A strategy to capture and characterize the synaptic transcriptome

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allows for identiﬁcations and is required speciﬁcally in the kinesin coimmunoprecipitations (CoIPs), but not in the Aplysia Target of Rapamycin (ApTOR) Ab (used as a nonspeciﬁc Ab control) CoIPs. Thus, the kinesin complex immunoprecipitated from CNS contains several RNA-binding proteins and is likely to contain RNAs destined for synaptic sites.

We next prepared RNAs from the ApKHC1 complexes isolated from Aplysia CNS and searched for the presence of Calcium/Calmodulin dependent protein kinase IIα (CaMK IIα) mRNA, a transcript reportedly transported by kinesin (14, 16). Western blot analysis of the communoprecipitated Kinesin Heavy Chain (KHC) complex revealed the presence of all three of these RNA-binding proteins (Fig. 1C) speciﬁcally in the kinesin communoprecipitations (CoIPs), but not in the Aplysia Target of Rapamycin (ApTOR) Ab (used as a nonspeciﬁc Ab control) CoIPs. Thus, the kinesin complex immunoprecipitated from CNS contains several RNA-binding proteins and is likely to contain RNAs destined for synaptic sites.

To adopt a genomics approach to identifying all RNAs present in the ApKHC1 complex, we ﬁrst used Aplysia microarrays containing probes corresponding to 56,000 unique neuronal transcripts described previously (18). Fig. 1E shows the results of the hybridization analysis carried out on this array showing ~200 RNAs (blue) with at least twofold enrichment in the kinesin complex over control (P ≤ 0.05) immunoprecipitates from CNS. The RNAs speciﬁcally represented in the complex included several neuropeptide precursors, kinases, phosphatases, ion channels, and regulatory factors involved in protein synthesis (Dataset S1, Tables S1, S2, and S3). In this collection, we identiﬁed several RNAs that are localized to neuronal processes of synaptic neurons, such as elongation factor 1α (19) and sensorin (20–23).

Results

Strategy for Identifying Synaptically Targeted RNAs: Isolation and Characterization of RNA Transport Complexes. We assumed that successful isolation of the RNA–protein complexes associated with the kinesin transport machinery would help identify the full repertoire of RNAs that are actively transported to synapses. Because the CNS contains both neuronal and nonneuronal cells, this approach will also yield RNAs found in the kinesin complex in nonneuronal cells, such as glia. We ﬁrst optimized conditions for isolating RNA transport complexes from the CNS of Aplysia based on a previously described protocol for preparation of kinesin transport complexes Aplysia Kinesin Heavy Chain 1 (ApKHC1) that contain several synaptic proteins (15). To examine whether the transport complexes prepared from CNS contain RNA cargos (Fig. 1A and B), we searched in the complex for the presence of three RNA-binding proteins known to be present at synapses—staufen, fragile X mental retardation protein (FMRP), and cytoplasmic polyadenylation element-binding protein (CPEB)—that have been associated with kinesin in the mouse (14, 16). Western blot analysis of the communoprecipitated Kinesin Heavy Chain (KHC) complex revealed the presence of all three of these RNA-binding proteins (Fig. 1C) speciﬁcally in the kinesin communoprecipitations (CoIPs), but not in the Aplysia Target of Rapamycin (ApTOR) Ab (used as a nonspeciﬁc Ab control) CoIPs. Thus, the kinesin complex immunoprecipitated from CNS contains several RNA-binding proteins and is likely to contain RNAs destined for synaptic sites.

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RNA deep sequencing (RNA-seq) Analysis of ApKHC1 Transport Complex Reveals the Complex Composition of Transported RNAs.

Our Aplysia microarray contained 50–60% of all of the genes predicted to be expressed in the CNS of Aplysia (18). An inherent limitation of microarrays is the inability to identify novel and low-abundance transcripts. In addition, the Aplysia genome has not yet been fully annotated, and a complete list of Aplysia protein-coding genes is not available. Thus, we used the Roche 454 pyrosequencing platform to directly identify all RNAs transported by kinesin. Using 454 sequencing followed by transcript assembly, we identified 5,657 nonredundant sequences (i.e., contigs) of RNAs associated with the kinesin complex prepared from CNS (Dataset S1, Table S4). Approximately 90% of the ~200 RNAs that we identified from the microarray experiments (described above) were found in this collection of RNAs (cf. Dataset S1, Tables S1, S2, S3, and S5), providing independent validation of the microarray results. Therefore, we are reasonably confident that the majority of transcripts identified by RNA-seq indeed comprise a significant portion of the kinesin transport complex. Our RNA-seq analysis has identified ~2,000 transcripts not previously represented in the Aplysia EST database.

Using BLAST searches (e-value <10^-5) in GenBank and SwissProt databases, among the 5,657 identified RNA sequences associated with the CoIP kinesin complex, we were able to identify 1,184 transcripts encoding predicted proteins with known homologs from other organisms (Fig. 2 and Dataset S1, Table S5). Based on their predicted physiological functions, these RNAs code for signaling molecules (15.8%), components of the protein synthesis machinery (13.5%), channels and receptors (5.9%), components of the protein degradation machinery (1.9%), metabolic and other processes (32.2%), and unknown proteins (24.3%). Importantly, the majority of known Aplysia secretory signaling molecules, including neuropeptide precursors and other secretory peptides (e.g., tolloid/bone morphogenetic proteins) (Dataset S1, Table S6) were found in the complex. In comparison, neuropeptide RNAs represent <0.5% of the total transcripts that we recently characterized from the CNS (18). These data suggest that there is substantial enrichment for this class of RNAs in the kinesin complex, and that in general, most neuropeptide RNAs may be transported to the synapses.

Consistent with previous findings on mRNA localization in the sensory neuron processes (18, 21), we identified transcripts encoding nearly all of the ribosomal proteins and the majority of components of the protein synthesis machinery and RNA-binding proteins, such as staufen and zinc finger dsRNA-binding proteins.
calmodulin, ion channels (e.g., hyperpolarization-activated ion channel, Phe-Met-Arg-Phe-NH2 (FMRFamide)-gated Na\(^+\) channel), neurotransmitter transporters (e.g., vesicular acetylcholine transporter), synaptic proteins (e.g., synaptotagmin-1, synaptobrevin, NMDA-type glutamate, other receptors, including seven transmembrane helix receptors), and cell adhesion molecules (e.g., neural cell adhesion molecule [NCAM]-related adhesion molecule). We also discovered several natural antisense/non-coding RNAs (e.g., natural antisense RNAs for beta tubulin, S6 kinase, protein kinase A type II) in the complex (Fig. S1), suggesting that they also might be transported and contribute to synaptic physiology and memory storage. We next compared recently published data on RNA localization in rat hippocampal neurons with that of our current dataset on kinesin-associated RNAs identified from *Aplysia* CNS. Bioinformatics analysis suggest that they share ∼40% Gene Ontology terms (Fig. S2 and Dataset S1, Table S10), suggesting a major overlap of signaling pathways present in hippocampal and *Aplysia* CNS neuronal processes.

**RNA Cargo-Enriched Microarray Analysis Suggests Enrichment of Specific RNAs in the ApKHC1 Complex from Aplysia CNS.** To determine whether there is a substantial enrichment of certain RNAs in the kinesin transport complex, we compared gene expression profiles of total RNAs expressed in the CNS and RNAs present in the ApKHC1 complex using *Aplysia* microarrays (18) supplemented with the ∼2,000 additional gene probes identified by 454 sequencing. We then used this kinesin cargo-enriched array to compare RNAs present in the immunoprecipitated complex from CNS and total RNA from CNS. Our microarray results suggest a significant enrichment of many RNAs in the kinesin immunoprecipitates, supporting our initial characterization (Fig. 2 B and C and Fig. S3). The enrichment of 317 transcripts in the kinesin immunoprecipitation (IP) complex (shown in red in Fig. 2 B and C) was statistically significant, with a median false discovery rate (FDR) <0.62%, which would correspond to approximately two false-positive results in the set. The functional assignment was based on homology searches and information obtained from EST sequencing. These transcripts in the kinesin IP complex included homologs for ash1 (asymmetric synthesis of HO endonuclease1), SFRS8 (splicing factor, arginine/serine-rich 8), cyclin-dependent kinase activator, cactin, and heat shock protein 60 (HSP60) (Dataset S1, Table S7). Of note, 40% of the transcripts that we identified as enriched in the kinesin complex compared with the total RNA were among the 2,000 additional features that we added to make the cargo-enriched array, thereby further validating our initial microarray and RNA-seq results.

We next studied the 317 transcripts identified as enriched in the ApKHC1 complex to identify biological pathways possibly mediated by these RNA cargos. Because *Aplysia* currently does not have a complete well-annotated genome and transcriptome, we identified human homologs of the *Aplysia* ESTs using *blat* searches against the human transcriptome. Out of 317 cargo-enriched transcripts (significant at 1% FDR), 147 transcripts produced hits in the human transcriptome (Dataset S1, Table S8). We further analyzed these 147 transcripts using the Expression Analysis Systematic Explorer (EASE) (30) to search for predominant biological themes in gene annotation databases and the Kyoto Encyclopedia of Genes and Genomes (KEGG) using *Aplysia* array projection into the human transcriptome, as a background for statistical estimations (Dataset S1, Table S9). From this analysis, we identified 16 homologous pathways, including pathways involved in basic cellular processes, such as protein export, calcium signaling, axon guidance, endocytosis, cytoskeletal regulation, and RNA splicing, and diseases, such as Huntington’s disease. Not surprisingly, our findings suggest that RNAs transported by kinesin could regulate several different physiological processes at the CNS synapses.

**Myosin Heavy Chain, an RNA Cargo of Kinesin, Is Required for the Establishment of LTF.** We next localized three candidate RNA cargos of ApKHC1 by RNA in situ hybridization, using a hybridization
probe for sensorin as a positive control. We found that synaptobrevin, α-tubulin, and myosin heavy chain (MHC) RNAs localized to sensory neuron processes (Fig. 3). α-tubulin has previously been found to be localized to sensory neurons (18, 21), which is of interest in understanding molecular motor-dependent transport and localization of proteins and RNAs to distal neuronal processes. MHCs are actin-dependent molecular motors critical for hippocampal long-term potentiation (LTP) (24, 25).

We next examined whether knockdown of ApKHC1 would affect the localization of Aplysia MHC (ApMHC). We injected ApKHC1 antisense oligonucleotides (15) into sensory neurons to disrupt kinesin-mediated transport and examined localization of MHC RNAs at the processes by in situ hybridization analysis. Analysis of the confocal projection images showed a ~30% decrease in ApMHC staining [unpaired t test, two-tailed P value = 0.014; t(7) = 3.28; mean fluorescence intensity ± SEM of ApMHC staining: control, 21.7 ± 2.03, (n = 4); ApKHC knockdown, 15.1 ± 0.78 (n = 5)] in the processes of sensory neurons that received ApKHC1 antisense oligonucleotide injection (Fig. 4 A and B).

We next examined whether repeated exposure to serotonin (5HT), a modulatory neurotransmitter that produces LTF at sensory and motor neuron synapses, regulates the expression of ApMHC RNA in sensory neurons. Quantitative RT-PCR (qRT-PCR) showed that exposure to five pulses of 5HT (5 × 5HT) increased ApMHC RNA levels in sensory neuron clusters (normalized fold increase after 1 h of 5HT exposure, 1.8 ± 0.13; after 6 h, 0.93 ± 0.37; n = 6; Student t test) (Fig. 5 A). Expression of ApC/EBP was used as a positive control for 5 × 5HT treatment.

To explore the significance of ApMHC up-regulation during memory storage, we studied the electrophysiological consequences of specific knockdown of ApMHC in sensory neurons during short-term facilitation (STF) and initiation and persistence of LTF of sensory and motor neuron synapses. We injected antisense oligonucleotides that specifically degrade ApMHC (unpaired t test, two-tailed P values: control vs antisense-injected, P = 0.0031; t (10) = 3.87; sense oligonucleotide vs antisense-injected, P = 0.0005; t (10) = 5.11; mean fluorescence intensity ± SEM of ApMHC staining: control, 19.5 ± 1.73 (n = 6); sense oligonucleotide, 20.8 ± 1.48 (n = 6); antisense oligonucleotide, 12.1 ± 0.84 (n = 6)] into sensory neurons and measured excitatory post synaptic potentials (EPSPs) (Fig. 4 C and D).
We began by examining STF. For this, ApMHC oligonucleotides (sense and antisense) were injected into sensory neurons cocultured with L7 motor neurons (31). At 4 h after oligonucleotide injection, the cultures were treated with 10 μM 5HT for 5 min. EPSPs were recorded (Fig. 5B) after 10 min of 5HT treatment. An uninfected sensory neuron synapsing with the same motor neuron was used as an internal control for injection. We found the following percent changes in mean EPSP amplitudes: control (untreated), −10.3 ± 7.9 (n = 7); antisense MHC oligonucleotide alone: 7.5 ± 6.4 (n = 10); sense MHC oligonucleotide alone, 1.9 ± 6.1 (n = 6); one pulse of 5HT (1x 5HT), 139.14 ± 51.5 (n = 7); antisense MHC + 1x 5HT, 137.2 ± 27.4 (n = 10); sense MHC + 1x 5HT, 145.2 ± 25.1 (n = 12). Our data shows that neurons that were exposed to one pulse of 5HT, 5HT + antisense ApMHC, and 5HT + sense ApMHC showed no statistically significant differences in EPSPs (F = 0.1924; P = 0.8264, repeated-measures ANOVA; P > 0.05, Tukey’s multiple-comparison test), suggesting that ApMHC RNA levels are not critical for STF.

To determine the role of ApMHC in LTF, we next injected antisense and sense oligonucleotides into sensory neurons in the coculture and then applied five pulses of 10 μM 5HT at 4 h after oligonucleotide injection. Measurements of EPSPs at 24 h and 48 h after the 5HT exposure revealed the following percent changes in mean EPSP amplitudes: 5HT alone: at 24 h, +57 ± 7.5 (n = 8); at 48 h, +51.4 ± 6.7 (n = 8); 5HT + ApMHC antisense oligonucleotide: at 24 h, +3.14 ± 8.5 (n = 12); at 48 h, +3.9 ± 11 (n = 12); 5HT + ApMHC sense oligonucleotide: at 24 h, +52.9 ± 14.7 (n = 8); at 48 h, +46 ± 10.7 (n = 8) (Fig. 5C). Neither the antisense nor the sense MHC oligonucleotides affected basal synaptic transmission. However, the antisense oligonucleotides blocked the 5HT-dependent increase in EPSPs [F(8, 88) = 4.3825; P = 0.00017, repeated-measures ANOVA; P < 0.001, Newman–Keuls post hoc test at both 24 h and 48 h], suggesting that ApMHC is required for the establishment of LTF (Fig. 5C).

We then asked whether inhibition of MHC would affect the persistence of LTF. We injected MHC antisense oligonucleotides at 24 h after induction of LTF with exposure to 5HT and measured EPSPs at 48 h. Interestingly, we found that MHC inhibition did not affect the persistence of LTF. Percent changes in EPSP amplitudes measured at 48 h after exposure to 5HT alone and to 5HT + antisense oligonucleotide injected at 24 h were not significantly different [5HT + ApMHC antisense oligonucleotide injected at 24 h, +73 ± 17.3 (n = 9); at 48 h, +58 ± 11 (n = 9); t (11) = 0.04; two-tailed t value = 0.9722, unpaired t test] (Fig. 5C). These results suggest that ApMHC levels are important for the initiation of LTF, but not for persistence of LTF at Aplysia sensory and motor neuron synapses.

Discussion
Characterization of ApKH1 Transport Complex Has Led to Identification of RNAs Actively Targeted to Synapses of Aplysia CNS. Several previous studies that characterized RNAs prepared from dissected processes of cultured neurons have identified distally localized RNAs in neurons (2, 18, 21, 26). The methodologies applied in those studies are not useful for directly identifying RNAs targeted to synapses of intact brain or brain regions, however. Our NextGen sequence analysis of RNAs isolated from kinesin immunoprecipitates allowed us to identify approximately 5,657 transcripts from Aplysia CNS, 1,184 of which were annotated using Gene Ontology terminology. This analysis will improve as the availability of Aplysia genome sequence and gene annotation increases. The RNAs identified in the kinesin complex constitute ~2–5% of the predicted transcriptome of the Aplysia genome. We also identified several naturally occurring antisense RNA transcripts with functions at synapses that remain to be determined. Our analyses suggest that the transported RNAs are surprisingly diverse and pose the question of why so many different RNAs are targeted to synapses.

One possible explanation is that these different mRNAs are stored at the synapses for later use. Translation of these RNAs might be regulated at specific synapses during the process of storing, maintaining, and reconsolidating long-term memories not only for modifying preexisting synaptic connections, but also for the
formation of new ones. Consistent with this idea, Si et al. (27, 28) have demonstrated learning-dependent activation of synthetically localized RNAs by polyadenylation, which is mediated by CPEB.

Role of Active Molecular Transport in Initiation and Persistence of Long-Term Memories. We previously found that microtubule-dependent kinesin motors are essential for the initiation of LTF, but not for its persistence (15). This finding led us to consider the possibility that during the persistence phase, actin/MHC-dependent delivery of cargos might be important. Contrary to our expectation, however, we found that MHC antisense oligonucleotides blocked initiation of LTF, but did not affect persistence of LTF when injected into sensory neurons at 24 h after injection. This result suggests that MHC levels are no longer critical during the persistence phase. Consistent with the requirement of ApMHC for the initiation of LTF, MHC has been found to be critical for LTP of the CA1 region of mouse hippocampus (24, 25). Taken together, our results lead us to conclude that during the early initiation phase of memory storage, the neuron uses active transport mechanisms, such as microtubule and actin-dependent motors, for delivery of cargos to synapses, but that once gene products arrive at their destination, either through microtubule and actin-dependent or independent of microtubule, no more critical for the persistence of memory storage. At this point, other mechanisms, including basal levels of transport, local regulation of translation, and local protein synthesis, become key determinants of persistence. Thus, these motors control the late phases of plasticity indirectly by supplying the population of mRNAs and proteins that are required for maintenance.

Experimental Procedures

Details of SHT stimulation, isolation of proteins and RNAs, cloning, antibodies, oligonucleotides, qRT-PCR, Western blot analysis, in situ hybridization, microinjection, and electrophysiology are provided in SI Experimental Procedures.

IP of Kinesin Complex and RNA Isolation. We modified previously described buffer conditions to isolate kinesin complexes from the Aplysia CNS (15). The modified buffer contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM KCI, 0.5% Nonidet P-40, 1 mM DTT, 1% ultrapure BSA (Ambion), 1% yeast tRNA, and inhibitors of proteins (Roche), phosphatases (Sigma-Aldrich), and RNAses (Ambion). All of the experiments were conducted at 4°C in an RNase-free environment. Precleared extracts prepared from two adult (50–90 g) Aplysia CNS (for one IP experiment, n = 1) were incubated with 20 μg of affinity-purified anti-kinesin Ab (15). After 6 h of incubation on a rotator, protein A/G beads (Pierce) were added to the immunoprecipitates, followed by another 1 h of rotation. The immunoprecipitates were washed three times with the same buffer, followed by buffer containing 1% Triton X-100 for 2 min. These washes were carried out in separate Eppendorf tubes (siliconized RNase-free) to minimize nonspecific binding of RNA to the plastic. After the third wash, the beads were incubated with Trizol to prepare RNA for Agilent Bioanalyzer, 454 sequencing, microarrays, and qRT-PCR analyses. Equivalent amounts of a rabbit polyclonal Ab against ApTor protein was used in control IPs.

cDNA Library Construction for 454 Sequencing. We used commercial kits (Marathon cDNA Amplification Kit, catalog no. 634913; Clontech) to ensure consistency and reproducibility of library preparations. Methods used for 454 library construction and sequencing have been described previously (29). Three independent biological replicates were sequenced. All original sequence reads were submitted to the National Center for Biotechnology Information’s Sequence Read Archive (project no. SRA009823.3). A total of 40,204 reads were generated, assembled, and used for analysis. The Velvet assembly (24) generated 5,657 unique transcripts.

Microarray Analysis. Microarray and sample preparation and hybridization were performed as described previously (18, 29). Custom microarrays supplemented with 2,000 additional features containing probes (Agilent) for kinesin-immunoprecipitated transcripts identified by 454 sequencing were used in later experiments.

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Supporting Information

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SI Experimental Procedures

5HT Stimulation and Pharmacologic Treatments. The central nervous system (CNS) was isolated from 80–100 g Aplysia californica and kept in L15 media supplemented with salts and glutamine. The CNS tissues were maintained at 8 °C for 14–16 h before serotonin (5HT) stimulation or any pharmacologic treatments. Treatment with 5HT (10 μM) was carried out as described previously (1). Pleural ganglia or sensory clusters were isolated for preparation of total RNA or proteins. Dissections were performed in ice-cold seawater to minimize injury-associated molecular changes.

RT-PCR and Western Blot Analysis. Semiquantitative RT-PCR, quantitative RT-PCR (qRT-PCR), and Western blot analysis and quantitation were done as described previously (2). Aplysia GAPDH or 18S rRNA primers were used as endogenous controls in the qRT-PCR experiments, and data were analyzed as described previously (3, 4).

mRNA in Situ Hybridization. In situ hybridization using digoxigenin (DIG)-labeled riboprobes was carried out as described previously (5). After hybridization, the sense and antisense RNA probes were visualized using a Roche Fluorescent Antibody Enhancer Kit for DIG detection. Images were acquired using an Olympus Fluoview 1000 confocal microscope with 20× objectives. Only projection images are shown in Figs. 3 and 4. For quantitation of the immunocytochemical data, we focused only on the major axons. Quantitative analyses of immunocytochemical data were carried out on confocal image stacks using MetaMorph (Molecular Devices).

Oligonucleotides and Antibodies. Sigma-Genosys synthesized all of the oligonucleotides described in this paper. A previously described affinity-purified polyclonal Ab (2) was used for immunoprecipitation (IP) of kinesin. Rabbit anti-staufen Ab (1:4,000; Abcam), mouse anti-fragile X mental retardation protein Ab (1:5,000; Millipore), and rabbit anti-cyttoplasmic polyadenylation element-binding protein Ab (1:5,000) (1) were used in the Western blot analyses.

Cloning of Probes for in Situ Hybridization. We designed specific primers based on EST sequence information to clone synaptobrevin, Aplysia myosin heavy chain (ApMHC), α-tubulin, and sensorin into a dual-promoter TOPO vector (Invitrogen). These plasmids were used to prepare DIG-labeled antisense RNA probes (Roche Applied Science) for in situ hybridization. After hybridization, the RNAs were detected using a Tyramide RNA detection system (PerkinElmer) following the manufacturer’s protocols. The following primers were used to clone synaptophysin, ApMHC, and α-tubulin: synapticbrevin (480 bp); forward, 5′ TCT GCC AGC AAT TTG ATA ATG TCA CCT TGT GGT 3′; reverse, 5′ GAC ATT CCT TCG CTC TAC GAG TTC AGA GTG 3′;α-tubulin (450 bp): forward, 5′ CCC GCC CCA CGG CAA GTA CAT GGC CTG CTG GTG 3′; reverse, 5′ CAC ATA TAT ATC TAG TAC TCC TCG CTC TCG 3′; ApMHC (330 bp): forward, 5′ CAA GTT CCG CAA GTC CAA GCT CCT GCT TGA AGT 3′; reverse, 5′ CTG TGA TTT CCA CCT TCA ATT 3′. Sensorin was cloned using previously described primers (2).

ApMHC Sense and Antisense Oligonucleotides. These oligonucleotides were designed based on the EST sequence data. The ApMHC EST was cloned from sensory neuron clusters using specific primers. Thiol-modified oligonucleotides (at the first three and last three positions) were synthesized (Sigma-Genosys) and gel-purified. The following oligonucleotides were used: MHC-S1 (sense oligonucleotide): 5′ ATG AAG ACC CAG CTC TGG GAA 3′; MHC-A1 (antisense oligonucleotide): 5′ TTC CGA CAG CTG CTT CAT 3′. These oligonucleotides were dissolved in nuclease-free water and pressure-injected into neurons at a concentration of 50 μg/mL.

Microarray Analysis. RNAs were labeled with Alexa Fluor 546 or Alexa Fluor 647 dye (Invitrogen) and reciprocally hybridized to the foregoing arrays (Agilent), which were developed by our group following the manufacturer’s recommendations. After hybridization, the raw hybridization signal intensities were normalized within the arrays using Agilent software following global normalization using the loess function from the Bioconductor affy package (6). Background filtering was performed with Agilent’s “Well Above Background” flag function using score modulation between the relevant groups of samples, and the differential gene expression analysis was done using Bioconductor samr package (7), followed by false discovery rate analysis (8). The resulting data were imported into the Spotfire DecisionSite for Functional Genomics (TIBCO) for data visualization and further review using principal components analysis, hierarchical clustering, and other methods. Differentially expressed genes were extracted as those demonstrating at least a twofold change in normalized intensities and q-values corresponding to fewer than one or two false-positive results in the set.

454 Sequencing of Kinesin Cargo Library. RNAs prepared from the Aplysia kinesin heavy chain-1 (ApKHCl) immunoprecipitates were reverse transcribed into cDNA with an anchored oligonucleotide dT primer (Clontech), followed by second-strand cDNA synthesis. The double-stranded cDNA was fragmented with the AluI restriction enzyme, and 454-specific adapters were ligated to the resulting fragments. Amplified fragmented DNA with ligated adapters was processed through emulsion-based clonal amplification (emPCR) and captured onto beads. The DNA-containing beads were placed on a Pico titer plate device for pyrosequencing using the 454 GS20 platform. We validated the quantitation, as well as the direction, of the change using absolute qRT-PCR and spiked-in controls. These data allowed us to quantify a given transcript by obtaining the number of reads specific to that transcript, expressed as percentage of the total number of sequences.

Cell Culture, Microinjection, and Electrophysiology. Cell culture and electrophysiology were performed as described previously (9). The electrophysiology experiments were not done blinded. For electrophysiology data, the criterion for basal EPSP was ≥10 mV. Our basal excitatory post-synaptic potentials (EPSPs) ranged from 10 to 20 mV. For long-term facilitation (LTF) measurements, percent increases of ≥40% were accepted for analyses. The antisense and sense oligonucleotide solutions for injection contained Fast Green dye. Presence of the dye in the cell body after pressure injection served as the criterion for injection. After basal EPSP measurements, the oligonucleotides (50 μg/mL) were pressure-injected into the cells. For both the short-term facilitation (STF) and LTF measurements, 5HT was applied at 4 h after oligonucleotide injection. For the LTF experiments, EPSPs were measured at 24 h and 48 h after five 10 μM 5HT
applications, whereas STF was measured at 10 min after one 10 μM 5HT application. For LTF persistence experiments, the MHC oligonucleotides were injected 24 h after induction of LTF with 5HT exposure, and EPSPs were measured at 48 h.


Fig. S1. Antisense RNAs associated with kinesin cargo. The percentage of antisense transcripts relative to sense transcripts for the given genes identified from RNA-seq experiments is shown.

Fig. S2. Venn diagram showing Gene Ontology category analysis of RNAs localized to rat hippocampal neurons (1) and kinesin-associated RNAs from Aplysia CNS.

Hierarchical clustering of the differentially expressed genes in total RNAs and Kinesin IPs from CNS.

Fig. S3. Hierarchical clustering of the differentially expressed genes in total RNAs and kinesin IPs from CNS. The heat diagram shows the enrichment of particular mRNAs in the ApKHC1 IP complex (Right; samples IP1–IP4) compared with the mRNA abundance in the Aplysia CNS (Left; samples T1–T4).

Table S1. One hundred thirty-nine annotated Aplysia transcripts differentially enriched in RNA IP samples identified from the microarray experiments

Table S2. Twelve 12 cytoskeleton- (bold) and 29 synapse-related RNAs enriched in the RNA IP samples

Table S3. Eleven neuropeptide precursor RNAs enriched in the RNA IP samples

Table S4. Assembly statistics for 454 sequencing of cDNAs prepared from the kinesin immunoprecipitates from Aplysia CNS

Table S5. Annotation of RNAs identified from 454 sequencing by homology searches using BLAST
Table S6. Neuropeptide precursor RNAs identified by 454 sequencing

Table S7. RNAs differentially enriched in kinesin IPs identified by dual-color microarray analysis of total RNAs prepared from the adult CNS of *Aplysia* and kinesin IPs

Table S8. Human homologs of the ESTs identified in the kinesin IP complex from *Aplysia* CNS

Table S9. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of 147 human homologs of ESTs identified in the kinesin IP complex from *Aplysia* CNS

Table S10. Gene Ontology terms shared by *Aplysia* and rat, with annotation

Other Supporting Information Files

Dataset S1 (XLSX)