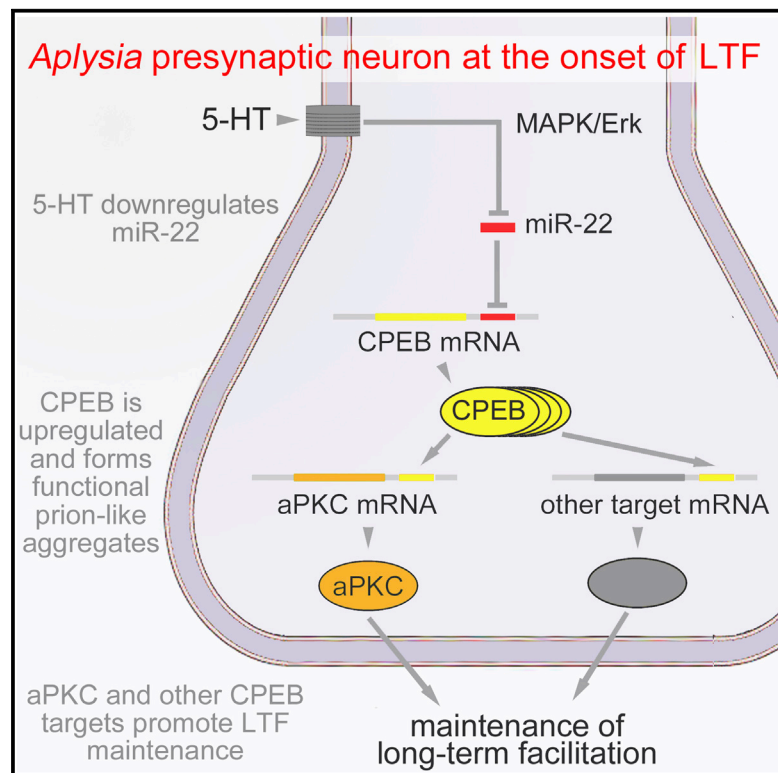


# MicroRNA-22 Gates Long-Term Heterosynaptic Plasticity in *Aplysia* through Presynaptic Regulation of CPEB and Downstream Targets

## Graphical Abstract



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## In Brief

Maintenance of memory-related long-term facilitation (LTF) of *Aplysia* synapses requires upregulation and prion-like activation of CPEB, a synaptic translational regulator. Fiumara et al. identify a presynaptic microRNA as a neurotransmitter-modulated regulator of CPEB levels and consequently of CPEB targets, including an atypical PKC with synergistic roles in LTF maintenance.

## Highlights

- Maintenance of long-term facilitation (LTF) in *Aplysia* requires upregulation of CPEB
- Serotonin-triggered downregulation of miR-22 permits the upregulation of CPEB in LTF
- Activation of CPEB regulates the translation of target synaptic mRNAs
- Atypical PKC, a CPEB target, synergistically promotes presynaptic LTF maintenance



# MicroRNA-22 Gates Long-Term Heterosynaptic Plasticity in *Aplysia* through Presynaptic Regulation of CPEB and Downstream Targets

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## SUMMARY

The maintenance phase of memory-related long-term facilitation (LTF) of synapses between sensory and motor neurons of the gill-withdrawal reflex of *Aplysia* depends on a serotonin (5-HT)-triggered presynaptic upregulation of CPEB, a functional prion that regulates local protein synthesis at the synapse. The mechanisms whereby serotonin regulates CPEB levels in presynaptic sensory neurons are not known. Here, we describe a sensory neuron-specific microRNA 22 (miR-22) that has multiple binding sites on the mRNA of CPEB and inhibits it in the basal state. Serotonin triggers MAPK/Erk-dependent downregulation of miR-22, thereby upregulating the expression of CPEB, which in turn regulates, through functional CPE elements, the presynaptic expression of atypical PKC (aPKC), another candidate regulator of memory maintenance. Our findings support a model in which the neurotransmitter-triggered downregulation of miR-22 coordinates the regulation of genes contributing synergistically to the long-term maintenance of memory-related synaptic plasticity.

## INTRODUCTION

Long-term plasticity of synaptic connections in the brain is one of the core mechanisms for the storage of both implicit and explicit memory, and for the persistent changes in the strength of specific sets of synapses, triggered by their homosynaptic activity or by heterosynaptic modulators (Kandel, 2001). In addition to defining cellular and molecular mechanisms underlying the induction and consolidation phases of memory-related long-term synaptic plasticity, recent studies have identified an addi-

tional maintenance phase for long-term memory (Si et al., 2003a, 2003b, 2010; Bailey et al., 2004).

Studies in invertebrates and vertebrates have identified the translational regulator cytoplasmic polyadenylating element binding protein (CPEB) as a key mediator of the long-term maintenance phase of behavioral memories and synaptic plasticity (Si et al., 2003a, 2003b, 2010; Keleman et al., 2007; Pavlopoulos et al., 2011; Krüttner et al., 2012; Majumdar et al., 2012; Fioriti et al., 2015). This RNA-binding protein regulates the cytoplasmic polyadenylation and translation of target mRNAs at synapses through a self-perpetuating, functional prion-like conformation triggered in *Aplysia* by the neuromodulator serotonin (5-HT) (Si et al., 2003a, 2003b, 2010). This prion-like process switches CPEB monomers from their native form to an alternative, self-propagating conformation in which CPEB forms aggregates that are persistently active (Si et al., 2003a), promoting the synaptic translation of dormant mRNAs induced by 5-HT and thus maintaining the growth of newly formed synapses essential for LTF (Si et al., 2003b, 2010).

The identification of CPEB as a protein with the properties of a functional prion raised new questions regarding the physiological regulation of its structural and functional dynamics (Fiumara et al., 2010; Pavlopoulos et al., 2011; Raveendra et al., 2013; White-Grindley et al., 2014). In particular, the ability of CPEB to become self-perpetuating and persistently active in response to heterosynaptic stimulation suggests that it is likely to be highly regulated. In fact, in *Aplysia* sensorimotor synapses activation of CPEB is triggered at the onset of LTF by a rapid synapse-specific increase in the expression level of CPEB induced by 5-HT (Si et al., 2003b). Interference with this process does not affect the induction of LTF but blocks its maintenance (Si et al., 2003b). However, the molecular mechanism by which 5-HT triggers the upregulation of CPEB necessary for conversion to a prionic mechanism required for maintenance of plasticity is not known.

In studying microRNAs in *Aplysia*, we previously found some that are strikingly downregulated by 5-HT at the onset of LTF

(Rajasethupathy et al., 2009). One of these, miR-124, regulates CREB1, a key transcription factor for the consolidation phase of LTF (Rajasethupathy et al., 2009). These findings raised the possibility that a similar regulatory mechanism may control the levels of CPEB and therefore its prionic switch, thereby regulating the maintenance of LTF.

To explore this possibility, we analyzed the set of *Aplysia* microRNAs that we have earlier characterized (Rajasethupathy et al., 2009) and found one candidate, miR-22, which has several consensus sites in the CPEB 3'-UTR. Here, we examine the role of miR-22 in the regulation of CPEB and LTF, and we find that miR-22 gates LTF at *Aplysia* sensorimotor synapses by regulating CPEB and therefore its downstream targets, which include a presynaptic kinase that has a synergistic role in the maintenance of long-term synaptic plasticity in *Aplysia*.

## RESULTS

### The 3'-UTR of the mRNA for *Aplysia* CPEB Contains Multiple Sites for miR22, a 5-HT-Regulated MicroRNA

Rajasethupathy et al. (2009) found that some microRNAs in the nervous system of *Aplysia* are rapidly downregulated by 5-HT, the heterosynaptic modulator that initiates LTF of sensorimotor synapses of the gill-withdrawal reflex. One of these, miR-124, controls LTF by regulating the transcription factor CREB (Rajasethupathy et al., 2009). To determine whether CPEB undergoes a similar regulation, we examined the 3'-UTR of its mRNA in search of consensus sites for known microRNAs expressed in the nervous system. We found one microRNA, miR-22, that has multiple putative canonical seed-sequence binding sites in the 3'-UTR of the CPEB mRNA (Figure 1A). Two additional features of miR-22 made it an attractive candidate for the physiological regulation of CPEB in LTF: miR-22 is nervous-system-specific (Rajasethupathy et al., 2009) and is downregulated by 5-HT (Figures 1B and 1C) in a MAPK/Erk-dependent manner (Figure 1D) as previously observed for miR-124 (Rajasethupathy et al., 2009). It is also expressed in neuritic processes of the presynaptic sensory neurons (Figure 1E), where CPEB exerts its fundamental role in the maintenance of LTF (Si et al., 2003b).

### miR-22 Regulates the Translation of CPEB in the Nervous System In Vivo

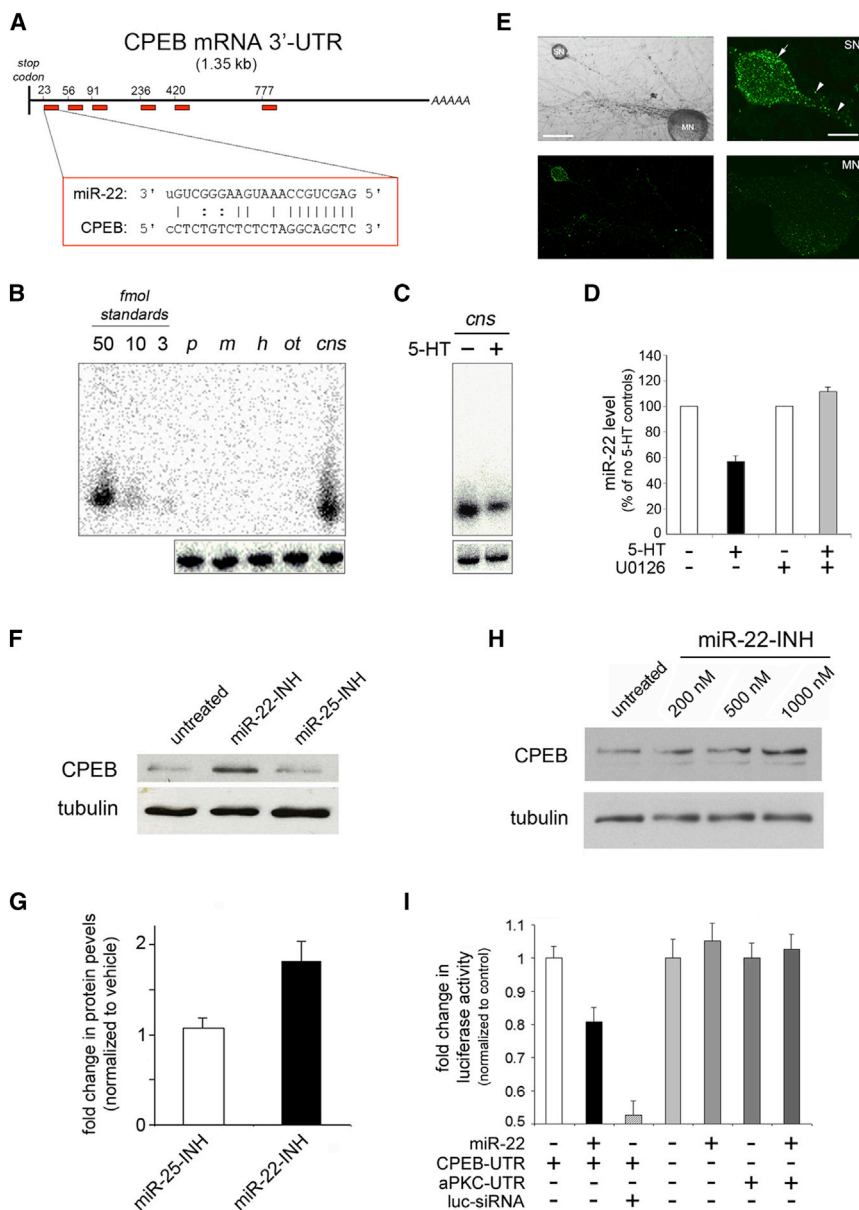
To determine whether miR-22 actually regulates the translation of CPEB in vivo, we artificially manipulated its levels in isolated ganglia of *Aplysia*. For this purpose, we covalently conjugated, to penetratin (pntr), a custom inhibitor of miR-22 containing a 2'-O-methyl backbone (miR-22-INH). We then exposed desheathed plural ganglia to miR-22-INH/pntr (~200  $\mu$ M) for 12–24 hr to reduce the levels of endogenous miR-22 in neurons (Rajasethupathy et al., 2009). We incubated control ganglia with a control pntr-conjugated microRNA inhibitor (miR-25-INH). After incubation, we lysed the ganglia and analyzed CPEB levels by western blot, using tubulin levels as a normalization control (Figure 1F). We found that inhibition of miR-22 caused a significant, dose-dependent, upregulation of CPEB (relative band intensity: miR-22

INH,  $1.81 \pm 0.22$ ,  $n = 8$ , versus control miR-25,  $1.07 \pm 0.12$ ,  $n = 6$ ;  $p < 0.01$ ,  $t$  test; all values normalized to the respective penetratin control groups; Figures 1G and 1H). This upregulation did not increase after exposure to 5-HT (data not shown). Overall, these findings indicate that miR-22 is an important regulator of CPEB levels.

To determine whether this effect is mediated by direct binding of miR-22 to the CPEB 3'-UTR, we performed a luciferase assay in which the portion of the UTR containing consensus sites for miR-22 was inserted as the 3'-UTR in a plasmid encoding for Renilla luciferase (psiCHECK-2). The same plasmid also encodes for firefly luciferase, which served as an internal control. We found that miR-22 significantly downregulated the Renilla luciferase activity (Figure 2I;  $p < 0.01$  versus control; one-way ANOVA, Newman-Keuls post hoc test). As a positive control, we used a small interfering RNA (siRNA) targeting luciferase, which induced as well a significant luciferase downregulation ( $p < 0.01$  versus control; Figure 2I). A negative control experiment showed that miR-22 did not affect luciferase activity when the consensus sequence of the CPEB UTR was not present ( $p = 0.52$  versus control; Figure 2I). Taken together, these findings indicate that miR-22 can bind the consensus region of the CPEB 3'-UTR directly.

### Increasing the Levels of miR-22 Impair LTF whereas Inhibition of miR-22 Enhances LTF

To determine whether the observed regulation of CPEB levels by miR-22 is physiologically relevant to CPEB's role in the maintenance of LTF, we altered miR-22 levels in sensory neurons and studied the effect of these manipulations on LTF of sensorimotor synapses (Figure 2A). LTF is a form of heterosynaptic plasticity that can be recapitulated in sensorimotor co-cultures by 5-HT application (Montarolo et al., 1986). We found that the presynaptic injection of a miR-22 mimic in sensorimotor co-cultures impairs the maintenance of LTF (Figure 2B), simulating what we had observed with antisense oligonucleotides to CPEB (Si et al., 2003b). ANOVA for repeated-measures revealed overall a significant effect of the treatment on facilitation ( $F_{(3,65)} = 4.63$ ,  $p < 0.01$ ) and an impairment of LTF that was particularly evident at 72 hr after 5-HT ( $p < 0.01$  for the miR-22 + 5-HT group versus the 5-HT group). Using in situ hybridization, we confirmed that mimics of miR-22 were able to increase the levels of miR-22 in sensory neurons (Figure 2A). The miR-22 mimic did not alter basal synaptic transmission (Figure 2C; basal EPSP =  $15.59 \pm 1.79$  mV,  $n = 30$ , in miR-22 mimic-injected cells versus  $13.54 \pm 1.55$  mV in control cells,  $p = 0.40$ ,  $t$  test). Injection of a microRNA mimic negative control also did not interfere with LTF or basal transmission (Figures 2D and 2E). Conversely, inhibition of miR-22 by miR-22-INH resulted in enhanced LTF ( $F_{(3,171)} = 10.33$ ,  $p < 0.01$ ) when compared to cells treated with a control microRNA inhibitor or with 5-HT alone (Figure 2F;  $p < 0.05$  in both cases). MiR-22-INH did not affect basal synaptic transmission (Figure 2G; basal EPSP =  $22.08 \pm 1.03$ ,  $n = 45$ , in miR-22-INH-treated cells versus  $21.45 \pm 0.98$ ,  $n = 47$ , in control cells,  $p = 0.65$   $t$  test). These findings indicate that the 5-HT-dependent downregulation of miR-22 has a physiologically relevant role in LTF, mediated, at least in part, through the presynaptic regulation of CPEB.



**Figure 1. MiR-22 Has Consensus Sites in the CPEB mRNA 3'-UTR and Regulates CPEB Protein Levels**

(A) Scheme of the CPEB 3'-UTR. Red segments represent miR-22 consensus sites. One of these is highlighted to show the perfect complementarity between the miR-22 seed region and the UTR.

(B) Top: northern blot for miR-22b. MiR-22 is detected in the CNS but not in other organs: hepatopancreas (p), heart (h), muscle (m), ovotestis (ot). Bottom: tRNA bands as loading controls.

(C) Northern blot for miR-22b in the nervous system (CNS) treated (+) or not (-) with 5-HT. MiR-22 is downregulated by 5-HT. Representative image of three independent experiments. Bottom: tRNA bands are shown as loading controls.

(D) Quantification of northern blot band intensities for miR-22b in the CNS treated (+) or not (-) with 5-HT, in the presence (+) or in the absence (-) of U0126, a MEK inhibitor that prevents the activation of MAPK/Erk. The significant miR-22 downregulation induced by 5-HT ( $p < 0.01$ ) is prevented in the presence of U0126. Data expressed as mean  $\pm$  SEM.

(E) In situ hybridization for miR-22 of sensorimotor cocultures. Top left: phase contrast micrograph of the co-culture with a sensory (SN) and a motor neuron (MN). Bottom left: in situ hybridization of the same co-culture. Top right: soma of the SN. Arrows and arrowheads indicate positive signals in the cell body and in neuritic processes, respectively. Bottom right: soma of the MN. Scale bars represent 100  $\mu$ M (left); 25  $\mu$ M (right).

(F) Western blot of pleural ganglia lysates after incubation for 24 hr with miR-22-INH. Control ganglia were incubated with miR-25-INH or left untreated. MiR-22-INH increases CPEB levels.

(G) Quantification of experiments shown in (F). Values are normalized to the respective penetratin control groups. Data expressed as mean  $\pm$  SEM.

(H) Dose dependency of the effect of miR-22-INH on CPEB levels. Tubulin levels are shown as loading controls (bottom).

(I) Luciferase reporter assay. MiR-22 mimics downregulate luciferase activity by binding to the CPEB 3'-UTR but not to the aPKC 3'-UTR. Data expressed as mean  $\pm$  SEM.

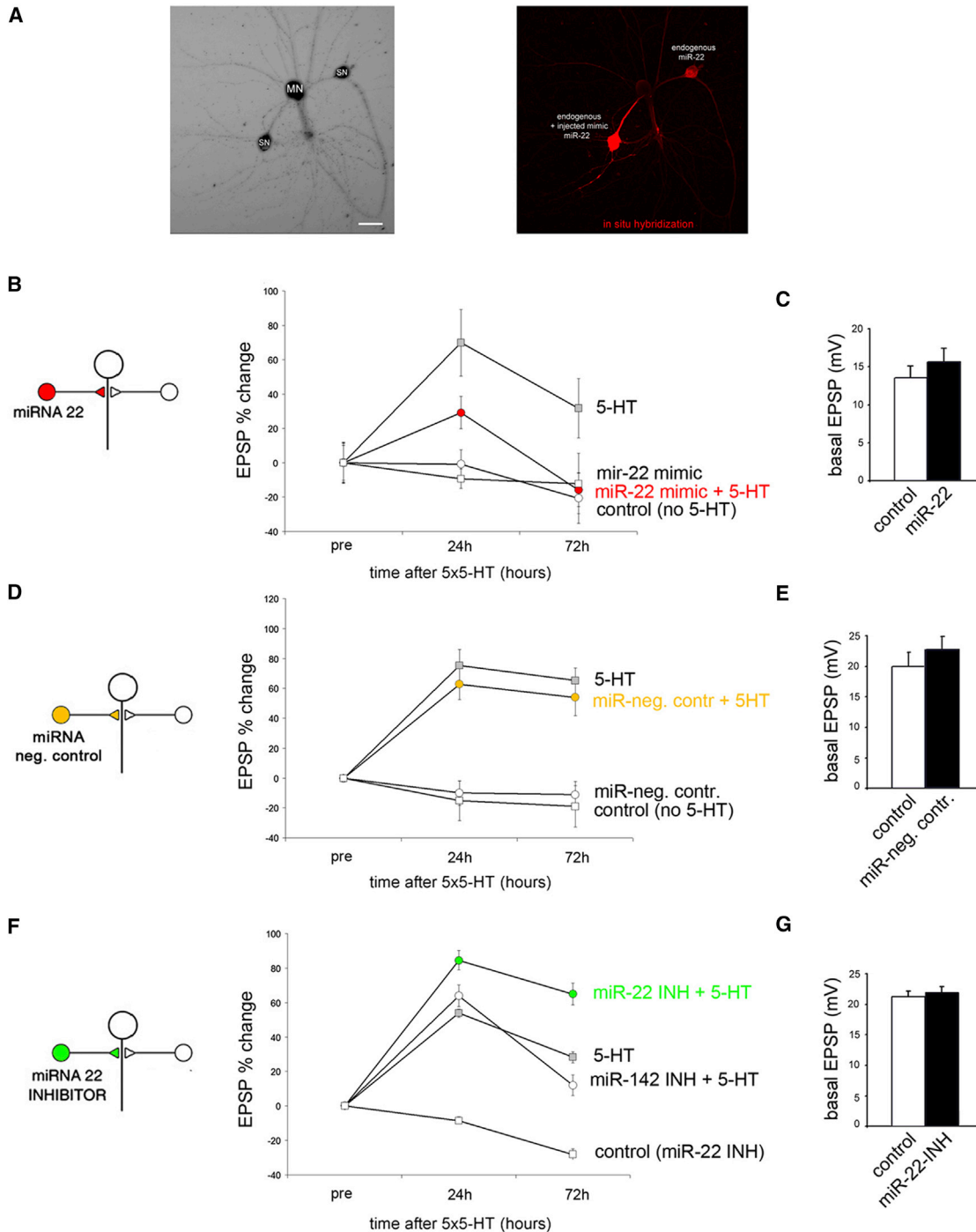
### miR-22 Also Regulates the Levels of aPKC, Another Candidate Mediator of LTF Maintenance

We then explored whether miR-22 might also regulate the expression of other potential mediators of the maintenance of LTF such as an atypical PKC isoform (aPKC) that is thought to be involved in the maintenance of some forms of long-term memory in *Aplysia* (Cai et al., 2011), similar to what found in *Drosophila* and in the rat (Drier et al., 2002; Shema et al., 2007, 2011). Unlike miR-22, aPKC is expressed both pre- and postsynaptically, as in *Drosophila* (Ruiz-Canada et al., 2004; Bougie et al., 2009). In fact, western blotting with two distinct antibodies raised against mammalian PKC- $\zeta$  (Figure 3A) shows that an aPKC is expressed in the pleural ganglia of *Aplysia* and apparently can also be cleaved to a shorter form (aPKM), consis-

tent with previous observations in *Drosophila* (Drier et al., 2002). When we tagged aPKC with GFP, we found that atypical PKC has a cell-wide distribution in sensory neurons and is present in the cell body and in synaptic varicosities, consistent with previous observations (Figure 3B) (Ruiz-Canada et al., 2004; Cheng et al., 2011). Based on these findings, we asked whether miR-22 may possibly also regulate a PKC, in conjunction with CPEB.

We first tested whether miR-22 regulates PKC levels in the nervous system and found that inhibition of miR-22 leads to an upregulation of the aPKC protein, similar to what observed for CPEB (Figures 3C and 3D; relative band intensity: miR-22 INH,  $1.54 \pm 0.20$  versus control miR-25 INH,  $1.11 \pm 0.04$   $n = 6$ ,  $p < 0.01$ ,  $t$  test).





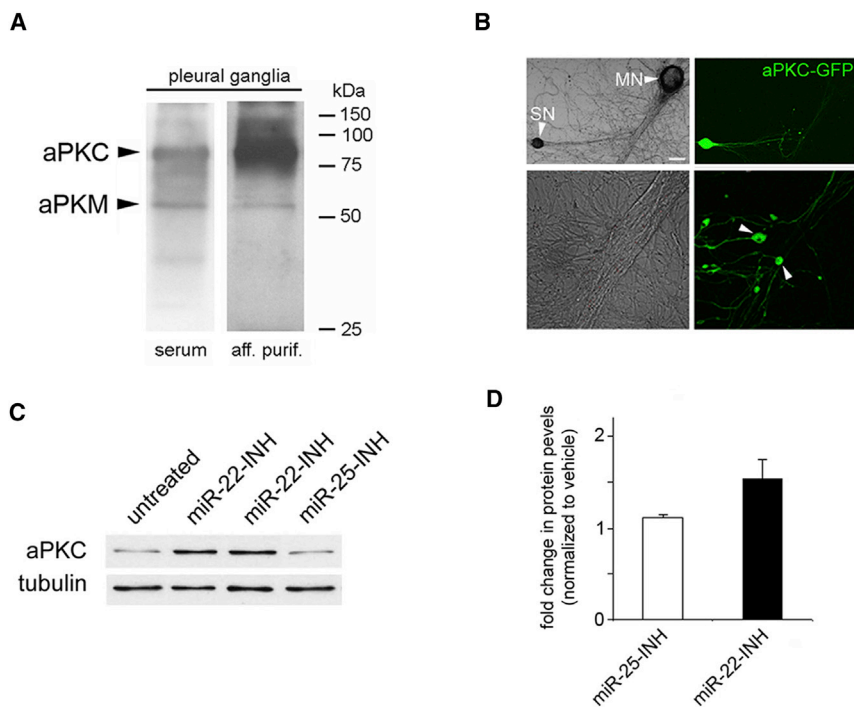
**Figure 2. Presynaptic Levels of miR-22 Regulate LTF of Sensorimotor Synapses**

(A) Left: phase-contrast micrograph of a co-culture of one motor neuron and two sensory neurons. The left sensory neuron was injected with a miR-22 mimic to increase the presynaptic levels of miR-22. Right: in situ hybridization for miR-22 of the same co-culture. MiR-22 levels are increased in the left sensory neuron. The right sensory neuron shows staining only for endogenous miR-22. Scale bar represents 100  $\mu$ M.

(B and C) Effect of miR-22 injection on 5-HT-induced LTF of sensorimotor synapses. MiR-22 mimic injection impairs the maintenance of LTF (B) but not basal transmission (C). Data expressed as mean  $\pm$  SEM.

(D and E) A microRNA mimic negative control does not interfere with LTF (D) nor with basal transmission (E). Data expressed as mean  $\pm$  SEM.

(F and G) MiR-22 inhibition enhances 5-HT-induced LTF of sensorimotor synapses (F) but does not alter basal transmission (G). Data expressed as mean  $\pm$  SEM.



**Figure 3. MiRNA-22 Regulates the Expression Levels of aPKC in Pleural Ganglia**

(A) Western blot of ganglia lysates using two antibodies directed against a conserved C-terminal epitope of the mammalian atypical PKC- $\zeta$ . Both an immune serum (left) and an affinity purified antibody (right) recognize a protein of the expected molecular weight of *Aplysia* aPKC (upper arrowhead) and a shorter truncated product (aPKM, lower arrowhead).

(B) Presynaptic overexpression of GFP-tagged aPKC in a sensorimotor co-culture. Left: phase contrast micrographs. Right: same fields imaged by confocal fluorescence microscopy. Top: low-magnification image of the entire co-culture. Scale bar represents 50  $\mu$ M. Bottom: magnified detail of the sensory neuron arborization: aPKC concentrates in presynaptic varicosities (arrowheads).

(C) Western blot of pleural ganglia lysates after incubation for 24 hr with a miR-22-INH. Control ganglia were incubated with miR-25-INH or left untreated. aPKC levels increase in the presence of miR-22-INH.

(D) Quantification (mean  $\pm$  SEM) of experiments like those shown in (C). All values were normalized to the respective penetratin control groups.

### An Extended 3'-UTR of *Aplysia* Atypical PKC Contains Functional CPE Elements and One Predicted Low-Affinity Site for miR-22

To determine whether this effect is due to direct binding of miR-22 to the aPKC mRNA, we examined the 3'-UTR deposited in GenBank (FJ869890.1). This sequence did not contain miR-22 consensus sites. However, we found evidence in the *Aplysia* EST database (*Aplysia* EST project: CNSN01-F-087156-501 and 1-MCCN20-F-015393-501; available at <http://aplysia.c2b2.columbia.edu/>) of cDNAs in which the aPKC mRNA extended beyond the putative polyadenylation site identified in the GenBank sequence, and these also overlapped with genomic sequences (Genbank: AAC02026854.1) that contained the last exon of aPKC, thus indicating the possible existence of a longer 3'-UTR.

Because 3'-RACE failed to amplify longer transcripts, we analyzed the genomic clone AAC02026854.1, which contains both the known aPKC 3'-UTR and the EST fragments and searched downstream for a canonical polyadenylation signal. We indeed found an AATAAA hexamer,  $\sim$ 3.5 kb downstream of the end of the GenBank UTR. We designed multiple reverse primers along this sequence before and after the polyadenylation hexamer and used them with forward primers in the coding region of aPKC, in the attempt to amplify an extended 3'-UTR by PCR. Using this strategy, we amplified from nervous system cDNA a transcript encompassing the full-length coding region of aPKC followed by a 4.3 kb long 3'-UTR (Figure 4A), terminating apparently  $\sim$ 50 bases after the hexamer, as reverse primers downstream failed to amplify any product.

We then searched for putative miR-22 consensus sites in the extended aPKC 3'-UTR and found one site with predicted low affinity. However, we observed multiple CPE elements, two of

which at a canonical distance from the polyadenylation hexamer, strongly indicating that the aPKC mRNA is subject to CPEB-mediated polyadenylation (Figure 4A). Cell-type-specific RT-PCR showed that the mRNA containing this extended UTR is expressed both in sensory and motor neurons and is physiologically polyadenylated in the nervous system (Figures 4B and 4C), similar to the actin mRNA, another CPE-containing CPEB target (Si et al., 2003b). To further confirm the actual functionality of the CPE elements, we performed a luciferase assay using a construct (CPE+, Figure 4D) in which a portion of the 3'-UTR encompassing the two CPE elements close to the polyadenylation hexamer was subcloned in the psiCHECK-2 plasmid downstream of the Renilla luciferase ORF. We also generated a control construct in which the two CPEs were deleted (CPE-). The CPE+ and CPE- constructs were each co-expressed together with CPEB for 36 hr and luciferase activity was then determined. The presence of CPEs significantly altered luciferase activity ( $0.39 \pm 0.04$  for CPE+ versus  $1 \pm 0.02$  for CPE-, values normalized to CPE-,  $p < 0.05$ , unpaired t test), similar to what previously observed for other CPEB targets (Pavlopoulos et al., 2011) indicating the functionality of the CPE elements in the 3'-UTR of aPKC. Furthermore, we found that the polyadenylation of the aPKC mRNA is enhanced by 5-HT in pleural ganglia (Figure 4E), as for the actin mRNA (Si et al., 2003b). By contrast, another luciferase assay did not show direct binding of miR-22 to the putative miR-22 consensus site in the aPKC 3'-UTR (Figure 1I). These findings indicate that the regulation of the translation of aPKC by miR-22 is not direct but mediated, at least in part, through CPE-related polyadenylation, consistent with what previously observed for *Drosophila* aPKC that is a target of the CPEB ortholog Orb2 (Mastushita-Sakai et al., 2010).

### aPKC Is Also Required Presynaptically for the Maintenance of LTF

Studies of the role of atypical PKC/Ms in synaptic plasticity have referred to post-synaptic actions of these kinases (Sacktor, 2011; Bougie et al., 2012). However, our findings that aPKC is a regulated target of miR-22 suggested the possibility that aPKC may also take part in the presynaptic regulation of LTF. To determine whether this is the case, we first used genetic approaches to alter the function of aPKC. We chose this approach considering the fact that the specificity of available pharmacological inhibitors for atypical PKCs, such as chelerythrine and ZIP, has been questioned recently (Wu-Zhang et al., 2012; Volk et al., 2013)—although they are apparently effective in blocking LTF in *Aplysia* (Cai et al., 2011)—and application of antisense oligonucleotides may not effectively block the activity of the kinase within the time window of the LTF protocol, because the kinase has a long half-life (Sacktor 2011) (Figure S1). Thus, we presynaptically overexpressed either GFP-tagged wild-type or dominant-negative forms of aPKC in sensorimotor cocultures to study their effect on LTF. Similar GFP-fusions have been previously used for in vivo studies of PKC localization and activity (Kajimoto et al., 2001). We generated dominant-negative form of aPKC (aPKC-DN) by mutating a critical phosphorylation site for PDK-1 in the activation loop (Figure 4F). Phosphorylation by PDK-1 is in fact required for activating both full-length and cleaved aPKC (Smith and Smith, 2002), and mutants in which this site is not phosphorylatable have a dominant-negative effect on the endogenous kinase (Lee, 2011). Phosphorylation of the PDK-1 site in aPKC is also modulated by 5-HT in *Aplysia* (Bougie et al., 2009).

We found that presynaptic overexpression of aPKC-DN impaired the maintenance of LTF after its initial induction at 24 hr, thus indicating a specific involvement of presynaptic aPKC in the late maintenance phase of LTF (Figure 4G). ANOVA for repeated-measures indicated an overall significant effect of aPKC-DN overexpression on the maintenance of facilitation ( $F_{(3,46)} = 21.26$ ,  $p < 0.01$ ) that was particularly marked at 48 and 72 hr after 5-HT ( $p < 0.01$  in both cases versus 5-HT). The presynaptic injection of an antisense oligonucleotide against aPKC designed to inhibit the synthesis of new aPKC after the 5-HT application did not cause significant inhibition of LTF (Figure S1). This indicates that the inhibition of atypical PKC molecules already present in the cell—that are those blocked through the dominant-negative approach—is necessary to impair the maintenance of LTF, as also indicated by pharmacological experiments with chelerythrine, an inhibitor that has some specificity for atypical PKC in *Aplysia* (Cai et al., 2011). Conversely, presynaptic overexpression of wild-type aPKC enhanced LTF (Figure 4H). ANOVA for repeated-measures indicated a significant effect of aPKC overexpression on facilitation ( $F_{(3,75)} = 56.24$ ,  $p < 0.01$ ) up to 72 hr ( $p < 0.01$  in all instances, aPKC+5HT group versus 5-HT group). The overexpression of either aPKC or aPKC-DN did not affect basal synaptic strength (Figures 4H and 4I; basal EPSP  $16.97 \pm 2.28$  mV,  $n = 11$  in aPKC-expressing cells versus  $18.71 \pm 1.82$  mV,  $n = 26$  in controls;  $10.90 \pm 1.25$  mV,  $n = 23$  in aPKC-DN-expressing cells versus  $9.58 \pm 1.05$  mV,  $n = 27$  in controls).

Taken together, these findings are consistent with a presynaptic role of aPKC in LTF, as part of a regulatory system activated by 5-HT and CPEB and gated by miR-22 (Figure 4L).

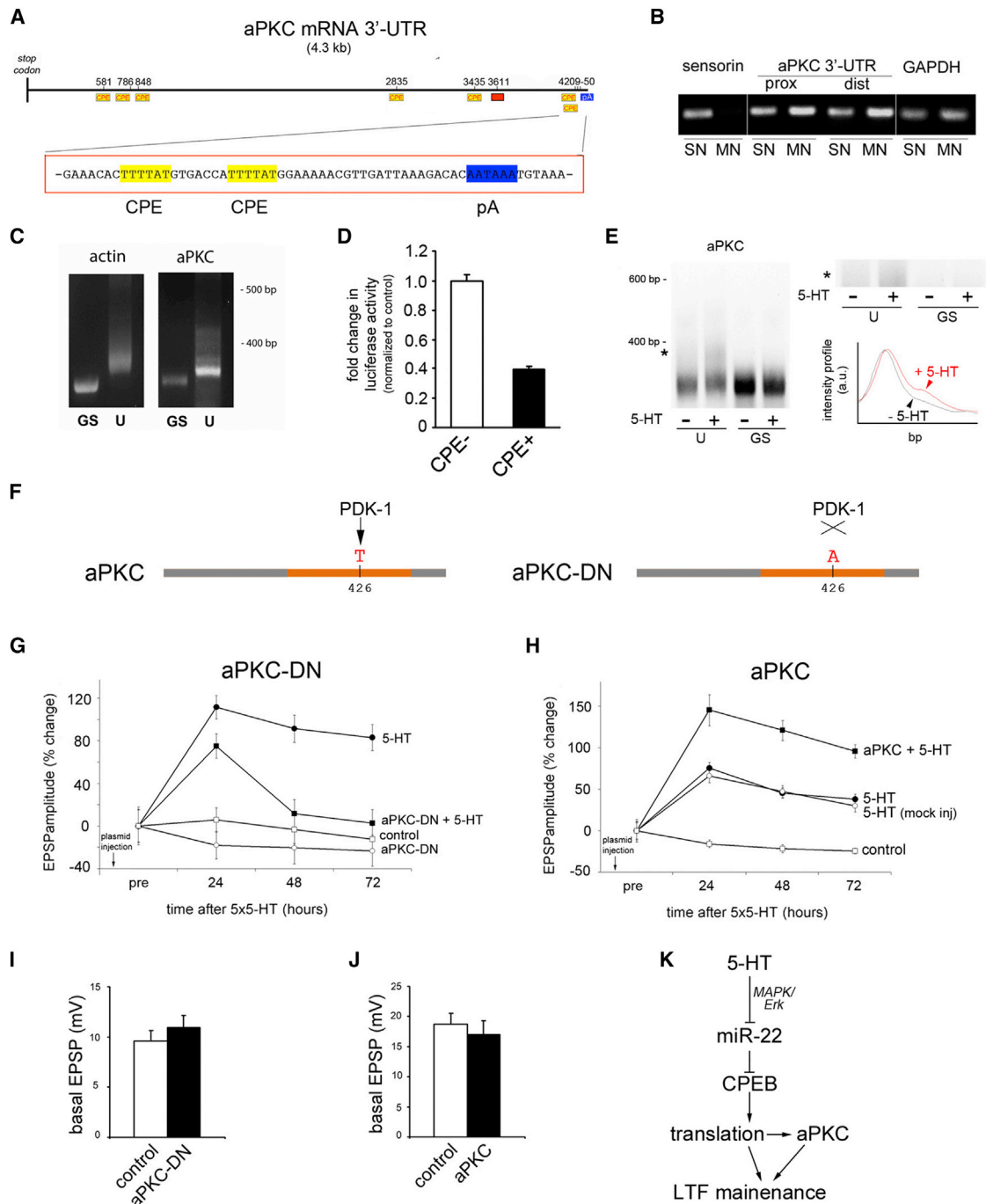
### DISCUSSION

The maintenance of LTF, which mediates long-term behavioral sensitization of the *Aplysia* gill-withdrawal reflex, requires a synapse-specific upregulation of CPEB, a functional prion-like regulator of local protein synthesis, which is essential for converting CPEB from a soluble to an aggregated functional prion form. (Si et al., 2003a, 2003b, 2010). We here asked: how is this upregulation to the functional prion form achieved?

We identified miR-22, a nervous system-specific microRNA in *Aplysia*, as a key regulator in the gating of LTF: miR-22 inhibits the mRNA for CPEB. The downregulation of miR-22 by 5-HT induces the initial upregulation and consequent aggregation of CPEB at the onset of LTF, which is in turn required for LTF to be maintained after its initial induction (Si et al., 2003b). The 5-HT-dependent downregulation of miR-22 is MAPK/Erk-dependent, as previously found for miR-124 (Rajasethupathy et al., 2009), thus showing that the same pathway mediates the 5-HT-dependent regulation of different miRNAs relevant to LTF.

### Translation-Dependent and Translation-Independent Regulatory Mechanisms of CPEB Function in LTF

The identification of CPEB as a functional prion-like molecule with a role in synaptic plasticity (Si et al., 2003a, 2003b, 2010; Keleman et al., 2007; Pavlopoulos et al., 2011; Krüttner et al., 2012) raises a number of biological questions regarding the regulation of its structural and functional dynamics in response to physiological stimuli. Recent studies have highlighted regulatory mechanisms intrinsic to the structure of this protein, as well as extrinsic regulatory mechanisms based on post-translational modifications (Fiumara et al., 2010; Pavlopoulos et al., 2011; Raveendra et al., 2013; White-Grindley et al., 2014). Since the conformational transitions of CPEB, like those of other Q/N-rich prions, are dependent on the expression level of the protein, it is of interest to understand how this conformational transition is dynamically regulated in neurons in response to synaptic signals. We here describe a microRNA-gated mechanism controlling the post-transcriptional upregulation of CPEB triggered by the neurotransmitter serotonin at the onset of LTF (Si et al., 2003b). Interference with this process is sufficient to block the maintenance of LTF after induction (Si et al., 2003b). Through its direct binding to the mRNA of CPEB, miR-22 represses the expression of CPEB. The 5-HT-dependent downregulation of miR-22 allows the upregulation of CPEB, which triggers its aggregation that is required for the maintenance of LTF. Rajasethupathy et al. (2009) previously described a similar mechanism whereby the 5-HT-dependent downregulation of miR-124 induces the upregulation of CREB-1 and, indirectly, of downstream genes acting synergistically in the induction phase of LTF. Another small non-coding RNA, a Piwi-interacting RNA (piRNA) regulated by serotonin, acts in a reverse way to silence CREB-2, an inhibitory constraint on CREB-1, thereby allowing CREB-1 to be



**Figure 4. aPKC Role in Presynaptic LTF**

(A) Scheme of the aPKC 3'-UTR. Yellow segments indicate CPE elements. The red segment indicates a miR-22 consensus site. The canonical polyadenylation signal hexamer is marked in blue. A sequence portion of the 3'-UTR (in the red box) shows two CPE elements neighboring the AATAAA hexamer.

(B) Cell-type-specific RT-PCR detects the extended 3'-UTR both in sensory and motor neurons. Sensorin transcripts are detected only in sensory neurons, whereas the ubiquitous gene GAPDH is expressed in both cell types.

(C) PCR-based *in vivo* polyadenylation assay of the aPKC mRNA (right) and of the actin mRNA (left) used as a control. A PCR product of defined length is amplified using gene-specific primers (GS). In both cases, a smeared product, indicative of a polyadenylation tail, is visible using a universal (U) reverse primer.

(D) Relative luciferase activity in cells expressing a construct (CPE+) in which a portion of the 3'-UTR encompassing two CPE elements is cloned downstream of the Renilla luciferase ORF versus a control construct in which the two CPEs were deleted (CPE-). The CPE+ and CPE- constructs were each co-expressed together with CPEB for 36 hr and luciferase activity was then determined. Values are normalized to the CPE- group. Data expressed as mean  $\pm$  SEM.

(legend continued on next page)



active for a longer time (Rajasethupathy et al., 2012). Together with these previous findings, our experiments define a multilayered system of translational control mediated by 5-HT regulation of small non-coding RNAs that gate the expression of these transcriptional and translational regulators—CREB1, CREB2, and CPEB—that are key molecular mediators of LTF induction and maintenance, ultimately controlling distinct temporal phases of LTF.

### miR-22 Gates Presynaptic LTF by Acting Directly on CPEB and Indirectly on Atypical PKC

We find that miR-22 also regulates aPKC expression in *Aplysia* ganglia indirectly, at least in part through CPEB-mediated polyadenylation, consistent with previous studies in *Drosophila* (Mastushita-Sakai et al., 2010). miR-22 is expressed in presynaptic sensory neurons but not in motor neurons. However, the activity of aPKC/M in synaptic plasticity has been studied primarily in the post-synaptic compartment (Sacktor, 2011; Bougie et al., 2012). Using genetic approaches based on the selective presynaptic overexpression of wild-type and dominant-negative forms of the kinase, we have now further defined the presynaptic role of this kinase in the maintenance of LTF, thus indicating that the 5-HT/miR-22 pathway controls LTF maintenance through CPEB and a downstream target with synergistic functions. Our analyses do not exclude that other microRNA-dependent mechanisms may be at work in the post-synaptic compartment during LTF.

In the mouse, the ascribed role of aPKCs in the maintenance of long-term plasticity and memory has been strongly challenged by gene knockout and other studies that revealed how at least two isoforms of atypical PKCs are dispensable for the maintenance of plasticity and memory (Volk et al., 2013; Lee et al., 2013; L.J. Volk et al., 2013, Soc. Neurosci., abstract), and the pharmacological inhibitors like chelerythrine and the ZIP peptide, which were thought to be specific for the atypical PKM-zeta isoform, lack that specificity (Lisman, 2012; Wu-Zhang et al., 2012; Volk et al., 2013; Lee et al., 2013; L.J. Volk et al., 2013, Soc. Neurosci., abstract). *Drosophila* and *Aplysia* have a simpler genetic organization of the PKC family and have only one atypical PKC isoform that can generate a PKM by proteolytic cleavage (Shieh et al., 2002; Bougie et al.,

2009, 2012). Dominant-negative genetic studies in these systems (Drier et al., 2002; Bougie et al., 2012), including our experiments, indicate that in invertebrates aPKC/M may indeed have a role in long-term plasticity. However, to address this issue in a more definitive way, a knockout approach in genetically tractable invertebrates such as *Drosophila* or a combined knockout of multiple aPKC/M isoforms (zeta and lambda/iota) in mammals are required. The presynaptic role for aPKC that we identify is also consistent with previous observations in *Drosophila* showing how presynaptic aPKC and PDK1 regulate synapse formation and function (Ruiz-Canada et al., 2004; Cheng et al., 2011).

Taken together, the results of our experiments support a model in which the presynaptic downregulation of miR-22 by 5-HT promotes the upregulation and activation of CPEB and downstream targets, including an atypical PKC, which function together synergistically in the maintenance of learning-related long-term synaptic plasticity.

## EXPERIMENTAL PROCEDURES

### Molecular Biology

The aPKC DNA sequence was PCR-amplified from *Aplysia* cDNA and cloned using the Gateway system (Invitrogen) to a suitable vector for aPKC-GFP expression. Mutagenesis was performed using the QuikChange-Multi Site-Directed mutagenesis kit (Agilent). The aPKC 3'-UTR was PCR-amplified from cDNA and then cloned into the Zero Blunt TOPO vector (Invitrogen).

### Northern Blot

Northern blot analysis was performed as previously described (Rajasethupathy et al., 2009). In brief, pleural ganglia were desheathed and kept in L-15 with glutamine for 24 hr at 18°C. Ganglia were then treated with serotonin or mock-treated with no serotonin, either in the presence or in the absence of U0126, a MEK inhibitor that blocks the activation of MAPK/Erk, as in Rajasethupathy et al. (2009). Total RNA was then extracted from the ganglia. Between 20 and 40 mg of total RNA were then loaded per gel lane, separated electrophoretically, and probed for miR-22 levels. The probes were 5'-<sup>32</sup>P-radiolabeled 21-nt or 22-nt oligodeoxynucleotides complementary to the miR-22 sequence, and the hybridization was performed at 42°C. To monitor equal loading of total RNA, the blots were reprobed with 5'-tgaggggacacctgggttcga-3' to detect tRNA.

### MicroRNA Inhibition and Western Blot

MicroRNA inhibition and western blot analyses were performed as previously described (Rajasethupathy et al., 2009).

(E) Polyadenylation assay of the aPKC mRNA after 5-HT treatment, representative of three independent experiments. A PCR product of defined length is amplified using gene-specific primers (GS). A smeared product, indicative of a polyadenylation tail, is visible using a universal (U) reverse primer. Top right: detail of the gel shown in the left panel at the level of the asterisk. Lower right: band intensity profiles for the two left lanes of the gel. Note the relative increase in intensity of products with longer polyA tails in the 5-HT lane (red trace).

(F) Left: scheme of the aPKC protein (gray bar) with the catalytic domain (orange) and the phosphorylation site in the activation loop (T426). Phosphorylation of T426 by PDK-1 is required for activation of aPKC. Right: scheme of the aPKC dominant-negative mutant (aPKC-DN). The phosphorylation site for PDK1 is mutated to alanine (T426A) to generate a non-phosphorylatable mutant that cannot be activated by PDK-1.

(G) Electrophysiological analysis of the effect of presynaptic overexpression of the GFP-tagged aPKC-DN mutant. EPSP amplitudes are measured before and after application of 5 × 5-HT to induce LTF. Synapses in which the presynaptic neuron overexpresses aPKC-DN show facilitation at 24 hr that is not maintained at later time points. Data expressed as mean ± SEM.

(H) Electrophysiological analysis of the effect of presynaptic overexpression of GFP-tagged wild-type aPKC. LTF is significantly enhanced by aPKC overexpression. Data expressed as mean ± SEM.

(I and J) Overexpression of aPKC-DN (I) or of aPKC (J) for 24 hr does not significantly alter basal EPSP amplitude with respect to non-overexpressing cells. Expressed as mean ± SEM.

(K) Schematic model of miR-22 gated presynaptic mechanisms implicated in LTF maintenance.

See also Figure S1.

### Luciferase Assay

Luciferase assays were performed 36 hr after transfection of appropriately modified versions of the psiCHECK-2 dual reporter plasmid (Promega) in HEK293 cells opportunistically co-transfected with miRNA duplexes or CPEB.

### Cell Culture, In Situ Hybridization, and Confocal Microscopy

Cell culture, in situ hybridization, and imaging of *Aplysia* sensory and motor neurons was performed as previously described (Montarolo et al., 1986; Rajasethupathy et al. 2009).

### Intracellular Injections, 5-HT Treatment, and Electrophysiology

Intracellular injections of miRNA mimic and inhibitors (Dharmacon) or plasmids, treatments with penetratin-conjugated inhibitors, and/or 5-HT and electrophysiological recordings were performed as previously described (Montarolo et al., 1986; Rajasethupathy et al., 2009; Fiumara et al., 2010).

### Statistics

Data are reported as mean values  $\pm$  SEM. Data analysis was performed using Excel (Microsoft) and Statistica (Statsoft) software. Student's *t* test, one-way ANOVA, and ANOVA for repeated-measures, followed by post hoc tests, were performed where appropriate and in all instances a *p* value  $<0.05$  was considered as statistically significant.

Detailed experimental procedures are in the [Supplemental Experimental Procedures](#).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and one figure and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.05.034>.

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