

The RNA binding protein CPEB regulates dendrite morphogenesis and neuronal circuit assembly in vivo

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Visual system development requires experience-dependent mechanisms that regulate neuronal structure and function, including dendritic arbor growth, synapse formation, and stabilization. Although RNA binding proteins have been shown to affect some forms of synaptic plasticity in adult animals, their role in the development of neuronal structure and functional circuitry is not clear. Using two-photon time-lapse in vivo imaging and electrophysiology combined with morpholino-mediated knockdown and expression of functional deletion mutants, we demonstrate that the mRNA binding protein, cytoplasmic polyadenylation element binding protein1 (CPEB1), affects experience-dependent neuronal development and circuit formation in the visual system of *Xenopus laevis*. These data indicate that sensory experience controls circuit development by regulating translational activity of mRNAs.

circuit integration | experience-dependent plasticity | in vivo imaging | visual system | whole-cell recording

During CNS development, neuronal structure, synaptic connections, and circuit functions change in response to sensory input. Although the regulation of mRNA translation and new protein synthesis have been implicated in some forms of neuronal plasticity in adult animals (1, 2), their role in experience-dependent sensory system plasticity has not been addressed (3). In the brain, the translation of many mRNAs is regulated by their association with ribonucleoprotein (RNP) granules, which are composed of core RNA binding proteins, translational machinery, and mRNAs (4). RNA binding proteins package specific mRNAs within RNP granules, regulate their transport into dendrites, and in principle, could temporally and spatially govern their translation in response to synaptic activity (4). Because of these features, mRNA binding proteins are in a unique position to control developmental events through the coordinated translation of a functionally related cohort of mRNAs (5, 6). Indeed, local protein synthesis affords neurons the ability to stabilize changes in synaptic connections and alter neuronal output (7). Recent studies indicate that cytoplasmic polyadenylation element binding protein 1 (CPEB1) is among a handful of RNA binding proteins that regulate dendritic protein synthesis and synaptic plasticity in vitro (8). Although studies in mutant mice implicate CPEB1 in some forms of synaptic and spine plasticity and in memory extinction (9–11), no studies have examined the potential role of CPEB1 in dendritic arbor development, experience-dependent structural plasticity, or the integration of neurons into a functional circuit in an intact animal.

CPEB1 (*Orb* in *D. melanogaster*) is a member of an evolutionarily conserved family of RNA binding proteins that is expressed in the brain (in vertebrates, CPEB1–4). While all members are defined by the presence of RNA recognition motifs in their carboxy-terminals, CPEB1 is the only member that targets mRNAs containing cytoplasmic polyadenylation elements (CPEs) in their 3' untranslated regions (12). Originally discovered in *Xenopus laevis* oocytes, CPEB1 binds CPE-containing mRNAs, regulates their microtubule-dependent dendritic trafficking, and represses their translation (13). Unlike vertebrate CPEB3 and 4, ApCPEB in *Aplysia* (14, 15), or *Drosophila* Orb2 (16), translational repression by CPEB1 is relieved by phosphorylation by either aurora kinase (17, 18) or calcium/calmodulin-dependent kinase II (CaMKII) (19), following

stimulation by progesterone in oocytes or glutamatergic synaptic activity in neurons. Phosphorylation of CPEB1 initiates a biochemical cascade that culminates in polyadenylation of the CPEB1-bound mRNAs and their translational derepression (13).

Distinct requirements for the functional domains of CPEB governing RNA transport and phosphorylation-dependent translational regulation have not been analyzed. Although CPEB mouse knockouts are deficient in some forms of plasticity (9, 10), because both CPEB's translational repression and mRNA transport functions are decreased in the knockout mice, it is unclear whether these distinct CPEB functions contribute specifically to the knockout phenotypes. Expression of a CPEB1 phosphorylation mutant in mouse Purkinje neurons prevented the late protein-synthesis-dependent phase of cerebellar long-term depression and altered dendritic spine morphology (11). This study suggests that CPEB-mediated translational derepression regulates synaptic plasticity, but it remains unclear whether the mRNA transport and translational regulation functions of CPEB play distinct roles in neuronal development and experience-dependent plasticity in vivo.

During *Xenopus* CNS development, glutamatergic signaling regulates the experience-dependent assembly and refinement of the visual circuitry. Dendritic arbor development of the retinorecipient neurons in the optic tectum is regulated by NMDA receptor and AMPA receptor signaling and affected by the visual experience of the animal (20, 21). The development of the dendritic arbor structure and maturation of tectal cell synaptic physiology progress coordinately and are governed by molecular pathways that include numerous proteins, such as α CaMKII (22) and brain-derived neurotrophic factor (BDNF) (23). Interestingly, the mRNA of both α CaMKII and BDNF are targets of CPEB1 (18, 24, 25), suggesting that CPEB may mediate aspects of activity-dependent visual system development. Indeed, studies in vivo have demonstrated that brief light exposure delivered to dark-reared rats produced CPEB-dependent increases in α CaMKII mRNA polyadenylation and protein synthesis that required NMDA receptor signaling (18, 25). We used a combination of in vivo imaging, electrophysiological measures of synaptic physiology, and evoked visual responses to assess the requirement for CPEB1 in the visual system development and plasticity. Furthermore, by expressing deletion mutants of CPEB1 we determined the relative importance of activity-dependent derepression of protein translation and mRNA trafficking in dendritic arbor development, synaptic physiology, and integration of neurons into functional circuits in vivo.

Results

CPEB1 is Required for Dendrite Development. To test whether CPEB1 plays a role in the elaboration of the dendritic arbor, we transfected

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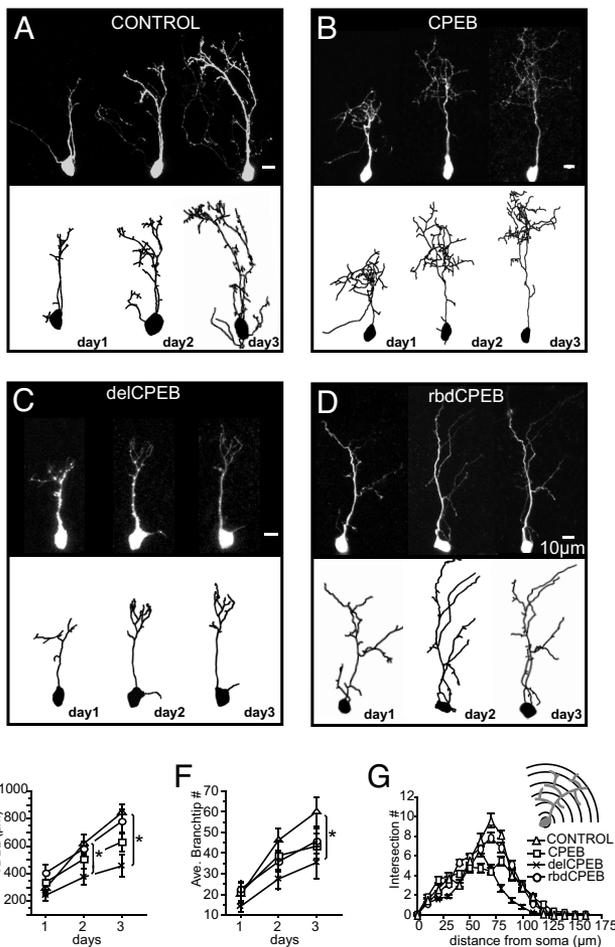


Fig. 1. CPEB is required for dendritic arbor growth in vivo. (A–D) Time-lapse in vivo two-photon images of tectal neurons and their three-dimensional (3D) reconstructions, collected daily over 3 days. (E–G) Quantification of dendritic morphology showing total dendritic branch length (TDBL) (E), branch-tip number (F), and 3D Sholl analysis (G). (E) Compared to control cells, the dendritic growth of delCPEB-expressing neurons, but not CPEB or rbdCPEB-expressing neurons, is significantly impaired by day 2 and 3. (F) By day 3, only delCPEB-expressing neurons have significantly fewer branch tips compared to control neurons. (G) Sholl analysis of 3-day cells illustrating the radial distribution of the branches of the dendritic arbor. Shown are the average number of branch intersections at 10- μm intervals beyond the soma. Control, $n = 11$; CPEB, $n = 12$; delCPEB $n = 10$; rbdCPEB = 12; *, $P \leq 0.05$. Values given in Table S2.

individual optic tectal neurons with control morpholinos and antisense morpholinos against CPEB1, using electroporation. Time-lapse images of transfected optic tectal neurons in an otherwise normal brain were collected once a day over 3 days, starting 1 day after electroporation [Fig. 1 A–D and supporting information (SI) Fig. S1]. An important feature of this experimental protocol is that neurons were already differentiated at the onset of these experiments; they had a dendritic arbor and synaptic inputs. Consequently, we assessed the effect of CPEB1 on experience-dependent circuit assembly, rather than neuronal differentiation or early stages of neuron development. Three-dimensional (3D) reconstructions show that although the dendritic arbors were matched in size on the first day of analysis, expression of morpholino antisense oligonucleotides directed against CPEB1 significantly decreased arbor development over 3 days and produced cells that were roughly two-thirds the size of the control morpholino group in their total dendritic branch length (TDBL) (see Fig. S1 and Table S1). Compared to the control morpholino cells, which grew $317 \pm 79\%$ of the starting size by day 3, the growth rate of the CPEB1

morpholino-expressing neurons was significantly inhibited and their TDBL reached just $156 \pm 45\%$ of their initial values ($P = 0.05$).

Knocking down CPEB1 protein with morpholinos did not allow us to distinguish whether CPEB1's function in microtubule-dependent mRNA transport or in activity-dependent polyadenylation was important for dendritic development. Based on previous studies of CPEB1 protein's functional domains (26–28), we designed experiments to distinguish the role of CPEB1 in the regulation of protein translation independently from its role in mRNA transport. We compared neurons expressing YFP, full-length CPEB1 (CPEB), and two previously characterized deletion mutants (Fig. S2). The first consisted of the CPEB RNA binding domain without the regulatory domains of the protein (rbdCPEB, $\Delta 1$ –258). This truncated CPEB1 protein interferes with both CPEB-dependent dendritic mRNA transport and protein synthesis (28). The second deletion mutant (delCPEB; $\Delta 124$ –258) lacks the phosphorylation site targeted by aurora kinase and αCaMKII . Because it is not activated by phosphorylation (26, 27), delCPEB can stably bind and transport mRNA (28) but interferes with the activity-dependent polyadenylation and translation initiation of CPEB mRNA targets. We verified in the retinotectal system that expression of delCPEB interferes with CPEB-dependent protein synthesis in a blind study that compared tissue plasminogen activator (tPA) immunofluorescence in tectal cells expressing delCPEB, CPEB1, or YFP alone with untransfected neighboring neurons (Fig. S3). With these tools, we address the question of whether CPEB1 is required for development of the visual system structure and function in *X. laevis* tadpoles.

While neurons expressing rbdCPEB, delCPEB, CPEB, and YFP alone were comparable in their TDBL and branch-tip number on the first day of imaging, delCPEB-expressing neurons grew at about half the rate of control cells, increasing only $104 \pm 35\%$ of their starting size compared to YFP control cells, which increase their TDBL by $240 \pm 40\%$ by day 3 ($P = 0.01$) (see Fig. 1). By the third day of imaging, delCPEB-expressing neurons had roughly half the TDBL and branch-tip number of control neurons (see Fig. 1 E and F and Table S2). Three-dimensional Sholl analysis (29) revealed that delCPEB-expressing neurons have fewer branches extending beyond 60 μm from the soma (see Fig. 1 F). In contrast, while rbdCPEB-expressing neurons did not significantly differ from the control cells in their TDBL or branch numbers over the 3 days ($126 \pm 39\%$) (see Fig. 1 E and F and Table S2), their growth rate was significantly slower than controls ($P = 0.03$). Neurons expressing CPEB were not significantly different from control cells with respect to TDBL, branch-tip number (see Fig. 1 E and F and Table S2) or growth rate ($169 \pm 54\%$ increase by day 3; $P = 0.08$). These data indicate that delCPEB, which is incorporated into dendritically targeted RNPs but cannot be phosphorylated and initiate mRNA polyadenylation downstream of extracellular signals, severely impairs dendritic arbor development. By contrast, cells expressing rbdCPEB, which lacks the microtubule-binding domain and therefore is retained in the cell body, can acquire normal dendritic arbor structure, although with slow growth rates.

delCPEB and rbdCPEB Expression Blocks Experience-Dependent Growth. Optic tectal neurons, like other CNS neurons, show experience-dependent structural plasticity. Exposure to NMDA receptor blockers reduces tectal-cell dendritic-arbor growth rates (30). Enhanced visual stimulation delivered to freely swimming tadpoles for 4 h increases synaptic drive onto optic tectal neurons (31) and significantly increases dendritic-arbor growth rate (21). This activity-dependent dendritic growth operates by an NMDA receptor-dependent mechanism (21). Although NMDA receptor signaling and downstream kinase activity increased CPEB-dependent mRNA polyadenylation and protein synthesis in the rodent visual system (18, 25) and hippocampal neurons (17, 19), CPEB1 has not yet been implicated in experience-dependent structural plasticity.

Interfering with CPEB function through the expression of del-

elaboration, while expression of mutants lacking functional domains of CPEB demonstrated selective roles for translational regulation and mRNA transport in experience-dependent structural plasticity and dendrite development. Whole-cell recordings of visual responses and spontaneous bursting activity in tectal neurons in the intact animal demonstrate that CPEB1 function is required for the integration of neurons into the visual circuit. The results support a model in which synaptic activity leads to phosphorylation of CPEB and translational derepression of target mRNAs (18, 19, 24, 35). The newly translated proteins then act as effectors to control experience-dependent modifications of dendritic arbor structure, synaptic connectivity, and ultimately the function and plasticity of the developing circuit.

Expression of CPEB or the deletion mutants likely sequesters mRNA from endogenous CPEB and the translation machinery. Additionally, because distinct spatial and temporal patterns of input activity may differentially regulate translational derepression by mRNA binding proteins (36), the expression of the CPEB1 constructs may also alter the threshold of input activity necessary for CPEB1-dependent protein synthesis and, therefore, place the transfected neurons at a specific disadvantage with respect to circuit integration compared to neighboring control tectal cells.

It is interesting to note that the deletion mutants of CPEB1 distinguished the requirements for the dendritic mRNA transport and phosphorylation-dependent polyadenylation functions of CPEB in the elaboration of dendritic arbors and in experience-dependent structural plasticity. Because the molecular structure of CPEB has been thoroughly examined (26, 27), further structure/function analysis with previously characterized deletion mutants of CPEB allowed us to identify which CPEB functions are most critical for different aspects of neuronal development and circuit function. Using time-lapse imaging, we found that expressing delCPEB, which interferes with CPEB's activity-dependent mRNA polyadenylation but leaves dendritic mRNA transport intact, impairs dendritic-arbor development and the integration of neurons into the visual circuit. By contrast, neurons expressing rbdCPEB, which have disrupted mRNA transport in addition to altered polyadenylation activity, can acquire normal dendritic arbor structure over 3 days, but fail to show rapid enhanced dendritic arbor growth rates following brief visual stimulation. The results suggest that failure of CPEB1 to transport mRNA into dendrites can be compensated over a timecourse of days, perhaps by transport of CREB mRNA targets by other RNA binding proteins or other means of regulating protein level, but that failure of CPEB1 to respond to synaptic activity is quite devastating for regulation of dendrite development over days, and for short-term structural plasticity in response to sensory input.

About 7% of brain mRNAs are estimated to be targets of CPEB1, although only a relatively small number have been confirmed experimentally (24). Potential mRNA targets include key plasticity genes, such as α CaMKII (18, 25), BDNF (24), tPA (37), engrailed1 (38), Homer (18), and insulin-receptor substrate p53 (11). Proteins from these mRNAs, plus many identified in a recent screen of CPE-containing mRNAs (24), are capable of altering synaptic strength and neuronal structure. Nevertheless, it is unlikely that the effects of CPEB1 manipulations that we document here can be attributed to misregulation of a single CPEB mRNA target. For instance, α CaMKII, BDNF, and Homer proteins have distinct but overlapping functions in the morphological and electrophysiological development and plasticity of optic tectal neurons (22, 23, 32, 39). The role that α CaMKII plays in CNS development and plasticity illustrates how activation of just one of the CPEB1 target mRNAs can have pleiotropic effects that have ramifications beyond its direct mRNA targets. Increasing or decreasing CaMKII activity affects synaptic plasticity and dendritic-arbor development of neurons in the *X. laevis* optic tectum (32) and mammalian systems, where it is also required for learning and memory (40). CaMKII phosphorylates CPEB1 in response to NMDA receptor activity (19)

and its own synthesis increases following plasticity-inducing stimuli (41), NMDA receptor activation, and CPEB1 activation (18). Because α CaMKII both activates CPEB1 and is also a downstream product of CPEB1 activity, α CaMKII may act in a feedback loop where synaptic input that activates α CaMKII can also derepress CPEB-mediated inhibition of α CaMKII synthesis (35). Therefore CPEB1-mediated control of α CaMKII synthesis alone is likely to have both immediate and long-lasting effects on functional and structural plasticity, as well as subsequent regulation of CPEB1, which likely work in concert with the other CPEB1 target mRNAs that are associated with synaptic and morphological plasticity (24).

Specific RNA binding proteins may coordinate the spatial and temporal translation of functionally related subpopulations of mRNAs (5, 6). Furthermore, different mRNA binding proteins bind different, but potentially overlapping sets of mRNAs, and may independently regulate their translation in response to specific inputs. For example, fragile X mental retardation protein, FMRP, is a dendritically targeted mRNA binding protein, which is activated in response to metabotropic glutamate receptor activity and is thought to regulate forms of long-term depression (42). It is interesting to note that *fmr1*, the gene encoding FMRP, has a CPE site and colocalizes with CPEB in RNP granules (43, 44), suggesting that synaptic activity that activates CPEB1 may also increase FMRP translation. Combined with evidence for posttranscriptional gene regulation by RNA binding proteins and microRNAs (5, 45), the examples described here show that local regulation of mRNA translation affords considerable spatial and temporal control over synaptic plasticity.

The results presented here demonstrate a requirement for CPEB1-mediated translational regulation for the integration of neurons into a functional circuit *in vivo*. Not only is CPEB1 function required for neurons to modify their morphological and synaptic development in response to changes in sensory input, but by interfering with CPEB1's ability to interpret activity-dependent signals, tectal neurons do not integrate properly and effectively lose their place in the development of an otherwise unaltered visual circuit.

Materials and Methods

Cell Transfection of Plasmid Constructs and Morpholino Antisense Oligonucleotides. Stage 46 to 48 albino *X. laevis* tadpoles were used for all experiments. All protocols were approved by the Cold Spring Harbor Laboratory Institutional Animal Care and Use Committee. For the electrophysiology and morpholino-imaging experiments, cells were transfected by electroporation as described previously (46). For the *in vivo* imaging experiments, single cells were targeted with electroporation as described previously (47) and in the *SI Methods*.

The lissamine-tagged morpholino antisense oligonucleotides against CPEB were designed by and purchased from GeneTools (Philomath, OR). The CPEB, delCPEB and rbd-eCFP (referred to as $\Delta 5$ and RBD in ref. 28) plasmid constructs were a generous gift of Dr. Joel D. Richter (University of Massachusetts Medical School, Worcester, MA). The original eGFP tags were replaced with eCFP and the genes were transferred to a bidirectional PCS2 plasmid. This plasmid contained two independent CMV promoters, one driving untagged, cytosolic eYFP to visualize the transfected cell (kindly provided by Dr. David Turner, University of Michigan, Ann Arbor, MI).

Two-Photon Imaging and Morphometric Analyses. Images were collected from anesthetized tadpoles (0.02% MS-222; Sigma) positioned under a glass coverslip in a Sylgard chamber. Animal preparation, laser sources, signal amplification, PMT specifications, and YFP/CFP filter sets have been described previously (48). Using the raw image stacks (1–1.5- μ m z interval), manual reconstructions of the dendritic arbor in three dimensions were generated and then analyzed for total dendritic branch length, branch-tip number, and 3D Sholl analysis (29); radius interval 1 μ m, averaged over 10- μ m bins) using Object-Image macros (written by Dr. E. Ruthazer, McGill University, Quebec).

Electrophysiology. Whole-cell patch clamp recordings of the AMPA mEPSCs were made from control and transfected cells and analyzed as described (32, 39). Control cells were from unelectroporated animals. For *in vivo* recordings, anesthetized tadpoles were transferred to the recording chamber where the tectum

was exposed by removing the overlying skin and making a dorsal midline cut to uncover the ventricular surface. The tadpole was stabilized with short pieces of tungsten wire (California Fine Wire) and perfused with extracellular saline containing 0.01 mM d-tubocurarine (Sigma) to prevent muscle movement. Cell access and patch quality were monitored throughout the experiment: average input resistance, 1.5 G Ω ; average series resistance, 50 Ω . Saline compositions are given in *SI Methods*.

AMPA mEPSC data and spontaneous events were analyzed with template matching (Axograph 4.6 software, or Clampfit 10.0, Molecular Devices). Complex events (bursts, consisting of a minimum duration 50 ms and amplitude of 10 pA) were detected using Clampfit 10.0. Light-evoked, -off responses were measured with custom software (Matlab, Mathworks; written by Dr. J. Demas, Cold Spring Harbor Laboratory, NY).

Visual Stimulation. A Luxeon III LED (530 nm) pigtailed to a 2-mm diameter optic fiber (n.a. = 0.5; Doric Lenses) was used to deliver a full-field stimulus to the eye while the responses from neurons of the contralateral optic tectum were assessed with whole-cell voltage clamp. The LED intensity was controlled with 10-dB neutral density filters that dropped into a housing coupled to the fiber (Oz Optics). The filters and output intensities were measured and verified with an IL 1400 radiometer/photometer, (International Light Technologies) and the radiant

flux used in the experiments ranged from 0.06 mW to 64 mW in 10-dB increments (referred to as relative intensities 0.01 to 10 in Fig. 4).

After obtaining whole-cell access and before they were presented with the visual stimulus protocol (described in *SI Methods* and Fig. S4), the tadpoles were dark-adapted for 6 min, during which spontaneous synaptic activity was recorded. The visual stimulation protocol was repeated up to four times, each repetition with a 10-dB (in some instances, 20 dB) increase in LED intensity.

Statistical Tests. Mann Whitney U tests were used to make comparisons between groups. Wilcoxon signed rank tests were used for paired data. The data from one cell in the delCPEB group, likely from an untransfected cell, satisfied Grubb's test for outliers ($P < 0.01$) and was removed from the light-evoked response and spontaneous-response experiments. Kolmogorov-Smirnov tests were used to compare distributions. In the quantification of the spontaneous events (see Fig. 4), 119 events were randomly chosen from each cell. Data presented in bar graphs are mean \pm SEM and are presented in Tables S1–S7.

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