Two mRNA-Binding Proteins Regulate the Distribution of Syntaxin mRNA in *Aplysia* Sensory Neurons

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Targeting mRNAs to different functional domains within neurons is crucial to memory storage. In *Aplysia* sensory neurons, syntaxin mRNA accumulates at the axon hillock during long-term facilitation of sensory-motor neuron synapses produced by serotonin (5-HT). We find that the 3' untranslated region of *Aplysia* syntaxin mRNA has two targeting elements, the cytosolic polyadenylation element (CPE) and stem-loop double-stranded structures that appear to interact with mRNA-binding proteins CPEB and Staufen. Blocking the interaction between these targeting elements and their RNA-binding proteins abolished both accumulation at the axon hillock and long-term facilitation. CPEB, which we previously have shown to be upregulated after stimulation with 5-HT, is required for the relocalization of syntaxin mRNA to the axon hillock from the opposite pole in the cell body of the sensory neuron during long-term facilitation, whereas Staufen is required for maintaining the accumulation of the mRNA both at the axon hillock after the treatment with 5-HT and at the opposite pole in stable, unstimulated sensory neurons. Thus, the cooperative actions of the two mRNA-binding proteins serve to direct the distribution of an mRNA encoding a key synaptic protein.

Key words: long-term synaptic plasticity; mRNA-binding proteins; RNA trafficking; double-stranded stem loop structures; long-term memory; cell culture

Introduction

The distribution of mRNAs changes during the production of long-term synaptic plasticity (St Johnston, 1995; Knowles et al., 1996; Kiebler and DesGroseillers, 2000; Job and Eberwine, 2001; Steward and Schuman, 2001). Interfering with these changes in hippocampal pyramidal cells disrupts long-term potentiation and blocks memory storage (Steward et al., 1998; Miller et al., 2002). In *Aplysia* sensory neurons, the distribution of syntaxin mRNA depends on whether new synapses are forming (Hu et al., 2003). When synapses first form with target neurons, syntaxin mRNA and protein accumulate at the axon hillock. As the synapses become stable, the mRNA and protein accumulate in the cell body opposite the axon hillock. The mRNA and protein again accumulate at the axon hillock as new synapses develop during long-term facilitation of sensory-motor neuron synapses (Bailey and Chen, 1988; Glanzman et al., 1990; Hu et al., 2003; Kim et al., 2003). More syntaxin mRNA and protein are then transported into neurites presumably to supply the new synapses. Conditions that block the long-term facilitation that underlies sensitization of defensive reflexes (Kandel, 2001) prevent the accumulation of the mRNA and interfere with the transport (Hu et al., 2003).

The distribution of an mRNA depends on defined nucleotide

sequences or structures (*cis*-acting elements) that are recognized by specific binding proteins [*trans*-acting (Kiebler and DesGroseillers, 2000)]. We now examine how two *trans*-acting proteins, the cytosolic polyadenylation element (CPE)-binding protein (CPEB) and Staufen, a double-stranded RNA-binding protein, regulate the distribution of the mRNA during long-term facilitation. The CPE was identified for its role in cytosolic polyadenylation, which activates the translation of dormant maternal mRNAs during oocyte maturation (Hake and Richter, 1994; Stebbins-Boaz et al., 1996; Walker et al., 1999). CPEB has been cloned in mollusks (Walker et al., 1999; Liu and Schwartz, 2003; Si et al., 2003) and in several vertebrates (Hake and Richter, 1994; Gebauer and Richter, 1996; Bally-Cuif et al., 1998; Welk et al., 2001). The binding protein, which is present at distal sites of neurons (Wu et al., 1998; Liu and Schwartz, 2003; Si et al., 2003), facilitates the transport of CPE-containing mRNAs in hippocampal neurons (Huang et al., 2003). Staufen, another RNA-binding protein critical for distributing mRNAs in neurons, recognizes double-stranded RNA stem-loop configurations (Ferrandon et al., 1994; Saunders and Barber, 2003). This protein is essential for *Drosophila* development, mediating the distribution of mRNAs in the egg and dividing neuroblasts (Schupbach and Wieschaus, 1986). In hippocampal neurons, Staufen is present in ribonucleoprotein particles (RNPs) (Kiebler et al., 1999; Tang et al., 2001; Macchi et al., 2003) that are moved along microtubules by kinesin motors (Kohrmann et al., 1999; Wickham et al., 1999; Mal-lardo et al., 2003; Kanai et al., 2004).

We find that disrupting the interaction between CPEB and Staufen with the 3' untranslated region (UTR) of syntaxin mRNA abolishes the serotonin (5-HT)-induced changes in the distribution

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of the mRNA and also blocks long-term facilitation. Interaction between CPEB and CPE is decisive for the accumulation at the axon hillock after the treatment with 5-HT, whereas Staufen seems to be needed to maintain the accumulation at the axon hillock in sensory neurons treated with 5-HT and at the opposite pole of the cell body in stable, unstimulated sensory neurons.

Materials and Methods

Animals and tissue preparation. *Aplysia californica* weighing 70–100 g were obtained from the Mariculture Resource Facility of the University of Miami (Miami, FL). Ganglia were dissected from animals anesthetized with Mg^{2+} (Schwartz and Swanson, 1987). RNA was isolated from tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription (RT) was done with random hexamers using Superscript II reverse transcriptase (Invitrogen). Protein was measured with the Micro BCA kit (Pierce, Rockford, IL) with bovine serum albumin as a standard. Immunoblotting was done as described by Liu and Schwartz (2003).

Cell culture and electrophysiology. Sensory neuron–L7 cocultures (consisting of one or two sensory neurons with one L7 motor neuron) were prepared as described previously (Rayport and Schacher, 1986; Schacher and Montarolo, 1991) and maintained in culture for 4–6 d. Sensory neurons were isolated from pleural ganglia dissected from mature animals (80–100 g); L7 motor cells were isolated from abdominal ganglia of juvenile animals (1–3 g). In some cultures, sensory neurons were plated alone. Standard electrophysiological techniques were used to monitor changes in the amplitude of the EPSP evoked in L7 with stimulation of each sensory neuron, before and after treatments as described by Schacher and Montarolo (1991). To produce long-term facilitation in cultures, neurons were pulsed with 5 μ M 5-HT five times for 5 min each at 20 min intervals after testing the EPSP amplitude (Montarolo et al., 1986). Cultures were incubated at 18°C and EPSP amplitudes tested again 24 h later.

Cloning Staufen. The sequences of degenerate primers, 5'CCNAC-NGTNGARYTNAAYGC and 5'GTDAYRAARTTYTTCATRTG, were based on conserved regions of human and *Drosophila* Staufen (GenBank accession numbers CAC14085 and AAF57752). These primers were used in RT-PCRs with a temperature profile of 94°C (30 s), 55°C (30 s), and 72°C (60 s) for 35 cycles with random hexamer-primed *Aplysia* neuronal cDNA as a template. A 530 bp DNA fragment was isolated that encoded a partial sequence. The full sequence was obtained by 5' and 3' rapid amplification of cDNA ends (RACE) performed with *Aplysia* sensory neuron cDNA and gene-specific primers, 5'TTGCCACGATAACGAG-GATCAGAC (for 3' RACE) and 5'CCCGTCATAATGCCGCCCAAAA (for 5' RACE), using the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA). The entire 5' UTR of the *Aplysia* Staufen gene was obtained using the First Choice RLM-RACE kit (Ambion, Austin, TX). This sequence has been deposited in GenBank (accession number AY764181).

Isolation of 3' UTR of syntaxin mRNA. A gene-specific primer (5' GGA-CACCAAAAAGGCGGTCAAGTA) was designed based on a partial sequence (GenBank accession number U03123), with only a short incomplete 3' UTR (75 nucleotides). 3' RACE was done using this primer with a PCR profile: 94°C (5 s) and 72°C (3 min) for 3 cycles; 94°C (5 s), 70°C (10 s), and 72°C (3 min) for 5 cycles; 94°C (5 s), 65°C (10 s), and 72°C (4 min) for 25 cycles yielding the complete syntaxin mRNA 3' UTR (GenBank accession number AY764183) that is 982 nucleotides and has a polyadenylation signal and cytoplasmic polyadenylation element near its poly(A) tail.

Site-directed mutagenesis. We used the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). Plasmids containing the 3' UTR of syntaxin mRNA were the parental vectors. A pair of primers, each complementary to opposite strands of the vector and containing the CPE deletion, was used in PCR with *PfuTurbo* DNA polymerase and the parental plasmid as templates. After plasmid was removed by *DpnI* digestion, the vector incorporating the desired mutations was transformed into *Escherichia coli* and purified for *in vitro* transcription.

In vitro transcription. Fragments of the syntaxin mRNA coding region and UTR containing the T7 promoter were amplified with the following

primer pairs and *Aplysia* neuronal cDNA as templates: (1) GAATTGTAATACGACTCACTATAGGGACTACCATTGAAACAATGATTGTA and TGTCCATCTTGCCGTCTCGATGTA (for the coding region; corresponding to nucleotides 37–60 and 886–910, respectively); (2) GAATTGTAATACGACTCACTATAGGGCCCTCCTCCCTCCCTCTAGTCTACT and GATAATGATTTTATATGACATCTTC (for the UTR; corresponding to nucleotides 1025–1049 and 1974–1999, respectively). The underlined sequence is the T7 promoter. Amplified DNA was the template for the *in vitro* transcription reactions using mMESSAGE mMACHINE capped transcription kit (Ambion). The resulting capped RNA was polyadenylated using a Poly(A) Tailing kit (Ambion) and purified with the MEGAclear kit (Ambion). For microinjection, the final capped and polyadenylated RNA was dissolved in buffered 0.1 M KCl at a concentration of 200 μ g/ml.

Antibodies. A rabbit anti-*Aplysia* Staufen antibody was raised and affinity purified by BioSource International (Hopkinton, MA). The inferred amino acid sequence of Staufen was analyzed using the Protean program from DNASTar (Madison, WI) to predict the surface probability and antigenic index. A peptide (NH₂-TSPKDSQPQKTSGRDSV-COOH corresponding to Thr301–Val318) was conjugated to keyhole limpet hemocyanin as an immunogen. This peptide has both a high surface probability and antigenic index.

Immunocytochemistry. After they were fixed in 0.1 M PBS, pH 7.4, containing 4% paraformaldehyde and 30% sucrose for 1 h and rinsed with 0.01 M PBS, pH 7.4, cultured neurons were blocked at room temperature with 0.01 M PBS, pH 7.4, containing 0.2% Triton X-100 and 3% normal goat serum for 1 h and incubated with the anti-Staufen antibody (1:1000 dilution) overnight at 4°C in 0.01 M PBS, pH 7.4, 1% normal goat serum, and 0.2% Triton X-100. After they were washed three times with 0.01 M PBS for 5 min, the neurons were treated at room temperature with Cy3-labeled goat anti-rabbit antibody (1:200 dilution) for 4 h and washed three times with 0.01 M PBS before photography. Preabsorption of primary antibody with the antigen peptide, omitting the primary or secondary antibodies, was done to test the specificity of immunostaining.

In situ hybridization. As described by Hu et al. (2002), the probes used were 5' biotin-CTTTGCTCTGGTACTTGACCGCCTTT (for syntaxin mRNA, complementary to nucleotides 925–940) and 5' biotin-TGGGGCCCTTGTTTACAATGCCATCAGTGCTGGCAC [for *Aplysia* cell adhesion protein (ApCAM); complementary to nucleotides 1078–1113]. After the cells were fixed and treated with protease K, a biotin-labeled probe (1.5 μ g/ml) was applied overnight at 42°C in 50% deionized formamide, 5 $\times$ SSC, 0.02% SDS, and 2% blocking reagent. The unbound probe was washed out with 2 $\times$ SSC (twice) at 42°C and with 0.1 $\times$ SSC (twice) at 50°C. Cells were then incubated in streptavidin-FITC (1:200 dilution; Invitrogen) for 4 h at 4°C. The unbound antibody was washed out with 0.1 M PBS (3 $\times$). Hybridization or immunocytochemical signals were visualized directly with fluorescence microscopy and imaged with a Nikon (Tokyo, Japan) Diaphot microscope attached to an SIT video camera (Dage-MTI, Michigan City, IN). The specificity of staining with the biotin-labeled antisense oligonucleotides probe was tested either by substituting a labeled sense probe for the antisense probe or by incubating with excess unlabeled antisense probe.

UGA¹CCUCCUCCUCCUCCUAGUCUACUCCUUAUUAUACA AUUGUUCUACGCGCGUGCACC
ACUGCUGUCCUUAACAAUCUGGACUACCAACACCGCAACAGCUACUACUACAACAGCAACA
AGCAACAGCAACAGCAACUGCAGCAGCAACUACUACGACAAUACUACAGCAACAGCAACGAAA
CACUACUACUACUACCCACUACUACUACUACUACUACUACUACUACUACUACUACUACUACUAC
UACUACUACUACUACUACUACGACUACUACUACUACUACUACUACUACUACUACUACUACUACUAC
UACUACUACUACUACUACGCUACUACUACUACUACUACUACUACUACUACUACUACUACUACUACUAC
UACCAACCAUACUACUACUACUACUACUACUACUACUACUACUACUACUACUACUACUACUACUAC
CACCACUACCAUACCAUACCAUACCAUACCAUACCAUACCAUACCAUACCAUACCAUACCAUACCAUAC
UACCAUACCAUACUACUACUACUACUACUACUACUACUACUACUACUACUACUACUACUACUACUAC
UAC
CUGUUAUGGCUUGCAGAGAUGAGUAGUAGACUACUAGUACUGUCCUUUGUACACUUUUCUAC
GGAUUGUUUUUAUCCAUUGCAACAAAAAGGUUCCUUUUUUUUCUGAUGUUGCAACAAACA
GGCAACAGGUUUUAACAAUCCUUUUUUCUUUUUUGCAGCUUACUAAUUUGUCUACUUCUACA
CGCUCAUUAUUUUUCUAGAAGUUGGGUUGUCCUUUUUUAAAAAACAUUUUGAUGAUAUUU
UUUUGUUUCUUGAUUUCUGUUGAAUUUUUGUUCUGAGUUUUUCCCAUACUUCUCUUAUU
UAUAGUGUUUGUAGAAAAAAAUUACGCAUUUUCUACAAUUCUACUUGAAGAUGUCA
UUUAAAAUACUUUAUUUUUUUUUUC + POLY (A) TAIL

Figure 1. The 3' UTR of *Aplysia* syntaxin mRNA. The stop codon is boxed. The CPE (UUUUAU) and polyadenylation signal (AAUAAA) are in bold and underlined. A poly(A) tail would be added after the terminal cytosine residue.

Pressure injection. Staufen antisense (CA-TGGGGTTGTACGTTGAATTATGC) and control (CGTATTAAGTTGATGTTGGGG-TAC, reversed sequence of the antisense) oligonucleotides and hnRNP L antisense oligonucleotides (CTCCAGTTGAATCATAGCA-GAC), dissolved in 0.5 M KCl, 10 mM HEPES-KOH, pH 7.4, at a concentration of 0.3 mM, were injected into sensory neurons with intracellular electrodes (resistance, 10–15 M Ω) using 50 ms pulses at 5–7 psi. The sensory neurons were hyperpolarized to -70 mV. Delivery of material into the neuron was confirmed by a small depolarization of 3–5 mV with each pressure pulse. Each cell was subjected to 50 pressure pulses given at 2 s intervals. Oligonucleotides were injected on day 1. The EPSP was recorded on day 2 before the treatment with 5-HT and on day 3 24 h after the treatment.

Immunoprecipitation/RT-PCR assay. Pleural and pedal ganglia were homogenized in buffer A (10 mM HEPES, pH 7.5, 40 mM KCl, 3 mM MgCl₂, 0.32 M sucrose, and 2 mM DTT) containing protease inhibitors (1:100 dilution; Sigma, St. Louis, MO) and 1 mg/ml tRNA. The homogenates were centrifuged at $14,000 \times g$ for 10 min at 4°C. The recovered supernatant was assayed for protein. Antibodies against *Aplysia* Staufen (1:50 dilution) were added to 0.5 mg of each sample and incubated for 2 h at 4°C. After protein G beads (Sigma) were added, the reaction mixture was incubated for 1 h. The beads were recovered by centrifugation, washed six times with $1 \times$ PBS containing 0.1% Triton X-100 and 0.5% NP-40, and washed twice with DEPC-treated H₂O. The RNA from the immunoprecipitated samples was purified using TRIzol reagents, treated with DNase I, and subjected to RT-PCR. The primer pairs were as follows: (1) 5'-GACTA-CATCGAGACGCCAAGA and 5'-CAGC-CTAGCGTGCCACCTATC for syntaxin; (2) 5'-gattacgtccctgcctttg and 5'-gatcgagtcgagcgtctct for 18S ribosomal RNA; and (3) 5'-aggatgatgtgagccgtttg and 5'-acgtgctgag-gttgcagag for ApCAM. The last two pairs of primers have been used before successfully (Liu et al., 2004).

Single-cell RT-PCR. We used a fine microelectrode to dissect and isolate the cell bodies and their neurites (Schacher et al., 1999). Total RNA for the cell bodies and neurites was isolated using TRIzol (Invitrogen). RT was then done using random primer and Superscript II reverse transcriptase, and the resulting cDNA served as templates for PCR. Gene-specific primer sets were used to amplify specific genes.

Quantification and data analysis. MCID software (version 7.0; Imaging Research, St. Catharines, Ontario, Canada) was used to quantify average pixel intensities for hybridized and immunostained signals from sensory neuron cell bodies (Hu et al., 2004b). Values for the overall differences in expression of mRNA or protein were determined by averaging pixel intensities in six areas of sensory neuron cytoplasm (25 μ m² each) positioned at 12:00, 2:00, 4:00, 6:00, 8:00, and 10:00 with respect to the center of the cell (Hu et al., 2003). For measuring differences in distribution, the middle of the axon hillock region is at the 6:00 position and staining intensity for each region of the cytoplasm is normalized to the staining intensity measured at the 12:00 position (Hu et al., 2003). Data are expressed as means $\pm$ SEM. Changes in EPSP amplitude were normalized to the initial amplitude for each culture. The effects of treatments were determined by ANOVA, and specific differences between treatments were determined by the Scheffe multi-comparison tests.

Figure 2. *Aplysia* Staufen gene. **A**, The predicted amino acid sequence of *Aplysia* Staufen. The double-stranded RNA binding domains are in bold and underlined. These domains are 61 and 63% similar to those of the brain-specific forms of mouse and human Staufen2. *Aplysia* Staufen has several phosphorylation sites for PKA (Ser317, Ser836, and Ser876) and for p42/44 MAPK (Ser302, Thr435, and Thr651), two signaling pathways activated by the treatment with 5-HT. **B**, An antibody against *Aplysia* Staufen specifically recognizes a 125 kDa component on immunoblotting. The peptide antigen used to generate the antibody prevents the antibody from binding to Staufen (+). **C**, *Aplysia* Staufen is enriched in nervous tissue. Equal amounts of total protein (20 μ g) from extracts of each tissue were fractionated by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. The gel shown is representative of three similar experiments. **D**, Staufen binds to syntaxin mRNA. Immunoprecipitation (IP) and RT-PCR was performed as described in Materials and Methods. The negative control experiment was performed without adding Staufen antibody (–Ab) during the immunoprecipitation step. For control of input, cDNA prepared from a total homogenate of pleural and pedal ganglia was used. Both 18S rRNA and syntaxin mRNA is present in the complex, whereas ApCAM mRNA is excluded.

Results

The 3' UTR of syntaxin mRNA contains putative binding sites for CPEB and Staufen

Unlike several other mRNAs examined (Hu et al., 2002, 2003), *Aplysia* syntaxin mRNA accumulates in the region opposite the axon hillock in sensory neurons with stable synapses; this mRNA moves to the axon hillock during the formation of new synapses that occurs in long-term facilitation (Hu et al., 2003). Because the distribution of an mRNA can be mediated by targeting elements in its 3' UTR (Ainger et al., 1993; Blichenberg et al., 1999; Zhang et al., 2001), we cloned the full-length 982-nucleotide-long 3' UTR of syntaxin mRNA. The sequence contains a consensus CPE (nucleotides 1690–1695) and a polyadenylation signal, AAUAAA (nucleotides 1985–1990), just before the start of the poly(A) tail (GenBank accession number AY764183) (Fig. 1).

As predicted by the GeneQuest program (Lasergene), the cloned 3' UTR can be folded into extensive stem-loop double-stranded configurations needed for binding Staufen (St Johnston et al., 1992; Saunders and Barber, 2003) (see supplemental Fig. 1,

Figure 3. The 3' UTR of syntaxin mRNA is required for the polar distribution of the mRNA. **A**, Accumulation of syntaxin mRNA either at the opposite pole in untreated sensory neuron cell bodies or at the axon hillock in 5-HT-treated neurons is blocked after injecting the 3' UTR of the mRNA. Cultures were treated with 5-HT 6 h after the injection of either the 3' UTR (UTR) or the coding region of syntaxin mRNA (orf) and fixed for *in situ* hybridization immediately after retesting the EPSPs 24 h after the treatment. Each panel is a pseudocolor representation of local fluorescent intensity (red, high intensity; blue, low intensity; the color bar represents the relative pixel intensity scale; see also Figs. 4–8). Injections of the ORF (top micrographs) do not interfere with the appropriate polar distribution, whereas injections with the 3' UTR (bottom micrographs) disperse the mRNA in both control- and 5-HT-treated cells. The axon hillock is located at the 6:00 position for each cell (see also Figs. 4–8). Scale bar, 25 μ m. The histogram summarizes the staining pattern for syntaxin mRNA in the sensory neuron cell body for each condition. The height of each bar represents the average staining intensity at each cytoplasmic position (2:00, 4:00, 6:00, 8:00, and 10:00) normalized to the staining intensity measured at the 12:00 position (opposite the axon hillock; see also histograms in Figs. 4, 6, and 8). Injecting ORF alone (control) results in relatively low staining intensity at the axon hillock (6:00; ~65%) compared with high staining at the axon hillock with ORF injections followed by treatment with 5-HT (~165%; $F = 9.748$; $p < 0.01$). Uniform staining throughout the cell is observed after UTR injections both for control-treated and 5-HT-treated cultures. Error bars indicate SEM. **B**, Injecting the 3' UTR blocks long-term facilitation. Representative EPSP traces were recorded before (0) and 24 h after the treatments. The increase in EPSP amplitude produced by 5-HT was abolished when sensory neurons were injected with the 3' UTR (UTR), but not after injection with the coding region (orf). Calibration: vertical bar, 20 mV; horizontal bar, 25 ms. EPSPs that are unchanged (0%) are indicated by the dashed lines in both panels (see also Figures 4B, 5B, 6B, 8E). ANOVA indicates a significant effect of treatment ($df = 3, 26$; $F = 7.993$; $*p < 0.001$). Individual comparisons indicate that long-term facilitation was produced when 5-HT was applied to sensory neurons injected with ORF but not with UTR ($F = 5.159$; $p < 0.01$ vs ORF alone, $F = 4.605$; $p < 0.02$ vs 5-HT and UTR). Error bars indicate SEM.

available at www.jneurosci.org as supplemental material). Because Staufen has been shown to be an important mRNA-binding protein in neurons (Kiebler et al., 1999; Tang et al., 2001; Macchi et al., 2003, 2004; Mallardo et al., 2003), we next cloned a Staufen gene from *Aplysia* nervous tissue (GenBank accession number AY764181), which is 6.2 kb long with an open reading frame (ORF) encoding 950 amino acid residues. The predicted protein, with a calculated molecular weight of 106,000 (Fig. 2A), is similar in size to its *Drosophila* homolog and has four regions with double-stranded RNA binding domains and a long N terminus. A small fifth region is also present at the C terminus (amino acids

896–916). Two mammalian homologs of Staufen (Stau1 and Stau2) have been described previously (Marion et al., 1999; Wickham et al., 1999; Macchi et al., 2004). The *Aplysia* protein is closer to Stau2, the brain-specific form. To test whether *Aplysia* Staufen interacts with syntaxin mRNA, we raised an antipeptide antibody that specifically recognizes a single M_r 125,000 component on immunoblotting (Fig. 2B), which is consistent with the calculated molecular weight. Immunoblot assays showed that Staufen is strongly expressed in nervous tissue (Fig. 2C). We then used a pull-down analysis with RT-PCR to determine whether Staufen interacts with mRNA. Staufen was immunoprecipitated using the antibody, and RNA was then isolated from the immunocomplex and treated with DNase I. Subsequent RT-PCR revealed that syntaxin mRNA and 18S ribosomal RNA is pulled down with Staufen. The coprecipitation is selective, because the neuron-specific mRNA that encodes ApCAM (Mayford et al., 1992) was not detected in the precipitate (Fig. 2D). The mRNA of ApCAM, like that of syntaxin, is concentrated at the axon hillock during long-term facilitation (Hu et al., 2002). The presence of ribosomal RNA in the precipitate suggests that syntaxin mRNA might be a component of an RNP (Mallardo et al., 2003).

The 3' UTR regulates the distribution of syntaxin mRNA

Because the 3' UTR of syntaxin mRNA contains elements that may bind both CPEB and Staufen, we injected excess amounts of the *in vitro*-synthesized 3' UTR into the cell body of sensory neurons with the aim of influencing the distribution of the mRNA. We reasoned that the injected RNA would prevent the interaction between the two binding proteins and syntaxin mRNA in the sensory neuron. A similar strategy has been used successfully to disrupt the interaction between binding proteins and their nucleic acid-binding elements (Dash et al., 1990; Ferrandon et al., 1994). We found that the injected 3' UTR disrupted the specific accumulation of syntaxin mRNA in both untreated sensory neurons ($n = 6$; opposite the axon hillock) and in neurons treated with 5-HT ($n = 6$; at the axon hillock) (Fig. 3A). Under both conditions, syntaxin mRNA became uniformly distributed throughout the cytoplasm (Fig. 3A, histogram). Control injections of the coding region (ORF; $n = 6$) did not affect the distribution of the mRNA in unstimulated cells ($n = 6$; opposite the axon hillock) or in cells treated with 5-HT ($n = 6$; at the axon hillock). Both types of injections did not change the overall amounts of syntaxin mRNA in sensory neuron cell bodies. These results suggest that the 3' UTR contains *cis*-acting elements that

both maintain the accumulations of the mRNA in unstimulated sensory neurons and the localization of the mRNA to the axon hillock after treatment with 5-HT.

Injecting the 3' UTR also blocked long-term facilitation (Fig. 3B). The change in EPSP amplitude (a measure of synaptic strength) after the treatment with 5-HT in cells injected with the 3' UTR was $15.9 \pm 6.4\%$ ($n = 9$) compared with $78.9 \pm 19.8\%$ when 5-HT was applied to the cells injected with the control ORF sequence ($n = 9$). Injection of the 3' UTR or ORF (UTR: $5.0 \pm 7.9\%$, $n = 6$; ORF: $4.3 \pm 4.9\%$, $n = 6$) did not affect baseline synaptic transmission. These observations suggest that the interactions between the binding proteins and *cis*-acting elements in the 3' UTR of syntaxin mRNA is essential for long-term facilitation.

The 3' UTR contains at least two *cis*-acting elements

To examine the roles of specific targeting elements in the 3' UTR of syntaxin mRNA in the distribution of the mRNA in stable as well as in 5-HT-treated neurons, we injected excess amounts of the CPE RNA (a 12-mer, UUGUUUUUAUCCA) into sensory neuron cell bodies with the expectation that this would block the interaction between CPEB and the CPE in the 3' UTR of syntaxin mRNA. As with the injections of the entire 3' UTR, injecting the CPE sequence blocked the accumulation of the mRNA at the axon hillock after the treatment with 5-HT ($n = 9$), whereas injecting control oligonucleotide (reverse sequence of the CPE RNA; $n = 9$) did not interfere with the accumulation (Fig. 4A). In contrast to the results obtained with the 3' UTR, injecting the CPE ($n = 6$) did not interfere with the accumulation of syntaxin mRNA in stable untreated cells; the mRNA remains situated opposite the axon hillock (Fig. 4A). The effect of injecting the CPE on the accumulation of the mRNA at the axon hillock is specific, because the distribution of the mRNA encoding the neuropeptide sensorin, which is also concentrated at the axon hillock (Hu et al., 2002, 2003), was not affected (Fig. 4A). Injecting the CPE also blocked long-term facilitation (a change in EPSP amplitude of $-1.3 \pm 4.6\%$, $n = 10$, compared with $52.6 \pm 6.1\%$, $n = 12$) but did not affect baseline synaptic transmission (a change in EPSP amplitude of $0.3 \pm 5.4\%$, $n = 9$, compared with $8.3 \pm 11.1\%$, $n = 6$ for neurons injected with control RNA) (Fig. 4B).

We next examined whether blocking the synthesis of CPEB protein affects the distribution of syntaxin mRNA. Injecting antisense oligonucleotides complementary to *Aplysia* CPEB mRNA (Si et al., 2003) abolished the accumulation of syntaxin mRNA at the axon hillock (Fig. 5A) and also blocked the long-term facilitation induced by 5-HT (Fig. 5B). It had no effect on the distribution of syntaxin mRNA in stable untreated cells, however (Fig. 5A).

Figure 4. The CPE regulates the movement of syntaxin mRNA to the axon hillock. **A**, Injecting the CPE blocked the 5-HT-induced accumulation of syntaxin mRNA at the axon hillock but did not affect the accumulation opposite the axon hillock in untreated sensory neurons. Sensory neurons were treated with 5-HT 6 h after the injection of oligonucleotides with control (reverse sequence of the CPE RNA) or the CPE RNA and fixed for *in situ* hybridization immediately after retesting the EPSPs 24 h after the treatment. Injecting the CPE did not affect the accumulation of syntaxin mRNA opposite the axon hillock or the accumulation of mRNA encoding the neuropeptide sensorin at the axon hillock in untreated sensory neurons (left micrographs). Injecting control oligonucleotides did not interfere with the 5-HT-induced accumulation of syntaxin mRNA or sensorin mRNA at the axon hillock (right micrographs). Injecting the CPE blocked the accumulation of syntaxin mRNA at the axon hillock after 5-HT but had no effect on the accumulation of sensorin mRNA at the axon hillock (middle micrographs). Scale bar, 25 μ m. The histogram summarizes the effects of CPE or control injections on syntaxin mRNA distribution in the sensory neuron cell body. After CPE injections, staining intensity at the axon hillock (6:00) remains low ($\sim 75\%$) relative to the accumulation opposite the axon hillock (12:00; normalized to 100%). Treatment with 5-HT after CPE produces a uniform distribution, whereas treatment with 5-HT after control injection produces a significant accumulation ($F = 5.269$; $p < 0.01$) at the axon hillock ($\sim 145\%$). **B**, Injecting the CPE blocks long-term facilitation. Representative EPSP traces were recorded before (0) the injection and 24 h after the indicated treatments. The increase in EPSP amplitude produced by 5-HT was abolished when sensory neurons were injected with oligonucleotides containing the CPE but not after injection with the control oligonucleotides. Injection of the CPE did not affect baseline synaptic efficacy. Calibration: vertical bar, 20 mV; horizontal bar, 25 ms. ANOVA indicates a significant effect of treatment ($df = 3, 33$; $F = 18.71$; $*p < 0.001$). Individual comparisons indicate that long-term facilitation was produced when 5-HT was applied to sensory neurons that were injected with the control oligonucleotides but not the CPE ($F = 6.833$, $p < 0.01$ vs control oligonucleotides alone; $F = 13.817$, $p < 0.01$ vs 5-HT and CPE). Error bars indicate SEM.

Thus, interfering with the interaction of CPEB with its targeting element (CPE) blocked both the accumulation of syntaxin mRNA at the axon hillock and long-term facilitation but failed to change the accumulation opposite the axon hillock in untreated neurons with stable synapses.

To distinguish the role of the CPEB from that of Staufen in regulating the distribution of syntaxin mRNA, we injected an altered 3' UTR with the CPE deleted. Our rationale was that injecting excess amounts of the altered 3' UTR would continue to interfere with the interaction of Staufen with the 3' UTR. Both complete (Fig. 3A) and altered 3' UTR sequences disrupted the accumulation opposite the axon hillock in stable untreated cells;

Figure 5. CPEB moves syntaxin mRNA to the axon hillock. *A*, Injecting antisense oligonucleotides complementary to *Aplysia* CPEB blocked the 5-HT-induced accumulation of syntaxin mRNA at the axon hillock. Sensory neurons were treated with 5-HT 24 h after the injection and fixed for *in situ* hybridization immediately after retesting the EPSPs 24 h after the treatment. Injecting control (reverse of antisense) or antisense oligonucleotides did not affect the accumulation of syntaxin mRNA opposite the axon hillock in untreated sensory neurons (left micrographs). Injecting antisense blocked the accumulation at the axon hillock that is observed after control injections (right micrographs). Scale bar, 25 μ m. *B*, Injecting antisense oligonucleotides

the mRNA was distributed uniformly throughout the cell body (Fig. 6*A*). After injection of the altered 3' UTR, treatment with 5-HT produced effects that are qualitatively different from those obtained with injections of the complete 3' UTR. Although the complete 3' UTR blocked both long-term facilitation (change in EPSP amplitude of only $0.9 \pm 5.4\%$; $n = 9$) (Fig. 3) and the accumulation at the axon hillock (Fig. 6*A*), the altered 3' UTR only reduced long-term facilitation moderately (an increase in EPSP amplitude of $37.5 \pm 4.5\%$; $n = 8$) compared with an increase of $78.9 \pm 19.8\%$ ($n = 9$) after control injections (Fig. 3), and moderately reduced the accumulation of syntaxin mRNA at the axon hillock after 5-HT (only 25% increase over controls in Fig. 6*A* compared with 50% increase over controls in Fig. 3*A*). These results suggest that targeting elements other than the CPE (presumably Staufen) maintain the distribution of the mRNA opposite the axon hillock in cells with stable synapses and anchor the 5-HT-induced accumulation at the axon hillock. In contrast, the CPE is crucial for the relocalization of syntaxin mRNA to the axon hillock.

Staufen maintains syntaxin mRNA accumulations

The distribution of Staufen protein is congruent with that of syntaxin mRNA: in untreated stable sensory neurons, both Staufen and syntaxin mRNA are located together in the region of the cell body opposite the axon hillock (Fig. 7*A*); after the treatment with 5-HT, both Staufen protein and syntaxin mRNA are expressed at their highest levels at the axon hillock (Fig. 7*A*). The change in distribution does not require a net increase in Staufen protein because the overall immunostaining intensity does not change significantly after the treatment with 5-HT. Immunocytochemistry also shows that *Aplysia* Staufen is present both in the cell body and neurites of sensory neurons (Fig. 7*B*). Single-cell RT-PCR analysis reveals that syntaxin mRNA is also present in neurites (Fig. 7*C*). This colocalization of protein with mRNA and the binding data shown in Figure 2*D* suggest that Staufen interacts with the mRNA and might regulate the localization of the mRNA.

Injecting antisense oligonucleotides complementary to the Staufen gene alters the distribution of syntaxin mRNA in both stable untreated (Fig. 8*A*) and 5-HT-treated sensory neurons (Fig. 8*B*); the mRNA was distributed uniformly throughout the cell body. Control injections had no effect (Fig. 8*A, B*). Injecting antisense oligonucleotides reduced the amount of Staufen protein in sensory neurons by 25%: 48 h after the injection and 24 h after treatment with 5-HT, the average intensity with control (reverse sequence of the antisense) was 54.1 ± 3.4 U and with antisense was 40.5 ± 3.5 U. This reduction in staining was significant ($df = 1, 16, F = 7.751, p = 0.015$). The effect of injecting Staufen antisense oligonucleotides is specific; it did not affect the distribution of ApCAM mRNA (Fig. 8*C*), an mRNA that does not interact with Staufen (Fig. 2). Furthermore, injection of antisense oligonucleotides complementary to the gene of another *Aplysia*

complementary to *Aplysia* CPEB (AS-CPEB) blocked long-term facilitation. Representative EPSP traces were recorded before (0) and 24 h after the indicated treatments. The increase in EPSP amplitude produced by 5-HT was abolished when sensory neurons were injected with AS-CPEB but not after injection with the control oligonucleotides (control). Injection of AS-CPEB did not affect baseline synaptic efficacy. Calibration: vertical bar, 20 mV; horizontal bar, 25 ms. ANOVA indicates a significant effect of treatment ($df = 3, 15, F = 8.665; *p < 0.002$). Individual comparisons indicate that long-term facilitation was produced when 5-HT was applied to sensory neurons that were injected with the control oligonucleotides but not with AS-CPEB ($F = 7.298, p < 0.01$ vs control oligonucleotides alone; $F = 5.303, p < 0.01$ vs 5-HT and injection of AS-CPEB). Error bars indicate SEM.

RNA-binding protein, hnRNP L (GenBank accession number AY764182), had no effect on the distribution of syntaxin mRNA (Fig. 8D). Thus, the constitutive expression of Staufen is essential for the localization of the syntaxin mRNA in both stable neurons (accumulation opposite the axon hillock) and in 5-HT-treated sensory neurons (accumulation at the axon hillock).

Blocking synthesis of Staufen protein also interferes with long-term facilitation. Injection of antisense oligonucleotides abolished long-term facilitation produced by 5-HT (the EPSP amplitude was reduced to $9.1 \pm 6.4\%$; $n = 8$) (Fig. 8E). Injection of control oligonucleotides had no effect on long-term facilitation (EPSP amplitudes increased by $71.3 \pm 15.8\%$; $n = 8$). Both control and antisense oligonucleotides did not significantly effect baseline synaptic transmission (Fig. 8E). The injection of antisense oligonucleotides complementary to the mRNA-binding protein hnRNP L also had no effect on the facilitation (data not shown). Thus, Staufen is essential for syntaxin mRNA accumulations and for the long-term facilitation produced by 5-HT.

Discussion

Syntaxin is important for synaptic function, participating in transmitter release (Chen and Scheller, 2001), vesicle fusion (Jahn et al., 2003), and the generation and maintenance of neuronal polarity (Foletti et al., 1999; Horton and Ehlers, 2003). We find that two RNA-binding proteins, CPEB and Staufen, influence the distribution of syntaxin mRNA in *Aplysia* sensory neurons. Accumulation of the mRNA at the axon hillock leads to increases in the amounts of syntaxin protein at the axon hillock for transport into the axon (Hu et al., 2003), where it can contribute to axon growth and synaptic function (Tang et al., 2001; Horton and Ehlers, 2003). Because axon growth and the formation of new synapses occur both early during the initial interaction between the sensory neuron with a target motor neuron and later during the formation of long-term facilitation of stable sensory-motor neuron connections (Bailey and Chen, 1988; Glanzman et al., 1989, 1990; Kim et al., 2003), increased syntaxin in the axon would be required for the formation, maintenance, and functioning of the new synapses (Hu et al., 2003; Kim et al., 2003; Grabham et al., 2005). Disrupting the accumulation of syntaxin mRNA at the axon hillock by interfering with specific interactions between *cis*-acting elements and *trans*-acting RNA-binding proteins blocked long-term facilitation (Figs. 3–8), indicating that the accumulation is important for the development of long-term facilitation.

Synthesis and accumulation of syntaxin protein correspond with the accumulation of its mRNA under all conditions (Hu et al., 2003). The mRNA is present in neurites of cells in culture, suggesting that

Figure 6. Two *cis*-elements in the 3' UTR regulate the distribution of syntaxin mRNA. **A**, The 3' UTR of syntaxin mRNA without the CPE contains the *cis*-acting element responsible for the polar distribution of the mRNA in the cell bodies of sensory neurons. Sensory neurons were injected with the complete 3' UTR of syntaxin (WT-UTR) or mutated 3' UTR with the CPE deleted (UTR-CPE) 6 h before treatment with 5-HT and fixed for *in situ* hybridization immediately after retesting the EPSPs 24 h after the treatment. Both injections dispersed syntaxin mRNA in untreated sensory neurons (left micrographs and histogram). After 5-HT, injection with WT-UTR blocked the accumulation at the axon hillock (bottom right micrograph and histogram), whereas injection with UTR-CPE resulted in a modest level of accumulation at the axon hillock ($\sim 25\%$ more than injection alone, $p < 0.05$) compared with other control injections (see top left micrograph in Figs. 3–5). Scale bar, 25 μm . **B**, Moderate level of long-term facilitation is produced by 5-HT after injecting sensory neurons with the altered 3' UTR (UTR-CPE). Representative EPSP traces were recorded before (0) and 24 h after the indicated treatments. The increase in EPSP amplitude produced by 5-HT was totally abolished when sensory neurons were injected with 3' UTR of syntaxin (WT-UTR) (see also Fig. 3), and the increase was smaller than expected (see Fig. 3) after injection with UTR-CPE. After injection of the control coding sequence (ORF), the increase in EPSP amplitude was higher ($\sim 79\%$; see Fig. 3). Injection of each UTR alone did not affect baseline synaptic efficacy. Calibration: vertical bar, 20 mV; horizontal bar, 25 ms. ANOVA indicates a significant effect of treatment ($df = 3, 27$; $F = 8.556$; $p < 0.002$). Individual comparisons indicate that long-term facilitation was produced when 5-HT was applied to sensory neurons that were injected with UTR-CPE, but not with WT-UTR ($F = 5.69$, $p < 0.01$ vs UTR-CPE alone; $F = 7.027$, $p < 0.01$ vs 5-HT and injection of WT-UTR). Error bars indicate SEM.

synthesis of the protein may also take place at distal neurites. The axon hillock may be a docking site for the incorporation of the mRNA into RNPs for transport to synapses. Staufen antibodies precipitate syntaxin mRNA and 18S ribosomal RNA together with Staufen protein, suggesting that the mRNA, Staufen, and ribosomes are all components of the same RNPs. CPEB is also present in neurites (Liu and Schwartz, 2003; Si et al., 2003) and may be needed to move the mRNA to distal sites where local translation of the mRNA would also contribute to synaptogenesis and synaptic function (Casadio et al., 1999; Schacher et al., 1999; Sun et al., 2001; Giustetto et al., 2003; Hu et al., 2003).

RNA interaction: do the *trans*-acting proteins have distinctive functions?

Staufen plays a critical role in accumulating syntaxin mRNA at specific sites: opposite the axon hillock in stable untreated sen-

Figure 7. Staufen colocalizes with syntaxin mRNA. **A**, Double-staining of Staufen protein and syntaxin mRNA in sensory neurons. The treatment with 5-HT affects the distribution of both Staufen protein and syntaxin mRNA. Both are concentrated opposite the axon hillock in untreated sensory neurons (top micrographs) and at the axon hillock after the treatment with 5-HT (bottom micrographs). Cultures were treated with 5-HT, fixed 24 h after the treatment, and processed for examining the distribution of Staufen protein (immunocytochemistry) and syntaxin mRNA (*in situ* hybridization) in the same sensory neurons. Scale bar, 25 μ m. See Figure 8, **A** and **B**, for histograms quantifying these distributions. **B**, A single sensory neuron cultured alone and processed for Staufen immunocytochemistry. Staufen protein is present in both cell body and neurites of sensory neurons. **C**, Syntaxin mRNA is present in neurites. RNA was isolated from the cell body of a single sensory neuron (SN-CB) and its axon and regenerated neurites contacting the axon and regenerated neurites of a single L7 (neurites). Samples were amplified by RT-PCR to detect each of the indicated sequences. The mRNA encoding *Aplysia* tolloid (BMP; GenBank accession number ACU57369), a metalloprotease, is expressed in the sensory neuron cell body but is not transported into the neurites (Schacher et al., 1999). Syntaxin mRNA is expressed in both the cell body and neurites.

sory neurons or at the axon hillock in 5-HT-treated neurons. During oogenesis, *Drosophila* Staufen is required for the accumulation of *oskar* mRNA at the posterior pole of the egg (St Johnston et al., 1991) and later is required for the accumulation of *bicoid* mRNA at the anterior pole (St Johnston et al., 1991; Ferrandon et al., 1994; Cha et al., 2001). In *Aplysia*, Staufen is distributed together with syntaxin mRNA: opposite the axon hillock in stable untreated sensory neurons and at the axon hillock in 5-HT-treated sensory neurons. Manipulations that disrupt the interaction of Staufen with syntaxin mRNA in *Aplysia* sensory neurons, intracellular injections of either Staufen antisense oligonucleotides or the 3' UTR of syntaxin mRNA, disrupt the accumulation of the mRNA under all conditions. We found that reducing Staufen levels by 25% is sufficient to interfere with the distribution of syntaxin mRNA and long-term facilitation. Although small, this decrease is similar in magnitude to the reduction in the regulatory subunit of protein kinase A (PKA) that is required for long-term facilitation (Hegde et al., 1993). In addition to syntaxin mRNA, Staufen may also interact with other mRNAs, the

distribution of which is important for changing synaptic function. We suggest that Staufen contributes to the formation of long-term synaptic plasticity by maintaining mRNAs at specific sites in the neurons. In accordance with the idea that this RNA-binding protein plays a role in the development of synaptic plasticity, Dubnau et al. (2003) found that *Drosophila* mutants of Staufen are unable to learn.

The RNA-binding protein CPEB has been shown not only to mediate the polyadenylation and translation of certain mRNAs critical for long-term synaptic plasticity but also to transport select mRNAs (Huang et al., 2003; Liu and Schwartz, 2003; Si et al., 2003). In cultured hippocampal neurons, the CPE directs the movement of mRNAs in a microtubule-dependent manner (Huang et al., 2003). In *Aplysia*, the CPE in the 3' UTR of syntaxin mRNA is critical for accumulation of syntaxin mRNA at the axon hillock during the development of long-term facilitation. Injection of CPE sequences or blocking the synthesis of CPEB protein with antisense oligonucleotides (Si et al., 2003) abolished both long-term facilitation and the accumulation of the mRNA at the axon hillock after the treatment with 5-HT. The effect of CPE injection was specific because sensorin mRNA accumulated at the axon hillock both in untreated cells and after 5-HT. These manipulations, however, did not affect the accumulation of syntaxin mRNA opposite the axon hillock in untreated cells. When the interaction with Staufen is interfered with following injections of the 3' UTR lacking the CPE or blocking new synthesis of Staufen, syntaxin mRNA does not accumulate at either pole of the neuron but is distributed uniformly. The importance of the CPE in the accumulation of the mRNA at

the axon hillock only after treatment with 5-HT is also supported by the observation that both the accumulation and the plasticity are attenuated but not abolished when sensory neurons are injected with the 3' UTR lacking the CPE. Under these conditions, the interaction with CPEB may be sufficient to move the uniformly distributed mRNA to the axon hillock. Alternatively, injection with the 3' UTR lacking the CPE may not affect the accumulation of syntaxin mRNA at the axon hillock to the same degree as reducing Staufen levels by injections with antisense or the full-length 3' UTR. This reduction may not be sufficient to interfere with some accumulation at the axon hillock after CPEB-mediated movement of the mRNA. We found that movement from the opposite pole to the axon hillock is dependent on intact microtubules, because nocodazole blocked the 5-HT-induced accumulation of syntaxin mRNA at the axon hillock (data not shown).

How do the actions of 5-HT lead to relocation of syntaxin mRNA from the opposite pole to the axon hillock? First, interaction between Staufen and syntaxin mRNA at the opposite pole of the neuron may be reduced through PKA and p42/44 mitogen-activated

protein kinase (MAPK) catalyzed phosphorylations activated by 5-HT (Greenberg et al., 1987; Martin et al., 1997; Hu et al., 2004a; Sharma and Carew, 2004). Hu et al. (2003) found that the change in the distribution of syntaxin mRNA after treatment with 5-HT is PKA dependent. Moreover, the activation of MAPK by 5-HT (Martin et al., 1997; Purcell et al., 2003) is mediated by the secretion of the sensory neuron-specific neuropeptide sensorin, and the secretion of this peptide also affects the distribution of syntaxin mRNA (Hu et al., 2004a,b). In *Xenopus* oocytes, the transient phosphorylation of Staufen by MAPK during meiotic maturation may be important for the release of RNAs from their accumulation at the vegetal cortex (Allison et al., 2004). *Aplysia* Staufen has several possible PKA and MAPK phosphorylation sites (Fig. 2). Phosphorylation by PKA or MAPK after the treatment with 5-HT may induce conformational changes in Staufen, resulting in the release of syntaxin mRNA for interactions with newly synthesized CPEB. The mRNA would be directed to the axon hillock by CPEB, induced by the treatment with 5-HT (Liu and Schwartz, 2003; Si et al., 2003) in a microtubule-dependent manner. At the axon hillock, syntaxin mRNA would interact with newly synthesized Staufen, anchoring the mRNA at this new location for potential transport to distal sites.

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Figure 8. Staufen is required for polar distribution of syntaxin mRNA. **A, B**, Injecting antisense oligonucleotides complementary to Staufen reduces the expression of Staufen (top left micrograph compared with bottom left micrograph) and disperses syntaxin mRNA in the cell bodies of sensory neurons in both control untreated and 5-HT-treated neurons (right micrographs and histograms). Sensory neurons were injected with antisense oligonucleotides complementary to the Staufen gene (nucleotides 244–268) and control oligonucleotides (reverse sequence of the antisense). Neurons were treated with 5-HT 24 h later and fixed 24 h after the treatment to examine with double staining the distribution of Staufen protein (immunocytochemistry) and syntaxin mRNA (*in situ* hybridization) in the same sensory neurons. Scale bar, 25 μ m. In untreated sensory neurons injected with control oligonucleotides, Staufen protein staining and syntaxin mRNA staining were lowest at the 6:00 position. After injection with antisense, protein and mRNA staining were uniform (histogram in **A**). After 5-HT and control injections, both Staufen protein and syntaxin mRNA staining were highest at the 6:00 position. Injection with antisense resulted in more uniform distribution for both protein and mRNA after treatment with 5-HT. **C**, Injection of Staufen antisense oligonucleotides did not disturb the distribution of ApCAM mRNA, which accumulates at the axon hillock under all conditions. **D**, Injecting antisense oligonucleotides complementary to another RNA-binding protein, HnRNP L, does not affect the accumulation of syntaxin mRNA opposite the axon hillock in untreated sensory neurons (top) or the 5-HT-induced accumulation of syntaxin mRNA to the axon hillock (bottom). **E**, Injecting Staufen antisense oligonucleotides (AS-STF) into the cell bodies of sensory neurons blocked long-term facilitation. Representative EPSP traces were recorded before the injection and 24 h after the treatment with 5-HT. The increase in EPSP after treatment with 5-HT was abolished when sensory neurons were injected with AS-STF but not control oligonucleotides (control). Injection of AS-STF alone did not affect baseline synaptic transmission. ANOVA indicates a significant effect of treatment ($df = 3, 24$; $F = 18.921$; $p < 0.001$). Individual comparisons indicate that long-term facilitation was produced when 5-HT was applied to sensory neurons injected with control oligonucleotides but not with AS-STF ($F = 8.545$, $p < 0.01$ vs control oligonucleotides alone; $F = 9.965$, $p < 0.01$ vs 5-HT and AS-STF). Error bars indicate SEM.

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