of genes different from CPEB2 and CPEB4. The nuclear import of Stat5b is not influenced by CPEB3 and NMDA. Because the fact that Stat5b activates transcription of EGFR gene and CPEB3 offsets its activity, it seems illogical for CPEB3 and Stat5b to import simultaneously to the nucleus. Moreover, CPEB3 and Stat5b interaction is not disrupted by NMDA treatment and a nuclear form
of CPEB3, CPEB3NLS, has stronger suppression effect on Stat5b-dependent transcription than CPEB3 in the promoter assays, so it is expected that NMDA-induced nuclear accumulation of the repressor CPEB3 is likely to further downregulate Stat5b-dependent EGFR gene transcription by NMDAR activation-induced neuronal toxicity, we can not directly demonstrate that EGFR transcription is downregulated by NMDA stimulation in a CPEB3-dependent manner. It remains to be identified the specific importins and non-canonical nuclear localization sequences in CPEB3 responsible for its import to further address the physiological significance of NMDA-induced nuclear accumulation of CPEB3.

Because pharmacological intervention of the kinase activity of EGFR in the brain affects spatial memory, EGFR is a PRP that modulates learning and memory. Hence, there should be caution regarding potential cognitive side-effects when using EGFR-targeted drugs to treat brain tumors. Since EGFR signaling leads to parallel activation of several processes, such as MAPK and PI3K-Akt signaling pathways that have been demonstrated to play important roles in plasticity and memory (50–53), even a subtle change in EGFR expression likely has significant impact on plasticity. In CPEB3-deficient neurons, the EGFR level is elevated. Interestingly, EGFR expression was found upregulated in forebrain regions of schizophrenics (54) and EGF administration in neonatal rats induced various behavioral hallmarks of schizophrenia (55). Generation of conditional CPEB3 knockout mice is in process to address whether aberrant EGFR expression will cause abnormal signaling and aberrant behaviors in mice.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS
The authors thank Peter Rotwein for Stat5b and IGF1 plasmids, Young-Sun Lin for the Gal4 system, Roger Tsien for cherry RFP plasmid, Pang-Hsien Tu for TDP43 antibody and Joel Richter for CPEB3 antibody. The authors appreciate Chun-Kuei Su for sharing stereotoxic instrument, Pan-Chyr Yang for advice on Iressa, Yu-Chia Lin and Ching-Pang Chang for assistance on behavior study. The authors thank Ya-Ping Lin in the institutional core for conducting ICAT analysis and the RNAi Core Facility in Academia Sinica for the Stat5b shRNA clone.

FUNDING
National Science Council and National Health Research Institute in Taiwan; National Research Program for Genomic Medicine Grants for the RNAi Core. Funding for open access charge: National Science Council in Taiwan.

Conflict of interest statement. None declared.

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