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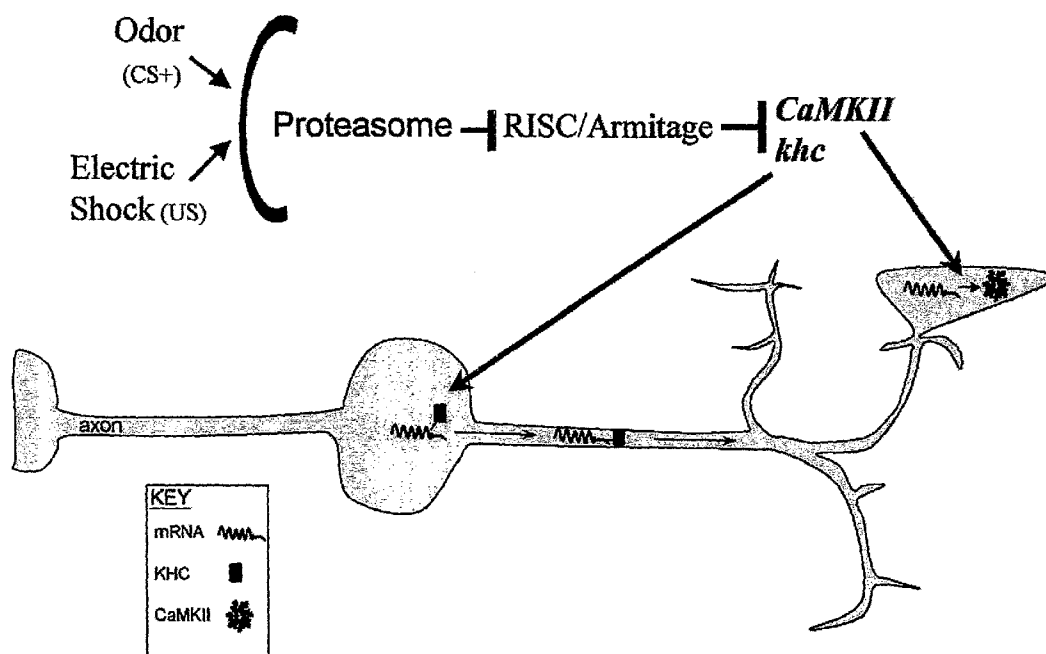
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(54) Title: COMPOSITIONS AND METHODS TO MODULATE MEMORY



(57) Abstract: Disclosed herein are compositions and methods for modulating memory. In one aspect, the regulation of protein expression of certain proteins related to both long-term and short-term memory is described. In one aspect, modulating the RISC pathway and associated targets is described.

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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE****A PCT PATENT APPLICATION****FOR****COMPOSITIONS AND METHODS TO MODULATE MEMORY****FIELD OF THE INVENTION**

[0001] The present invention pertains to compositions and methods for modulating memory. In particular, the invention relates to the modulation of the RNA-induced silencing complex ("RISC") pathway as a means to enhance or repair memory.

**BACKGROUND OF THE INVENTION**

[0002] It has long been known that the establishment of long-lasting forms of memory requires protein synthesis, a feature of memory in both vertebrates and invertebrate. A number of cellular changes appear to accompany protein synthesis and the formation of memory, which modify synapse and circuit function and, in net effect, behavior. But it is not yet known how protein synthesis is deployed across the nervous system and contributes to the formation of a particular memory. Of special interest is protein synthesis localized to the synapse, since such synthesis may confer selective synaptic change and the stable modification of a circuit. An interesting question is whether protein synthesis localized to specific synapses whose modification alters the performance of a circuit, or is it a widespread feature of a system primed to store information by other more localized means.

[0003] A well-defined system for the study of memory is the olfactory/electric shock paradigm of *Drosophila*. A memory of odor associated with electric shock, revealed by tactic avoidance behavior, can be induced in phases that include short-term (STM) and long-term memory (LTM). These phases of memory are distinguished by their dependence on training

protocol, genetic pathway and protein synthesis. A requirement for protein synthesis in olfactory LTM was demonstrated long ago and recently reinforced by the identification of long-term memory mutants as genes with functions in mRNA transport and translation. These genes encode proteins such as the Stauf RNA-binding protein, the transcriptional regulator CREB, and the regulator of protein synthesis known as CPEB, all of which are homologs of mammalian proteins with parallel functions in regulating synaptic function in the brain, and with roles in memory. The study of short-term (protein synthesis-independent) memory in *Drosophila* has also identified the same biochemical pathways utilizing the synthesis of the signaling molecule cAMP as are at work in memory in mammals and the well known model for memory studies, *Aplysia*. These observations have established an overall and precise conservation of memory mechanisms between *Drosophila* and mammals, which includes humans.

[0004] Protein expression is critical to both long-term and short-term memory. Equally important is where protein expression occurs. The modulation of protein expression can affect memory in individuals. There is a clear need to understand and manipulate the protein expression that is central to memory. The ability to affect memory can assist individuals whose memory has been negatively impacted by disease or old age.

#### BRIEF SUMMARY OF THE INVENTION

[0005] The present invention is directed toward compositions and methods for modulating memory. In one aspect, the invention pertains to regulating protein expression of certain proteins related to both long-term and short-term memory. In a particular aspect, the invention relates to the modulation of the RISC pathway.

[0006] One embodiment of the present invention relates to compositions and methods employed to affect memory. In one aspect, the invention is directed toward modulating the RISC pathway through the use of one or more cholinergic pharmacological agents and combinations thereof. In one aspect, the pharmacological agent modulates proteasome-mediated degradation of one or more components of RISC. In a particular aspect the component of RISC is Armitage. Other components of RISC are listed in Table 1. In one

aspect, the agent is a proteasome activator. Proteasome activators include 11S Regulator ( $\alpha$  and  $\beta$  subunits), 19S Regulatory complex, REG $\alpha$ , REG $\beta$ , REG $\gamma$ , PA28, PA28 $\gamma$  and PA700. Proteasome activators increase proteasome degradation of components of RISC. These RISC components include Mov10, Ago2, Dicer, TRBP, DGCR8, FMRP and combinations thereof.

[0007] Another aspect of the invention relates to compositions and methods of modulating one or more RISC target proteins. In one aspect, the target protein is Calmodulin-dependent Kinase. In a particular aspect, the Calmodulin-dependent Kinase is CaMKII. In another aspect, the target protein is Kinesin Heavy Chain or Staufin. Other target proteins are listed in Tables 2, 3, and 4.

[0008] Both muscarinic and nicotinic cholinergic pharmacological agents can be used to modulate the RISC pathway and associated target proteins. As used herein, a cholinergic agent is any chemical which functions to enhance the effects mediated by acetylcholine. These include acetylcholine's precursors and cofactors, acetylcholine receptor agonists, and cholinergic enzymes such as the anticholinesterases. In one aspect, the cholinergic agent is a nicotine. In another aspect, the cholinergic agent is acetylcholine. Other cholinergic agents include muscarine, bethanechol, carbachol, Cevimeline, Pilocarpine, suberylcholine, succinylcholine, anabasine, decamethonium, and Aceclidine. Cholinesterase inhibitors also can be used to modulate the RISC pathway and associated target proteins. Cholinesterase inhibitors include Ambenonium, Donepezil, Edrophonium, Galantamine, Neostigmine, Physostigmine, Pyridostigmine, Rivastigmine, and Tacrine.

[0009] In another embodiment, the invention is directed toward modulating the RISC pathway and associated target proteins through the use of anti-microRNA (miRNA) oligonucleotides. The anti-miRNA oligonucleotides disrupt RISC activity by binding to miRNA's, effectors that guide the RISC proteins to regulate the synthesis of protein from particular genes.

[0010] For a better understanding of the present invention, together with other and further objects thereof, reference is made to the accompanying drawings and detailed description and its scope will be pointed out in the appended claims.

## BRIEF DESCRIPTION OF THE DRAWINGS

[0011] FIGURE 1 shows synaptic CaMKII is regulated by neural activity: (a) a schematic view of the adult *Drosophila* brain, illustrating the dendritic and axonal arbors of the Projection Neurons (PNs) of the olfactory system. PN cell bodies, indicated in the region of enlarged inset, lie adjacent to the antennal lobe (AL), the first-order processing center for olfactory sensory input. PN dendrites extend into the AL, a neuropil consisting of sensory axon termini and local interneurons specifically connected in synaptic structures known as glomeruli. The PN axons project along the internal antennal cerebral tract (iACT), branching into the mushroom body **calyx**, where they form synapses with the Kenyon cells, and the lateral horn (LH). Expression of the presynaptic vesicle marker n-Synaptobrevin::GFP (**n-Syb::GFP**; P. Salvaterra, City of Hope) specifically in PNs labels their dendrites in the AL, and axon termini in the calyx and lateral horn (green color in the inset); (b) in the calyx, choline acetyltransferase (ChAT; red color in **B**, shown alone in **B'**) localizes to the PN presynaptic terminals along with n-Syb::GFP (green color in **B**). The *UAS-n-syb::GFP* transgene is driven in the PNs by *GHI46-GAL4*; (c) in the calyx, CaMKII protein (anti-CaMKII, blue color in **C**, shown alone in **C'**) localizes to the PN presynaptic terminals, along with Chat (red color in **C**); (d) in the antennal lobe (AL), CaMKII is strongly concentrated in the dendritic neuropil of the glomeruli; (e) a high magnification view of a few glomeruli reveals concentration of CaMKII (blue color in **E**, shown alone in **E''**) at postsynaptic sites labeled with anti-Discs Large (DLG) antibody (red color in **E**, shown alone in **E'**; Koh *et al.*, 1999); (f) dendritic CaMKII (red color in **F**, shown alone in **F'**) does not, in contrast, display significant co-localization with the presynaptic marker, n-syb::GFP (green color in **F**, shown alone in **F''**); (g) nicotine induction of synaptic CaMKII expression in the antennal lobe. Adult brains were placed in AHL with (**G'**, +**nic**) or without (**G**) 100  $\mu$ M nicotine or 50  $\mu$ M ACh for 10-20 minutes and stained with anti-CaMKII antibody. The CaMKII level is rapidly increased by nicotine or acetylcholine incubation (see quantification in **J**); (h) adult brain specimens explanted and treated as in **G** (with nicotine, **H'**) and stained with anti-DLG antibody; (i) adult brain specimens explanted and treated as in **G** (with nicotine, **I'**) and stained with anti-ARD antibody, labeling a nAChR  $\beta$ -subunit; (j) quantitative analysis of CaMKII, ARD and DLG fluorescence following explant into AHL media containing nicotine, acetylcholine (ACh) or neither. Fluorescence was determined under constant image

acquisition conditions, from a total of ~12 specimens for each condition. Error bars indicate the SEM.; and (k) Western analysis of adult brains following explant into culture media, with or without nicotine, as described for **G**. Nicotine incubation increased the total CaMKII protein level 4-fold, without affecting the level of  $\alpha_1$ -tubulin (**tub**, lower panel).

[0012] FIGURE 2 demonstrates dendritic expression of CaMKII is mediated by its 3'UTR:

Animals expressing one of the three transgenic constructs, *EYFP<sup>3'UTR</sup>*, *CaMKII::EYFP<sup>NUT</sup>* or *CaMKII::EYFP<sup>3'UTR</sup>* in a subset of Projection Neurons (with the *GHI46-GAL4* driver) were stained with antibodies, as indicated, and examined by confocal microscopy; (a) a control cytoplasmic *UAS-GFP* construct weakly labels glomeruli (green color in **A**, shown alone in **A'**). The glomeruli were additionally stained with MAbnc82 (red color in **A**; Laissue *et al.*, 1999). Two glomeruli, DL3 and DA1, are indicated for reference (see **E**, **F** and **H**); (b) with a fusion of EGFP to the *CaMKII* 3'UTR (*EYFP<sup>3'UTR</sup>*), strong punctate GFP labeling is found in the antennal lobe (AL; **B**) and calyx (**B'**), (c) with the *CaMKII* 3'UTR absent in *CaMKII::EYFP<sup>NUT</sup>*, labeling of the AL is weak, but the calyx is strongly labeled (**C'**); (d) with the *CaMKII::EYFP<sup>3'UTR</sup>* construct, with the *CaMKII* 3'UTR present, both the AL (**D**) and calyx (**D'**) are strongly labeled; (e, f, & g) high magnification views of the fluorescence of *EYFP<sup>3'UTR</sup>* (**E**) *CaMKII::EYFP<sup>NUT</sup>* (**F**) and *CaMKII::EYFP<sup>3'UTR</sup>* (**G**) in single glomeruli (GFP shown in green color in **E**, **F** and **G**, alone in **E'**, **F'** and **G'**). In all cases, co-localization with the post-synaptic marker ARD (nAChR) is observed (anti-ARD in red in **E**, **F**, and **G**; shown alone in **E''**, **F''**, and **G''**), as well as co-localization with endogenous CaMKII (blue color in **E** and **F**; shown alone in **E'''** and **F'''**); (h) a high magnification view of cytoplasmic GFP expression in a single glomerulus, DA1 reveals distinction from *EYFP<sup>3'UTR</sup>* expression; (i, j, & k) localization of *EYFP<sup>3'UTR</sup>* (**I**) *CaMKII::EYFP<sup>NUT</sup>* (**J**) and *CD8::GFP* (**K**) in the calyx. Both the *EYFP<sup>3'UTR</sup>* and *CaMKII::EYFP<sup>NUT</sup>* products (GFP fluorescence in green color) are strongly localized to presynaptic axon terminals (arrowheads), which are co-labeled with anti-Chat (red color in all panels). However, *CD8::GFP* displays generalized membrane localization, and is not concentrated in the synaptic bouton.

[0013] FIGURE 3 shows that neural activity induces 3'UTR-dependent expression of CaMKII: (a-f) adult brains were removed from animals expressing the one of the transgenes *EYFP<sup>3'UTR</sup>* (A, B) *CaMKII::EYFP<sup>NUT</sup>* (C, D) or *CD8::GFP* (E, F) and incubated for 20 minutes in culture medium alone (A, C, E) or with 100  $\mu$ M nicotine (B, D, F). In all cases, transgene expression was localized to PNs with the *GHI46-GAL4* driver. After incubation, specimens were stained with anti-GFP antibody and imaged under quantitative conditions for GFP and anti-GFP fluorescence, shown in rows as indicated. GFP fluorescence requires a slow maturation of the nascent polypeptide. Hence we utilized anti-GFP staining for quantification; (g) quantitative analysis of ~12 specimens for each experimental condition reveals that nicotine incubation induces an average 3.4 fold increase in the expression of *EYFP<sup>3'UTR</sup>*, and little or no increase with the *CaMKII::EYFP<sup>NUT</sup>* and *CD8::GFP* transgenes. Error bars are for SEM; (h-k) high-magnification views of single glomeruli after incubation in culture medium alone (H), or with nicotine (I), ACh (J) or KCl (40  $\mu$ M; K) to stimulate neural activity. Brains were isolated from animals harboring the *EYFP::CaMKII<sup>3'UTR</sup>* transgene, driven by *GHI46-GAL4*. Anti-GFP fluorescence reveals a significant increase in the size and intensity of post-synaptic puncta (arrowheads) after incubation with nicotine, ACh or KCl.

[0014] FIGURE 4 shows a pattern of synaptic protein synthesis associated with a long-term memory: Animals harboring the *EYFP<sup>3'UTR</sup>* or cytoplasmic EYFP (No UTR) and the *GHI46-GAL4* driver were subjected to 'spaced training' or the variations indicated (see Experimental Procedures for details). The animals were grown at 25°C and humidity through eclosion, trained as indicated, maintained at 17°C for 22 to 24 hours post-training, and tested for LTM. The brains of animals with a positive PI score were isolated in PBS, stained with MAbnc82 to label glomeruli and imaged in a single operational day under quantitative conditions. The graph depicts the  $\Delta F/F$  change in EYFP fluorescence measured for each identified glomerulus, which is an average derived from confocal stacks through individual glomeruli accumulated from 8 brains per experiment. Each experiment was performed from 3-5 trials. The training variations represented are:

OCT: *EYFP<sup>3'UTR</sup>* expression, Octanol (OCT) as CS+, methylcyclohexanol (MCH) as CS-  
 MCH: *EYFP<sup>3'UTR</sup>* expression, MCH as CS+, OCT as CS-.

No UTR, ES/OCT: UAS-EGFP (cytoplasmic) expression, OCT as CS+, MCH as CS-.

**Unpaired, ES/MCH:** *EYFP*<sup>3'UTR</sup> expression, MCH as CS+, no CS- used.

**Es:** Spaced presentation of electric shock (US), without odorant.

Sample data images are presented for the 'OCT as CS+' (above the graph) and 'MCH as CS+' (below the graph) experimental conditions. Fold increase was calculated as  $\Delta F/F = (F_1 - F_0)/F_0$ . One-way ANOVA (with Bonferroni correction) was performed to test level of significance among the different populations of specimens (n=9). Differences, where present, were highly significant ( $p < 0.0001$ ). Error bars are for SEM.

[0015] FIGURE 5 shows data of *CaMKII* mRNA transport along dendrites in response to neural activity and training: The bacteriophage MS2 RNA tracking system (Rook *et al*, 2000) was adapted to *Drosophila* (see Experimental Procedures) and used to monitor *CaMKII* mRNA localization in the adult brain: (a & b) the MS2::GFP fusion protein (green color in A, B; detected by anti-GFP staining in A', B') was expressed in the adult brain under control of *GHI46-GAL4*. When co-expressed with an MS2 binding site-tagged full-length *CaMKII* cDNA (*UAS-ms2bs-CaMKII*), GFP-positive puncta are enriched in the antennal lobe neuropil (arrowheads in B, B'); (c & d) adult brains harboring *UAS-MS2::GFP::nls* and the *UAS-ms2bs-CaMKII* transgene were explanted into culture medium with (D) or without (C) 50  $\mu$ M ACh. The ACh incubation resulted in a significantly higher frequency of GFP-positive puncta, and higher overall fluorescence in glomeruli (quantification in K); (e) animals harboring *UAS-MS2::GFP::nls*, *UAS-ms2bs-CaMKII* and homozygous for the *armi*<sup>72.1</sup> mutation display an enhanced level of GFP-positive puncta in glomeruli (arrowhead), similar to that observed following incubation with ACh (compare with D); (f) puncta labeled by *UAS-MS2::GFP::nls* and *UAS-ms2bs-CaMKII* (anti-GFP labeling, red color in F, shown alone in F') display co-localization with KHC (anti-KHC staining, blue color in F, shown alone in F''). Correlation coefficient = 0.75; (g & h) the frequency of GFP-positive puncta is significantly increased in the antennal lobe of *UAS-MS2::GFP::nls*, *UAS-ms2bs-CaMKII* animals subjected to spaced training (H) compared to naïve animals of the same genotype (G). Enhanced neuropil localization was not observed after a 3-minute single training episode (not shown); (i & j) brains harboring *UAS-MS2::GFP::nls* and the MS2 binding site-tagged mouse  $\alpha$ *CaMKII* 3'UTR transgene were explanted into culture media with (J) or without (I) 100  $\mu$ M nicotine for 20 minutes. Nicotine incubation dramatically increases the localization of GFP to glomeruli; and (k) quantitative analysis of GFP-positive puncta fluorescence in

glomeruli in the various conditions described in **B, D, E, H** and **J**, determined from averaging fluorescence intensity from at least 10 specimens, and calculating  $\Delta F/F$  relative to  $F$  for the relevant control specimens represented in **A, C, G** and **I**. Error bars are for SEM.

[0016] FIGURE 6 depicts data showing Armitage regulates synaptic synthesis of CaMKII: (a- f) in all panels, EYFP fluorescence was recorded under quantitative conditions from wildtype (**A, C, E**) or *armi<sup>1</sup>/armi<sup>72.1</sup>* (**B, D, F**) animals harboring the transgenes *EYFP::CaMKII<sup>Nut</sup>* (**A, B**), *EYFP<sup>3'UTR</sup>* (**C, D**) or *CD8::GFP* (**E, F**), in all cases driven by *GH146-GAL4*. Fluorescence from *EYFP::CaMKII<sup>Nut</sup>* and *EYFP<sup>3'UTR</sup>* was significantly increased in *armi* (see **G**) but unchanged for *CD8::GFP* expression; (g) quantification of antennal lobe dendritic fluorescence for n=7-8 brains of each genotype (\*\* indicates significance by the two-sample t-test of  $p < 0.05$ ); (h & h') affinity-purified anti-Armi antibody reveals punctate Armi expression concentrated at the PN presynaptic terminals of the calyx (green color in **H**, shown alone in **H'**), (i) an adult brain from an animal expressing *UAS-EGFP::Armi* under *elav-GAL4* control displays punctate dendritic localization of EGFP::Armi to the antennal lobe (AL) glomeruli; (j) Armi protein (~160 KD, red arrowhead) isolated from adult brain is revealed by Western analysis, and absent from *armi<sup>72.1</sup>/armi<sup>72.1</sup>* adult brain.; and (k) putative miR-280 target sites on the *CaMKII* and *KHC* 3'UTRs. Both sites display a perfect match for the first 7-8 bases at the 5'-end of miR-280. A binding site for miR-289 is also found on the *CaMKII* 3'UTR, as indicated. A straight bar (|) indicates homology, and ':' indicates G, U pairing. Non-pairing bases are shown in subscript. Gaps were introduced for optimal alignment.

[0017] FIGURE 7 presents data showing auto-regulation of Armi expression and the suppression of CaMKII and KHC synthesis: (a) **Left panel:** Western analysis of adult brain isolated from animals expressing GFP::Armi under control of the pan-neural driver *elav-GAL4* (+) display reduced CaMKII protein compared to brain isolate from *elav-GAL4* (-) animals. **Right panel:** Western analysis of adult brain from *armi<sup>72.1</sup>/+*, *armi<sup>72.1</sup>/armi<sup>72.1</sup>* animals, and *armi<sup>+</sup>* animals expressing the *UAS-GFP::Armi* transgene. The blot was stained to reveal Armi protein. Armi is absent in *armi<sup>72.1</sup>/armi<sup>72.1</sup>* mutants and animals expressing GFP::Armi in the brain; (b & c) expression of *UAS-GFP::Armi* by the *elav-GAL4* driver (**C, C'**) results in a reduction of CaMKII expression (anti-CaMKII, red color in **B, C**; shown

alone in B', C') in the antennal lobe glomeruli. Expression of *UAS-src::GFP* by *elav-GAL4* (B, B') has no such effect; (d & e) in subsets of PNs that express GFP::Armi by the *GHI46-GAL4* driver at elevated levels (yellow outline; green color in D, alone in D''), CaMKII expression (red color in D, shown alone in D') is strongly reduced. KHC expression (blue color in D, shown alone in D''') is similarly reduced. When *UAS-CD8::GFP* is present instead (E-E'''), strongly GFP-positive cells display normal CaMKII and KHC levels; (f & g) Armi displays dosage sensitive expression and effects on CaMKII expression. Pan-neural (*elav-GAL4* driven) expression of GFP-Armi (F and G) results in significant GFP fluorescence in only subsets of cells. The level of GFP fluorescence in such cells is significantly greater in an *armi<sup>72.1</sup>/armi<sup>72.1</sup>* genetic background (compare the outlined area in G'' and F''). Notably, CaMKII expression (anti-CaMKII, red color in F, G, shown alone in F', G') is markedly increased in the *armi<sup>72.1</sup>/armi<sup>72.1</sup>* specimen, but less so in the cells distinguished by high GFP::Armi expression (yellow outline in G').

[0018] FIGURE 8 shows Proteasome-dependent regulation of Armi and CaMKII expression: (a - b) brains harboring *elav-GAL4*, *UAS-GFP::Armi* with (C, D) or without (A, B) the temperature-sensitive proteasome subunit *UAS-DTS5* transgene (Speese *et al.*, 2003). GFP fluorescence from the GFP::Armi transgene is shown in green in A-B and alone in A'-B'. When animals carrying *UAS-DTS5* are maintained at 17°C (C), proteasome activity is inhibited and antennal lobe GFP fluorescence is ~3-fold greater (see E) than in animals grown at 17°C without the *UAS-DTS5* transgene (A, A'). E; (e) quantitative analysis of GFP fluorescence in the experiment described for A-D. (). When *UAS-DTS5* is present and active, fluorescence is greatly increased (DTS17, 17°C); (f-h) adult brains harboring *elav-GAL4* and *UAS-GFP::Armi* were explanted into culture medium for 20 minutes with (G) or without (F) 100 µM nicotine. With nicotine present, PNs (yellow outline) display reduced GFP::Armi expression (compare F' with G'), while CaMKII expression is strongly induced (G). When the brains were pre-incubated with 100 µM lactacystin prior to nicotine incubation, the loss of GFP::Armi fluorescence and the induction of CaMKII expression were both blocked (H, H'); (i) Western analysis of adult brain following explant into culture medium with or without nicotine (nic), as indicated. Twenty minutes incubation with nicotine resulted in the near complete elimination of Armi, while an  $\alpha$ -tubulin control (tub) was unchanged; (j) quantitative analysis of antennal lobe fluorescence from specimens prepared as described for

F-H. Fluorescence from GFP::Armi expression and anti-CaMKII staining was averaged following imaging of glomeruli in 6-8 brains; (k) Armi is stabilized by inhibition of the proteasome. Western analysis of adult brain following explant into culture medium with or without nicotine and/or lactacystin, as indicated. Nicotine incubation alone results in near complete loss of Armi, as in I. When pre-incubated with 100  $\mu$ M lactacystin prior to nicotine, Armi remained at the normal level; (l) the induction of CaMKII (CKII) expression was blocked by a 40 minute pre-incubation with the proteasome inhibitor lactacystin prior to nicotine addition in culture. Neither nicotine or the combination of nicotine and lactacystin affected  $\alpha$ -tubulin (tub) expression; (m) stocks of the indicated genotypes were prepared genetically and grown for training as described in the Experimental Procedures. In animals bearing *CaMKII-GAL4/+* (CKIIGal4), both the short-term (STM) and long-term (LTM) paradigms produced normal Performance Index (PI) scores. However, animals bearing both *CaMKII-GAL4* and the *UAS-CaMKII<sup>hpn</sup>* transgene (CKII<sup>hpn</sup>) displayed normal STM, but significantly reduced LTM. Similarly, *armi<sup>72.1</sup>/+* animals displayed normal STM and LTM scores, but *armi<sup>72.1</sup>/Df(3L)E1* siblings displayed normal STM and deficient LTM, like animals expressing the *CaMKII<sup>hpn</sup>* transgene. A phenotype was observed with *armi<sup>72.1</sup>/armi<sup>72.1</sup>* homozygotes (not shown). The LTM defect of *armi* mutant animals was rescued by the *GFP::Armi* transgene, expressed pan-neurally by the *elav-GAL4* driver, whereas animals expressing GFP::Armi in an *armi/+* background displayed normal STM and LTM. Error bars are for SEM. Two-sample t-test with unequal variance was performed for all statistical analysis ( $p < 0.05$ ); and (n) adult brain expression of the *CaMKII* hairpin transgene *UAS-CaMKII<sup>hpn</sup>* (hpin) resulted in near elimination of CaMKII protein (CKII). Wildtype (CS) is shown in the left lane.

[0019] FIGURE 9 is a model for the control of synaptic protein synthesis in the establishment of a stable memory: An integrated signal resulting from coincident odor (CS+) and electric shock (US) triggers the proteasome-mediated degradation of Armitage, releasing RISC pathway suppression of target gene expression, both at the synapse and cell body. In the case of Kinesin Heavy Chain, increased synthesis may facilitate synaptic transport of mRNA, including *CaMKII*. In addition, *CaMKII* and other target mRNAs may be regulated directly at the synapse.

[0020] FIGURE 10 shows wild-type and *aub<sup>HN</sup>/aub<sup>QC42</sup>* adult brains were stained with anti-CaMKII antibody and examined by confocal microscopy. Regulation of EYFP-CaMKII transgenes by *aubergine* was also examined by measuring EYFP fluorescence under quantitative conditions and data analysis in ImageJ.

- A) A wildtype (CS) brain stained with anti-CaMKII.
- B) Adult *aub<sup>HN</sup>/aub<sup>QC42</sup>* brains were stained in parallel to those in panel A, and imaged under the same conditions. The level of CaMKII in *aub/aub* is substantially higher than in the wildtype (A; n=10 specimens).
- C) Brains harboring the mushroom body (MB)-specific *247-Gal4* driver and *UAS-EYFP::CaMKII<sup>3'UTR</sup>* were imaged under quantitative conditions. EYFP fluorescence is seen predominantly in the MB neuropil. Only the  $\beta$  lobe is shown.
- D) In the *aub<sup>HN</sup>/aub<sup>QC42</sup>* background, the level of *EYFP::CaMKII<sup>3'UTR</sup>* expression in the MB neuropil is significantly higher than in the wild-type (C; quantification in G).
- E) Adult brains harboring *247-Gal4, UAS-EYFP<sup>3'UTR</sup>* were imaged under quantitative conditions. EYFP fluorescence is seen predominantly in the MB neuropil. Only the  $\beta$  lobe is shown.
- F) In the *aub<sup>HN</sup>/aub<sup>QC42</sup>* background, the level of *EYFP<sup>3'UTR</sup>* expression in the MB neuropil is significantly higher than in the wildtype background (E; quantification in G).
- G) Average fluorescent intensity of the genotypes described in panels C-F. Statistical significance is measured by the two-sided t-test with unequal variance ( $p < 0.001$ ). Significant differences are indicated by \*\*. Error bars are for SEM.

[0021] FIGURE 11A shows Dig-U labeled LNA probe localization of dmMir280 in the presynaptic terminals of the axonal terminals at the Mushroom Body Calyx, where olfactory memory is processed and stored.

[0022] FIGURE 11B shows Dig-U labeled LNA probe localization of dmMir280 in the neuropils of the Antennal Lobe (AL) and Mushroom Body (MB), where olfactory long-term memory is processed and stored.

## DETAILED DESCRIPTION OF THE INVENTION

[0023] The present invention is directed toward compositions and methods for modulating memory. In one aspect, the invention pertains to regulating protein expression of certain proteins related to both long-term and short-term memory.

[0024] All references cited herein are incorporated in their entirety by reference.

[0025] One embodiment of the present invention relates to compositions and methods employed to affect memory. In one aspect, the invention is directed toward modulating the RISC pathway through the use of one or more cholinergic pharmacological agents and combinations thereof. In one aspect, the pharmacological agent modulates proteasome-mediated degradation of one or more components of RISC. In a particular aspect the component of RISC is Armitage. Other components of RISC are listed in Table 1.

[0026] The compositions and methods that modulate the RISC pathway can be used to treat or alleviate the symptoms of Fragile X mental retardation Syndrome. FMRP, the protein responsible for the Fragile X mental retardation Syndrome, is an RNA-binding protein involved in localization and translation of neuronal mRNAs. The Drosophila homolog, Fmr1, is associated with the RISC complex.

[0027] Another aspect of the invention relates to compositions and methods of modulating one or more RISC target proteins. In one aspect, the target protein is Calmodulin-dependent Kinase. In a particular aspect, the Calmodulin-dependent Kinase is CaMKII. In another aspect, the target protein is Kinesin Heavy Chain. Other target proteins are listed in Tables 2, 3, and 4.

[0028] Both muscarinic and nicotinic cholinergic pharmacological agents can be used to modulate the RISC pathway and associated target proteins. In one aspect, the cholinergic agent is a nicotine. In another aspect, the cholinergic agent is acetylcholine. One skilled in the art is familiar with cholinergic agonists, such as esters of choline including, acetylcholine, pilocarpine, carbachol and bethanechol chloride. It is well appreciated in the art that there

are muscarinic and nicotinic cholinergic agonists. Any suitable pharmacology text can be consulted including, but not limited to, the Physician's Desk Reference. (See, Physician's Desk Reference, 54th ed., Medical Economics Company, Inc., the entire teaching of which is incorporated herein by reference.)

[0029] Another embodiment of the invention involves increasing concentrations or activity of acetylcholine by inhibiting its metabolism through the use of Ach esterase inhibitors, such as neostigmine or physostigmine. Other acetylcholinesterase inhibitors include donepezil (Aricept<sup>TM</sup>), rivastigmine (Exelon<sup>TM</sup>) and galantamine (Reminyl<sup>TM</sup>). Galantamine also enhances the action of acetylcholine on some receptors in the brain. Another neuro-receptor antagonist is memantine (Ebixa<sup>TM</sup>).

[0030] Another embodiment of the invention involves modulating the activity of proteasomes through the use of proteasome activators. Proteasome activators enhance the peptidase activity of proteasomes, which results in the hydrolysis of protein substrates including various protein components of RISC. Thus, proteasome activators can release RISC pathway suppression of target gene expression at synapses and in the cell body. Examples of proteasome activators include 11S Regulator ( $\alpha$  and  $\beta$  subunits), 19S Regulatory complex, REG $\alpha$ , REG $\beta$ , REG $\gamma$ , PA28, PA28 $\gamma$  and PA700.

[0031] Proteasome activators can be used to treat deficits of contextual learning and long-term potentiation. The ubiquitin-proteasome cascade is required for mammalian long-term memory formation. Moreover, mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation.

[0032] Proteasome activators can also be used to treat or alleviate the symptoms of Parkinson's disease. Parkin (PARK2) has a RING finger domain (signature sequence of Ubiquitin ligase). Mutations in the ubiquitin ligase Parkin results in Autosomal Recessive Juvenile Parkinsonism (AR-JP). Mutation in PARK2 is also associated with 50% of patients of autosomal recessive Parkinson's disease (APRD), which is the most common form of PD. Moreover, recent findings of defects in the ubiquitin-proteasome system in hereditary and

sporadic forms of the illness suggest that environmental proteasome inhibitors are candidate PD-inducing toxins.

[0033] Proteasome activators can also be used to treat or alleviate the symptoms of Alzheimer's disease, Brainstem Lewy body, Bunia Bodies (in ALS), Nuclear inclusions (in Huntington's disease), Spinocerebellar Ataxia, and Spinal and Bulbar Muscular dystrophy (SBMA). The aggregated disease-specific proteins of these diseases or conditions inhibit the activity of the ubiquitin-proteasome system, and proteinaceous deposits associated with these diseases or conditions have been shown to be immunopositive for ubiquitin.

[0034] In addition to treating neurodegenerative diseases, the compositions and methods of the invention can be used in the treatment of cancer, including B-cell malignancies. Specifically, proteasome inhibitors induce apoptosis, have *in vivo* antitumor efficacy, and sensitize malignant cells and tumors to the pro-apoptotic effects of conventional chemotherapeutics and radiation therapy. In addition, anti-miRNA oligonucleotides can be used in the treatment of cancer, including colon cancer, Burkitt's lymphoma, B-cell lymphoma, and lung adenocarcinoma.

[0035] Long-lasting forms of memory require protein synthesis. The present invention involves neural activity directing mRNA of  $\text{Ca}^{2+}$ , Calmodulin-dependent Protein Kinase II (CaMKII) to post-synaptic sites, where it is translated. These features of CaMKII expression are recapitulated during the induction of a long-term memory, and produce a pattern of local protein synthesis specific to the memory. The synaptic synthesis of CaMKII is regulated by components of the RISC pathway, including the SDE3 helicase Armitage, which is specifically required for the establishment of long-lasting memory. Armitage is regulated by neural activity, which triggers its rapid Proteasome-mediated degradation. Armitage is a RNA helicase. The homologous human RNA helicase RISC component is MoV10 like 1 protein. The present invention, in part, shows that the degradative control of RISC underlies the synaptic pattern of protein synthesis associated with the establishment of a stable memory.

[0036] Since the mammalian CaMKII is found at synapses, where its synthesis is induced by neural activity (reviewed by Richter and Lorenz, 2002), investigators turned their

attention to the homologous *CaMKII* gene in *Drosophila*. The *Drosophila* CaMKII protein is involved in neuromuscular synaptic plasticity and memory in the courtship-conditioning paradigm (Griffith *et al.*, 1993; Koh *et al.*, 1999). CaMKII is localized to both pre- and post-synaptic sites in the adult *Drosophila* brain (Figure 1B-1F). We focused our attention on the olfactory system because of its well-described neural components, circuitry and paradigms for the establishment of memory (Davis, 2004). This system consists of sensory neurons and interneurons that together form an early receptive and processing circuit, with synapses localized in bilaterally symmetric centers known as the antennal lobes (Figure 1A). The first order interneurons, called Projection Neurons (PNs), collect sensory input in a stereotyped array of synaptic structures known as glomeruli, which harbor PN dendritic postsynaptic sites. The PNs direct output to two distinct brain centers via branching axons that project to the 'calyx' of the mushroom body and to the lateral horn. PNs receive cholinergic input via dendritically localized nicotinic acetylcholine receptors (nAChRs; Figure 1I), and signal via the release of acetylcholine from choline acetyltransferase (Chat)-positive boutons in the calyx (Figure 1B and 1C). The PN dendrites are also predicted to form reciprocal synapses with local interneurons (Ng *et al.*, 2002; Wilson *et al.*, 2004). CaMKII protein is localized to puncta on PN dendrites (Figure 1E and 1F) and at the pre-synaptic boutons of PN axonal branches in the mushroom body calyx (Figure 1C) and lateral horn.

[0037] To determine how cholinergic activity might affect CaMKII expression, these synapses were activated in a CNS explant culture by acetylcholine (ACh) or nicotine, an agonist of nAChRs. Brains were explanted and incubated in bath culture for 20 minutes, processed for immunohistochemistry to detect CaMKII, and examined by confocal microscopy under quantitative conditions (Figure 1G-1I). On average (n=10, Figure 1J), brains in which cholinergic synapses were activated displayed a 77-122% increase in CaMKII immunofluorescence in dendritic glomeruli (Figure 1G and 1J; nicotine, 122%, p=0.0014; ACh, 77%, p=0.007). In a time course experiment, the increase in CaMKII level was detectable within 5-10 minutes of nicotine exposure (data not shown). The increase was widespread in the brain, and reflected in a ~4 fold increase of CaMKII protein on Western blot analysis of adult brains incubated with nicotine (Figure 1K). The effect on CaMKII protein was specific, as other synaptic proteins such as Discs Large (Dlg; Koh *et al.*, 1999) and the nAChR subunit ARD (Chamaon *et al.* 2002) were not affected by nicotine or acetylcholine incubation (Figure 1H-1I and 1J).

[0038] Two mechanisms that could account for the rapid induction of synaptic CaMKII protein by neural activity would be the synthesis of new protein and the selective localization or stabilization of existing protein. Investigators showed that the dendritic expression of CaMKII relies on the activity-dependent translation of a localized mRNA, but that axonal expression does not.

[0039] The mouse CaMKII mRNA displays dendritic localization and activity dependent synaptic translation, regulatory features conferred by sequences located in its 3'UTR (Rook *et al.*, 2000; Wu *et al.*, 1998). To determine whether this is also the case for the *Drosophila* CaMKII, the 3'UTR was inserted downstream of the coding sequence for EYFP to create the reporter construct, *UAS-EYFP<sup>3'UTR</sup>*. An additional pair of constructs was made harboring a translational fusion of EYFP to CaMKII, where the 3'UTR<sup>CaMKII</sup> was present (*UAS-CaMKII::EYFP<sup>3'UTR</sup>*) or absent (*UAS-CaMKII::EYFP<sup>NT</sup>*). These constructs were expressed specifically in PN's using the *GAL4, UAS* binary system (Brand and Perrimon, 1993).

[0040] In *EYFP<sup>3'UTR</sup>* transgenic animals, EYFP fluorescence (Figure 2B, 2E and 2I) displayed a striking synaptic localization at PN terminals in the antennal lobe and calyx. In the calyx, EYFP was concentrated at PN pre-synaptic terminals, co-localized with ChAT (Figure 2I, arrowhead). On antennal lobe dendritic arbors, EYFP was concentrated at post-synaptic sites, co-localized with the nAChR  $\beta$ -subunit ARD (Figure 2E, arrowhead). This distribution to axon and dendrite roughly matched that of endogenous CaMKII protein (Figure 1B-1F). A cytoplasmic EYFP reporter lacking CaMKII sequences was, in contrast, distributed uniformly to cell bodies, axons and dendrites (Figure 2A' and 2H). Membrane-targeted CD8::GFP (Lee and Luo, 1999) was likewise distinct from the synaptic concentration of *EYFP<sup>3'UTR</sup>* expression (Figure 2K).

[0041] The CaMKII::EYFP fusion protein synthesized from mRNA harboring the 3'UTR (*CaMKII::EYFP<sup>3'UTR</sup>*) displayed synaptic localization in axons and dendrites like that of *EYFP<sup>3'UTR</sup>* (Figure 2D and 2G). However, the same fusion protein made from mRNA lacking the 3'UTR (*CaMKII::EYFP<sup>NT</sup>*) was strongly localized to axonal pre-synaptic sites in the calyx (Figure 2C and 2J), but only a low level was detected in the antennal lobe, localized

post-synaptically with ARD (Figure 2C and 2F). These observations indicate that sequence(s) in the *CaMKII* 3'UTR are required for the localization of CaMKII protein to dendritic synapses.

[0042] The dependence of CaMKII dendritic localization on its 3'UTR is consistent with the idea that synaptic protein synthesis is responsible for the rapid elevation of CaMKII protein at dendritic synapses by neural activity. This was examined by monitoring the dendritic expression of EYFP from the reporter *EYFP*<sup>3'UTR</sup> in explant culture (Figure 3A, B). Synaptic EYFP was rapidly induced by the addition of nicotine or ACh to the culture medium. Following a five-minute incubation with nicotine, *EYFP*<sup>3'UTR</sup> expression increased by 30%, in parallel with the increase in endogenous CaMKII protein. EYFP fluorescence and anti-GFP antibody immunoreactivity reached a plateau at a 280% total increase after 20 minutes in the presence of nicotine (Figure 3G; and data not shown). In contrast, *CaMKII::EYFP*<sup>NUT</sup> expression was virtually unchanged in the presence of nicotine or ACh (Figure 3C and 3D). Also, no change was observed when the control fluorescent proteins 'cytoplasmic EYFP', dsRed or CD8::GFP were expressed under similar conditions (Figure 3E and 3F; data not shown). The activity-induced expression of the *EYFP*<sup>3'UTR</sup> reporter was evident in the dendritic synapses of the antennal lobe, but not at pre-synaptic terminals in the calyx, where little or no increase in EYFP fluorescence was found. Thus the expression and rapid induction of CaMKII at dendritic sites by neural activity would be most easily explained by a 3'UTR-dependent regulation of synaptic protein synthesis.

[0043] Synaptic protein synthesis is thought to play a role in the establishment of a long-term memory (LTM; Steward and Schuman; 2003). In *Drosophila*, an olfactory LTM is induced by 'spaced training', a protocol involving paired odor (CS+) and electric shock (US), presented coincidentally at spaced intervals. A second odor (CS-) follows the CS+ odor in each interval, but is not paired with shock. An LTM appears after several hours and lasts beyond 24 hours, as assayed by tactic behavior in a T-maze (Tully and Quinn, 1985; Beck *et al.*, 2000). investigators followed this protocol, and used *CaMKII EYFP*<sup>3'UTR</sup> to report synaptic protein synthesis in animals that developed a stable memory of paired odor and shock.

[0044] The analysis focused on the antennal lobe glomeruli because these are well-characterized structures that can be reproducibly identified and display homogeneous synaptic

activity (Vosshall *et al.*, 2000; Ng *et al.*, 2002; Wang *et al.*, 2003). Furthermore, modification of the first-order antennal lobe synapses might be an early step in the storage of a memory (Faber *et al.*, 1999; Menzel *et al.*, 1999; Yu *et al.*, 2004). The analysis (Figure 4) revealed an odorant-specific pattern of synaptic protein synthesis associated with the induction of a long-term memory.

[0045] Animals harboring the *EYFP*<sup>3'UTR</sup> transgene under control of the PN-specific *GHI46-GAL4* driver were trained and dissected at times from 22-24 hours post-training. The brains of trained and untrained animals were processed for microscopy in parallel, which included staining with the antibody MA5nc82 (Figure 2A) to permit identification of glomeruli in confocal Z-series data sets. For each glomerulus, a stack of 6-8 images was recorded and analyzed via a thresholding protocol to isolate pixel groups composing individual synaptic puncta, and ignore non-synaptic fluorescence (Figure 4; see Experimental Procedures). The summed pixel intensities were averaged to obtain a glomerulus intensity score. An average glomerulus increase  $\Delta F/F$  in *EYFP*<sup>3'UTR</sup> fluorescence was calculated from intensity scores obtained from 5-8 independent brains for each experiment, and each experiment was repeated from 3 to 5 times. In each experiment, LTM was verified by examining the animal's performance in the T-maze.

[0046] The analysis was restricted to a subset of glomeruli that included those with a primary response to the odorants octanol (OCT) and methylcyclohexanol (MCH; Wang *et al.*, 2003; Wilson *et al.*, 2004). Only select glomeruli displayed training-dependent increases in *EYFP*<sup>3'UTR</sup> fluorescence, while others did not; their identity depended on the odorant (CS+) paired with shock. When OCT was the CS+, only the glomeruli D and DL3 displayed increased fluorescence, by 115% and 108%, respectively (Figure 4). When MCH was the CS+, fluorescence greatly increased in glomeruli DA1 and VA1, by 95% and 70%, respectively, while there were more modest but significant increases in glomeruli DM6 and VC2 (Figure 4). The glomerulus-specific increases were noted as early as 4 hours post-training. The induction of *EYFP*<sup>3'UTR</sup> fluorescence required the spaced training protocol; no fluorescence increase was observed when odorant and/or electric shock were unpaired or left out. There was no statistically significant fluorescence increase when the *CaMKII* 3'UTR was not present, in animals expressing a cytoplasmic EYFP reporter or *CaMKII::EYFP*<sup>NUT</sup> (Figure 4; data not shown). In these experiments, the data was analyzed for statistical

significance by various methods. In the final analysis, the significance of glomeruli-specific fold increases was determined by ANOVA ( $p < 0.05$ ). These observations indicate that an odor-specific induction of synaptic protein synthesis followed the spaced training paradigm, an induction that required the coincident presentation of the conditioned and unconditioned stimuli.

[0047] If CaMKII is synthesized at synapses in response to neural activity or the induction of memory, its mRNA should be localized there. To examine this, we utilized an mRNA tracking system based on the bacteriophage coat protein MS2 and its RNA binding site (Rook *et al.*, 2000; Forrest and Gavis, 2003). The fusion protein MS2::GFP::nls is ordinarily concentrated in the nucleus due to its nuclear localization signals (nls). It can however be diverted elsewhere by binding to an MS2 binding site (MS2-bs) tagged mRNA. Three mRNA's were tagged; the complete *Drosophila CaMKII* cDNA, its 3'UTR alone, and the mouse  $\alpha$ CaMKII 3'UTR, which has been shown to undergo dendritic localization and synaptic translation (Rook *et al.*, 2000; Wu *et al.*, 1998).

[0048] GFP fluorescence was examined when the *UAS-MS2::GFP::nls* reporter was expressed in projection neurons (PNs) with or without a transgene encoding a tagged mRNA. When any of the three tagged mRNA's was present, a punctate pattern of GFP fluorescence was observed in glomeruli that was not observed with *MS2::GFP::nls* alone (Figure 5A, 5B). Quantitative analysis revealed that the tagged *CaMKII* mRNA increased the number of GFP-positive puncta and the intensity of glomerular fluorescence by 75% (Figure 5K;  $p < 0.001$ ). Recently it has been observed that mRNA in dendrites, including the mouse  $\alpha$ CaMKII, is localized to particles containing the motor protein Kinesin (Kanai *et al.*, 2004). *See also*, Miller, S., *Neuron*, 36(3):507-19 (2002). Consistent with this observation, the MS2::GFP dendritic puncta were labeled by an antibody directed against the major kinesin heavy chain, KHC (Figure 5F; Pearson's correlation coefficient = 0.75; Brendza *et al.* 2002).

[0049] Adult brains harboring *MS2::GFP::nls* and one of the tagged *CaMKII* mRNA constructs were explanted into culture media containing nicotine or ACh. A significant increase in glomerular GFP fluorescence was observed, in the form of puncta, compared to explants maintained in the absence of a cholinergic agonist (compare Figure 5C and 5D, 5I and 5J, 5K;  $p < 0.001$ ). The effect of cholinergic stimulation was modest (2.4 fold) with the

*Drosophila* CaMKII mRNA, and more robust (4.25 fold) with the tagged mouse  $\alpha$ CaMKII 3'UTR. These observations indicate that in *Drosophila*, like mammals, neural activity increases the rate of mRNA movement to the synapse.

[0050] When animals carrying the *MS2::GFP::nls* and *ms2bs-CaMKII* transgenes were subjected to the spaced training protocol, the number of GFP-labeled puncta in glomeruli was increased, with an average effect on overall fluorescence intensity of 2.25 fold (n=10 specimens, p<0.001; compare Figure 5G and 5H; Figure 5K). The induction of dendritic puncta appeared widespread, without apparent glomerular specificity (e.g. Figure 5H). A more detailed analysis will be required to rule out specificity entirely.

[0051] RISC silences gene expression by RNA interference, a process including the targeted degradation of endogenous mRNAs or their non-destructive silencing. The RISC-mediated translational silencing of *oskar* mRNA controls its expression in the developing oocyte. An SDE3 class RNA helicase, Armitage (Armi; Cook, *et al.*, 2004) acts as part of RISC to control *oskar* translation and to regulate cytoskeletal organization, via control of Kinesin Heavy Chain (KHC) translation. Both the *oskar* and *Khc* 3'UTR's have putative binding sites for the microRNA (miRNA) miR-280 (Cook *et al.*, 2004). Interestingly, a remarkably similar miR-280 binding site is found in the CaMKII 3'UTR (Figure 6K). This site, and a site for miR-289 (Figure 6K) satisfy the important predictive rule that 7 of the 8 nucleotides at the 5' end of an miRNA are cognate (Enright *et al.*, 2003; Jing *et al.*, 2005; Lai *et al.*, 2003; Lai *et al.*, 2004; Stark *et al.*, 2003).

[0052] Armi is expressed in multiple neuronal populations of the adult brain, including the projection neurons (PNs) and Kenyon cells, where it is distributed in puncta in cell bodies, dendrites and at axon terminals (Figure 6H and 6J; data not shown). A GFP::Armi fusion protein, expressed in PN's, displayed a similar punctate distribution in glomeruli (Figures 6I) and at axon terminals in the calyx. It is noted that when *UAS-GFP::armi* was expressed in all neurons by the pan-neural driver *elav-GAL4*, GFP expression varied considerably between neurons, variation not observed with GFP reporters such as *UAS-CD8::GFP*. As described below, the variation of GFP::Armi expression is evidently due to post-transcriptional regulation, which includes degradation by the proteasome.

[0053] The GFP::Armi fusion protein retains *armi*<sup>+</sup> activity (Cook *et al.*, 2004), such that neurons with high levels of GFP::Armi might have greater *armi*<sup>+</sup> activity than those with low or undetectable levels. It was observed that the pattern of GFP::Armi expression was inversely related to the pattern of CaMKII and KHC expression in the brain (Figure 7 and 8). To explore this relationship further, UAS-GFP::*armi* was targeted to particular subsets of neurons, such as PN's with the *GHI46-GAL4* driver. Again, neurons that expressed GFP::Armi at high levels displayed a proportionately reduced expression of CaMKII and KHC, with fewer puncta detectable in cell bodies and dendrites (Figure 7D). By comparison, a control UAS-CD8-GFP transgene had no such effect (Figure 7E). The suppression of CaMKII expression by ectopic GFP::Armi was verified by Western blot analysis of adult brains (Figure 7A, left panel).

[0054] Conversely, the *EYFP*<sup>3'UTR</sup> reporter construct, which harbors a predicted miR-280 binding site, displayed increased expression in *armi* mutant animals (Figure 6C and 6D). *EYFP*<sup>3'UTR</sup> expression was increased by an average of 1.5 fold in these animals (Figure 6G). The enhanced fluorescence was localized to large dendritic puncta, reminiscent of *EYFP*<sup>3'UTR</sup> expression following explant into nicotine-containing media (compare Figures 3I and 6D). Additionally, *EYFP*<sup>3'UTR</sup> expression in *armi* animals did not appear further increased by explant into nicotine-containing media, suggesting that the pathway for induction of CaMKII expression was fully de-repressed (data not shown). The *CaMKII::EYFP*<sup>NUT</sup> transgene, which lacks the 3'UTR, also displayed elevated expression in the *armi* mutant background (Figure 6A, 6B and 6G), but other GFP constructs, such as CD8::GFP, did not (Figure 6E, 6F and 6G). In addition, RT-PCR analysis comparing wildtype and *armi* mutant adult brains did not detect a difference in the level of transgenic mRNAs, and thus indicated regulation at the post-transcriptional level (data not shown).

[0055] Several observations indicate that an auto-regulatory circuitry controls Armi function. As pointed out above, the level of UAS-GFP::*Armi* expression displayed wide variance among neuronal populations when driven uniformly by *GAL4* (Figure 7F). Notably, this transgene harbors a complete *armi* cDNA, including its UTRs (Cook *et al.*, 2004) and may harbor targets for translational control. Second, a Western analysis of animals expressing UAS-GFP::*Armi* revealed the absence of endogenous Armi protein from the adult brain (Figure 7A, right panel). Conversely, many neurons expressed GFP::Armi at a higher

level in an *armi/armi* genetic background than in an *armi/+* background (compare Figure 7F'' and 7G''). In either the *armi/armi* or *armi/+* genetic backgrounds, the expression of CaMKII is reduced in neurons that strongly express GFP::Armi; and in contrast, a high level of CaMKII is observed in *armi/armi* neurons that express GFP::Armi weakly (Figure 7G').

[0056] Since Armi regulates KHC expression in PNs (Figure 7D), it could affect the synaptic transport of *CaMKII* mRNA. When examined with the MS2::GFP system (Figure 5E), *armi*<sup>72.1</sup> homozygotes indeed displayed a 2-fold increased level of GFP-positive puncta in PN dendrites, which depended on co-expression with the tagged *CaMKII* mRNA (Figure 5K; n=7, p<0.05). This suggested that Armi regulation of CaMKII expression reflects a coordinated program involving multiple miRNA targets, including mRNA transport and translational suppression at the synapse, where Armi protein is found.

[0057] Additional RISC component mutants were examined for effects on CaMKII expression in order to assess whether Armitage acts via its RISC function. Aubergine, one of the Argonaute proteins in *Drosophila*, has been implicated in RISC assembly and oskar and Stellate regulation, like armitage (Cook et al., 2004; Tomari et al., 2004). In aubergine mutant brains, the *CaMKII::EYFP*3'UTR transgene displayed a significantly increased level of synaptic GFP fluorescence (Figure 10). Native CaMKII, detected via anti-CaMKII staining, was similarly increased. Western analysis for CaMKII expression in adult brain from wt, *armi* and *dcr-2* mutants showed that in both *armi* and *dcr-2* mutant brains, CaMKII level is 5-10 fold higher than normal.

[0058] Another central RISC component is the RNA endonuclease Dicer. The *Drosophila* Dicer-2 protein is the homolog of human Dicer. In the *Drosophila* Dicer-2 mutant, the expression of CaMKII protein was greatly increased, as was the expression of the *CaMKII::EYFP*<sup>3'UTR</sup> reporter. Thus Armi, Dicer-2 and Aubergine act as part of the RISC pathway to control synaptic CaMKII expression via its 3'UTR regulatory sequences.

[0059] If mRNA silencing by Armitage plays a role in LTM, one would expect this pathway to be regulated by processes involved in neural function. We had observed CaMKII expression in *armi* mutants appeared de-repressed, lacking normal regulation of synaptic CaMKII by cholinergic activity. Therefore, we determined whether Armi activity is neural

activity-dependent. When explanted into culture medium in the presence of nicotine, the level of GFP::Armi fluorescence rapidly decreased, while there was a correlated increase in CaMKII expression in the same neurons (Figure 8F-H and 8J). Overall, a short incubation with 100  $\mu$ M nicotine reduced GFP::Armi fluorescence in glomeruli by  $\sim$ 3.5 fold, while CaMKII expression increased by  $\sim$ 4.5 fold (Figure 8J). By Western analysis, we observed that incubation in nicotine-containing medium resulted in the nearly complete elimination of Armi protein (Figure 8I).

[0060] Given the rapid loss of Armi, a proteasome-dependent mechanism could be involved. Consistent with this notion, GFP::Armi fluorescence was increased by the expression of a dominant temperature-sensitive mutant of the proteasome  $\beta$ -subunit (DTS5; Speese *et al.*, 2003). At 17°C, where this protein product inhibits proteasome activity, the level of GFP::Armi was 3.2 fold higher than in animals lacking the DTS5 transgene (compare Figure 8A and 8C, 8E,  $p < 0.0001$ ,  $n = 8$  for each). By comparison, when specimens harboring the DTS5 transgene were shifted to the inactive temperature (35°C for 30 mins), GFP::Armi expression returned to normal (Figure 8D). This effect was not observed in the absence of the DTS5 transgene (Figure 8B). Nor did the activity of DTS5 effect the level of the CD8::GFP fusion protein (data not shown). In explant culture, prior incubation with the proteasome inhibitor lactacystin blocked the nicotine-induced loss of GFP::Armi expression (Figure 8F-H) and the loss of native Armi protein from adult brains, as determined by Western analysis (Figure 8K).

[0061] Given the role of the proteasome in Armi control, we determined whether it also regulates CaMKII expression. When explanted into culture medium containing nicotine, the overall level of CaMKII protein in isolated adult brains increased dramatically (Figures 1G and 8L, center lane). This induction was mostly prevented if the explanted brains were first incubated in culture medium containing lactacystin, before nicotine (Figure 8L, right lane). This effect was mirrored by anti-CaMKII staining of glomeruli (Figure 8H), where lactacystin blocked the increase seen with nicotine alone (Figure 8G). Therefore, like the degradation of Armi, cholinergic activity requires the function of the proteasome to induce synaptic CaMKII synthesis.

[0062] Several *armi* hypomorphic alleles display normal adult viability and behavior. This included behavior in the T-maze assay, where they showed nearly normal odor sensitivity and avoidance (data not shown). Investigators tested odor acuity by allowing flies to choose between air and odor and odor sensitivity by testing their balance between the odors OCT and MCH (see Experimental Procedures; Waddell *et al.*, 2000). These animals were also tested for normal electric shock avoidance behavior.

[0063] Given their normal performance in these tests, we examined *armi* mutant animals for STM and LTM. Mutant animals (*armi*<sup>72.1</sup> homozygotes or *armi*<sup>72.1</sup>/*Df(3L)E1*) displayed a normal level of memory in the short-term paradigm (average of three trials; *Armi*<sup>-</sup>, PI=0.55 versus *Armi*<sup>+</sup>, PI=0.58;  $p>0.1$ ; Figure 8M). In contrast, animals of these genotypes tested with spaced training were profoundly deficient in LTM (*Armi*<sup>-</sup>, PI=0.085 versus *Armi*<sup>+</sup>, PI=0.31;  $p<0.05$ ). Expression of the GFP::*Armi* transgene rescued the LTM of *armi*<sup>72.1</sup>/*armi*<sup>72.1</sup> animals to a normal value (Figure 8M).

[0064] *Armi* might function in LTM via regulation of CaMKII, or might have multiple essential targets for memory. Therefore we tested CaMKII-deficient animals for olfactory LTM. Previously, animals with a partial loss of CaMKII activity, induced by a transgenic protein inhibitor, were found to be deficient in courtship conditioning (Griffith *et al.*, 1993). To achieve a more complete and tissue specific loss of CaMKII, we made a construct that generates a *CaMKII* hairpin RNA (*UAS-CaMKII*<sup>hpn</sup>). When expressed in the brain, the *CaMKII*<sup>hpn</sup> induced the near complete elimination of CaMKII in adult animals (Figure 6N). Animals expressing *UAS-CaMKII*<sup>hpn</sup> in all CaMKII-positive neurons (with the *CaMKII-GAL4* driver) retained normal short-term memory (PI=0.653; *CaMKII-GAL4* alone, PI=0.64), but displayed a near complete loss of LTM (PI=0.07 versus *CaMKII-GAL4* alone, PI=0.3;  $p<0.001$ ). Elimination of CaMKII specifically from a subset of PN's or different subsets of mushroom body Kenyon cells did not result in a similar loss of LTM (data not shown). Thus, RISC regulation of CaMKII in these cell populations accounts for the loss of LTM in *armi* mutant animals.

[0065] The present invention is directed, in part, to how mRNA transport and synaptic protein synthesis occur in relation to the establishment and maintenance of a memory. One perspective, derived from work in *Aplysia* (Martin *et al.*, 1997), suggests that protein

synthesis is a global feature of a system primed for local change in synaptic efficacy. A distinct view is that synaptic protein synthesis is part and parcel of local synaptic change, restricted to synapses whose altered function forms a memory code (Kelleher *et al.*, 2004; Steward and Schuman, 2003; e.g. Si *et al.*, 2003). These views are derived from experiments both in mammalian neural explant and cell culture and from studies of the invertebrate *Aplysia*. The conservation of memory mechanisms in diverse systems demonstrates a general conservation of the basic mechanisms of synaptic change underlying memory.

[0066] Using a reporter based on the 3'UTR of *Drosophila CaMKII* (*EYFP<sup>3'UTR</sup>*), we observed induction of synaptic protein synthesis in several *Drosophila* brain centers in response to the 'spaced' training paradigm of repetitive paired odor and electric shock (Figure 4; data not shown). There were however local patterns of memory specificity, identifiable in glomeruli of the antennal lobe, where synapses of similar function are clustered. When the odorant OCT was paired with electric shock, protein synthesis occurred exclusively in the D and DL3 glomeruli. When the odorant MCH was paired with shock, the DA1 and VA1 glomeruli displayed a robust enhancement of synaptic protein synthesis. Notably, these animals were exposed to both odorants during training; the pattern of synthesis depended on the odorant paired with the shock. There was no significant induction of protein synthesis when the animals were exposed to odor and shock in non-overlapping order, or either stimulus alone. These results were derived from the analysis of populations of trained and naïve animals, at sites in the CNS where a robust signal from the *EYFP<sup>3'UTR</sup>* reporter produced data of high statistical significance. In order to generate an extensive and detailed synaptic protein synthesis map, single animals are being imaged continuously from training to response in order to resolve small changes at particular synapses.

[0067] The patterns of synaptic protein synthesis, as reflected in the expression of the *CaMKII EYFP<sup>3'UTR</sup>* reporter, did not mirror patterns of synaptic activity revealed in prior studies of animals exposed to odor or electric shock (Wang *et al.*, 2003; Wilson *et al.*, 2004; Ng *et al.*, 2002; Yu *et al.*, 2004). Nor was synaptic protein synthesis induced by odor or shock presented alone or without pairing (Figure 4). Notably, the projection neurons respond to cholinergic input via nicotinic receptors, and we have shown that this input triggers synaptic protein synthesis in explant culture (Figure 3). It is however unlikely that this *in vitro* induction replicates the trigger for synaptic protein synthesis utilized *in vivo*, which might

involve activation of other receptor systems, with or without correlated cholinergic activity. Therefore, synaptic protein synthesis is not normally induced by activity *per se*, but relies on an integrated trigger, provided by some receptor or combination of receptor systems capable of integrating multiple sensory inputs.

[0068] Studies in the honeybee (Faber *et al.*, 1999; Menzel *et al.*, 1999) suggested that the antennal lobe is an early way station for a memory code. Likewise, the *Drosophila* experiments of Yu *et al.* (2004) revealed a few antennal lobe glomeruli that display enhanced odor-evoked synaptic activity for a short time following a single episode of paired odor and electric shock. These transient effects on glomerular synaptic activity were undetectable later, at times when the behavioral read-outs of short-term memory were still robust. It was thus proposed that memory is held elsewhere for the longer term. Remarkably, the glomeruli that display a training-induced transient modification of the odor-evoked activity pattern (Yu *et al.*, 2004) are those that displayed enhanced synaptic protein synthesis in the spaced training episodes that induce an LTM (Figure 4). Therefore, the mechanism that integrates a single paired odor and shock to activate new glomerular activity may also induce synaptic protein synthesis in the case of multiple, spaced, paired events.

[0069] There are two possible functions for protein synthesis in glomeruli that hold an early transient memory trace. One is to stably modify synaptic activity in these glomeruli. However, the plasticity observed by Yu *et al.* (2004) is transient. In addition, animals subjected to the spaced training protocol displayed normal patterns of glomerular activity hours later (Yu *et al.*, 2004). Nonetheless, long-lived change in odor-evoked synaptic activity may have been beneath detection. An alternative view is that glomerular protein synthesis produces a signal that transfers memory to a distal brain center, such as the mushroom body, where memory may be more indelibly stored (Heisenberg, 2003; Pascual *et al.*, 2001). As in *Aplysia* interneurons that respond to serotonin-mediated sensitization (Martin *et al.*, 1997), synaptic protein synthesis at PN dendritic synapses might facilitate long-term potentiation at other, distant synapses of the cell, such as those of the axonal branches of the calyx or lateral horn. An important next step will be to map the requirement for protein synthesis in the establishment of an LTM to particular neurons in the olfactory pathway.

[0070] Long-term olfactory memory requires the activity of proteins that transport mRNA (Staufen) and regulate translation (Orb/CPEB, Pumilio; Dubnau *et al.*, 2003). The presence of potential recognition motifs for CPEB, Pumilio and Staufen in the *CaMKII* 3'UTR (our unpublished observations) suggests that these proteins may regulate CaMKII in a manner analogous to mammalian  $\alpha$ CaMKII regulation in the hippocampus (Richter *et al.*, 2002). Indeed, we observed clear parallels between the regulation of *Drosophila* CaMKII and  $\alpha$ CaMKII expression, including 3'UTR-dependent dendritic protein synthesis and mRNA translocation in response to neural activity (Figure 5).

[0071] Investigators identified potential binding motifs for the miRNAs miR-280 and miR-289 in the CaMKII mRNA (Figure 6). The former site is similar to miR-280 binding sites found in the *oskar* and *Kinesin heavy chain (Khc)* mRNAs. In the oocyte, *oskar* regulation employs the RNA helicase Armitage and other RISC components to produce miRNA-mediated translational silencing. Armitage expression was found in the adult brain, where it displayed a punctate localization to dendrites and axon termini, including those of antennal lobe projection neurons (Figure 6). Investigators found that Armitage regulates *CaMKII* translation in the adult brain (Figures 6 and 7). Ectopic expression of an *armi*<sup>+</sup> transgene (*GFP::Armi*) silenced endogenous CaMKII in a cell-autonomous fashion. Conversely, in loss-of-function *armi* mutants, CaMKII expression, in particular expression dependent upon the 3'UTR, was induced. We also observed CaMKII mis-regulation in mutants for the RISC component *aubergine* (Figure 10; Tomari *et al.*, 2004). Aubergine is an endonuclease. The homologous human endonuclease RISC component is Ago2. Therefore, translation of *CaMKII*, like *oskar* (Webster *et al.* 1997), is regulated by at least two tiers of control.

[0072] A second avenue for Armitage/RISC control of synaptic protein synthesis can be the regulation of dendritic mRNA transport. Armitage has multiple targets, including *Khc* (Cook *et al.*, 2004), which were found to be regulated by *armi* in the adult brain (Figure 7). Increased KHC expression might underlie the enhanced transport of *CaMKII* mRNA to synapses observed in *armi* mutant animals (Figure 5). Binding sites for miR-280 and miR-305 are also found in the *Staufen* 3'UTR (Rajewsky and Socci, 2004). We have indeed found that the Armitage protein regulates Staufen expression. Significantly, data indicate that the

establishment of memory is associated with the induction of mRNA transport to synapses (Figure 5), which might indeed be integral to memory formation (Dubnau *et al.*, 2003).

[0073] Armi and CaMKII are required for long-term memory, but not short-term memory, which is normal in absence of these functions (Figure 8). Hence the neural systems involved in acquiring and encoding short-term memory are normal in these animals. Armi could indeed act entirely via its regulation of CaMKII, but there are likely other significant targets of Armi/RISC control.

[0074] Proteasome-mediated degradation of Armitage can be the link between sensory experience and release from miRNA-mediated translational silencing (Figures 8 and 9). Investigators found that cholinergic induction of CaMKII synthesis was accompanied by the degradation of Armi, and both were blocked by the inhibition of proteasome activity (Figure 8). Therefore, a novel mechanism for the formation of a stable memory, involves an integrated sensory input triggered proteasome-mediated degradation of a RISC factor, which releases synaptic protein synthesis and mRNA transport from microRNA suppression (Figure 9).

[0075] Known RISC assembly and pathway members, including homologs (for example, the *Drosophila* one that is known and mammalian or other homologs) are shown in Table 1.

**Table 1: RISC components:**

| Protein    | Function                                             | Human Ortholog         |
|------------|------------------------------------------------------|------------------------|
| Dicer1     | RNaseIII, dsRNA-binding                              | Dicer<br>(single gene) |
| Dicer2     | RNaseIII, helicase,<br>dsRNA-binding                 |                        |
| Drosha     | RNaseIII endonuclease                                | TRBP*                  |
| R2D2       | dsRNA-binding (partner of Dcr-2)                     |                        |
| R3D1-L     | dsRNA-binding (partner of Dcr-1)                     |                        |
| Loquacious | dsRNA-binding<br>(paralog of R2D2, partner of Dcr-1) |                        |
| Pasha      | dsRNA-binding (partner of Drosha)                    | DGCR8                  |
| Ago1       | endonuclease                                         | Ago2                   |
| Ago2       | endonuclease                                         |                        |

|           |                             |              |
|-----------|-----------------------------|--------------|
| Fmr1/Fxr  | RNA-binding                 | FMRP         |
| Vig       | RNA-binding                 |              |
| Tsn       | Nuclease                    |              |
| Armitage  | RNA helicase                | MoV10 like 1 |
| Aubergine | endonuclease (Ago2 paralog) | Ago2         |

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\* TRBP is the human immunodeficiency virus trans-activating response RNA-binding protein), which contains three double-stranded, RNA-binding domains and is an integral part of Dicer-Ago2 containing RISC complex (involved in miRNA processing and RISC assembly).

[0076]        **Recent findings related to Armitage homolog in humans (Mov10), ubiquitin-proteasome system in human brain disorders.** Human protein Mov10 has structural and functional homology to Drosophila Armitage. Mov10 a human homolog of Armitage is required for miRNA mediated degradation of target mRNAs and for efficient RNAi (Meister G, Landthaler M, Peters L, Chen PY, Urlaub H, Luhrmann R, Tuschl T: **Identification of novel argonaute-associated proteins.** *Curr Biol* 2005, **15**:2149-2155. Epub 2005 Nov 2110).

[0077]        MoV10 can be coprecipitated with miR-16, a highly abundant miRNA in HEK293 cells, along with Arganaute protein Ago2. However, the Mov10 immunoprecipitate did not contain Dicer activity, indicating that like Armitage Mov10 might also function downstream of the Dicer cleavage step (Meister G, Landthaler M, Peters L, Chen PY, Urlaub H, Luhrmann R, Tuschl T: **Identification of novel argonaute-associated proteins.** *Curr Biol* 2005, **15**:2149-2155. Epub 2005 Nov 2110; Cook HA, Koppetsch BS, Wu J, Theurkauf WE: **The Drosophila SDE3 homolog armitage is required for oskar mRNA silencing and embryonic axis specification.** *Cell* 2004, **116**:817-829). It is also possible that both Armitage and Mov10 transiently associates with the RISC complex for efficient RNAi after Dicer mediated processing of miRNAs.

[0078]        The RNA interference pathway utilizes protein complexes and short RNA molecules, the latter which serve to target a protein complex to specific messenger RNA molecules by a base-pairing interaction. The biochemical pathway is highly conserved across phylogeny, most significantly, from fruit fly to human. One element of the biochemical pathway is focused on producing the short RNA molecules, siRNAs (exogenously supplied) or miRNAs (genomically encoded). A second element of the pathway utilizes these targeting

molecules to either degrade or repress the translation of specific mRNAs, thus preventing their expression as proteins.

[0079] There are three core RISC complexes in *Drosophila*. One is the Dicer-1/Loq/R3D1 complex involved predominantly in miRNA processing and gene silencing by translation repression. The second is the Dicer-2/r2d2 complex that has the predominant role in the siRNA-mediated pathway, which degrades target mRNA's. The third complex Drosha/Pasha functions within the nucleus to generate pre-miRNA from the long hair-pin RNA precursors synthesized from genomic loci. There is only one Dicer-type protein in Humans, mouse and *C. elegans*. Therefore, the relative functional significance of the single Dicer protein in humans in the two pathways (miRNA vs. siRNA) is unclear. It is possibly determined by associated proteins that alter its functionality.

[0080] Ago proteins, containing the PIWI, PAZ structural domains, are the signature subunits of RISC. The PIWI domains of Ago proteins are considered to harbor the endonuclease activity of RISC, underlying their target degradation functions.

[0081] Proteins with the RNA-binding domain similar to the Vasa intronic gene (Vig) and the ortholog of the human fragile-X-mental-retardation protein (FMR; known also as Fxr or Fmr-1) are associated with RISC. Notably, the FMR protein has well-established functions in regulating synaptic plasticity, a local correlate of memory formation. The *Drosophila* FMR homolog has been demonstrated to have analogous functions to the human protein. Intriguingly, FMRP has been found to be associated with the BC200 non-coding RNA transcript in human brain. BC200 transcription reduction has been observed in the brains of Alzheimer's disease patients. The current focus of Alzheimer's research and therapeutic efforts is on the amelioration of plaques formed of an aberrantly processed form of the protein APP. Thus, the RISC pathway in memory formation offers a new entry point into Alzheimer's treatment, either by the path of boosting remaining neural function, or by directly intervening in an aspect of Alzheimer's pathology.

**Table 2: Proteins that are regulated by the RISC pathway including homologs.**

| <u>miRNA</u>   | <u>Targets</u>                                                                                |
|----------------|-----------------------------------------------------------------------------------------------|
| miR-280        | oskar                                                                                         |
| miR-280        | Kinesin-Heavy chain                                                                           |
| miR-280        | CaMKII                                                                                        |
| miR-280        | Staufen                                                                                       |
| miR-289        | CaMKII                                                                                        |
| Bantam         | hid (pro-apoptotic gene)                                                                      |
| miR-6          | hid                                                                                           |
| miR-2/13       | hid                                                                                           |
| miR-196        | Hox genes                                                                                     |
| miR-7 and 2a/b | Notch-target genes:<br>Basic helix-loop-helix (bHLH)<br>repressor and Bearded family<br>genes |

[0082] A number of mammalian miRNA targets have neural functions, including functions related to synaptic plasticity, e.g. Brain Derived Neurotrophic Factor, BDNF (Tables 3 and 4). The functional relevance of the target proteins was determined using Drosophila and mouse behavioral paradigms. Added support has been obtained from studies using human primary neuronal cell cultures.

**Table 3: miRNA and their Neuronal Targets in Drosophila and Human nervous system**

| <b>Drosophila miRNA</b>                                | <b>Drosophila Target genes</b> | <b>Hu-miRNA</b> | <b>Human Gene Target</b> | <b>Function in Memory or Neurological Disease</b> |
|--------------------------------------------------------|--------------------------------|-----------------|--------------------------|---------------------------------------------------|
| miR-280                                                | CaMKII                         | hsa-let-7e      | CaMKII alpha Chain       | Involved in Dm and mouse memory                   |
| miR-289                                                | CaMKII                         | hsa-let-7f      | CaMKII alpha Chain       | Involved in Dm and mouse memory                   |
| miR-280, miRs-274, 317, 310, 285, 303, 318, 87, 5, 274 | Staufen                        | hsa-miR-105     | Staufen                  | Involved in Dm memory                             |
| miR-280                                                |                                | hsa-miR-142_3p  | Staufen Homolog 2        | Involved in Dm memory                             |

|         |              |                            |                                                                                                                                                                                                    |                                                           |
|---------|--------------|----------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------|
|         | Disc Large   | hsa-miR-105                | Disc-large homolog                                                                                                                                                                                 | Likely Involved in Dm memory                              |
| miR-289 | Pumilio      | hsa-miR-136<br>hsa-miR-182 | Pumilio Homolog 2                                                                                                                                                                                  | Involved in Dm memory                                     |
| miR-280 | oskar        |                            |                                                                                                                                                                                                    |                                                           |
|         | Eph Receptor | hsa-let-7a                 | Eph Related Receptor Tyrosine Kinase                                                                                                                                                               | Involved in Dm and mouse memory (has been shown in mouse) |
|         |              | hsa-miR-137                | Guanine Nucleotide-binding Protein G(S) Alpha subunit                                                                                                                                              | Involved in Dm memory                                     |
|         |              | hsa-let-7a                 | AMYLOID BETA PRECURSOR PROTEIN-BINDING PROTEIN 2; AMYLOID BETA PRECURSOR PROTEIN (CYTOPLASMIC TAIL)-BINDING PROTEIN 2; PROTEIN INTERACTING WITH APP TAIL 1                                         | Alzheimer's disease                                       |
|         |              | hsa-miR-148                | AMYLOID BETA A4 PROTEIN PRECURSOR (APP) (ABPP) (ALZHEIMER'S DISEASE AMYLOID PROTEIN) (CEREBRAL VASCULAR AMYLOID PEPTIDE) (CVAP) (PROTEASE NEXIN-II) (PN-II) (APPI) (PREA4) [CONTAINS: SOLUBLE APP- | Alzheimer's disease                                       |

|  |  |              |                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                 |
|--|--|--------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------|
|  |  |              | ALPHA (S-APP-ALPHA); SOLUBLE APP-BETA (S-APP-BETA); C99; BETA-AMYLOID PROTEIN 42 (BETA-APP42); BETA-AMYLOID PROTEIN 40 (BETA-APP40); C83; P3(42); P3(40); GAMMA-CTF(59) (GAMMA-SECRETASE C-TERMINAL FRAGMENT 59) (AMYLOID INTRACELLULAR DOMAIN 59) (AID(59)); GAMMA-CTF(57) (GAMMA-SECRETASE C-TERMINAL FRAGMENT 57) (AMYLOID INTRACELLULAR DOMAIN 57) (AID(57)); GAMMA-CTF(50) (GAMMA-SECRETASE C-TERMINAL FRAGMENT 50) (AMYLOID INTRACELLULAR DOMAIN 50) (AID(50)); C31] |                                 |
|  |  | hsa-miR-134  | Tau                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | Alzheimer's disease             |
|  |  | hsa-let-7a   | CPEB (cytoplasmic polyadenylation element binding protein)                                                                                                                                                                                                                                                                                                                                                                                                                 | Involved in Dm and mouse memory |
|  |  | hsa-miR-130b | CREB binding Protein                                                                                                                                                                                                                                                                                                                                                                                                                                                       | Involved in Dm and mouse memory |
|  |  | hsa-miR-130b | Translation Initiation Factor 4E                                                                                                                                                                                                                                                                                                                                                                                                                                           | Involved in Dm and mouse        |

|                       |                              |                            |                                                          |                                 |
|-----------------------|------------------------------|----------------------------|----------------------------------------------------------|---------------------------------|
|                       |                              |                            |                                                          | memory                          |
|                       |                              | hsa-let-7a                 | MAPK                                                     | Involved in Dm and mouse memory |
|                       |                              | hsa-let-7a                 | Cadherin isoforms                                        |                                 |
|                       |                              | hsa-let-7a                 | Amyloid Beta A4 precursor binding protein (FE65 Protein) |                                 |
| miR-280, miR-282, 9-b | Ubiquitin Conjugating Enzyme | hsa-let-7a                 | Ubiquitin Conjugating Enzyme E2 B                        |                                 |
|                       |                              | hsa-miR-106a               | Ubiquitin                                                |                                 |
|                       |                              | hsa-let-7c                 | Muscarinic Acetylcholine Receptor M1                     |                                 |
|                       |                              | hsa-miR-105                | NMDA receptor                                            | Involved in Dm and mouse memory |
| miR-280               | Kinesin Heavy Chain (KHC)    | hsa-let-7c                 | Kinesin Like Protein (KLP)                               |                                 |
|                       |                              |                            | Voltage Dependent R-type Ca <sup>2+</sup> channel        |                                 |
|                       |                              | hsa-let-7d<br>hsa-miR-130b | Fragile-X mental Retardation protein (FMRP)              | Fragile X mental Retardation    |
|                       |                              | hsa-miR-182                | Fragile-X mental Retardation protein 2 (FMRP-2)          | Fragile X mental Retardation    |
|                       |                              |                            | Glycine Receptor Alpha-2 Chain                           |                                 |
|                       |                              |                            | Homer Homolog                                            |                                 |
|                       |                              | hsa-let-7e                 | Calmodulin                                               | Role in mouse and Dm memory     |
|                       |                              | hsa-miR-106a               | Ubiquitin                                                |                                 |

|  |  |                |                                                                                                                    |                                                                                                      |
|--|--|----------------|--------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------|
|  |  | hsa-miR-136    | Beta-catenin                                                                                                       | mouse brain development                                                                              |
|  |  | hsa-miR-136    | Pleckstrin Homology domain Containing, Family A                                                                    |                                                                                                      |
|  |  | hsa-miR-137    | Synaptotagmin I                                                                                                    |                                                                                                      |
|  |  | hsa-miR-142_3p | Poly A binding Protein                                                                                             |                                                                                                      |
|  |  | hsa-miR-144    | Neural Cell Adhesion Molecule 1 (N-CaM 140KD)                                                                      |                                                                                                      |
|  |  | hsa-miR-132    | Acetylcholinesterase collagenic tail peptide precursor (AChE Q subunit) (Acetylcholinesterase-associated collagen) | Involved in memory (attributed from AD drug therapy) (acetylcholine breakdown in the synaptic cleft) |

[0083] The sequences for the human miRNA's listed in Table 3 are shown below:

3' \_UUGAUAUGUUGGAUGAUGGAGU\_5':hsa-let-7a [SEQ ID NO. 1]  
3' \_UUGGUAUGUUGGAUGAUGGAGU\_5':hsa-let-7c [SEQ ID NO. 2]  
3' \_uGAUACGUUGGAUGAUGGAGa\_5':hsa-let-7d [SEQ ID NO. 3]  
3' \_UGAUAUGUUGGAGGAUGGAgU\_5':hsa-let-7e [SEQ ID NO. 4]  
3' \_UUGAUAUGUUAGAUGAUGGAGu\_5':hsa-let-7f [SEQ ID NO. 5]  
3' \_uGUCCUCAGACUCGUAACu\_5':hsa-miR-105 [SEQ ID NO. 6]  
3' \_CGAUGGACGUGACAUCGUGAAAa\_5':hsa-miR-106a [SEQ ID NO. 7]  
3' \_UACGGGAAAGUAGUAACGUGAc\_5':hsa-miR-130b [SEQ ID NO. 8]  
3' \_gGGAGACCAGUUGGUCAGUGU\_5':hsa-miR-134 [SEQ ID NO. 9]  
3' \_aGGUAGUAGUUUUGUUUACCUCa\_5':hsa-miR-136 [SEQ ID NO. 10]  
3' \_GAUGCGCAUAAGAAUUCGUUAU\_5':hsa-miR-137 [SEQ ID NO. 11]  
3' \_AGGUAU-UUCAUCCUUUGUGAUGu\_5':hsa-miR-142\_3p [SEQ ID NO. 12]  
3' \_GAUCAUGUAGUAGAUUAUGACAU\_5':hsa-miR-144 [SEQ ID NO. 13]  
3' \_UGUUUCAAGACAUCACGUGACu\_5':hsa-miR-148 [SEQ ID NO. 14]  
3' \_ACACUCAAGAUGGUAACGGUUu\_5':hsa-miR-182 [SEQ ID NO. 15]  
3' \_gCUGGUACCGACAUCUGACAAU\_5':hsa-miR-132 [SEQ ID NO. 16]

Table 4: Targets of Mammalian miRNAs

| Category                                    | Seed    | miRNAs                      | Ensembl ID | Gene Name                                                           |
|---------------------------------------------|---------|-----------------------------|------------|---------------------------------------------------------------------|
| Regulation of transcription/<br>DNA binding | AGUGCAA | miR-130,-130b               | 169057     | Methyl-CPG-binding protein 2 ( <i>MECP2</i> )                       |
|                                             | GUGCAAA | miR-19a                     | 169057     | " "                                                                 |
|                                             | AAAGUGC | miR-20,-106                 | 101412     | Transcription factor <i>E2F1</i>                                    |
|                                             | GAGGUAG | <i>let-7(a-g,i)</i> ,miR-98 | 100823     | DNA-(apurinic or apyrimidinic site) lyase ( <i>APEN</i> )           |
|                                             | GAAAUGU | miR-203                     | 125347     | Interferon regulatory factor 1 ( <i>IRF-1</i> ),                    |
|                                             | ACAGUAC | miR-101                     | 134323     | <i>N-MYC</i> protooncogene protein                                  |
|                                             | GAGGUAU | miR-202                     | 134323     | " "                                                                 |
|                                             | AAUCUCA | miR-216                     | 065978     | Nuclease sensitive element binding protein 1 ( <i>YB-1</i> )        |
|                                             | UAAGGCA | miR-124a                    | 163403     | Microphthalmia-associated transcription factor                      |
|                                             | CCUGGUG | miR-138                     | 054598     | Forkhead box protein C1 ( <i>FKHL7</i> )                            |
| Signal                                      | AAAGUGC | miR-20,-106                 | 103479     | Retinoblastoma-like protein 2 ( <i>RBR-2</i> )                      |
|                                             | UCCAGUU | miR-145                     | 151702     | Friend leukemia integration 1 transcription factor ( <i>FLI-1</i> ) |
|                                             | GCAGCAU | miR-103,-107                | 137309     | High mobility group protein HMG-I/HMG-Y ( <i>HMG-I(Y)</i> )         |
|                                             | GGAAGAC | miR-7                       | 136826     | Kruppel-like factor 4 ( <i>EZF</i> )                                |
|                                             | UAAGGCA | miR-124a                    | 168610     | Signal transducer and act. of transcription 3 ( <i>STAT3</i> )      |
| transduction/<br>cell-cell<br>signaling     | UGGUCCC | miR-133,-133b               | 010610     | T cell surface glycoprotein CD4 precursor                           |
|                                             | UCACAUU | miR-23a,-23b                | 107562     | Stromal cell-derived factor 1 precursor ( <i>SDF-1</i> )            |
|                                             | GCUACAU | miR-221,-222                | 157404     | Mast/stem cell growth factor receptor precursor ( <i>C-KIT</i> )    |
|                                             | GGAUGU  | miR-1,-206                  | 176697     | Brain-derived neurotrophic factor precursor ( <i>BDNF</i> )         |
|                                             | UAAGGCA | miR-124a                    | 154188     | Angiopoietin-1 precursor ( <i>ANG-1</i> )                           |
|                                             | GGCAGUG | miR-34                      | 143400     | Notch homolog protein 1 precursor ( <i>HNI</i> )                    |
|                                             | CCCUGAG | miR-125a,-125b              | 128342     | Leukemia inhibitory factor precursor ( <i>LIF</i> )                 |
|                                             | AGUGCAA | miR-130,-130b               | 184371     | Macrophage colony stimulating factor-1 precursor ( <i>MCSF</i> )    |
|                                             | UCACAGU | miR-27a                     | 184371     | " "                                                                 |
|                                             | AAUACUG | miR-200b                    | 008710     | Polycystin 1 precursor                                              |
| Other                                       | GAAAUGU | miR-203                     | 122641     | Inhibin beta A chain precursor ( <i>EDF</i> )                       |
|                                             | AUUGCAC | miR-25,-92                  | 065559     | Dial spec. mitogen-activated protein kinase kinase 4                |
|                                             | GCUGGUG | miR-138                     | 070886     | Ephrin type-a receptor 8 precursor ( <i>HEK3</i> )                  |
|                                             | GUAAACA | miR-30(a-c)                 | 156052     | Guanine nucleotide-binding protein G(1),alpha-2 subunit             |
|                                             | AUUGCAC | miR-25,-92                  | 156052     | " "                                                                 |
|                                             | GAGAACU | miR-146                     | 175104     | TNF receptor-associated factor 6 ( <i>TRAF6</i> )                   |
|                                             | GGCUCAG | miR-34                      | 166484     | Mitogen-activated protein kinase 7 ( <i>ERK4</i> )                  |
|                                             | GAGAUGA | miR-143                     | 166484     | " "                                                                 |
|                                             | AGCUGCC | miR-22                      | 166484     | " "                                                                 |
|                                             | GCAGCAU | miR-103,-107                | 141433     | Pituitary adenylate cyclase act. polypeptide precursor              |
|                                             | GUGCAAA | miR-19a,-19b                | 171862     | Phosphatidylinositol-3,4,5-trisphos. 3-phosphatase ( <i>PTEN</i> )  |
|                                             | AGUGCAA | miR-130,-130b               | 130164     | Low-density lipoprotein receptor precursor ( <i>LDLR</i> )          |
|                                             | GGAUGU  | miR-1,-206                  | 160211     | Glucose-6 phosphate 1-dehydrogenase ( <i>G6PD</i> )                 |
|                                             | UUGGCAC | miR-96                      | 101986     | Adrenoleukodystrophy protein ( <i>ALDP</i> )                        |
|                                             | AGCACCA | miR-29b,-29c                | 168542     | Collagen alpha 1(III) chain precursor                               |
|                                             | AGCACCA | miR-29b,-29c                | 114270     | Collagen alpha 1(VII) chain precursor                               |
|                                             | AUUGCAC | miR-25,-92                  | 168090     | <i>COP9</i> subunit 6                                               |
|                                             | AAGUGCU | miR-93                      | 168090     | " "                                                                 |
|                                             | AAAGUGC | miR-20,-106                 | 168090     | " "                                                                 |
|                                             | CCCUGAG | miR-125a,-125b              | 160613     | Proprotein convertase subtilisin/kexin type 7 precursor             |

"Seed" designates the recognition sequence.

From: Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. *Cell*. 2003 Dec 26;115(7):787-98.

[0084] The genes encoding miRNAs are much longer than the processed miRNA molecule; miRNAs are first transcribed as primary transcripts or pri-miRNA and processed to short, 70-nucleotide stem-loop structures known as pre-miRNA in the cell nucleus. This processing is performed in animals by a protein complex known as the Microprocessor

complex, consisting of the nuclease Drosha and the double-stranded RNA binding protein Pasha (Denli AM, Tops BB, Plasterk BB, Ketting RF, Hannon GJ, *Nature* 432(7014):231-5 (2004)). These pre-miRNAs are then processed to mature miRNAs in the cytoplasm by interaction with the endonuclease Dicer, which also initiates the formation of the RNA-induced silencing complex (RISC) (Bernstein E, Caudy AA, Hammond SM, Hannon GJ, **Role for a bidentate ribonuclease in the initiation step of RNA interference**, *Nature* 409(6818):363-6 (2001)).

[0085] When Dicer cleaves the pre-miRNA stem-loop, two complementary short RNA molecules are formed, but only one is integrated into the RISC complex. This strand is known as the guide strand and is selected by the argonaute protein, the catalytically active RNase in the RISC complex, on the basis of the stability of the 5' end (Preall JB, He Z, Gorra JM, Sontheimer EJ, **Short interfering RNA strand selection is independent of dsRNA processing polarity during RNAi in *Drosophila***, *Curr Biol* 16(5):530-5 (2006)). The remaining strand, known as the anti-guide or passenger strand, is degraded as a RISC complex substrate (Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R, **Human RISC couples microRNA biogenesis and posttranscriptional gene silencing** *Cell* 123(4):631-40 (2005)). After integration into the active RISC complex, miRNAs base pair with their complementary mRNA molecules and induce mRNA degradation by argonaute proteins, the catalytically active members of the RISC complex. It is as yet unclear how the activated RISC complex locates the mRNA targets in the cell, though it has been shown that the process is not coupled to ongoing protein translation from the mRNA (Sen GL, Wehrman TS, Blau HM, **mRNA translation is not a prerequisite for small interfering RNA-mediated mRNA cleavage** *Differentiation* 73(6):287-93 (2005)).

[0086] The RNA-induced silencing complex could be disrupted by interfering with any of the steps in the formation or processing of miRNAs. For example, by inhibiting transcription of pri-miRNA or inhibiting or disrupting the processing of pri-miRNA to pre-miRNA. This could be achieved by disrupting the Microprocessor complex, inhibiting the nuclease activity of Drosha or inhibiting binding of Pasha. RISC could also be disrupted by blocking transport of pre-miRNA from the nucleus to the cytoplasm or by inhibiting the endonuclease activity of Dicer. RISC could also be disrupted by interfering with the interaction between the processed miRNA and argonaute.

[0087] Micro-RNAs (miRNA) are involved in inhibition of protein translation by binding to the 3'UTR of target messenger RNA (mRNA). In the nervous system, local synthesis of proteins occurs at synapses, in order to regulate their activity. Protein synthesis at synapses is associated with their plasticity, and is required for the formation of long-term memory. At a particular synapse, depending on the stimulus, specific proteins are predictably synthesized. Our work shows for the first time that the RISC pathway, including microRNAs (miRNA), regulates this type of protein synthesis, and therefore that this pathway has an integral function in memory formation. Our data predicts the localization of specific miRNAs at synapses. Specific miRNAs silence synaptic protein synthesis, until an activating input is received, resulting in reversing the translation inhibition. Thus, the localization of specific miRNA at the synapse and their regulation is a hallmark of specificity of synaptic activation and underlying behavior and consolidation of memory.

[0088] 86 miRNAs were also isolated from Rat cortical neurons and found associated with polyribosomes, further suggesting a role in translation regulation of mRNA in mammalian neurons. (Kim et al., *PNAS*, 101(1):360-365 (2004)).

[0089] In Humans, it is predicted that there are ~800 distinct miRNAs; their characterization to date remains rudimentary. Matching predicted human miRNAs to binding sites on the mRNAs of synaptic proteins, will identify miRNAs involved in the modulation of memory.

#### **Distinct classes of Small non-coding RNAs (ncRNAs) in insect and mammalian cells**

[0090] Recent studies to delineate genome-wide transcription using "Genome-Tiling" microarrays have identified many small non-coding RNAs that are expressed, with presumed roles in structural, catalytic and regulatory processes of other RNA, but devoid of any code for proteins (Mattick JS: **The functional genomics of noncoding RNA**. *Science* 2005, **309**:1527-1528; Johnson JM, Edwards S, Shoemaker D, Schadt EE: **Dark matter in the genome: evidence of widespread transcription detected by microarray tiling experiments**. *Trends Genet* 2005, **21**:93-102). Lacking significant understanding of their role in cellular physiology (apart from microRNAs), these small RNA species have been

christened as “non-coding RNAs” (Costa FF: **Non-coding RNAs: new players in eukaryotic biology**. *Gene* 2005, **357**:83-94).

[0091] Two distinct classes of non-coding small RNAs have recently been identified in tissues derived from broad range of species, including mammalian brain. These are:

1. rasiRNA (repeat-associated small interfering RNA) (Aravin AA, Lagos-Quintana M, Yalcin A, Zavolan M, Marks D, Snyder B, Gaasterland T, Meyer J, Tuschl T: **The small RNA profile during *Drosophila melanogaster* development**. *Dev Cell* 2003, **5**:337-350; Vagin VV, Sigova A, Li C, Seitz H, Gvozdev V, Zamore PD: **A distinct small RNA pathway silences selfish genetic elements in the germline**. *Science* 2006, **313**:320-324. Epub 2006 Jun 2029; Megosh HB, Cox DN, Campbell C, Lin H: **The Role of PIWI and the miRNA Machinery in *Drosophila* Germline Determination**. *Curr Biol* 2006, **16**:1884-1894. Epub 2006 Aug 1831)
2. snoRNAs (small-nucleolar RNA).

[0092] rasiRNA: This class of small non-coding RNAs (24-29 nucleotides long) were identified in *Drosophila*, with predominant expression in the germ-line (Aravin AA, Lagos-Quintana M, Yalcin A, Zavolan M, Marks D, Snyder B, Gaasterland T, Meyer J, Tuschl T: **The small RNA profile during *Drosophila melanogaster* development**. *Dev Cell* 2003, **5**:337-350). Aravin et al., (2003) identified 178 repeat-associated small interfering RNAs (rasiRNAs), transcribed from transposable elements, satellite and microsatellite DNA suggesting that small RNAs participate in defining chromatin structure. rasiRNAs are most abundant in testes and early embryos, where regulation of transposon activity is critical. Armitage, a non-DEAD-box helicase, and its mammalian homolog are required small interfering RNA (siRNA) induced RNAi (Cook HA, Koppetsch BS, Wu J, Theurkauf WE: **The *Drosophila* SDE3 homolog armitage is required for oskar mRNA silencing and embryonic axis specification**. *Cell* 2004, **116**:817-829; Meister G, Landthaler M, Peters L, Chen PY, Urlaub H, Luhrmann R, Tuschl T: **Identification of novel argonaute-associated proteins**. *Curr Biol* 2005, **15**:2149-2155. Epub 2005 Nov 2110).

[0093] Recently, Vagin et al., (Vagin VV, Sigova A, Li C, Seitz H, Gvozdev V, Zamore PD: **A distinct small RNA pathway silences selfish genetic elements in the germline**. *Science* 2006, **313**:320-324. Epub 2006 Jun 2029) reported a requirement for

Armitage and Aubergine (a Argonaute protein family member in *Drosophila*) in maturation of rasi-RNA. This study suggests, in addition to a role in siRNA mediated gene silencing, Armitage and Aubergine are involved in a non-coding RNA pathway—independent of classic siRNA (small interfering RNA) and microRNA dependent silencing pathways. Although, whether rasiRNA is expressed in brain has not been investigated, our earlier findings of a role for Armi and Aub in memory formation in *Drosophila* (Ashraf SI, McLoon AL, Sclarsic SM, Kunes S: **Synaptic protein synthesis associated with memory is regulated by the RISC pathway in *Drosophila***. *Cell* 2006, **124**:191-205), predicts a potential role for rasiRNAs in behavioral plasticity.

[0094] Small nucleolar RNA (snoRNA): This class of non-coding RNA (ncRNA) resides in the nucleolus and functions to modify other RNA molecules. There are two classes of snoRNAs—box C/D and box H/ACA. These small RNAs associate with proteins to form ribonucleoprotein particles (snoRNPs) that function to modulate the house keeping RNAs (ribosomal RNA, tRNAs etc.) (Cao X, Yeo G, Muotri AR, Kuwabara T, Gage FH: **Noncoding RNAs in the mammalian central nervous system**. *Annu Rev Neurosci* 2006, **29**:77-103). Recently, however, one brain specific snoRNA, MBII-52, have been implicated in regulating splicing of serotonin receptor 2C (5-HT-2C) mRNA, failing which results in Prader-Willi syndrome (PWS), a neurobehavioral disorder with neonatal mental disorder (Kishore S, Stamm S: **The snoRNA HBII-52 regulates alternative splicing of the serotonin receptor 2C**. *Science* 2006, **311**:230-232. Epub 2005 Dec 2015).

#### **RISC and neurological disorders:**

[0095] FMRP, the protein responsible for the Fragile X mental retardation Syndrome, is an RNA-binding protein involved in localization and translation of neuronal mRNAs. The *Drosophila* homolog, Fmr1, is associated with the RISC complex.

#### **Proteasome and neurological disorders:**

[0096] The ubiquitin-proteasome cascade is required for mammalian long-term memory formation. Lopez-Salon, M., *et al.*, Eur J Neurosci, 14:1820-6 (2001). Moreover, mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. Jiang, Y.H., *et al.*, Neuron, 21:799-811 (1998).

#### **Role of Ubiquitin-proteasome pathway in Fragile-X-mental retardation and Alzheimer's disease**

[0097] In a recent report, Meister et al. (2006), observed synthesis of Fragile X mental retardation protein (FMRP) upon induction of metabotropic Glutamate receptor mediated long-term depression (mGluR-LTD) (Hou L, Antion MD, Hu D, Spencer CM, Paylor R, Klann E: **Dynamic translational and proteasomal regulation of fragile X mental retardation protein controls mGluR-dependent long-term depression**. *Neuron* 2006, 51:441-454). The authors reported rapid increase in FMRP in the soma and dendrites of pyramidal cells upon treatment with selective group I mGluR agonist (RS) 3,5, dihydroxyphenylglycine (DHPG), which is sensitive to protein synthesis inhibitor anisomycin and Proteasome inhibitor MG132 and lactacystin. Thus, induction of mGluR-LTD is associated with both increased translation of FMRP and Proteasome dependent degradation. Intriguingly, FMRP was found associated with Ago2 protein complex and miRNAs, presumably for its role in RNAi and synaptic synthesis of proteins. In support of this model, Meister et al. (2006), also found increase in proteins whose mRNAs are predicted to bind FMRP (Hou L, Antion MD, Hu D, Spencer CM, Paylor R, Klann E: **Dynamic translational and proteasomal regulation of fragile X mental retardation protein controls mGluR-dependent long-term depression**. *Neuron* 2006, 51:441-454).

[0098] Supporting a critical role of the ubiquitin-proteasome system (UPS) in human brain disorders, Gong et al., (2006) recently reported that ubiquitin hydrolase Uch-L1 can rescue Amyloid (A $\beta$ ) induced decreases in synaptic function and contextual memory (Gong B, Cao Z, Zheng P, Vitolo OV, Liu S, Staniszewski A, Moolman D, Zhang H, Shelanski M, Arancio O: **Ubiquitin hydrolase Uch-L1 rescues beta-amyloid-induced decreases in synaptic function and contextual memory**. *Cell* 2006, 126:775-788). This study reported that Uch-L1 is required for normal synaptic and cognitive function. Uch is an enzyme that

enhances the recycling of ubiquitin, but also with some ubiquitin ligase activity. Previous studies found increased accumulation of ubiquitinated proteins and Uch-L1 is associated with neurofibrillary tangles in postmortem brains of Alzheimer's disease patients (Choi J, Levey AI, Weintraub ST, Rees HD, Gearing M, Chin LS, Li L: **Oxidative modifications and down-regulation of ubiquitin carboxyl-terminal hydrolase L1 associated with idiopathic Parkinson's and Alzheimer's diseases.** *J Biol Chem* 2004, **279**:13256-13264. Epub 12004 Jan 13213; de Vrij FM, Fischer DF, van Leeuwen FW, Hol EM: **Protein quality control in Alzheimer's disease by the ubiquitin proteasome system.** *Prog Neurobiol* 2004, **74**:249-270). LDN-57444 (LDN), a reversible inhibitor of Uch-L1 reduced long-term potentiation and contextual fear memory, as was also observed due to A $\beta$  overexpression in culture and in double transgenic mice (APP/PS1) overexpressing APP (K670N:M671L) and PS1 (M146L). In addition, intraperitoneal injections with a fusion Uch-1 protein improved learning in APP/PS1 mice overtime. Thus, this study provides a strong link between UPS, synaptic plasticity and memory dysfunction (Gong B, Cao Z, Zheng P, Vitolo OV, Liu S, Staniszewski A, Moolman D, Zhang H, Shelanski M, Arancio O: **Ubiquitin hydrolase Uch-L1 rescues beta-amyloid-induced decreases in synaptic function and contextual memory.** *Cell* 2006, **126**:775-788).

[0099]        Parkinson's disease: Parkin (PARK2) has a RING finger domain (signature sequence of Ubiquitin ligase). Mutations in the ubiquitin ligase Parkin results in Autosomal Recessive Juvenile Parkinsonism (AR-JP). Mutation in PARK2 is also associated with 50% patients of autosomal recessive Parkinson's disease (APRD), which is the most common for of PD. Thus, a direct pathogenetic link exists between the Proteasome function and the resulting disease.

[0100]        In the following examples, the aggregated disease-specific proteins inhibit the activity of the ubiquitin-proteasome system. For example, proteinaceous deposits associated with the following neurodegenerative diseases are also immunopositive for Ubiquitin:

- Alzheimer's disease
- Brainstem Lewy body (neuropathological hallmark of PD)
- Bunias Bodies (in ALS)
- Nuclear inclusions in (Huntington's)
- Spinocerebellar Ataxia
- Spinal and Bulbar Muscular dystrophy (SBMA)

[0101] Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by a CAG trinucleotide repeat expansion in the HD gene. The expanded repeats are translated into an abnormally long polyglutamine tract close to the N-terminus of the HD gene product ('huntingtin'). Studies in humans and mouse models suggest that the mutation is associated with a deleterious gain-of-function. Several studies have suggested that the large huntingtin protein is cleaved to produce a shorter N-terminal fragment containing the polyglutamine expansion, and that the polyglutamine expansion causes the protein fragment to misfold and form aggregates (inclusions) in the nuclei and processes of neurons. It is likely that neurotoxicity is caused by the misfolded protein in its soluble form, and/or in aggregates, and/or in the process of aggregation. One potential mechanisms for neurotoxicity is the inhibition of proteasome activity. See, David C. Rubinsztein and Jenny Carmichael, *Expert Reviews in Molecular Medicine*, 5: 1-21 Cambridge University Press (2003).

[0102] Environmental toxins have been implicated in the etiology of Parkinson's disease. Recent findings of defects in the ubiquitin-proteasome system in hereditary and sporadic forms of the illness suggest that environmental proteasome inhibitors are candidate PD-inducing toxins. Adult rats were systemically injected six doses of naturally occurring (epoxomicin) or synthetic (Z-Ile-Glu(OtBu)-Ala-Leu-al [PSI]) proteasome inhibitors over a period of 2 weeks. After a latency of 1 to 2 weeks, animals developed progressive parkinsonism with bradykinesia, rigidity, tremor, and an abnormal posture, which improved with apomorphine treatment. This animal model induced by proteasome inhibitors closely recapitulates key features of PD and may be valuable in studying etiopathogenic mechanisms and putative neuroprotective therapies for the illness. See, McNaught KS, Perl DP, Brownell AL, Olanow CW, *Ann Neurol.*, 56(1):149-62 (2004).

[0103] Proteasome activators enhance the peptidase activity of proteasomes, *i.e.* the hydrolysis of peptide substrates. One skilled in the art is familiar with proteasome activators. See *e.g.*, Wojcik, C., *et al.*, *Eur. J. Cell Biol.*, 77:151 (1998); Kuehn, L. and Dahlmann, B., *Mol. Biol. Rep.*, 24:89 (1997); Dubiel, W., *et al.*, *J. Biol. Chem.*, 267:22369 (1992); and Ma, C.-P., *et al.*, *J. Biol. Chem.*, 267:10515 (1992).

[0104] The proteasome can also be targeted in the treatment of cancer, including B-cell malignancies. In preclinical cancer models, proteasome inhibitors induce apoptosis, have *in vivo* antitumor efficacy, and sensitize malignant cells and tumors to the pro-apoptotic effects of conventional chemotherapeutics and radiation therapy. Interestingly, transformed cells display greater susceptibility to proteasome inhibition than nonmalignant cells. Therefore, proteasome inhibition holds promise as a novel approach to the treatment of cancer. *See e.g.*, Voorhees, PM, *et al.*, Clin Cancer Res, 9(17):6316-25 (2003); and Schenkein, D, Clin Lymphoma, 3(1):49-55 (2002).

[0105] One skilled in the art is familiar with proteasome inhibitors, such as epoxomicin, lactacystin and bortezomib (VELCADE™). It is well appreciated in the art that there are proteasome inhibitors. Any suitable pharmacology text can be consulted including, but not limited to, the Physician's Desk Reference. (See, Physician's Desk Reference, 54th ed., Medical Economics Company, Inc., the entire teaching of which is incorporated herein by reference.)

**Table 5: miRNAs in Cancer**

| <b>miRNA</b> | <b>Organism</b> | <b>Type of Cancer</b>          |
|--------------|-----------------|--------------------------------|
| miR-143      | Human           | Colon                          |
| miR-145      | Human           | Colon                          |
| miR-155/BIC  | Human           | Burkitt and B-cell<br>Lymphoma |
| let-7        | human           | Lung-adenocarcinoma            |

**Involvement of MicroRNAs in Cancer and human brain disorders**

[0106] Several recent studies have implicated microRNAs in human cancer. In one remarkable finding authors reported human miR-373 and miR-372 as potential oncogenes that neutralize p53-mediated CDK inhibition and promote tumorigenic growth in human testicular germ cells (Voorhoeve PM, le Sage C, Schrier M, Gillis AJ, Stoop H, Nagel R, Liu YP, van Duijse J, Drost J, Griekspoor A, et al.: **A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors.** *Cell* 2006, 124:1169-1181). In other studies, several miRNAs have been implicated in cancers of lung, breast, brain, liver, colon cancer, and leukemia, with possible roles as oncogenes or tumor suppressors (Zhang B, Pan X, Cobb GP, Anderson TA: **microRNAs as oncogenes and tumor suppressors** *Developmental Biology* (2006), doi10.1016/j.ydbio.2006.08.028). The role for small non-

coding RNAs in human psychiatric and neurodegenerative disorders has also been speculated (Perkins DO, Jeffries C, Sullivan P: **Expanding the 'central dogma': the regulatory role of nonprotein coding genes and implications for the genetic liability to schizophrenia.** *Mol Psychiatry* 2005, **10**:69-78; Rogaev EI: **Small RNAs in human brain development and disorders.** *Biochemistry (Mosc)* 2005, **70**:1404-1407). Expression of large number of non-coding RNAs is predicted, some targeting thousands of mRNA for silencing protein synthesis, and thus might be involved several human complex genetic disorders (Miranda KC, Huynh T, Tay Y, Ang YS, Tam WL, Thomson AM, Lim B, Rigoutsos I: **A Pattern-Based Method for the Identification of MicroRNA Binding Sites and Their Corresponding Heteroduplexes.** *Cell* 2006, **126**:1203-1217).

[0107] Any of the identified compounds of the present invention can be administered to a subject, including a human, by itself, or in pharmaceutical compositions where it is mixed with suitable carriers or excipients at doses therapeutically effective to prevent, treat or ameliorate a variety of disorders, including those characterized by that outlined herein. A therapeutically effective dose further refers to that amount of the compound sufficient result in the prevention or amelioration of symptoms associated with such disorders. Techniques for formulation and administration of the compounds of the instant invention may be found in Goodman and Gilman's The Pharmacological Basis of Therapeutics, Pergamon Press, latest edition.

[0108] The compounds of the present invention can be targeted to specific sites by direct injection into those sites. Compounds designed for use in the central nervous system should be able to cross the blood-brain barrier or be suitable for administration by localized injection.

[0109] Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount effective to prevent development of or alleviate the existing symptoms and underlying pathology of the subject being treating. Determination of the effective amounts is well within the capability of those skilled in the art.

[0110] For any compound used in the methods of the present invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the  $IC_{50}$  (the dose where 50% of the cells show the desired effects) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans.

[0111] A therapeutically effective dose refers to that amount of the compound that results in the attenuation of symptoms or a prolongation of survival in a subject. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the  $LD_{50}$  (the dose lethal to 50% of a given population) and the  $ED_{50}$  (the dose therapeutically effective in 50% of a given population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between  $LD_{50}$  and  $ED_{50}$ . Compounds which exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the  $ED_{50}$  with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of a patient's condition. Dosage amount and interval can be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the desired effects.

[0112] In case of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

[0113] The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

[0114] The pharmaceutical compositions of the present invention can be manufactured in a manner that is itself known, *e.g.*, by means of conventional mixing,

dissolving, granulating, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

[0115] Pharmaceutical compositions for use in accordance with the present invention thus can be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

[0116] For injection, the agents of the invention can be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barriers to be permeated are used in the formulation. Such penetrants are generally known in the art.

[0117] For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject to be treated. Pharmaceutical preparations for oral use can be obtained solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinyl-pyrrolidone (PVP). If desired, disintegrating agents can be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

[0118] Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions can be used, which can optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments can be

added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

[0119] Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds can be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers can be added. All formulations for oral administration should be in dosages suitable for such administration.

[0120] For buccal administration, the compositions can take the form of tablets or lozenges formulated in conventional manner.

[0121] For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoromethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit can be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.*, gelatin for use in an inhaler or insufflator can be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

[0122] The compounds can be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form, *e.g.*, in ampoules or in multidose containers, with an added preservatives. The compositions can take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

[0123] Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the

active compounds can be prepared as appropriate oily injection suspension. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions can contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension can also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

[0124] Alternatively, the active ingredient can be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

[0125] The compounds can also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

[0126] In addition to the formulations previously described, the compounds can also be formulated as a depot preparation. Such long acting formulations can be administered by implantation (*e.g.*, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds can be formulated with suitable polymeric or hydrophobic materials (*e.g.*, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, *e.g.*, as a sparingly soluble salt.

[0127] A pharmaceutical carrier for the hydrophobic compounds of the invention is a co-solvent system comprising benzyl alcohol, a non-polar surfactant, a water-miscible organic polymer, and an aqueous phase. Naturally, the proportions of a co-solvent system can be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components can be varied.

[0128] Alternatively, other delivery systems for hydrophobic pharmaceutical compounds can be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds can be delivered using a sustained-release system, such as

semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various of sustained-release materials have been established and are well known to those skilled in the art. Sustained-release capsules can, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization can be employed.

[0129] The pharmaceutical compositions also can comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include, but are not limited to, calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

[0130] Many of the compounds of the invention can be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts can be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, *etc.* Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms.

[0131] Suitable routes of administration can, *e.g.*, include oral, rectal, transmucosal, transdermal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

[0132] Alternatively, one can administer the compound in a local rather than systemic manner, *e.g.*, via injection of the compound directly into an affected area, often in a depot or sustained release formulation.

[0133] Furthermore, one can administer the compound in a targeted drug delivery system, *e.g.*, in a liposome coated with an antibody specific for affected cells. The liposomes will be targeted to and taken up selectively by the cells.

[0134] The compositions can, if desired, be presented in a pack or dispenser device which can contain one or more unit dosage forms containing the active ingredient. The pack

can, e.g., comprise metal or plastic foil, such as a blister pack. The pack or dispenser device can be accompanied by instruction for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier can also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition. Suitable conditions indicated on the label can include treatment of a disease such as described herein.

## EXAMPLES

### *Drosophila stocks and genetics*

[0135] Fly stocks were maintained at 25°C on standard cornmeal agar medium under a constant 24 hr. light/dark cycle. *Aubergine* (*aub<sup>HN</sup>* & *aub<sup>QC42</sup>*) and *Df(3L)E1* strains were obtained from the Bloomington Stock Center (Indiana). Transformant lines carrying *UAS-GFP::Armi*, and *armi* mutant lines were generously provided by H. Cook and W. Theurkauf (U.Mass., Worcester). The *GHI46-GAL4* strain was obtained from Dr. R. Stocker (Berlin). The strain *y,w<sup>67c23</sup>* was used for *P*-element-mediated transformation.

### *Immunohistochemical methods*

[0136] Adult brains were dissected in PBS, and processed for immunohistochemistry as described by Kunes *et al* (1993). Antibodies were used at the following dilutions: mouse  $\alpha$ -CaMKII (1:100; Takamatsu *et al.*, 2003), rabbit  $\alpha$ -CaMKII (1:4000; Koh *et al.*, 1999),  $\alpha$ -ARD (1:50),  $\alpha$ -Elav (1:100),  $\alpha$ -Armi (1:200) and  $\alpha$ -KHC (1:50),  $\alpha$ -mouse Cy3 (1:100),  $\alpha$ -rat Cy5 (1:200) and  $\alpha$ -rabbit Cy5 (1:500). The brains were mounted in 70% glycerol containing paraphenylenediamine for confocal microscopic analysis.

### *Explant conditions*

[0137] Adult brains were exposed by the removal of cuticle from adult heads while submersed in 'adult hemolymph' medium (AHL; Wang *et al.*, 2003) and further incubated as described in the text. When present, nicotine [(-) Nicotine, Sigma] was dissolved in AHL at 100  $\mu$ M, and adjusted to pH7.0-7.4. Acetylcholine (Acetylcholine chloride ~99%, Sigma) was utilized at a concentration of 50  $\mu$ M. The specimens were incubated at room temperature for 5-20 minutes as indicated, washed three times in AHL and fixed in 4% paraformaldehyde.

For experiments involving proteasome inhibition, specimens were incubated for 40 minutes in lactacystin (100  $\mu$ M in AHL; 0.2 mg, Sigma) before further manipulation.

[0138] The *UAS-DTS5* strain (Speese *et al.*, 2003) was crossed to *elav-GAL4*, *UAS-GFP::armi* or *GHI46-GAL4*, *UAS-GFP::Armi* animals and grown at 17°C (permissive temperature) through development. Animals were dissected in AHL and, where indicated, incubated at 35°C for 30 minutes before fixing in 4% paraformaldehyde. Sibling flies that remained at permissive temperature were dissected at room temperature and immediately fixed.

#### **Behavioral assays**

[0139] Short and long-term olfactory memory tests were performed in the T-maze apparatus according to the protocols of Dubnau *et al.* (2003), Pascual *et al.* (2001), Tully *et al.* (1985) and Yu *et al.* (2004)). For the behavioral experiments, most transgenic lines were backcrossed to Canton S (obtained from C. Quinn, MIT) for 3-4 generations prior to use. Animals were used between 2-4 days post-eclosion.

#### **Fluorescence intensity measurements**

[0140] Brains were imaged by confocal microscopy (on a Zeiss LSM510) with constant optical settings between control and experimental samples, which were all typically examined in a single session. Mean fluorescence intensities were calculated by averaging the intensity of several ( $n \approx 10$ ) brains. The intensity of specific channels was then measured in Image J software and analyzed with Microsoft Excel and STATA 8.0 software. A Pearson's Correlation coefficient was determined with Image J, when co-localization of different markers was evaluated.

#### **Image quantification and statistical analysis after olfactory conditioning**

[0141] Adult brains were isolated at times noted post-training or without training, fixed and stained with antibodies, as indicated in the text. At least 10 control and 10 experimental animals were dissected for each behavioral assay, and assays were repeated as indicated in the text. Images were collected as Z-series stacks, maintaining constant optical parameters throughout the analysis. The images were individually analyzed with Image J software.

[0142] The EYFP fluorescent intensity was determined for particularly glomeruli, using mABnc82 antibody staining to permit systematic glomerulus identification. In order to isolate pixel groups containing synaptic puncta, a specific threshold was given to a particular day's control and experimental samples. Gaussian Blur was used to smooth edges and enhance contrast between neighboring puncta. We then measured the black/white ratio in a *region of interest* (ROI). To evaluate the significance of the values obtained from this processing, the data was analyzed with STATA 8.0 statistical software and graphs were plotted in Microsoft Excel. For every condition specified, experiments were repeated 3-5 times and a compilation of values were compared and when appropriate (judged by a positive learning score for a given experiment) combined and averaged to obtain mean fluorescence intensity for the image stack that contained a particular glomerulus. For statistical analysis, we used the two-sided t-test with unequal variance (significance level  $p < 0.05$ ), and measured 95% confidence intervals to measure the precision and significance of tests. ANOVA (with Bonferroni correction) was used to compare the significance of a 'fold-increase ( $\Delta F/F$ )' observed in a given glomerulus from 5 individual training episodes.

### **Recombinant DNA methods**

#### *1) CaMKII RNAi transgene*

[0143] The *CaMKII* hairpin construct was generated following the method described by Negeri et al. (2002). A 612 base pair long section of the *CaMKII* cDNA sequence was ligated with a 528 base pair long inverted sequence matching the 5' end of the longer fragment. The forward sequence was cloned using primers:

CamForRI: 5' CGGAATTCCACCAGCAGCCTGTACGCGTT3' [SEQ ID NO. 17] and

CamForNOT: 5' AAGCGGCCGCGCCGGCAAATCCAAACCA3' [SEQ ID NO. 18];

The reverse sequence was cloned using:

CamRevNOT: 5' AAGCGGCCGCGAATAACTCCACATGCCCA3' [SEQ ID NO. 19] and

CamRevXho: 5' CGCTCGAGCACCAGCAGCCTGTACGCGTT3' [SEQ ID NO. 20].

#### *2) EGFP-MS2-nls and MS2bs-CaMKII 3' UTR constructs*

[0144] To construct *P[UAS-GFP-MS2-nls]*, the *EGFP-MS2-nls* sequence (Rook, 2000 #59) was ligated between the XhoI and KpnI sites in SK (Stratagene, LaJolla, CA). The

resultant plasmid was cleaved with XhoI and KpnI. The *EGFP-MS2-nls* fragment was ligated into the *pUSAT* vector (Brand and Perrimon, 1993).

[0145] To construct *P[UAS-MS2bs-CaMKII<sup>3'UTR</sup>]* transgene was made by excising the MS2bs-8UTR region from the RSV-MS2-8UTR plasmid with BamHI and NotI and inserting it into the pBS-KS vector. A *Drosophila CaMKII 3'UTR* with flanking BgIII and NotI sites was generating via PCR amplification from cDNA using primers:

CamXho: 5'CTCGAGTTTTTATTATTATCTTTAAAAATTC3' [SEQ ID NO. 21] and

Cam3UbgI: 5'GCAGATCTTAGTGGGCATTAATCAATGG3' [SEQ ID NO. 22]

and ligated into the TOPO plasmid. The *dCaMKII 3'UTR* was excised using BgIII and NotI and ligated into *pUAST-EYFP*, then later excised and ligated between the BgIII and NotI sites in the *KS MS2bs* plasmid. The fused *MS2bs-dCaMKII 3'UTR* was excised with BamHI and NotI and ligated between the BamHI and NotI sites of *pUAST*.

[0146] To construct *P[CaMKII::EYFP<sup>NUT</sup>]*, the *CaMKII* ORF was amplified from a cDNA (cDNA 415, (Takamatsu, et al. 2003) clone with two primers, one bearing a BamHI site on the 5'-end and another bearing an EcoRI on the 3' end. EYFP was amplified from *pEYFP-C1* (Clontech) with primers carrying RI on the 5'-end and BgIII on the 3' end. They were ligated into the pBS-KS+ vector. An EcoRI fragment containing the fusion construct was sub-cloned into the EcoRI site of *pUAST*. The *P[EYFP::CaMKII]* full length fusion protein was generated following a similar strategy.

### **Drosophila miRNA-280 Localization**

[0147] *Drosophila* miRNA-280 is localized to the synapse of brain circuit involved in learning and memory. RNA *in situ* hybridization with LNA probes (Thomsen R, Nielsen PS, Jensen TH: **Dramatically improved RNA in situ hybridization signals using LNA-modified probes.** *RNA* 2005, 11:1745-1748. Epub 2005 Sep 1721) reveals such localization in antennal lobe, mushroom body (MB), neuropil and synapses of the MB calyx (FIGURES 11A and 11B).

## LITERATURE CITED

- Bailey, C. H., Kandel, E. R. and Si, K. (2004). The persistence of long-term memory: a molecular approach to self-sustaining changes in learning-induced synaptic growth, *Neuron* 44, 49-57.
- Beck, C. D., Schroeder, B. and Davis, R. L. (2000). Learning performance of normal and mutant *Drosophila* after repeated conditioning trials with discrete stimuli, *J Neurosci* 20, 2944-53.
- Bentwich, I., Avniel, A., et al. Identification of hundreds of conserved and nonconserved human microRNAs. *Nature Genetics* 2005 July 19; 37(7): 766-770.
- Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes, *Development* 118, 401-15.
- Burbea, M., Dreier, L., Dittman, J. S., Grunwald, M. E., and, and Kaplan, J. M. (2002). Ubiquitin and AP180 regulate the abundance of GLR-1 glutamate receptors at postsynaptic elements in *C. elegans*, *Neuron* 35, 107-20.
- Chamaon, K., Smalla, K. H., Thomas, U. and Gundelfinger, E.D. (2002). Nicotinic Acetylcholine receptors of *Drosophila*: three subunits encoded by genomically linked genes can co-assemble into the same receptor complex. *J. Neurochem.* 80, 149-57.
- Ciechanover, A and Brundin, P. The Ubiquitin system in Neurodegenerative diseases: Sometimes the Chicken, sometimes the Egg. *Neuron*. 2003 Oct 9; 40: 427-446.
- Colledge, M., Snyder, E. M., Crozier, R. A., Soderling, J. A., Jin, Y., Langeberg, L. K., Lu, H., Bear, M. F., and, and Scott, J. D. (2003). Ubiquitination regulates PSD-95 degradation and AMPA receptor surface expression, *Neuron* 40, 595-607.
- Cook, H. A., Koppetsch, B. S., Wu, J. and Theurkauf, W. E. (2004). The *Drosophila* SDE3 homolog *armitage* is required for *oskar* mRNA silencing and embryonic axis specification, *Cell* 116, 817-29.
- Costa, F. F. Non-coding RNAs: new players in eukaryotic biology. *Gene*. 2005; 357:83-94.
- Davis, R. L. (2004). Olfactory learning. 44, 31-48.
- DiAntonio, A., and, and Hicke, L. (2004). Ubiquitin-dependent regulation of the synapse, *Annu Rev Neurosci* 27, 223-46.
- Dubnau, J., Chiang, A. S., Grady, L., Barditch, J., Gossweiler, S., McNeil, J., Smith, P., Buldoc, F., Scott, R., Certa, U., et al. (2003). The *staufen/pumilio* pathway is involved in *Drosophila* long-term memory, *Curr Biol* 13, 286-96.

- Ehlers, M. D. (2003a). Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system, *Nat Neurosci* 6, 231-42.
- Ehlers, M. D. (2003b). Eppendorf 2003 prize-winning essay. Ubiquitin and the deconstruction of synapses, *Science* 302, 800-1.
- Enright, A. J., John, B., Gaul, U., Tuschl, T., Sander, C. and Marks, D. S. (2003). MicroRNA targets in *Drosophila*, *Genome Biol* 5, R1.
- Faber, T., Joerges, J. and Menzel, R. (1999). Associative learning modifies neural representations of odors in the insect brain, *Nat Neurosci* 2, 74-8.
- Forrest, K. M. and Gavis, E. R. (2003). Live imaging of endogenous RNA reveals a diffusion and entrapment mechanism for nanos mRNA localization in *Drosophila*., *Curr Biol* 13, 1159-68.
- Forstemann, K., Tomari, Y., et al., Normal microRNA maturation and Germ-line stem cell maintenance requires Loquacious a double-stranded RNA-binding domain protein. *PLOS Biology*. 2005 July; 3(7): e236.
- Ge, X., Hannan, F., Xie, Z., Feng, C., Tully, T., Zhou, H., and, and Zhong, Y. (2004). Notch signaling in *Drosophila* long-term memory formation, *Proc Natl Acad Sci U S A* 101, 10172-6.
- Griffith, L. C., Verselis, L. M., Aitken, K. M., Kyriacou, C. P., Danho, W. and Greenspan, R. J. (1993). Inhibition of calcium/calmodulin-dependent protein kinase in *Drosophila* disrupts behavioral plasticity, *Neuron* 10, 501-9.
- Heisenberg, M. (2003). Mushroom body memoir: from maps to models, *Nat Rev Neurosci* 4, 266-75.
- Huang, Y. S., Carson, J. H., Barbarese, E. and Richter, J. D. (2003). Facilitation of dendritic mRNA transport by CPEB, *Genes Dev* 17, 638-53.
- Jing, Q., Huang, S., Guth, S., Zarubin, T., Motoyama, A., Chen, J., Di Padova, F., Lin, S. C., Gram, H. and Han, J. (2005). Involvement of microRNA in AU-rich element-mediated mRNA instability, *Cell* 120, 623-34.
- Joiner M, A., and, and Griffith, L. C. (1997). CaM kinase II and visual input modulate memory formation in the neuronal circuit controlling courtship conditioning, *J Neurosci* 17, 9384-91.
- Kanai, Y., Dohmae, N. and Hirokawa, N. (2004). Kinesin transports RNA: isolation and characterization of an RNA-transporting granule., *Neuron* 43, 513-25.

- Kelleher, R. J., 3rd., Govindarajan, A. and Tonegawa, S. (2004). Translational regulatory mechanisms in persistent forms of synaptic plasticity, *Neuron* 44, 59-73.
- Koh, Y. H., Popova, E., Thomas, U., Griffith, L. C. and Budnik, V. (1999). Regulation of DLG localization at synapses by CaMKII-dependent phosphorylation, *Cell* 98, 353-63.
- Kunes, S., Wilson, C. and Steller, H. (1993). Independent guidance of retinal axons in the developing visual system of *Drosophila*, *J Neurosci* 13, 752-67.
- Lai, E. C., Tam, B. and Rubin, G. M. (2005). Pervasive regulation of *Drosophila* Notch target genes by GY-box-, Brd-box-, and K-box-class microRNAs, *Genes Dev* 19, 1067-80.
- Lai, E. C., Tomancak, P., Williams, R. W. and Rubin, G. M. (2003). Computational identification of *Drosophila* microRNA genes, *Genome Biol* 4, R42.
- Lai, E. C., Wiel, C. and Rubin, G. M. (2004). Complementary miRNA pairs suggest a regulatory role for miRNA:miRNA duplexes, *RNA* 10, 171-5.
- Laissue, P. P., Reiter, C., Hiesinger, P. R., Halter, S., Fischbach, K. F. and Stocker, R. F. (1999). Three-dimensional reconstruction of the antennal lobe in *Drosophila melanogaster*, *J Comp Neurol* 405, 543-52.
- Lee, T., and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis, *Neuron* 22, 451-61.
- Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. *Cell*. 2003 Dec 26;115(7):787-98.
- Lisman, J., Schulman, H., and Cline, H. The molecular basis of CaMKII function in synaptic and behavioural memory. *Nat Rev Neurosci*. (2002) 3(3):175-90.
- Martin, K. C., Casadio, A., Zhu, H., Yaping, E., Rose, J. C., Chen, M., Bailey, C. H., and Kandel, E. R. (1997). Synapse-specific, long-term facilitation of aplysia sensory to motor synapses: a function for local protein synthesis in memory storage, *Cell* 91, 927-38.
- Menzel, R. and Giurfa, M. (1999). Cognition by a mini brain, *Nature* 400, 718-9.
- Miller, S., Yasuda, M., Coats, J.K., Jonesy, Y., Martone, M.E., and Mayford, M. (2002). Disruption of dendritic translation of CaMKIIalpha impairs stabilization of synaptic plasticity and memory consolidation. *Neuron* 36(3):507-19.
- Negeri, D., Eggert, H., Gienapp, R., and Saumweber, H. (2002). Inducible RNA interference uncovers the *Drosophila* protein Bx42 as an essential nuclear cofactor involved in Notch signal transduction, *Mech Dev* 117, 151-62.

- Ng, M., Roorda, R. D., Lima, S. Q., Zemelman, B. V., Morcillo, P. and Miesenbock, G. (2002). Transmission of olfactory information between three populations of neurons in the antennal lobe of the fly., *Neuron* 36, 463-74.
- Pascual, A. and Preat, T. (2001). Localization of long-term memory within the *Drosophila* mushroom body, *Science* 294, 1115-7.
- Rajewsky, N., and Socci, N. D. (2004). Computational identification of microRNA targets, *Dev Biol* 267, 529-35.
- Richter, J. D., and Lorenz, L. J. (2002). Selective translation of mRNAs at synapses, *Curr Opin Neurobiol* 12, 300-4.
- Rook, M. S., Lu, M., and Kosik, K. S. (2000). CaMKIIalpha 3' untranslated region-directed mRNA translocation in living neurons: visualization by GFP linkage, *J Neurosci* 20, 6385-93.
- Si, K., Giustetto, M., Etkin, A., Hsu, R., Janisiewicz, A. M., Miniaci, M. C., Kim, J. H., Zhu, H. and Kandel, E. R. (2003). A neuronal isoform of CPEB regulates local protein synthesis and stabilizes synapse-specific long-term facilitation in aplysia, *Cell* 115, 893-904.
- Sontheimer, E.J. Assembly and function of RNA silencing complexes. *Nature Reviews Molecular Cell Biology*. 2005, 6: 127-138.
- Speese, S. D., Trotta, N., Rodesch, C. K., Aravamudan, B. and Broadie, K. (2003). The ubiquitin proteasome system acutely regulates presynaptic protein turnover and synaptic efficacy, *Curr Biol* 13, 899-910.
- Stark, A., Brennecke, J., Russell, R. B. and Cohen, S. M. (2003). Identification of *Drosophila* MicroRNA targets, *PLoS Biol* 1, E60.
- Steward, O., Wallace, C. S., Lyford, G. L., and Worley, P. F. (1998). Synaptic activation causes the mRNA for the IEG Arc to localize selectively near activated postsynaptic sites on dendrites, *Neuron* 21, 741-51.
- Steward, O. and Schuman, E. M. (2003). Compartmentalized synthesis and degradation of proteins in neurons, *Neuron* 40, 347-59.
- Takamatsu, Y., Kishimoto, Y. and Ohsako, S. (2003). Immunohistochemical study of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II in the *Drosophila* brain using a specific monoclonal antibody, *Brain Res* 974, 99-116.
- Tomari, Y., Du, T., Haley, B., Schwarz, D. S., Bennett, R., Cook, H. A., Koppetsch, B. S., Theurkauf, W. E. and PD., Z. (2004). RISC assembly defects in the *Drosophila* RNAi mutant armitage., *Cell* 116, 831-41.

- Tully, T. and Quinn, W. G. (1985). Classical conditioning and retention in normal and mutant *Drosophila melanogaster*, J Comp Physiol [A] 157, 263-77.
- Tully, T., Preat, T., Boynton, S. C. and Del Vecchio, M. (1994). Genetic dissection of consolidated memory in *Drosophila*. Cell 79, 35-47.
- Vosshall, L. B., Wong, A. M. and Axel, R. (2000). An olfactory sensory map in the fly brain, Cell 102, 147-59.
- Waddell, S., Armstrong, J. D., Kitamótó, T., Kaiser, K. and Quinn, W. G. (2000). The amnesiac gene product is expressed in two neurons in the *Drosophila* brain that are critical for memory, Cell 103, 805-13.
- Wang, J. W., Wong, A. M., Flores, J., Vosshall, L. B. and Axel, R. (2003). Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain, Cell 112, 271-82.
- Webster, P. J., Liang, L., Berg, C. A., Lasko, P., and Macdonald, P. M. (1997). Translational repressor bruno plays multiple roles in development and is widely conserved, Genes Dev 11, 2510-21.
- Wilson, R. I., Turner, G. C. and Laurent, G. (2004). Transformation of olfactory representations in the *Drosophila* antennal lobe., Science 303, 366-70.
- Wu, L., Wells, D., Tay, J., Mendis, D., Abbott, M. A., Barnitt, A., Quinlan, E., Heynen, A., Fallon, J. R., and Richter, J. D. (1998). CPEB-mediated cytoplasmic polyadenylation and the regulation of experience-dependent translation of alpha-CaMKII mRNA at synapses, Neuron 21, 1129-39.
- Yu, D., Ponomarev, A. and Davis, R. L. (2004). Altered representation of the spatial code for odors after olfactory classical conditioning; memory trace formation by synaptic recruitment, Neuron 42, 437-49.

## WHAT IS CLAIMED IS:

1. A method of modulating memory comprising disrupting the RNA-induced silencing complex (RISC).
2. The method of claim 1, comprising administering to a subject a cholinergic agent.
3. The method of claim 2, wherein said cholinergic agent is selected from the group consisting of a nicotinic agonist, a muscarinic agonist, a cholinesterase inhibitor and combinations thereof.
4. The method of claim 3, wherein said cholinergic agent is a muscarinic or nicotinic agonist selected from the group consisting of acetylcholine, nicotine, muscarine, bethanechol, carbachol, Cevimeline, Pilocarpine, suberylcholine, succinylcholine, anabasine, decamethonium, Aceclidine and combinations thereof.
5. The method of claim 3, wherein said cholinesterase inhibitor is selected from the group consisting of Ambenonium, Donepezil, Edrophonium, Galantamine, Neostigmine, Physostigmine, Pyridostigmine, Rivastigmine, Tacrine and combinations thereof.
6. The method of claim 1, comprising administering to a subject an agent which modulates the activity of a small RNA.
7. The method of claim 6, wherein said RNA is selected from the group consisting of miRNA, rasiRNA, ncRNA and snoRNA.
8. The method of claim 7, wherein said RNA is miRNA.
9. The method of claim 8, wherein said agent is an oligonucleotide which specifically binds to an miRNA.
10. The method of claim 9, wherein said miRNA is selected from the group consisting of hsa-let-7e, hsa-let-7f, hsa-miR-105, hsa-miR-142\_3p, hsa-miR-136, hsa-miR-182, hsa-let-7a, hsa-miR-137, hsa-miR-148, hsa-miR-134, hsa-miR-130b, hsa-miR-106a, hsa-let-7c, hsa-let-7d, hsa-miR-144, hsa-miR-132, miR-1, and miR-206.

11. The method of claim 1, comprising administering to a subject an agent which modulates the biosynthesis of a small RNA.

12. The method of claim 11, wherein said RNA is selected from the group consisting of miRNA, rasiRNA, ncRNA and snoRNA.

13. The method of claim 1, comprising administering to a subject a proteasome activator.

14. The method of claim 13, wherein said proteasome activator is selected from the group consisting of 11S Regulator ( $\alpha$  and  $\beta$  subunits), 19S Regulatory complex, REG $\alpha$ , REG $\beta$ , REG $\gamma$ , PA28, PA28 $\gamma$  and PA700.

15. The method of claim 13, wherein said proteasome activator increases proteasome degradation of one or more components of the RNA-induced silencing complex.

16. The method of claim 15, wherein said one or more components of the RNA-induced silencing complex is selected from the group consisting of Mov10, Ago2, Dicer, TRBP, DGCR8, FMRP and combinations thereof.

17. The method of claim 1, wherein said disrupting RNA-induced silencing complex increases protein expression of one or more RNA-induced silencing complex target proteins.

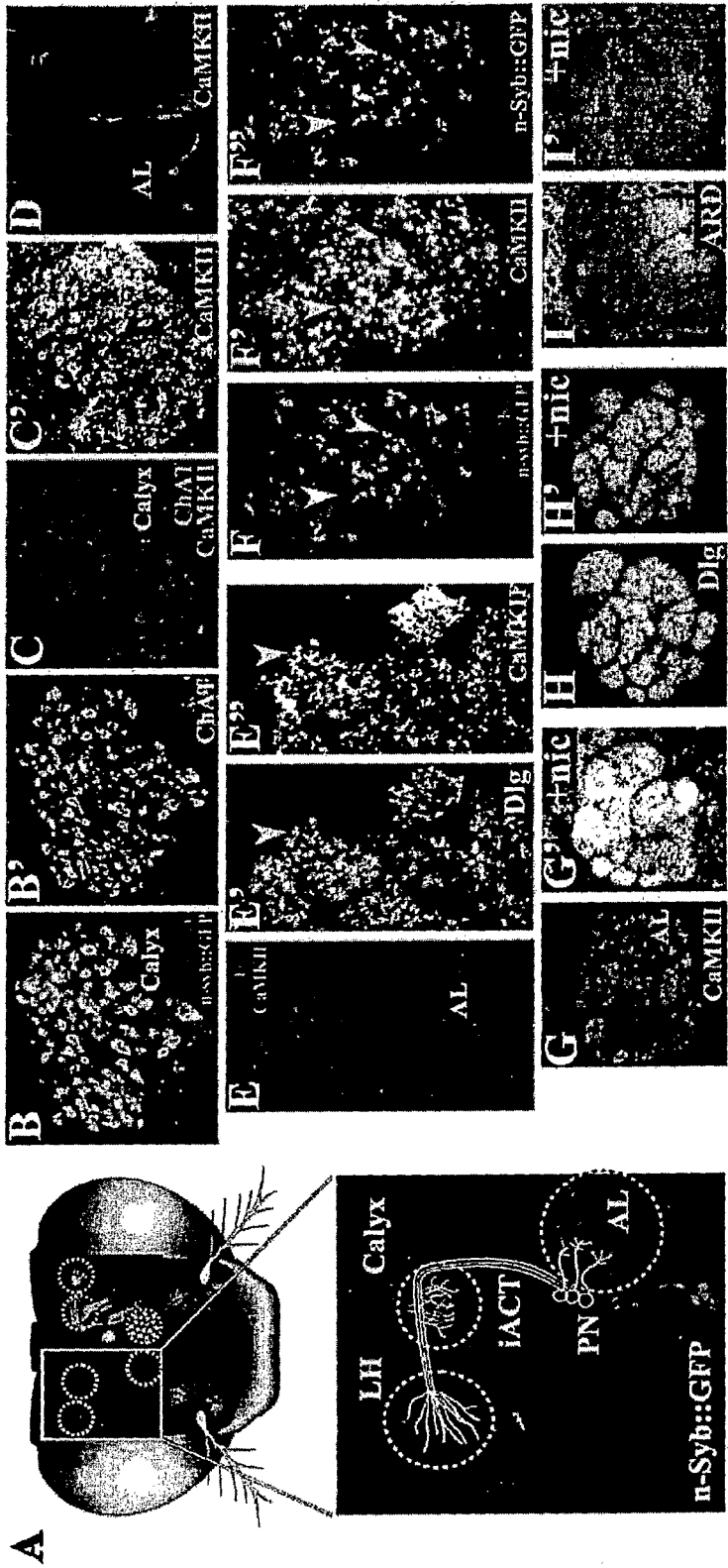
18. The method of claim 17, wherein said one or more target proteins is selected from the group consisting of CaMKII alpha chain, Staufen, Staufen Homolog 2, Disc-large homolog, Pumilio Homolog 2, Eph Related Receptor Tyrosine Kinase, Guanine Nucleotide-binding Protein G(S) Alpha subunit, Amyloid Beta Precursor Protein-Binding Protein 2, Amyloid Beta A4 Protein Precursor, Tau, CPEB, CREB binding protein, Translation Initiation Factor 4E, MAPK, Cadherin isoforms, FE65 Protein, Ubiquitin Conjugating Enzyme E2 B, Ubiquitin, Muscarinic Acetylcholine Receptor M1, NMDA receptor, KLP, Voltage Dependent R-type Calcium channel, FMRP, FMRP-2, Glycine Receptor Alpha-2 Chain, Homer Homolog, Calmodulin, , Beta-catenin, Pleckstrin Homology domain containing

Family A, Synaptotagmin I, Poly A binding protein, N-CaM 1, AChE Q subunit and combinations thereof.

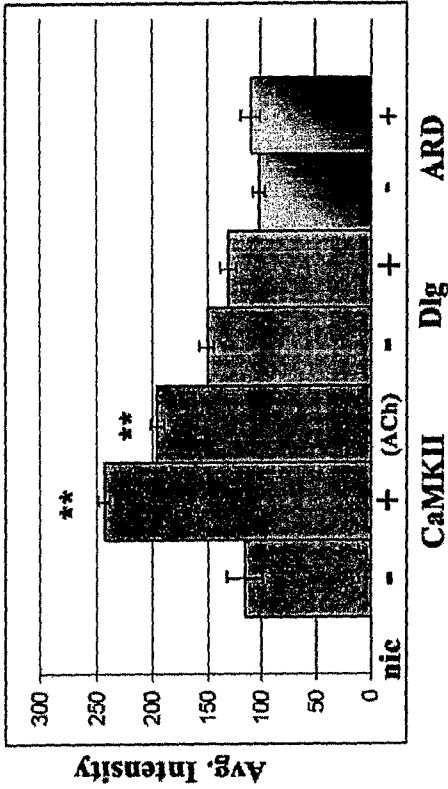
19. A method of treating Alzheimer's disease comprising disrupting the RNA-induced silencing complex.

20. A method of treating Fragile X mental retardation comprising disrupting the RNA-induced silencing complex.

Figure 1



**J**



**K**

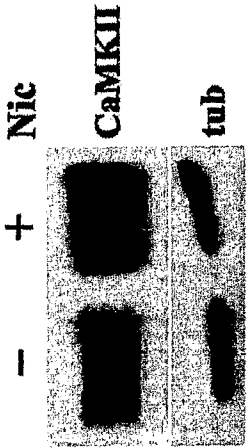


Figure 2

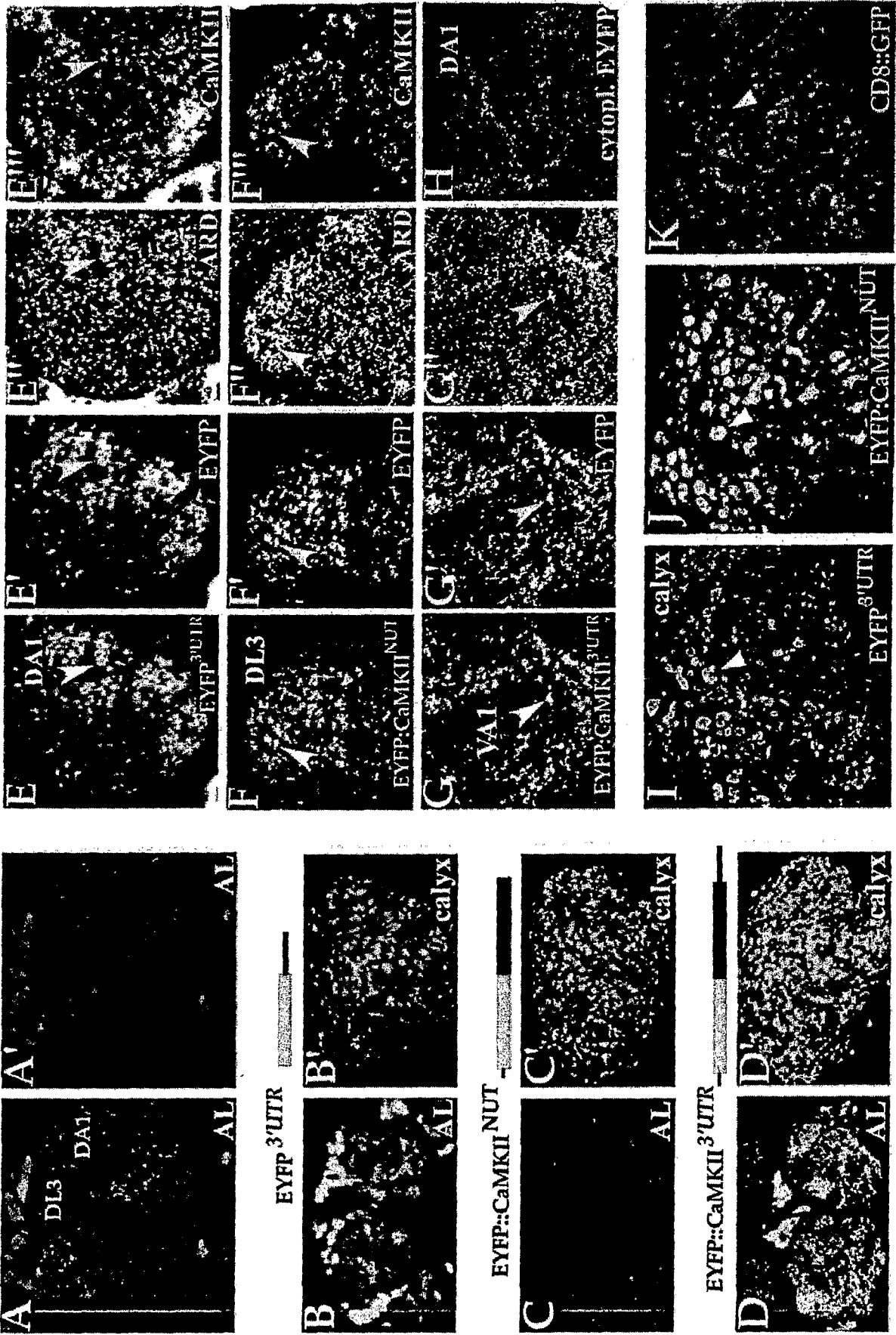


Figure 3

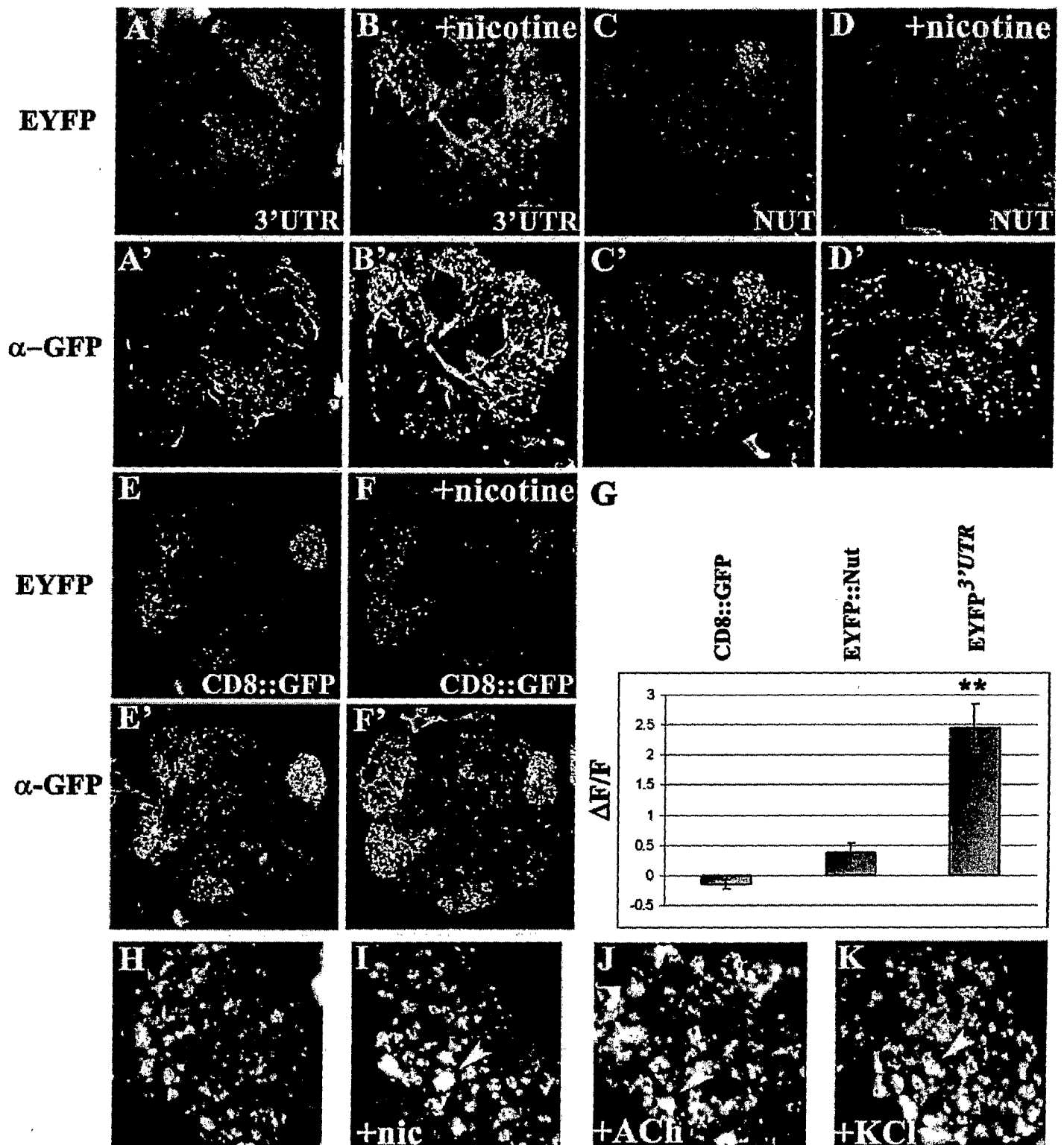


Figure 4

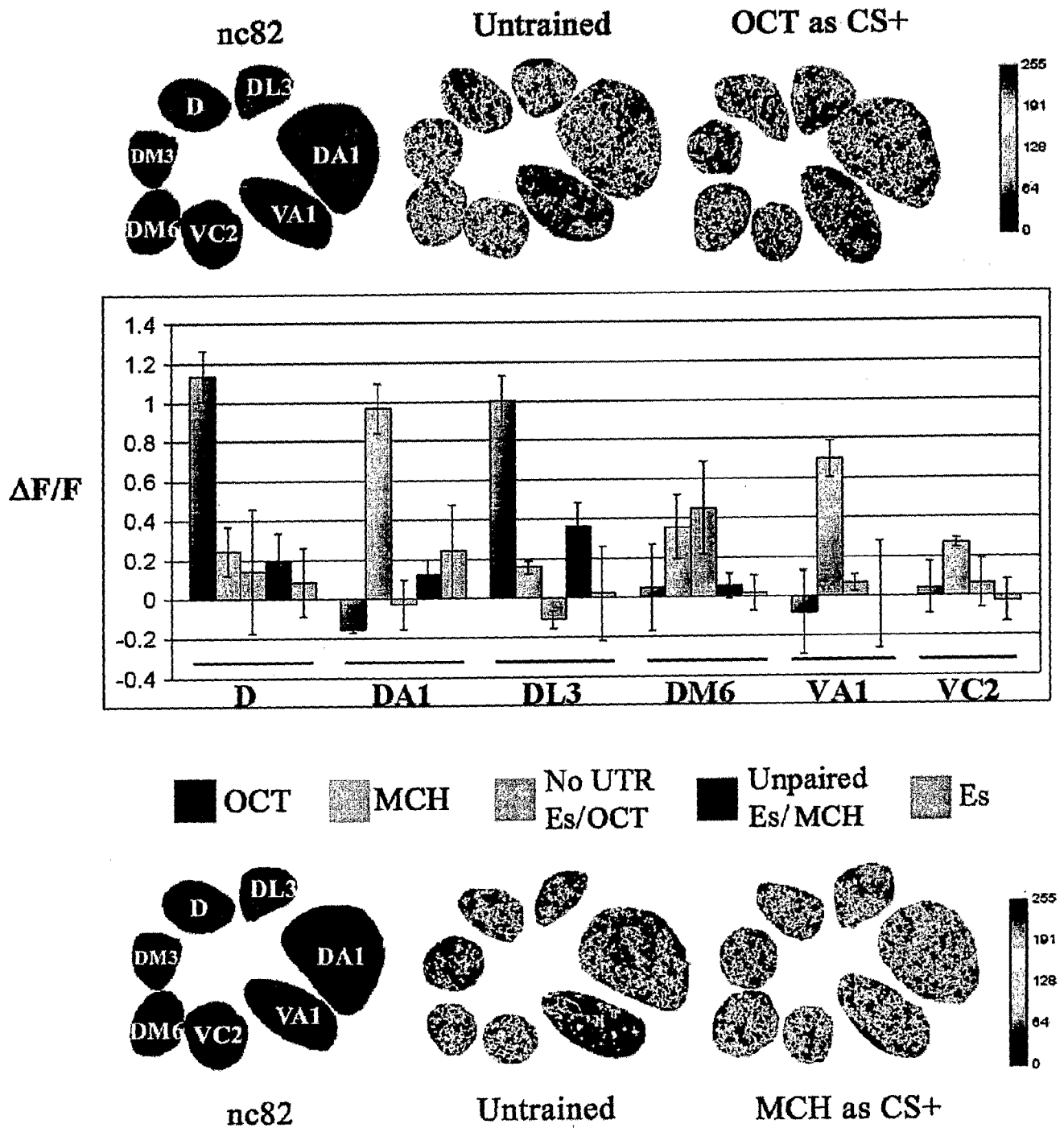


Figure 5

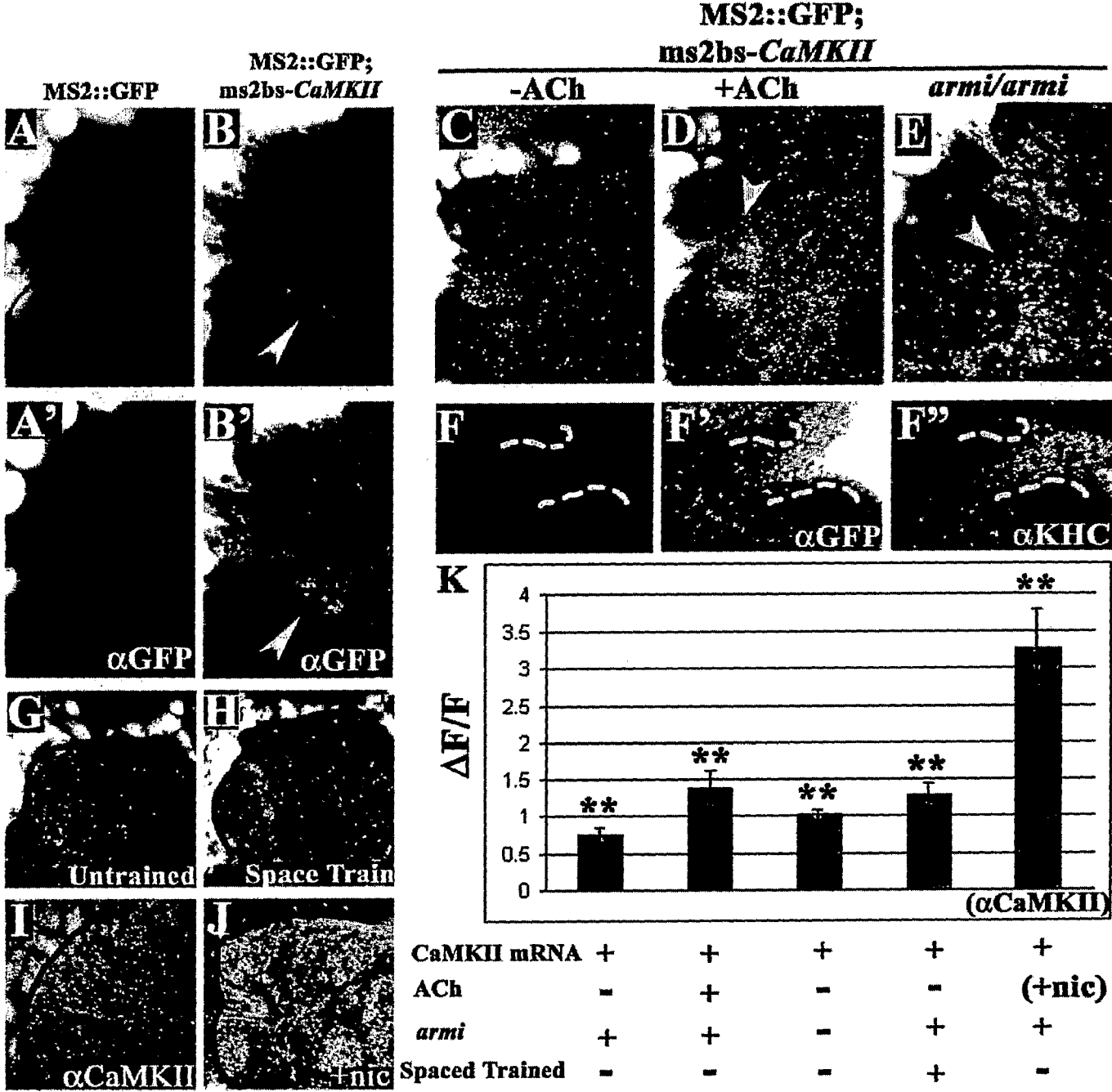


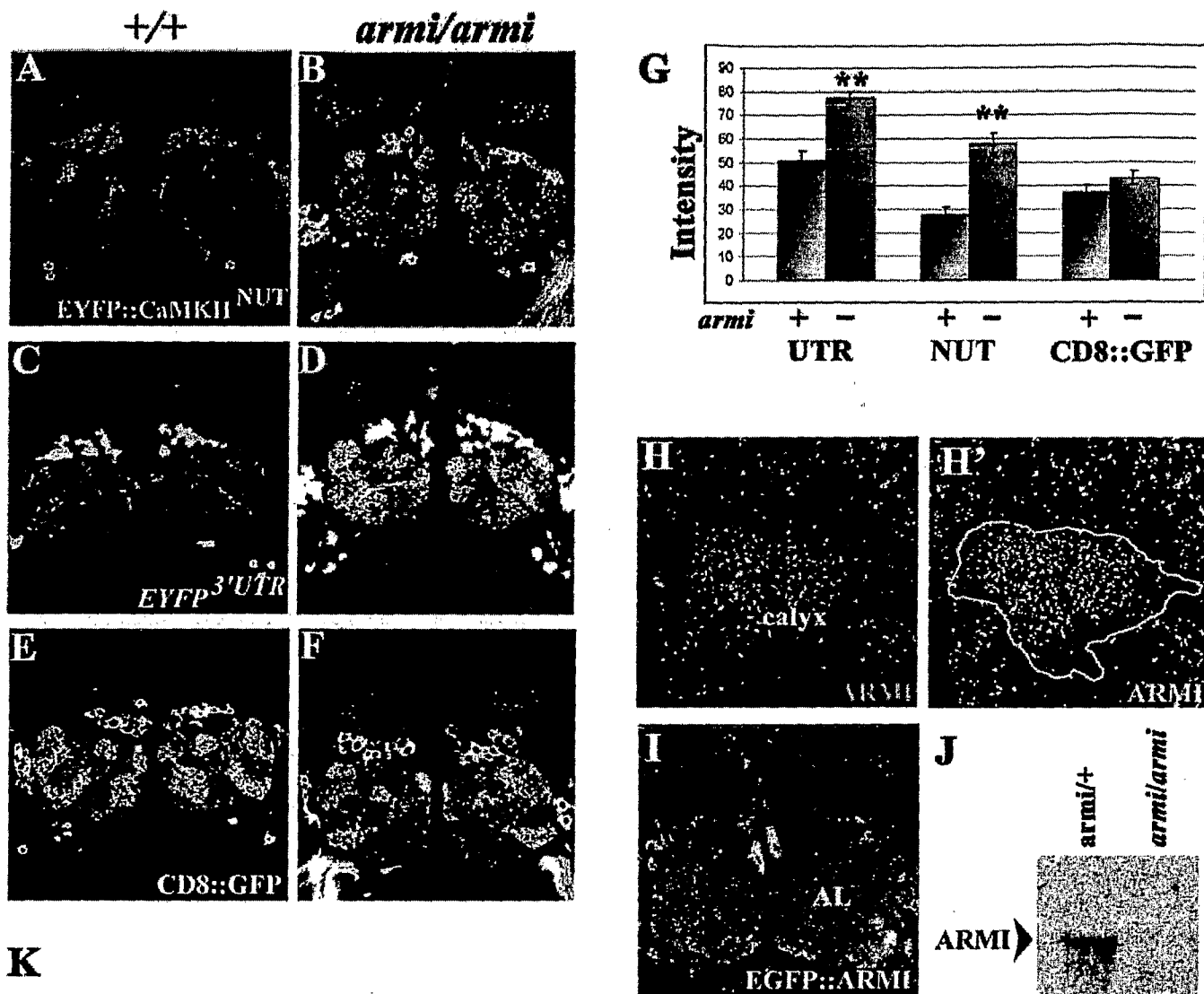
**Figure 6**

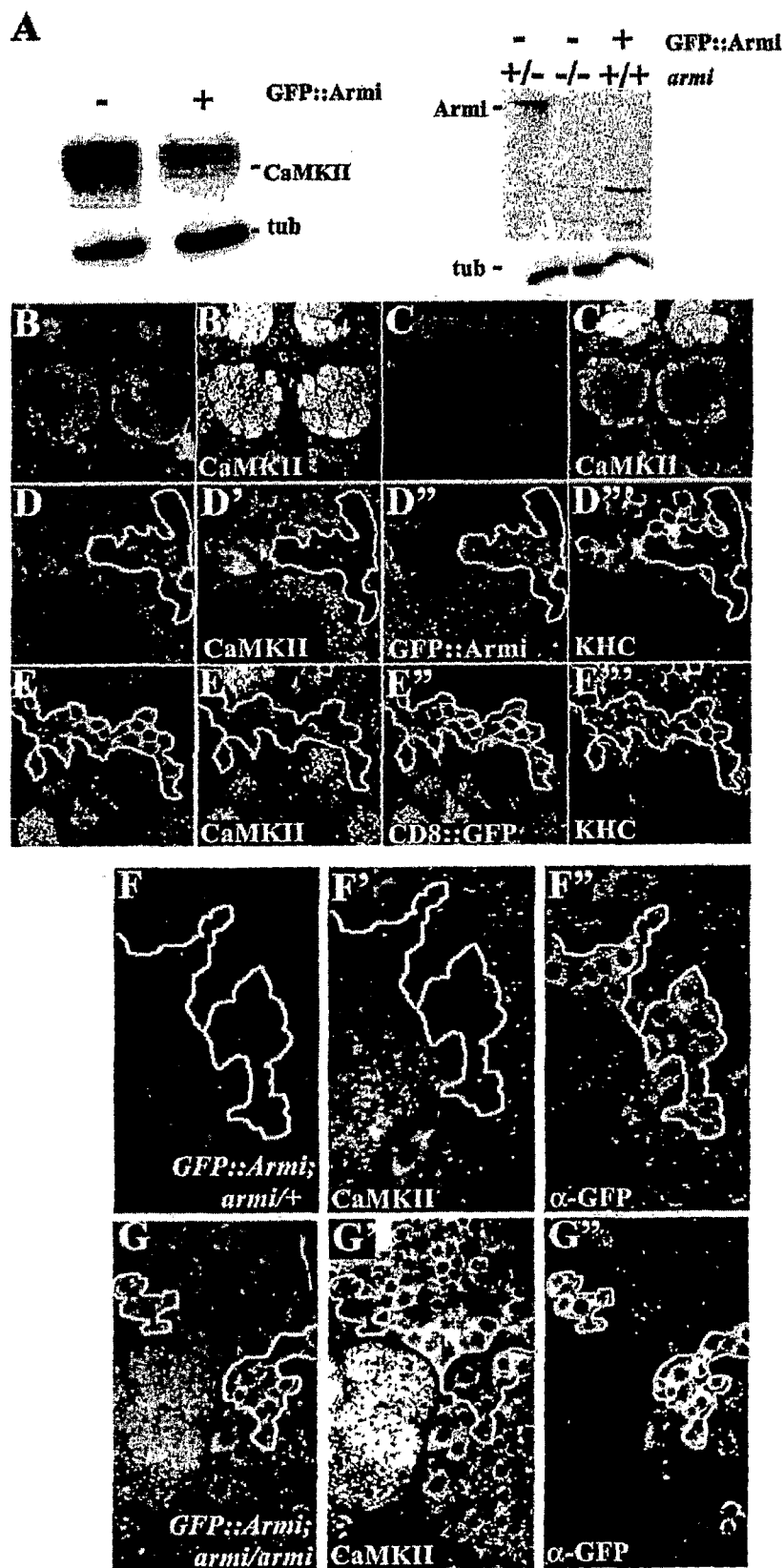
**Figure 7**

Figure 8

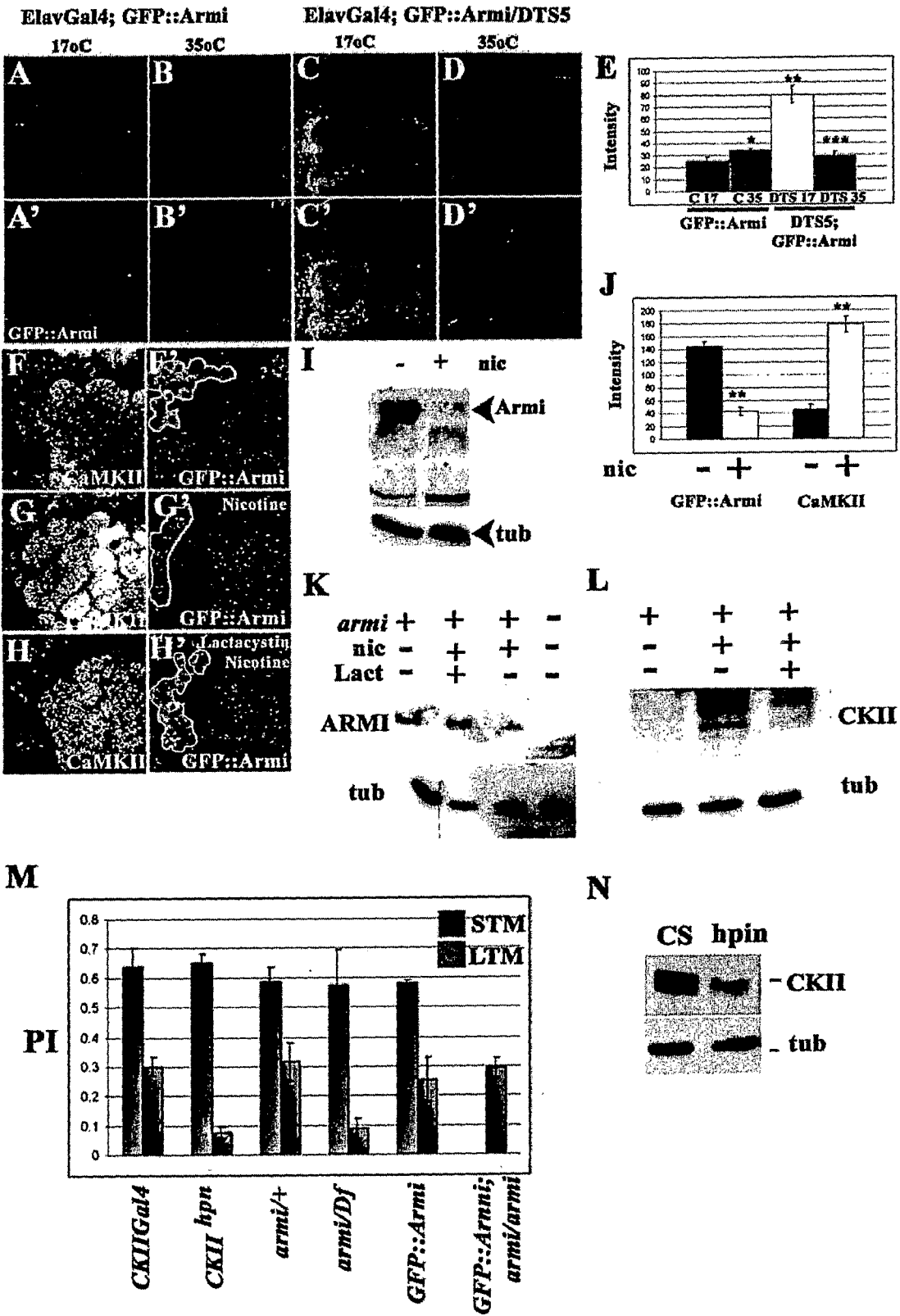


**FIGURE 9**

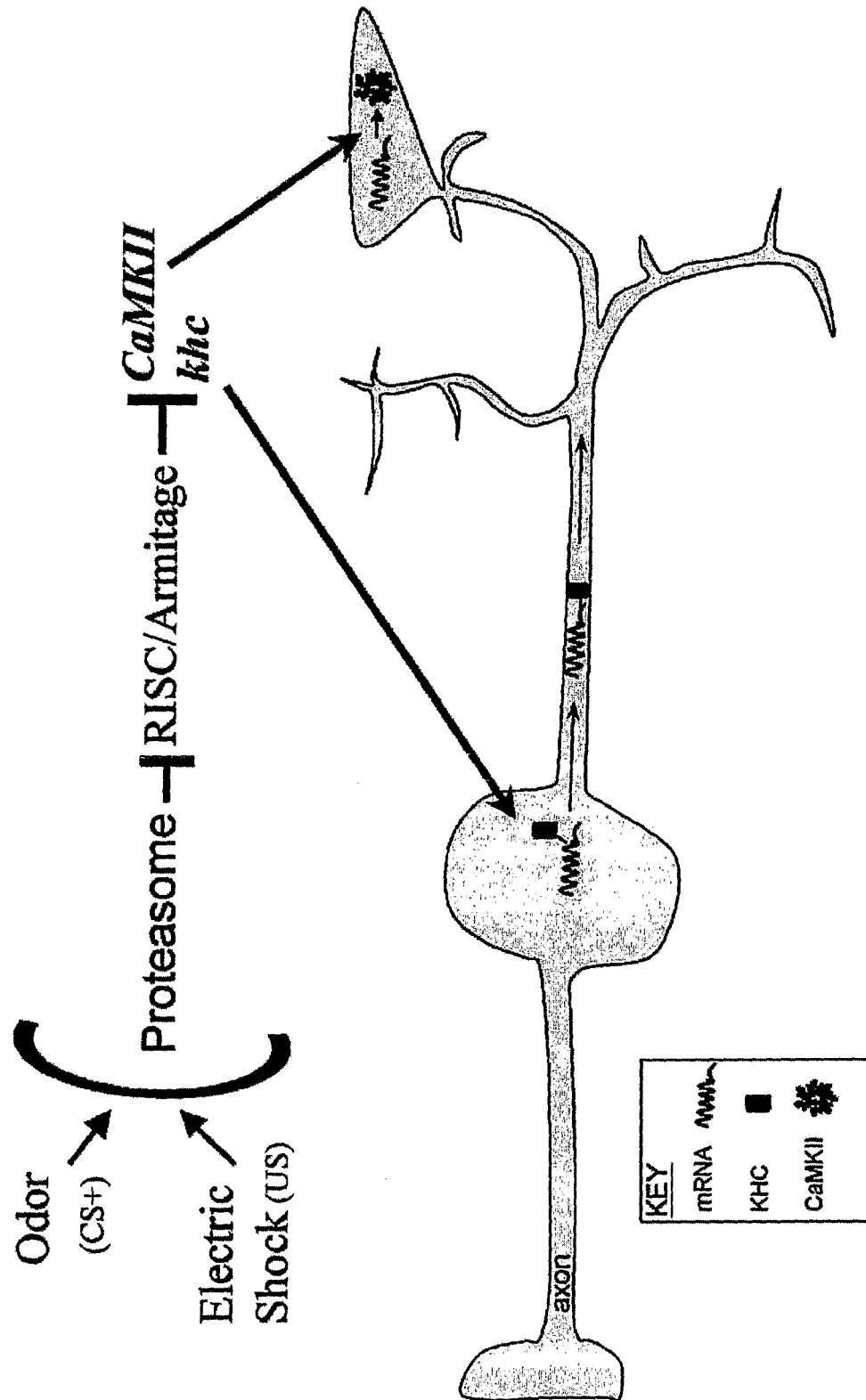


FIGURE 10

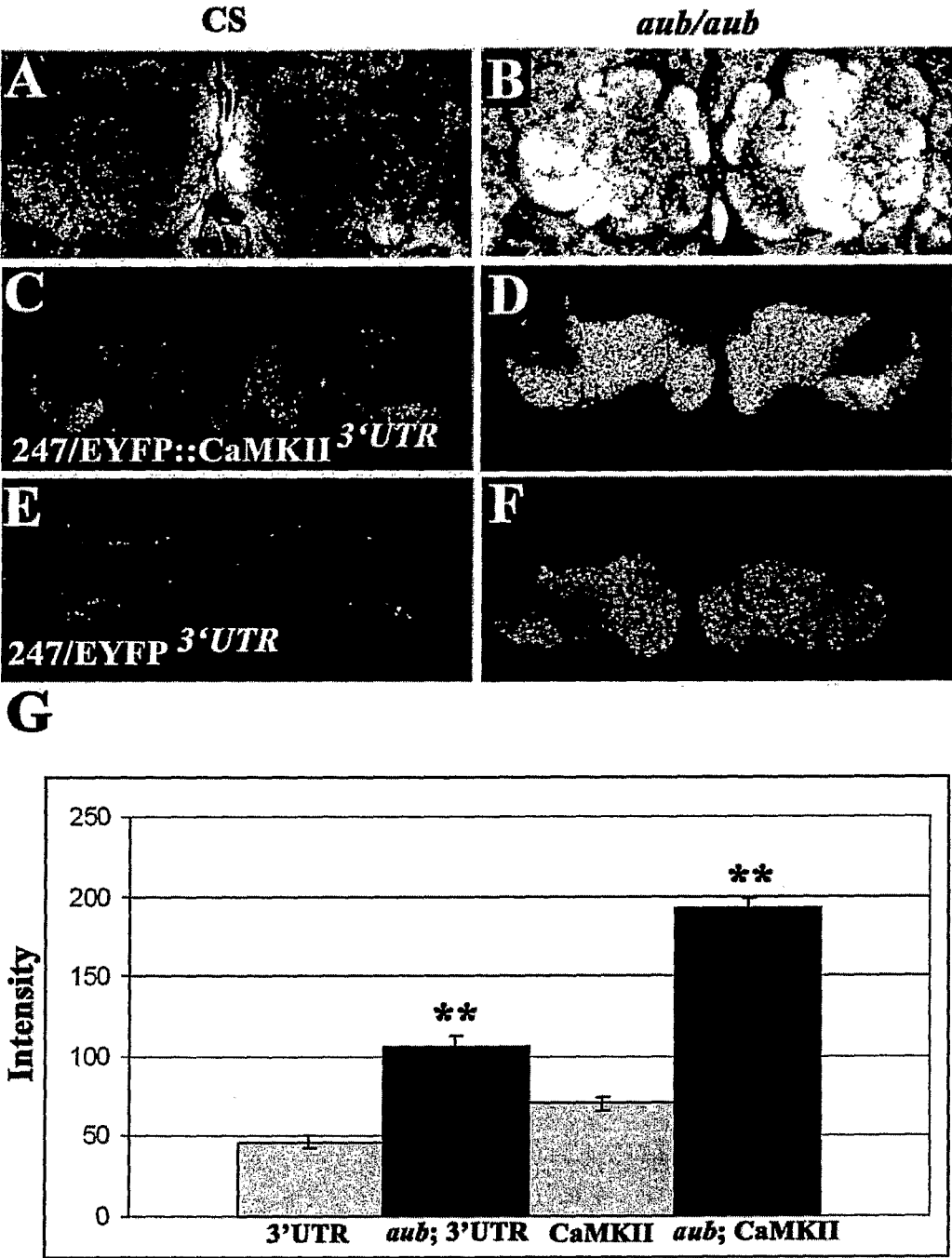


FIGURE 11A



FIGURE 11B

