Cellular/Molecular

A Molecular Circuit Composed of CPEB-1 and c-Jun Controls Growth Hormone-Mediated Synaptic Plasticity in the Mouse Hippocampus

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Cytoplasmic polyadenylation element binding protein 1 (CPEB-1) resides at postsynaptic sites in hippocampal neurons in which it controls polyadenylation-induced translation. CPEB-1 knock-out (KO) mice display defects in some forms of synaptic plasticity and hippocampal-dependent memories. To identify CPEB-1-regulated mRNAs, we used proteomics to compare polypeptides in wild-type (WT) and CPEB-1 KO hippocampus. Growth hormone (GH) was reduced in the KO hippocampus, as were the GH signaling molecules phospho-JAK2 and phospho-STAT3. GH mRNA and pre-mRNA were reduced in the KO hippocampus, suggesting that CPEB-1 controls GH transcription. The transcription factor c-Jun, which binds the GH promoter, was also reduced in the KO hippocampus, as was its ability to coimmunoprecipitate chromatin containing the GH promoter. CPEB-1 binds c-Jun 3′ untranslated region CPEs *in vitro* and coimmunoprecipitates c-Jun RNA *in vivo*. GH induces long-term potentiation (LTP) when applied to hippocampal slices from WT and CPEB-1 KO mice, but the magnitude of LTP induced by GH in KO mice is reduced. Pretreatment with GH did not reverse the LTP deficit observed in KO mice after theta-burst stimulation (TBS). Cordycepin, an inhibitor of polyadenylation, disrupted LTP induced by either GH application or TBS. Finally, GH application to hippocampal slices induced JAK2 phosphorylation in WT but not KO animals. These results indicate that CPEB-1 control of c-Jun mRNA translation regulates GH gene expression and resulting downstream signaling events (e.g., synaptic plasticity) in the mouse hippocampus.

Key words: **CPEB-1; c-Jun; growth hormone; mRNA translation; plasticity; hippocampus**

Introduction

Experience-induced alterations in synaptic connections (synaptic plasticity) are thought to underlie learning and memory (Kandel, 2001). Activity-dependent protein synthesis promotes these alterations (Steward and Schuman, 2003; Sutton and Schuman, 2005), which could involve the recruitment of mRNAs onto polysomes. One mechanism that regulates mRNA translation in the synapto-dendritic compartment involves the cytoplasmic polyadenylation element binding protein (CPEB-1), a sequence-specific RNA binding protein (Mendez and Richter, 2001; Theis et al., 2003; Huang et al., 2006) that interacts with the cytoplasmic polyadenylation element (CPE), a U-rich structure in the 3′ untranslated regions (UTRs) of certain mRNAs. Once phosphorylated, CPEB-1 stimulates polyadenylation and translation (Mendez et al., 2000; Barnard et al., 2004; Kim and Richter, 2006; Kim and Richter, 2007); in synapto-dendrites, CPEB-1 phosphorylation is mediated by NMDA receptor activation (Wu et al., 1998; Huang et al., 2002; Atkins et al., 2004).

Analysis of CPEB-1 knock-out (KO) mice supports a role for this protein in certain types of synaptic plasticity and learning and memory. Theta-burst stimulation (TBS) of Schaffer collateral CA1 hippocampal neurons results in a decrease in long-term potentiation (LTP). Moreover, hippocampal long-term depression elicited by 1 Hz stimulation is more enduring in the KO mice compared with wild type (WT) (Alarcon et al., 2004). CPEB-1 KO mice also have alterations in hippocampus-dependent behavioral tasks (Berger-Sweeney et al., 2006), which together with some of the electrophysiological parameters noted above may require protein synthesis (Berman and Dudai, 2001; Vianna et al., 2001; Pedreira and Maldonado, 2003; Santini et al., 2004).

We used two-dimensional gel electrophoresis and mass spectrometry to identify proteins with altered abundance levels in the hippocampus of CPEB-1 KO versus WT mice. Growth hormone (GH) was the most dramatically changed; it was reduced by ~10-fold in the KO hippocampus. GH modulates synaptic efficacy of hippocampal neurons and itself is regulated during memory for-

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mation (Donahue et al., 2002, 2006; Mahmoud and Grover, 2006). GH can signal through janus kinase 2 (JAK2) phospho-Y1007/Y1008 and signal transducer and activator of transcription 3 (STAT3) phospho-Y205 (Zhu et al., 2001), both of which were reduced in the KO hippocampus. GH mRNA and pre-mRNA were also reduced, suggesting that GH transcription is directly or indirectly regulated by CPEB-1. The mouse GH promoter contains binding sites for c-Jun whose mRNA contains CPEs that are bound by CPEB. c-Jun levels were reduced in the KO hippocampus, as was its ability to coimmunoprecipitate chromatin containing the GH promoter. GH stimulated LTP in hippocampal slices from WT and KO mice, although LTP expression in the KO was reduced. LTP expression was also altered when the slices were incubated with cordycepin, which inhibits polyadenylation. These data suggest that c-Jun mRNA translation is regulated by CPEB-1 and that GH transcription via c-Jun could be partially responsible for the changes in synaptic plasticity and behavior in the CPEB-1 KO mouse. Moreover, GH stimulation of LTP may involve cytoplasmic polyadenylation; this polypeptide may thus act in autocrine and paracrine manner to stimulate CPEB-1 activity.

Materials and Methods

Animals, cell culture, and biochemistry. CPEB-1 KO mice (Tay and Richter, 2001) were backcrossed onto the C57BL/6 background for four to nine generations. The brain was removed from males only and chilled on ice, and the hippocampus was manually dissected from the surrounding tissues and snap frozen in liquid nitrogen until use. Hippocampal protein was extracted as described by Davis et al. (2006), separated on two-dimensional gels, and stained with SYPRO-Ruby. Spots were determined to be significantly different as assessed by Progenesis Discover and Pro software (Nonlinear Dynamics). Normalized spot volume was determined by principle component analysis; pairwise comparisons of spots between WT and CPEB-1 KO hippocampus were performed with the Student's *t* test in the Progenesis Discovery software (Davis et al., 2006). Selected spots were excised, and their identity was determined by mass spectrometry. All proteomics were conducted by the University of Massachusetts Medical School Proteomics facility. For Western blotting, protein extracts were prepared according to Cao et al. (2005), or, for hippocampal slices, flash-frozen tissue was disrupted by sonication in 1% SDS or directly in sample buffer. CPEB-1 antibody has been described by Tay et al. (2003) or purchased from Affinity Bioreagents; c-Jun antibody was a gift from Roger Davis (University of Massachusetts Medical School, Worcester, MA) or was purchased from Cell Signaling Technologies.

RNA was extracted from hippocampal tissue using Trizol (Invitrogen) and treated with RQ1 RNase-free DNase (Promega). cDNA was amplified with *Taq* polymerase (Qiagen) in a reaction mix that contained 10 mM dATP, dGTP, and dTTP but 0.2 mM dCTP as well as trace amounts of [α-^{32}P]dCTP. For each primer pair, the optimal cycle number was empirically determined. Primer sequences were as follows: c-Jun, 5′-CCTTCTACGACGATGCCCTCAA-3′ and 5′-GGGGTCGGTGTAGTGGTGATGT-3′; GH, 5′-CTGGCTGCTGACACCTACAAAG-3′ and 5′-TGTTGGTGAAAATCCTGCTGAG-3′; GH intron, 5′- GGGCAGGAGTATGGGGTAGGAC-3′ and 5′-TTTCTCCTGCCCTCCTGTCTCT-3′; neurofilament (NF), 5′-CCTCAAGTCTATCCGCACACAA-3′ and 5′-TGCTTCTCGTTAGTGGCGTCTT-3′. The PCR products were separated on denaturing polyacrylamide gels.

Chromatin immunoprecipitation (ChIP) from hippocampal tissue was performed as described previously (Chen-Plotkin et al., 2006). Two hippocampal pairs were pooled for each genotype. Genomic DNA coimmunoprecipitating with c-Jun was identified by PCR with the following primers: c-Jun sites 1 and 2 in the GH promoter, 5′-GAGGAGGAACAATAGGAGGAA-3′ and 5′- TCTGTCTCTTTGTCTGTCCATC-3′; c-Jun site 3 in the GH promoter, 5′-GCAAACATGGCTGGCTCACTCT-3′ and 5′-TCTTTTTGGACCCTGGAGTTCT-3′; GH intron, 5′- GGGCAGGAGTATGGGGTAGGAC-3′ and 5′-TTTCTCCTGCCCTCCTGTCTCT-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) exon, 5′-GCTAAGGCTACACAGACAAACTC-3′ and 5′-AGGAGCAGAGAGCCAGTTTGTTA-3′.

RNA–protein coimmunoprecipitation was modified from the procedure of Tenenbaum et al. (2000, 2002). Hippocampal tissue from CPEB-1 KO and WT littermates was rinsed with ice-cold PBS and homogenized in lysis buffer containing 10 mM HEPES, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 0.5% NP-40, 1 mM DTT, 3 μl/ml RNase inhibitor (RNAguard), 0.2% vanadyl ribonucleoside complex, 10 μl/ml of 10 mM phenylmethylsulfonyl fluoride, and 10 μl of a 10 mg/ml stock of pepstatin A, aprotinin, and leupeptin. One hundred microliters of this extract was diluted with 400 μl of NT2 (50 mM Tris, pH 7.4, 150 mM KCl, 1 mM $MgCl_2$, plus the RNase and protease inhibitors noted above). Five micrograms of antibody was added, and the reaction was mixed end-over-end overnight at 4°C. Twenty microliters of Dynabeads M-280 were washed with NT2 supplemented with 5% BSA and 0.05% NP-40 and then added to the extract, which was incubated with tumbling for 4 h at 4°C. The beads were then washed five times with NT2 supplemented with 0.05% NP-40, and the protein was digested with 0.1% SDS and 0.3 mg/ml proteinase K. The RNA was then phenol extracted and subjected to reverse transcription (RT)-PCR as described above. The RNA gel shift was conducted according to Hake et al. (1998) using an *in vitro* transcribed region of the c-Jun 3′ UTR that contains the CPEs.

Electrophysiology. Transverse hippocampal slices (400 μm) from wild-type and CPEB-1 KO mice (2.5–4 months old) were incubated at room temperature with oxygenated artificial CSF (ACSF) (in mM: 119 NaCl, 4 KCI, 1.5 $MgSO_4$, 2.5 $CaCI_2$, 26.2 $NaHCO_3$, 1 NaH_2PO_4, and 11 glucose) and allowed to equilibrate for 60 min. The slices were then placed in a submerged chamber with ACSF at 28 ± 1°C for at least 30 min before recording. Field EPSPs (fEPSPs) were recorded at CA3–CA1 synapses via stimulation of the Schaffer collateral axons with a bipolar electrode CBAPD75 (FHC) and recording with a 4–5 MΩ glass pipette (A-M Systems). The stimulation intensity (square pulse, 50 μs duration) was adjusted to give fEPSP slopes of ~40% of maximum. Baseline and post-stimulation responses were sampled once per minute at this intensity. The LTP protocol for theta-burst stimulation was a single theta-burst episode, which consisted of nine bursts of four pulses at 100 Hz with 200 ms interburst intervals.

For some experiments, to ensure that a large portion of hippocampal slice CA1 neurons be subjected to TBS treatment, the stimulating and recording electrodes were positioned at the beginning (CA2/CA1 border) and the ending (CA1/subiculum border) of the CA1 area, respectively. For all experiments, two slices from approximately the same hippocampal area were recorded in parallel, one control and one stimulated. At the end of the recordings, slices were collected and immediately frozen on dry ice. The CA1 area was the dissected out under a microscope and stored at −80°C. For GH-treated material, hippocampal slices were incubated at room temperature in ACSF and allowed to equilibrate for 90 min before GH application (22 ng/ml). Slices were collected at the indicated times (slices from WT and KO mice were treated in parallel), frozen on dry ice, and stored at −80°C.

Statistics. Statistical analysis for the electrophysiology was performed using a two-tailed two-population Student's *t* test (Microcal Origin statistical tool). Data are reported as mean ± SE in the figures and as mean ± SD in the text. The experimental *n* equals the number of animals; at least two slices were recorded per animal.

Drugs. Recombinant human growth hormone (Cell Sciences), reconstituted in water at 100 μg/ml, was bath applied onto the slices (at 22 ng/ml) for the times indicated in each experiment. Cordycepin (3′-deoxyadenosine) (Sigma) was dissolved in 50% ethanol for a final concentration of 200 μg/ml to bathe slices (2% ethanol). Control experiments were performed in ACSF plus 2% ethanol. The pretreatment process (30 min) was performed to ensure an optimal effect of the drug in slices.

Results

Growth hormone is deficient in the CPEB-1 KO hippocampus

CPEB-1 KO mice have defects in some types of synaptic plasticity (Alarcon et al., 2004) and hippocampal-dependent memories

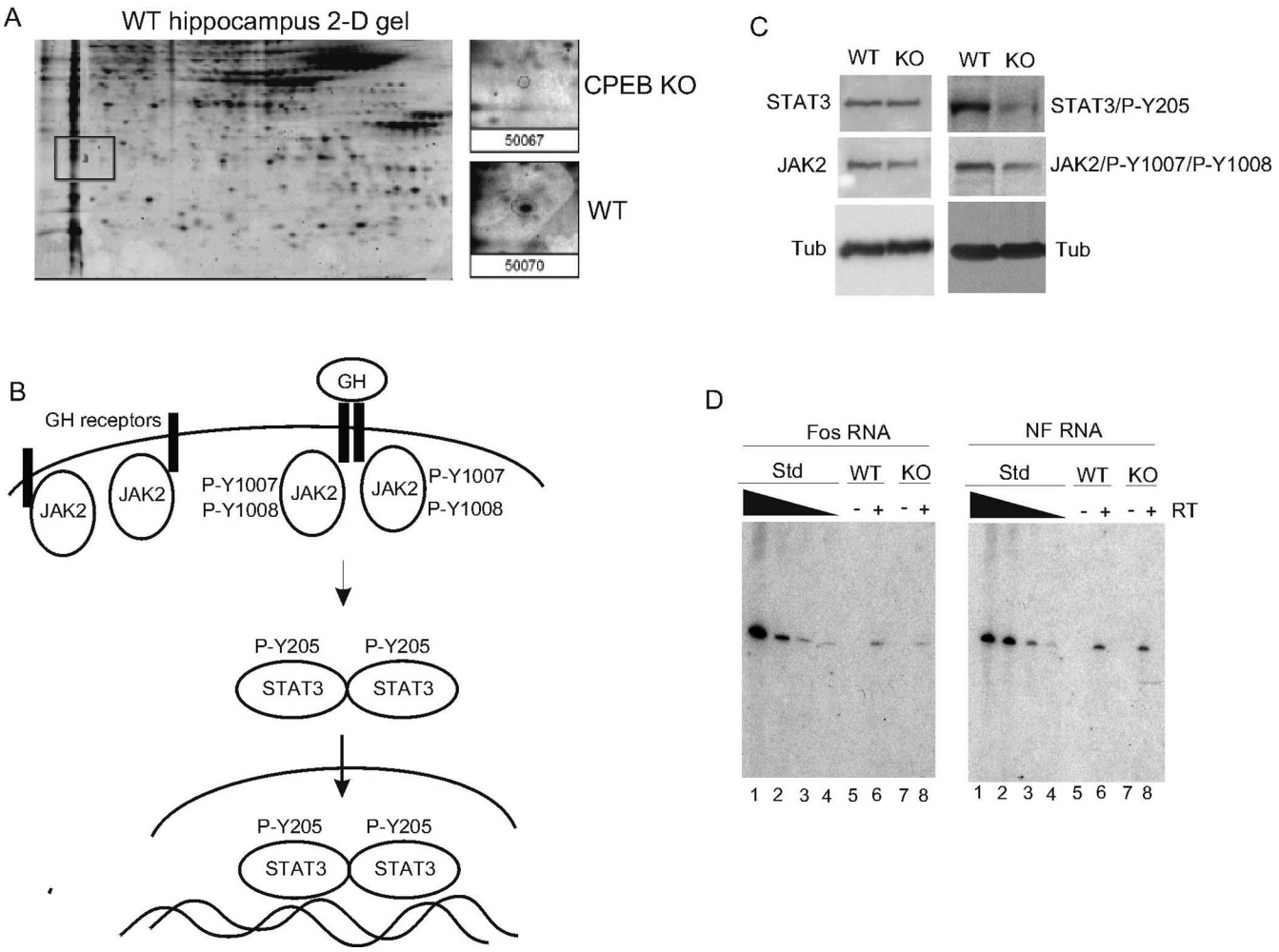

Figure 1. Growth hormone expression and signaling is reduced in the CPEB-1 KO mouse hippocampus. ***A***, Representative SYPRO-Ruby-stained two-dimensional gel and the detection of a spot that was reduced ~10-fold in the KO hippocampus. Mass spectrometry identified this spot as GH. Gels were analyzed in triplicate using tissue from one pair of WT and KO littermates. ***B***, Schematic diagram of GH signaling. GH binds transmembrane receptors, which, when autophosphorylated, induce the activation of the kinase JAK2 by phosphorylating residues of Y1007 and Y1008. JAK2 then phosphorylates Y205 of STAT3, which causes it to homodimerize or heterodimerize and be translocated to the nucleus in which it binds DNA and stimulates transcription. ***C***, Hippocampal extracts from WT and CPEB-1 KO mice were immunoblotted and probed for STAT3 and JAK2, as well as for phospho-Y205 of STAT3, phospho-Y1007 and phospho-Y1008 of JAK2, and tubulin (Tub). Representative blots are shown of an analysis of at least two pairs of animals. ***D***, RNA from WT and CPEB-1 KO hippocampus was subjected to semiquantitative RT-PCR for Fos and NF RNAs. A serial twofold dilution of input RNA from WT hippocampus is shown in lanes 1–4 (Std). In some cases, the RT step was omitted and served as a negative control. Three replicates of this experiment were performed using RNA from three different age-matched WT and KO pairs, and the differences in the Fos signal between WT and KO hippocampus were statistically significant ($p < 0.025$, one-tailed paired-sample *t* test).

such as extinction (Berger-Sweeney et al., 2006). To identify molecular targets of CPEB-1 that could be responsible, at least in part, for these phenotypes, total hippocampal protein from WT and CPEB-1 KO male littermates was resolved by two-dimensional PAGE using two different isoelectric point ranges and stained with SYPRO-Ruby (Fig. 1*A*). The protein gels, analyzed in triplicate, were scanned, and the relative amount of signal in each spot was quantified. One protein that was consistently lower in the KO hippocampus by ~10-fold (Fig. 1*A*) was excised and identified by mass spectrometry to be GH (also referred to as somatotropin). Two other proteins were consistently altered in the KO hippocampus: αb crystalline (lower by approximately fivefold), and collapsin response mediator protein 3 (CRMP3) (increased by approximately twofold). Because the importance of αb crystalline and CRMP3 for neuronal activities is unknown, we focused on GH, which has been reported to influence synaptic activity (Mahmoud and Grover, 2006). Although the primary site of GH synthesis is the pituitary, an analysis of that tissue revealed that the level of GH was not changed in the KO (data not shown).

GH signals to cells by interacting with and causing the dimerization and phosphorylation of its trans-membrane receptor, which then activates a JAK/STAT signaling cascade. Although GH can activate a number of JAKs and STATs, the cascade can involve the phosphorylation of JAK2 Y1007/1008 and the phosphorylation of STAT3 Y-205 (Zhu et al., 2001). Phospho-STAT3 then homodimerizes or heterodimerizes and is transported to the nucleus in which it stimulates transcription (Fig. 1*B*) (Moutoussamy et al., 1998; Herrington and Carter-Su, 2001). To determine whether GH-dependent signaling is altered in the KO mouse, we examined several tyrosine-phosphorylated JAKs and STATs. Although we could detect no change in JAK1 phospho-Y1022/1023, STAT3 phospho-Y5727, or STAT5 phospho-Y694 (data not shown), we found that STAT3 phospho-Y205 and JAK2 phospho-Y1007/1008 were reduced in the hippocampus of KO versus WT animals (Fig. 1*C*). Finally, we determined whether one downstream target gene of STAT3 that has been shown to be GH responsive (Liao et al., 1997), Fos, was also reduced in the CPEB-1 KO hippocampus. Compared with NF RNA as a control,

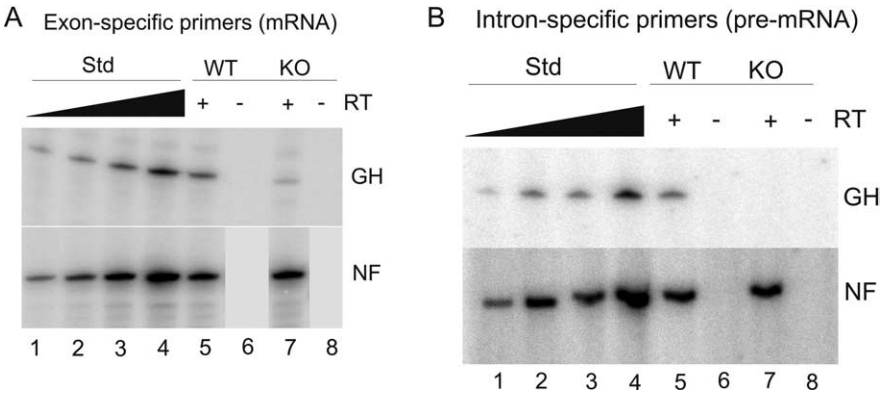

Figure 2. Reduced GH RNA and pre-mRNA in the CPEB-1 KO hippocampus. ***A***, RNA from WT and CPEB-1 KO hippocampus was subjected to semiquantitative RT-PCR with exon-specific primers for GH and NF RNAs. Input consisted of a serial twofold dilution of RNA from the WT hippocampus. In some cases, the RT step was omitted. ***B***, RNA from WT and CPEB-1 KO hippocampus was subjected to semiquantitative RT-PCR with intron-specific primers for GH and NF RNAs. Standard (Std) consisted of a serial twofold dilution of RNA from the WT hippocampus. In some cases, the RT step was omitted.

Fos RNA levels were reduced by a modest 30% in the hippocampus of unstimulated animals; although this amount was variable, it was statistically significant ($p < 0.025$, one-tailed paired sample t test) (Fig. 1*D*). Because Fos is an immediate early gene whose transcription increases substantially on certain types of synaptic stimulation, we also examined the relative levels of Fos RNA in the hippocampus of animals injected with kainate, a seizure-causing agent (Theis et al., 2003). Although Fos levels increased >10-fold after kainate treatment, there was no discernable difference between WT and KO animals (data not shown). Thus, although CPEB-1 influences the basal level of Fos RNA, it has no effect on the amount of this mRNA after synaptic stimulation and seizure.

GH mRNA and pre-mRNA are reduced in the CPEB-1 KO hippocampus

Although the primary site of GH gene expression is the pituitary, it is also synthesized in the hippocampus (Donahue et al., 2006). To determine whether CPEB-1 might affect GH RNA levels, total RNA from the hippocampus of WT and CPEB-1 KO mice was subjected to semiquantitative RT-PCR using exon-specific GH primers. GH mRNA, like the protein, was reduced in the hippocampus of the CPEB-1 KO mouse. NF RNA levels were the same in WT and KO hippocampus (Fig. 2*A*).

Because the GH 3′ UTR contains no obvious CPE, we reasoned that the reduction of GH mRNA in the KO hippocampus might be a secondary effect and that factors affecting GH RNA synthesis or stability could be direct targets of CPEB-1 activity. To assess this possibility, primers specific for introns 1 and 2 of GH pre-mRNA were used for semiquantitative RT-PCR. Like GH protein and mRNA, GH pre-mRNA was also reduced in the KO hippocampus. As before, NF RNA was the same in WT and KO hippocampus (Fig. 2*B*). These results suggest that GH gene transcription is inhibited in the CPEB-1 KO hippocampus, leading us to speculate that CPEB-1 might control the expression of a factor that in turn controls GH transcription.

c-Jun levels are reduced in the CPEB-1 KO hippocampus

We examined the GH promoter for binding sites of transcription factors whose mRNAs contain CPEs. One primary candidate was Pit-1, which controls GH transcription in the pituitary. Although Pit-1 RNA contains CPEs, we could detect no Pit-1 mRNA or protein in the hippocampus (data not shown). Another transcription factor we considered was c-Jun. The GH promoter contains two c-Jun consensus binding sites (TGAGTCA) located ~3.9 and 4.3 kb upstream of the start of transcription; a third nonconsensus site (TGAGTTCA) is ~0.5 kb upstream of the start of transcription (Fig. 3*A*). The two consensus c-Jun sites also reside near putative nuclear factor of activated T-cells (NFAT) transcription factor binding sites; NFAT and AP-1 (i.e., c-Jun homodimers or c-Jun/Fos heterodimers) are thought to cooperate in promoting transcription (Macian et al., 2001). Moreover, the mRNA encoding c-Jun contains CPEs (Fig. 3*B*). Most importantly, the hippocampus contains c-Jun protein in relative abundance, which was reduced by ~50% in the CPEB-1 KO mouse (Fig. 3*C*). To investigate whether the GH promoter is bound by c-Jun, we performed ChIP experiments using an antibody specific for *c*-Jun protein. WT and CPEB-1 KO hippocampus were treated with formaldehyde to covalently crosslink chromatin proteins to DNA; the DNA was then sonicated to an average length of 0.5 kb (0.2–1 kb), followed by c-Jun immunoprecipitation, crosslink reversal, and PCR detection of specific DNA sequences. Figure 3*D* shows that, in the WT hippocampus, c-Jun antibody immunoprecipitated DNA in the GH promoter that contains the two c-Jun consensus sites; this did not occur in the CPEB-1 KO hippocampus. The c-Jun antibody did not immunoprecipitate DNA containing the nonconsensus c-Jun site in the GH promoter (c-Jun 3), a GH intron, or a GAPDH exon; these latter two sequences served as negative controls. Moreover, a nonspecific antibody against a hemagglutinin epitope did not immunoprecipitate any of the DNA sequences from WT or KO hippocampus. These data demonstrate that c-Jun binds the GH promoter and may control GH transcription.

To determine whether c-Jun RNA could be a direct target of CPEB-1 regulation, ribonucleoproteins from WT and CPEB-1 KO hippocampus were subjected to CPEB-1 antibody coimmunoprecipitation, followed by semiquantitative RT-PCR for c-Jun and NF RNAs. c-Jun RNA was coimmunoprecipitated with CPEB-1 from WT but not KO hippocampus (Fig. 3*D*, left). Only background levels of NF were coimmunoprecipitated from WT and KO hippocampus. In the absence of reverse transcriptase, no c-Jun or NF amplification products were detected from WT or KO hippocampus (Fig. 3*D*, right). Finally, a gel shift assay shows that the c-Jun 3′ UTR was bound by recombinant CPEB-1 *in vitro* but not when CPE-containing RNA was added to the gel shift mixture (Fig. 3*E*). Thus, in the wild-type hippocampus, CPEB-1 control of c-Jun mRNA translation results in GH transcription.

GH-induced LTP is deficient in CPEB-1 KO mice

As reported previously by others, bath application of GH to hippocampal slices produced a robust LTP in Schaffer collateral to CA1 pyramidal neuronal synapses of WT mice (Mahmoud and Grover, 2006). However, the same treatment in slices from KO animals produced LTP with a reduction of ~40% (Fig. 4*A*,*B*; note the 90–120 min time interval; WT, 170 ± 18%; KO, 139 ± 15%; $p = 0.01$), suggesting that LTP expression in response to GH-signaling activation depends partially on CPEB function. To further explore the nature of this deficiency, we next investigated the effect of GH pretreatment on LTP induced by TBS. We showed previously that TBS-induced LTP is deficient in CPEB-1 KO mice (Alarcon et al., 2004)

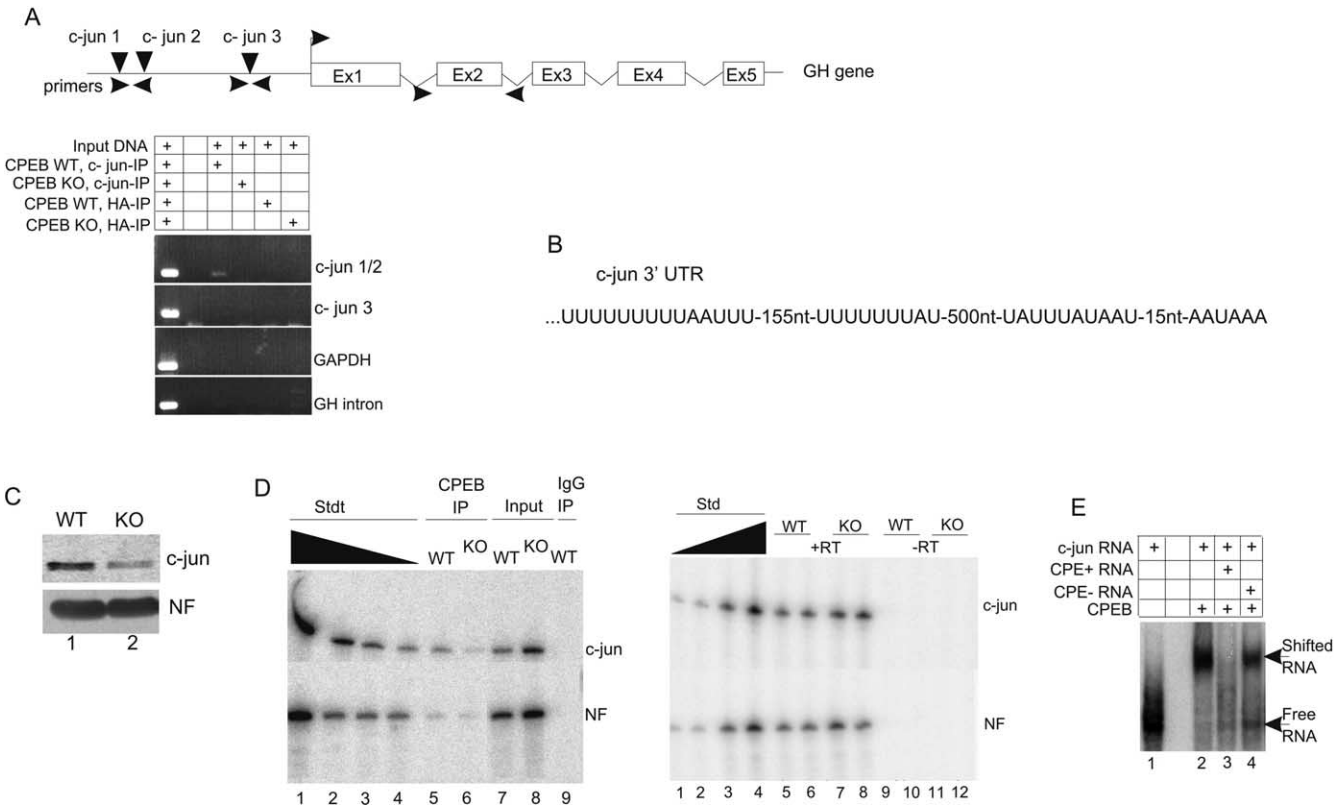

Figure 3. CPEB-1 regulates c-Jun RNA translation. ***A***, Illustration of the GH gene showing two consensus c-Jun sites (~4 kb upstream of the start of transcription) and a third nonconsensus c-Jun site (~0.5 kb upstream). The primers used for PCR to detect various regions of DNA are indicated. Hippocampus from two WT or CPEB-1 KO animals was subjected to a ChIP assay with c-Jun antibody. After formaldehyde-induced DNA–protein crosslinking and sonication to shear the DNA, c-Jun was immunoprecipitated (IP), the crosslinks were reversed, and the DNA was subjected to PCR with primers specific for c-Jun sites 1 and 2, c-Jun site 3, a GH intron, and a GAPDH exon. HA, Hemagglutinin. ***B***, Relevant sequence of the c-Jun 3′ UTR. The putative CPEs and the AAUAAA hexanucleotide are noted. ***C***, Immunoblot of c-Jun and NF from hippocampal extracts derived from WT and CPEB-1 KO hippocampus. A representative blot is shown from an analysis of two pairs of animals. ***D***, Extracts derived from WT and CPEB-1 KO hippocampus were subjected to immunoprecipitation with CPEB-1 antibody, followed by RNA extraction and semiquantitative RT-PCR for c-Jun and NF RNAs. Ten percent of the input material, before immunoprecipitation, is also shown. Extracts from WT hippocampus was also immunoprecipitated with a nonspecific IgG, which served as an additional negative control. Standard (Std) consisted of a serial twofold dilution of RNA from the WT hippocampus. The right panel shows relative c-Jun RNA levels in the absence of immunoprecipitation from WT and CPEB-1 KO hippocampus when the RT step was both included and omitted. ***E***, RNA gel shift of the c-Jun 3′ UTR with CPEB-1. Recombinant CPEB-1 was mixed with ^{32}P-labled c-Jun 3′ UTR and, in lanes 3 and 4, with excess unlabeled CPE-containing or CPE-lacking RNA, which was followed by electrophoresis in a nondenaturing gel. The RNA–protein complex (shifted RNA) and free RNA are noted.

and corroborated these early findings here (Fig. 4*C1*; TBS, 90–120 min time interval; WT, 158 ± 15%; KO, 116 ± 6%; $p = 0.0004$). Pretreatment of hippocampal slices with GH lessened the subsequent expression of LTP induced by TBS in WT mice (Fig. 4*C2*; 90–120 min time interval; WT, 119 ± 14%), suggesting that both GH-LTP and TBS-LTP share common expression mechanisms. Most interestingly, GH pretreatment did not affect the subsequent LTP induced by TBS in KO mice (Fig. 4*C2*; 90–120 min time interval; 121 ± 16%). This LTP was similar in magnitude to both the occluded TBS-LTP in WT mice (Fig. 4*C2*; WT vs KO, $p = 0.88$) and the deficient TBS-LTP observed in KO mice without GH pretreatment (Fig. 4*C1*, KO vs *C2*, KO; $p = 0.55$). These results suggest a connection between the deficient LTP induced by TBS in KO mice, the reduced LTP induced by TBS in WT mice after GH treatment, and the reduced LTP induced by bath application of GH in KO mice: that is, an ablated GH-signaling pathway linked to CPEB-1 function.

To further investigate the connection between CPEB-1 function and the LTP deficits associated with GH cascade activation, we used the polyadenylation blocker cordycepin (3′-deoxyadenosine). Because of the 3′-deoxy moiety, this nucleoside analog acts by blocking poly(A) elongation. Pretreatment with cordycepin dramatically affected both types of LTP, TBS-LTP (Fig. 4*D1*; 90–120 min time interval; control, 168 ± 13%; cordycepin, 138 ± 16%; $p = 0.037$) and GH-LTP (Fig. 4*D2*; 90–120 min time interval; control, 176 ± 14%; cordycepin, 148 ± 9%; $p = 0.008$), indicating that polyadenylation is a critical process for the expression of each form of LTP.

Finally, we prepared hippocampal slices to investigate whether TBS or GH could stimulate elements of the GH signaling cascade. In the dissected, stimulated CA1, TBS did induce the expression of c-Jun (Fig. 5*A*), although we could not detect signals with the phospho-JAK2 or phospho-STAT3 antibodies because of their relatively low abundance and the small size of the tissue sample. When GH was applied to slices, we observed an increase in phospho-JAK2 with no change in the level of JAK2. Moreover, this increase was not observed in the CPEB-1 KO hippocampus (Fig. 5*B*). Unfortunately, we could not reliably detect a phospho-STAT3 signal in these slices. Nonetheless, these data show that GH signaling in the hippocampus requires CPEB-1.

Discussion

Our two-dimensional gels revealed that, compared with WT hippocampus, three proteins were changed in the KO: GH and αb crystalline were decreased (~10-fold and ~5-fold, respectively), whereas CRMP3 was increased (~2-fold). Although GH production is often associated with the pituitary, it is also synthesized in the hippocampus (Sun et al., 2005; Donahue et al., 2006), and indeed we detected GH mRNA and pre-mRNA in that tissue. GH RNA and pre-mRNA were reduced in the KO hippocampus, and

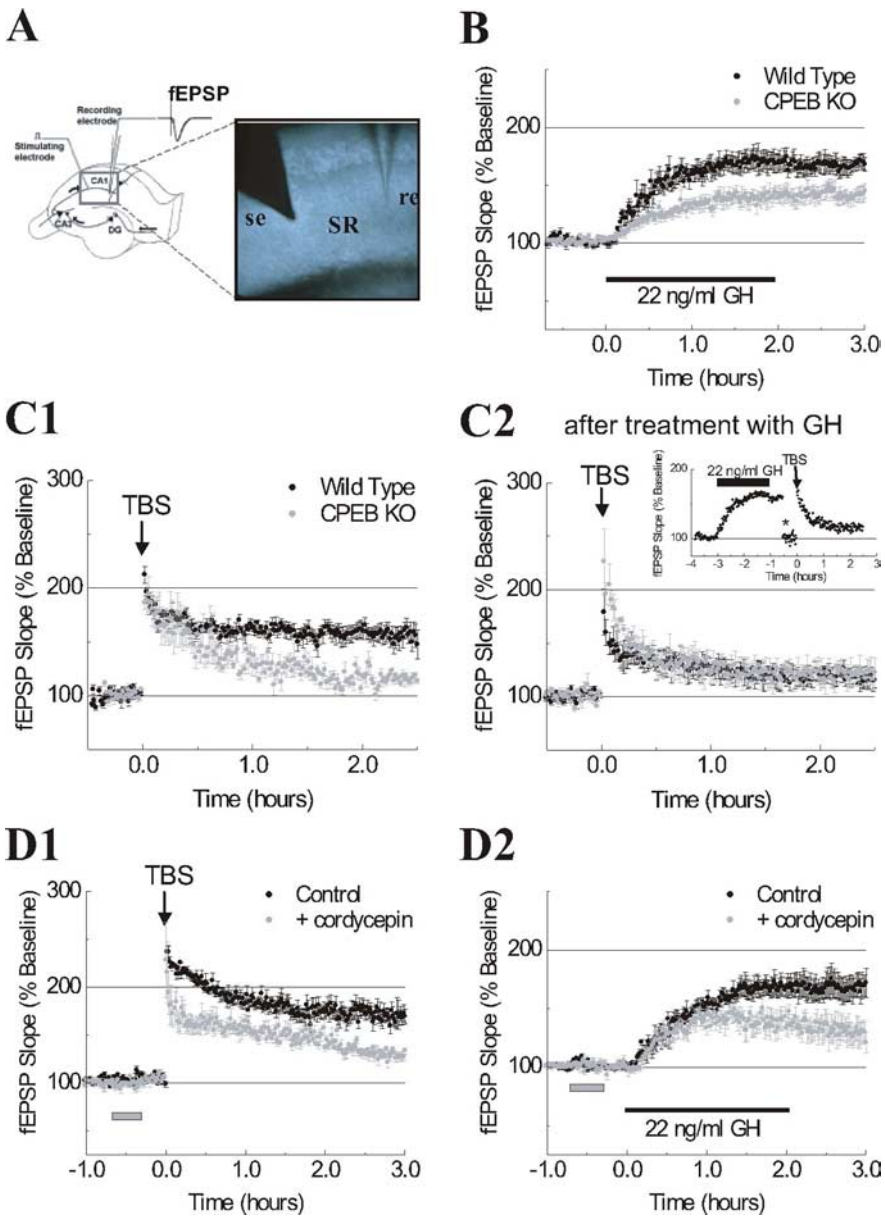

Figure 4. Analysis of GH-induced LTP. ***A***, Illustration of the hippocampal mouse slice preparation and micrograph showing the placement of the stimulating (se) and recording (re) electrodes in the stratum radiatum (SR) of area CA1. DG, Dentate gyrus. ***B***, Bath application of recombinant human GH (22 ng/ml) onto slices elicits LTP in wild-type and CPEB-1 KO mice. Note that, at 90–120 min, the amplitude of the LTP in KO animals is 55% of that in the wild-type animals ($n = 5$ each for wild type and KO). ***C1***, TBS (arrow) induces a deficient LTP in KO mice (90–120 min interval; $n = 4$ each for wild type and KO). ***C2***, Pretreatment with GH fails to rescue deficient LTP in KO mice. The inset shows the complete experiment: after GH-induced LTP reached a plateau, the stimulation strength of the test pulses was lowered to baseline levels (*); 30 min later, LTP was induced with TBS (arrow) (90–120 min interval; $n = 4$ each for wild type and KO). ***D1***, LTP induced by TBS is dramatically impaired by pretreatment (30 min) with the polyadenylation blocker cordycepin (90–120 min interval, 200 μg/ml; $n = 5$ each for control and cordycepin). ***D2***, Cordycepin pretreatment also impairs LTP induced by GH (90–120 min interval; $n = 5$ each for control and cordycepin).

molecules downstream of the GH receptor, phospho-JAK2 and phospho-SAT3, were also reduced. These results suggested that an mRNA encoding a transcription factor that controls GH expression might be directly regulated by CPEB-1. The GH promoter contains several transcription factor binding sites, and the mRNA encoding c-Jun harbors CPEs. On immunoblots, c-Jun was reduced by approximately twofold in the KO versus WT hippocampus. Sequences containing c-Jun sites in the GH promoter were coimmunoprecipitated with c-Jun from WT but not KO hippocampus. c-Jun RNA was bound by CPEB-1 and coimmunoprecipitated with it from WT but not KO hippocampus. These data suggest that CPEB-1 regulation of c-Jun mRNA translation controls downstream events that culminate in changes in synaptic plasticity and hippocampal-dependent memories.

GH plays a role in neural function because dwarf mice with pituitary GH deficiencies are resistant to the cognitive decline that is commonly associated with aging (Kinney et al., 2001; Kinney-Forshee et al., 2004). Recent data suggests that GH directly affects synaptic efficacy; for example, Mahmoud and Grover (2006) examined synaptic transmission in CA1 synapses in the presence or absence of GH and observed an enhancement of NMDA- and AMPA-dependent synaptic responses. Additionally, upregulation of GH expression is observed after learning (Donahue et al., 2006). Given that GH is produced in the hippocampus, we speculate that it operates in an autocrine manner as well as communicating with neighboring cells. Thus, GH secretion could increase the efficacy of synaptic transmission among cells in their immediate environment.

The reduction in c-Jun and phospho-STAT3 in the KO hippocampus might be expected to result in reduced expression of many genes. However, our proteomics analysis as well as an investigation using microarrays to detect changed RNA levels in the WT and KO hippocampus revealed few alterations of more than twofold (Berger-Sweeney et al., 2006). Although there might be several low-abundance mRNAs whose levels change substantially in the KO hippocampus, it seems more likely that GH represents one of the few genes whose expression is profoundly affected by the loss of CPEB-1. Although the loss of GH probably contributes to the electrophysiological and behavioral phenotypes observed in the KO mouse, it is unlikely to be solely responsible for them; modest changes in the expression of other genes in the KO hippocampus may also contribute. Moreover, because stimuli that elicit LTP and long-term depression induce the synthesis of many of the same proteins (Kelleher et al., 2004), it has been argued that the productive capture or utilization of proteins at synapses, rather than their selective synthesis, controls synaptic strength (Govindarajan et al., 2006).

Mahmoud and Grover (2006) demonstrated that GH induced a form of LTP in hippocampal slices. We have extended these results to show that GH does not do so in the presence of cordycepin, an inhibitor of polyadenylation. Although we have not demonstrated that GH induces polyadenylation, these results imply that it does and that this process is necessary to establish LTP.

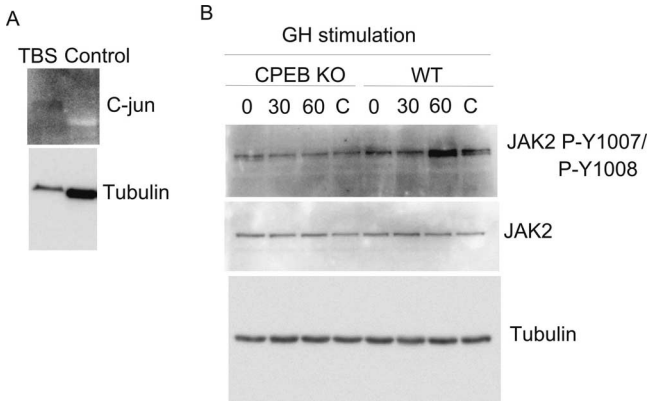

Figure 5. Stimulation of elements of the GH signaling cascade. ***A***, Hippocampal slices were subjected to TBS and then probed on a Western blot for c-Jun and tubulin. ***B***, WT and KO hippocampal slices were treated with GH for the times indicated. Extracts were then prepared and Western blots were probed for JAK2, phospho-Y1007 and phospho-Y1008 JAK2, and tubulin. The blot is representative of an analysis of two different pairs of WT and CPEB-1 KO animals.

Similarly, we provide evidence on the nature of the deficit in electrically induced LTP by theta-burst stimulation observed in the KO, namely, that of an altered GH signaling cascade.

The results presented here raise three additional issues, one of which is the mechanism by which CPEB-1 controls c-Jun mRNA translation. A likely possibility is cytoplasmic polyadenylation because CPEB-1 controls this process in germ cells and the brain (Richter, 2001; Klann and Richter, 2006). We have attempted to determine whether the c-Jun poly(A) tail is altered in WT versus KO hippocampus but have detected no change. However, c-Jun RNA has multiple AAUAA motifs, indicating that there may be multiple 3′ UTRs; if such is the case, our attempts to detect cytoplasmic polyadenylation would have been obscured (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Moreover, perhaps only a small amount of c-Jun RNA is regulated by CPEB-1, in which case changes in poly(A) would be masked by the c-Jun that is not bound by CPEB-1. Finally, there may be some indirect effects of CPEB-1 on c-Jun expression that do not involve changes in poly(A). This possibility leads to the second issue, which is the subcellular location of CPEB-1-dependent regulation of c-Jun RNA. Although CPEB-1 resides at synapses and in the cell body (Wu et al., 1998), we were particularly interested to determine whether c-Jun RNA might be regulated at synapses, in which CPEB-1 controls αCaMKII mRNA (Huang et al., 2002). Unfortunately, we were unable to observe a c-Jun RNA signal in dendrites. However, we did detect c-Jun protein in dendrites by immunostaining, but it usually did not colocalize with synaptophysin, a synaptic marker (data not shown). One intriguing possibility is that c-Jun is transported to the nucleus after synthesis in the synapto-dendritic compartment. It is noteworthy that the transcription factor NF-κB (Meffert et al., 2003) and proteins that mediate nuclear import, importin α and β, are transported from dendrite to nucleus in an activity-dependent manner (Thompson et al., 2004).

Finally, the two transcription factors that are regulated by CPEB-1, c-Jun (directly) and c-Fos (indirectly), are products of immediate early genes that heterodimerize to form the transcription factor AP-1. AP-1 controls the expression of genes in the hippocampus, including TIMP-1, which modifies synaptic plasticity (Kaczmarek et al., 2002). Thus, the expression of several genes that are dependent on CPEB-1-controlled translation of c-Jun mRNA could comprise a circuit of factors that modify synaptic plasticity. Indeed c-Fos, as well as CPEB-1, has been linked to memory extinction (Santini et al., 2004).

Figure 6 presents a speculative model that incorporates several of the observations reported here into the overall context of CPEB-mediated plasticity. CPEB controls the translation of c-Jun mRNA translation in neurons, at least some of which might occur in the synapto-dendritic compartment. This locally synthesized c-Jun could then be transported to the nucleus in which, probably in concert with other factors, it binds the GH promoter to induce the transcription of this gene. GH mRNA would then likely be translated in the cell body; GH would be secreted from the cell in which it could stimulate plasticity of its own synapses (autocrine) and synapses of neighboring cells (paracrine). However, GH-induced plasticity requires CPEB, as demonstrated by the data in Figures 4 and 5. Because GH has been shown to modulate NMDA receptor expression (Le Greves et al., 2006), changes in postsynaptic NMDA receptors may accompany GH activity. Additionally, Mahmoud and Grover (2006) show that GH-induced LTP is mediated by both NMDA and AMPA receptors. Thus, we speculate that a molecular circuit including CPEB, c-Jun, and GH comprises a positive feedback loop that mediates synaptic plasticity. This model also provides a framework for future studies addressing cytoplasmic polyadenylation and protein synthesis-dependent synaptic plasticity.

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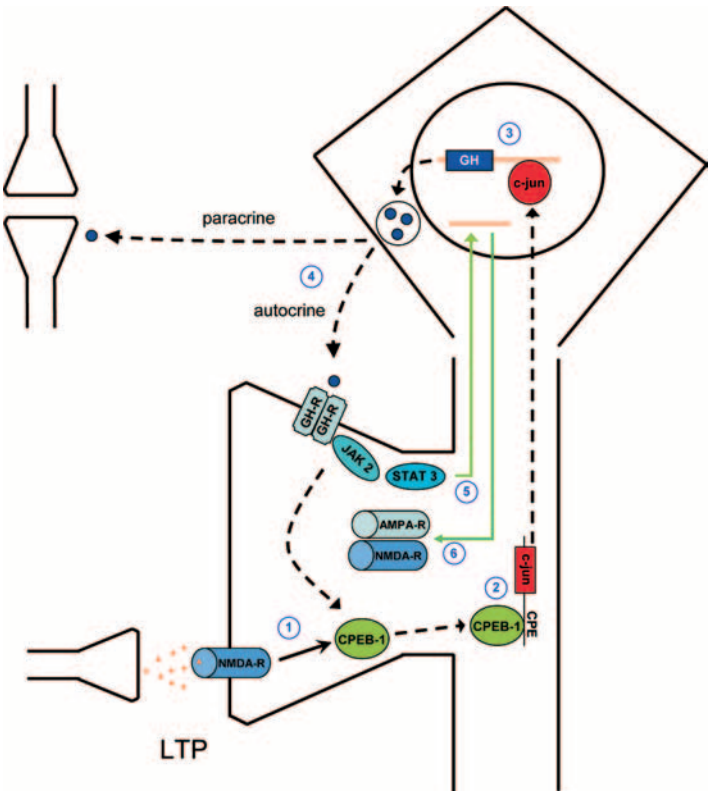

Figure 6. Model for CPEB-1-dependent molecular circuitry in neurons. CPEB-1 in the synapto-dendritic compartment controls local c-Jun mRNA translation (1, 2). c-Jun protein is then transported to the nucleus, in which it activates the transcription of GH and perhaps other genes as well (3). Secreted GH interacts with receptors on the same cell and nearby cells in which it modifies plasticity and other signaling events (4). Activation of GH receptors triggers transcription (5) and possibly polyadenylation by way of CPEB-1. Newly formed NMDA and AMPA receptors maintain LTP (6).

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