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Reflectins: The Unusual Proteins of Squid Reflective Tissues

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Although we have emphasized the role of DLPFC in suppression, stopping retrieval is a complex act that recruits the full network identified in our overall analysis. This experiment does not identify the contributions of these regions, but research on attention suggests several possibilities. The ACC may play a key role in suppression, signaling the need for control by DLPFC (*10*) in response to memory intrusions and/or mediating the influence of DLPFC on the medial-temporal lobe (MTL). These possibilities are consistent with the dense bidirectional projections of ACC with MTL structures (*26*). Activations in the PMDr, pre-SMA, and IPS are often observed when prepotent motor responses need to be overridden (*9*). However, PMDr and preSMA receive multimodal inputs (*27*) and are activated by visual selective attention (*28*) and by purely cognitive tasks that demand updating in memory and require no motor output (*27*). These considerations indicate that this network serves a general function that may include controlling perceptually and memorially focused attention.

The current findings begin to specify central features of a neurobiological model of memory control that people may use to adapt their mental environment in response to traumatic experiences (*1*, *29*, *30*). Although controlling traumatic memories is difficult, intrusive remindings of trauma and the intensity of the associated emotional response to trauma-related stimuli diminish over time for most people (*31*). This remission may reflect in part the cumulative inhibitory effects of the voluntary suppression mechanism revealed here, perhaps in tandem with systems involved in the extinction of conditioned emotional responses (*32*) or in the cognitive reappraisal of traumatic memories (*33*).

Whether suppression can produce complete and lasting amnesia for an unwanted memory remains unknown. However, this work confirms the existence of an active process by which people can prevent awareness of an unwanted past experience and specifies the neural systems that underlie it. This process causes forgetting. Thus, the current findings provide the first neurobiological model of the voluntary form of repression proposed by Freud, a model that integrates this otherwise controversial proposal with widely accepted and fundamental mechanisms for controlling behavior.

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Reflectins: The Unusual Proteins of Squid Reflective Tissues

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A family of unusual proteins is deposited in flat, structural platelets in reflective tissues of the squid *Euprymna scolopes*. These proteins, which we have named reflectins, are encoded by at least six genes in three subfamilies and have no reported homologs outside of squids. Reflectins possess five repeating domains, which are highly conserved among members of the family. The proteins have a very unusual composition, with four relatively rare residues (tyrosine, methionine, arginine, and tryptophan) comprising ~57% of a reflectin, and several common residues (alanine, isoleucine, leucine, and lysine) occurring in none of the family members. These protein-based reflectors in squids provide a marked example of nanofabrication in animal systems.

The biological world is an arena of nanofabrication, one that can be tapped for information about constraints on the design and production of small-scale materials. Among the most intricate of natural nanoscale materials are those that modulate light, such as the lenses, irises, and reflectors of animals (*1*).

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Reflective tissues are prevalent across the animal kingdom, being particularly conspicuous in species that live in the visually homogeneous pelagic environments of the ocean. In these habitats, reflectors often function in camouflage by modulating incident sunlight or bioluminescence (*2*, *3*).

Reflectivity in animal tissues is achieved by the deposition of flat, insoluble, structural platelets of high refractive index that alternate in layers with materials of low refractive index. This arrangement creates thin-film interference, which results in reflection of some or all of the incident light (*4*). In aquatic animals, reflector platelets are most often

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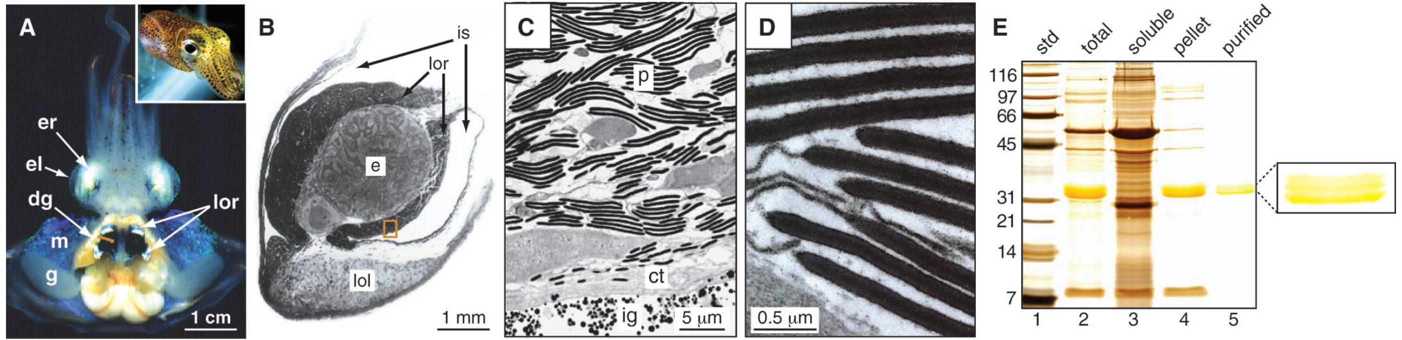

Fig. 1. Reflective tissues in *E. scolopes*. (**A**) The locations of reflective (dg, digestive gland; er, eye reflector; lor, light organ reflector; m, mantle) and nonreflective (el, eye lens; g, gill) tissues of *E. scolopes* are revealed by a ventral dissection of an adult animal (inset). (**B**) A light micrograph of a cross section [located at the orange line in (A)] of the light organ. The central epithelium (e) is surrounded by the reflector (lor), which is in turn surrounded by ink sac diverticula (is). Lens tissue (lol) is located on the ventral surface of the light organ. (**C**) TEM image of the boxed area in (B). Stacks of electron-dense reflector platelets (p) abut connective tissue (ct) and the ink sac with its secreted ink granules (ig). (**D**) Higher magnification TEM image of the LOR platelets. (**E**) Silver-stained SDS-PAGE gel of protein extracts from LORs (*12*). Lane 1, molecular mass markers (std) expressed in kD; lane 2, the total homogenate of LORs (total); lane 3, the supernatant fraction of LORs extracted in phosphate-buffered saline (PBS) (soluble); lane 4, the supernatant of pellets from PBS-extracted LORs re-extracted with 2% SDS (pellet); lane 5, purified reflectins (purified). Five μg of protein were loaded in samples for lanes 2 to 4; 3 μg were loaded in lane 5. The expanded area shows the presence of three protein species in a higher magnification of reflectin bands.

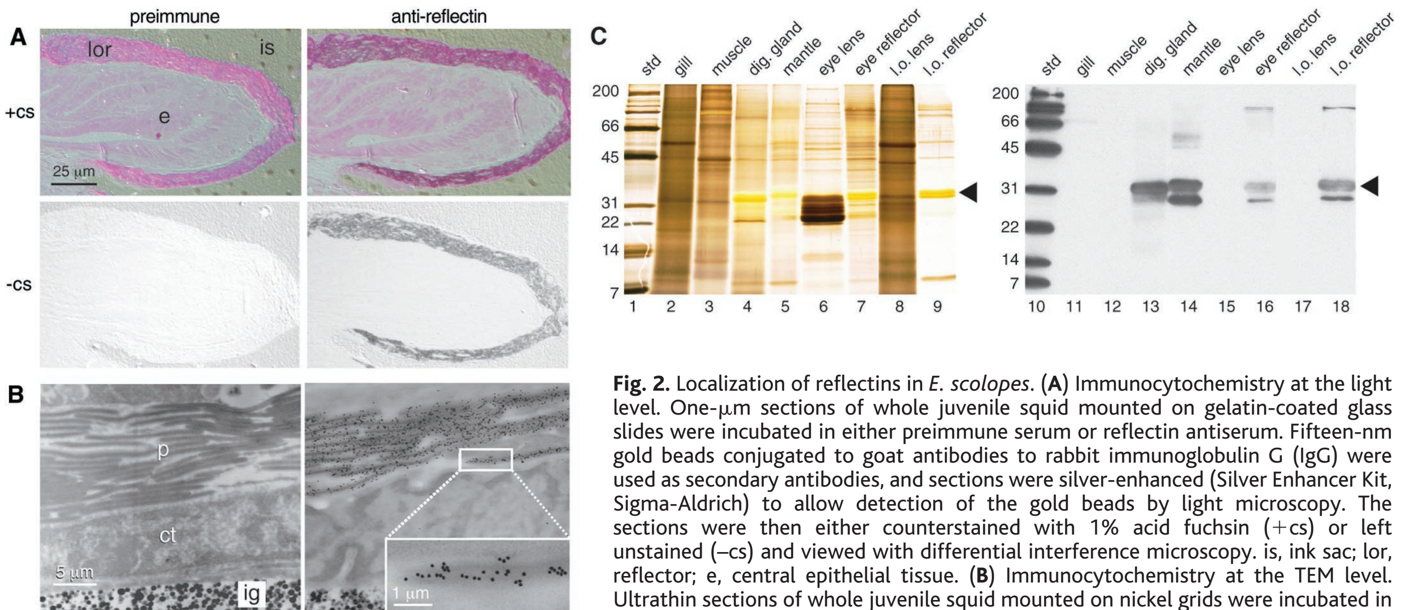

Fig. 2. Localization of reflectins in *E. scolopes*. (**A**) Immunocytochemistry at the light level. One-μm sections of whole juvenile squid mounted on gelatin-coated glass slides were incubated in either preimmune serum or reflectin antiserum. Fifteen-nm gold beads conjugated to goat antibodies to rabbit immunoglobulin G (IgG) were used as secondary antibodies, and sections were silver-enhanced (Silver Enhancer Kit, Sigma-Aldrich) to allow detection of the gold beads by light microscopy. The sections were then either counterstained with 1% acid fuchsin (+cs) or left unstained (–cs) and viewed with differential interference microscopy. is, ink sac; lor, reflector; e, central epithelial tissue. (**B**) Immunocytochemistry at the TEM level. Ultrathin sections of whole juvenile squid mounted on nickel grids were incubated in either preimmune serum or reflectin antiserum. Fifteen-nm gold beads conjugated to goat antibodies to rabbit IgG were used as secondary antibodies. Inset: Higher magnification showing the labeling of an individual platelet. p, platelets; ct, connective tissue; ig, ink granules. (**C**) Silver-stained SDS-PAGE (lanes 1 to 9) and immunoblot analyses (lanes 10 to 18) of 2% SDS-extracted proteins from pellets of aqueous-buffer extractions of reflective and nonreflective squid tissues (*12*); 2.5 μg of total protein were loaded per lane. The arrowheads indicate the position on the gels where reflectins resolve. std, molecular mass standards in kD; dig., digestive; l.o., light organ.

composed of purine crystals, particularly guanine and hypoxanthine (*5*). In contrast, cephalopod reflector platelets do not contain these purines, and studies of their biochemical and biophysical characteristics have suggested that they are composed of protein (*5*, *6*). However, the composition of cephalopod reflector platelets has not been definitively characterized (*6*, *7*).

The Hawaiian bobtail squid *Euprymna scolopes* (Cephalopoda: Sepiolidae) (Fig. 1A) is similar to other cephalopod species that have been studied (*6*–*9*) in having both variably reflective tissues, such as the skin of the mantle, and statically reflective tissues, such as those associated with the eye, digestive gland, and light organ. The reflector of the bilobed light organ is a particularly well-developed tissue (Fig. 1, A to D) that modulates the luminescence produced by a population of the symbiotic bacterium *Vibrio fischeri* (*10*, *11*). On each side of the adult light organ, symbiont-containing epithelial tissue comprises a core that is surrounded by the thick silvery reflector. Together with a muscle-derived lens, these dioptrics function to direct the bacterial luminescence ventrally (*11*). Consistent with reflectors in other animals, the light organ reflector (LOR) tissue is composed of a thick layer of platelets (Fig. 1, C and D).

Extractions of total protein from the *E. scolopes* LOR revealed an abundant set of three polypeptides that resolved between 33 and 36 kD on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and were a characteristic golden-yellow color upon silver staining (Fig. 1E) (*12*). These polypeptides, which we called reflectins, were not detected in the aqueous soluble fraction of the LOR, but they were abundant species in the supernatant of the SDS-solubilized pellet, composing ~40% of the proteinaceous component of the LOR.

To localize the reflectins within the LOR, polyclonal antibodies were generated against

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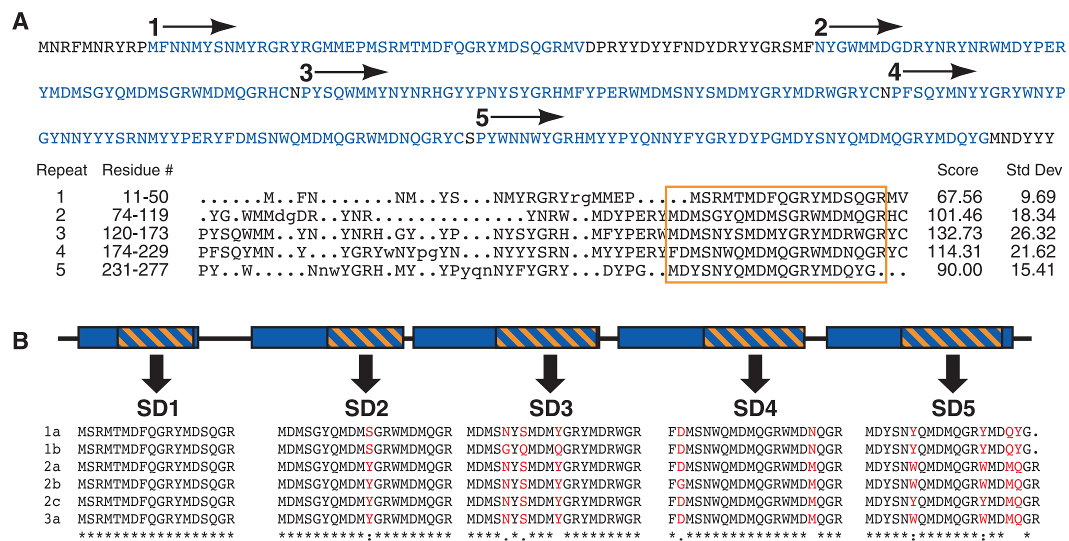

Fig. 3. Reflectins are composed of repeating domains, as predicted by Rapid Detection and Alignment of Repeats (RADAR) (*12*). (**A**) Top: The entire amino acid sequence of reflectin 1a, with repeat regions indicated in blue and amino acids excluded from the repeats in black. Numbers and arrows indicate the number and direction of the repeats. Bottom: The RADAR output, showing the score of each repeat unit when scored against the whole repeat and the number of standard deviations (Std Dev) above the mean for shuffled sequences scored against the same profile. The fourth repeat was used as the template repeat. (**B**) Schematic showing the positioning of the repeats (blue boxes) and the conserved subdomains (hatched boxes, SD1 to SD5) from a representative reflectin. The subdomains are also outlined by the orange box in the RADAR alignment in (A). The subdomain amino acid alignments among all reflectins are shown under each subdomain; positions in which there is a change in amino acid among the reflectins are highlighted in red.

gel-purified reflectin proteins (Fig. 1E, lane 5) and used in immunocytochemical and immunoblot analyses. The reflectin antibodies strongly recognized the LOR, but not the bacteria-containing epithelium, the ink sac, or the lens of the light organ (Fig. 2A). Using immunogold localization by transmission electron microscopy (TEM), we determined that the reflectins in the LOR were directly associated with the LOR platelets (Fig. 2B); no labeling was detected in the surrounding connective tissue or ink granules (Fig. 2B). Higher magnification TEM images of the LOR demonstrated that the antibodies cross-reacted specifically with the electron-dense platelets but not with the inter-platelet region (Fig. 2B, inset).

Proteins with similar molecular mass, biochemistry, and antigenicity to the LOR reflectins were found in all reflective tissues of *E. scolopes.* Silver-stained SDS-PAGE gels revealed that the characteristic golden-yellow bands at 33 to 36 kD were detectable in reflective tissues. In addition, these proteins cross-reacted with antibodies to LOR reflectins in Western blot analyses (Figs. 1A and 2C). Cross-reactive bands were also found at other molecular weights (26, 55, and 120 kD), and corresponding golden-yellow bands were present on the SDS-PAGE gels of these tissues. Because the antibodies were generated from a very discrete region of a gel and these other bands only occur in reflective tissues, it is likely that the antibodies were cross-reacting with other members of the reflectin family or with closely related proteins. Cross-reactivity was low or undetectable in nonreflective tissues (the gills, muscle, eye lens, and light organ lens) (Fig. 2C).

The sequences of three tryptic peptides (fig. S1A) from tryptic digestion of gel-purified reflectins (Fig. 1E, lane 5) were used to identify reflectin cDNAs from predicted translations of *E. scolopes* cDNA and expressed sequence tag (EST) library clones. Rapid amplification of cDNA ends–polymerase chain reaction (RACE-PCR) conducted on light organ cDNA pools with reflectin primers (table S1) identified six similar reflectin cDNAs (fig. S1 and table S2), suggesting that several genes encoding reflectins were expressed in the light organ. Sequencing of PCR products from the amplification of genomic DNA provided evidence that all six reflectin genes amplified from the cDNA pools were represented in the genome of a single individual. None of the reflectin genes amplified from genomic DNA possessed introns. Only one entry in the nucleotide databases had similarity to the *E. scolopes* reflectins, a gene sequence from the European squid *Loligo forbesi* (*13*) that encodes "methionine-rich repeat protein 1" (fig. S1, A and B, and table S2).

The derived amino acid sequences of the six full-length clones were aligned, demonstrating that the reflectins are highly (85.0 to 98.6%) similar and that they group into three subfamilies (fig. S1, A and B, and table S2). Analysis of their structure revealed some unusual characteristics. Reflectins possess a highly unusual amino acid composition (fig. S1C); six amino acids (Y, M, R, N, G, and D) (*14*) compose more than 70% of the total, and four other amino acids (A, I, L, and K) are absent. Although their SDS solubility suggested that they may be membrane-associated, further analyses demonstrated that they are not predicted to possess hydrophobic or charged clusters, transmembrane domains, or glycosylphosphatidylinositol anchors (*12*). However, each reflectin is composed of five repeating domains (Fig. 3). When these repeats are aligned (Fig. 3A, lower), a core subdomain of 18 to 20 amino acids is revealed; we defined the subdomains by the presence of a repeating motif $[M/FD(X)_5MD(X)_5MD(X)_{3/4}]$ that occurs in 4 of the 5 subdomains. In these subdomains, 21 of 23 methionine residues occur in the same relative position in the repeat. The subdomains are enriched in amino acids M, R, G, D, S, and Q, and depleted in Y, N, W, P, and F relative to the whole protein (table S3). Inter- and intraprotein subdomain alignments demonstrated that individual subdomains from different reflectins (e.g., subdomain SD1 from reflectin 1a versus SD1 from reflectin 2a) are more similar to each other (80 to 100%) than are different subdomains (e.g., SD1 from reflectin 1a versus SD2 from reflectin 1a) of the same reflectin (55 to 70%) (Fig. 3B). These data suggest greater functional constraint on the sequence within a subdomain across the family.

The identification and characterization of the reflectins confirm that, although the majority of animal reflective tissues are composed of purine platelets, cephalopod reflector platelets are proteinaceous. Reflectins, a protein family with skewed amino acid compositions, repeating domains, and localized deposition, are thus far restricted to cephalopods. They represent a marked example of natural nanofabrication of photonic structures in these animals. Protein-based nanofabrication is a frontier area in biomimetics, in which protein structures are engineered to be used as biomaterials, molecular devices, and biosensors (*15*). Future analysis of reflectins should inspire the "bottom-up" synthesis of nanostructured supramolecular devices, especially those used in spectroscopic and optic

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applications (*1*). For example, reflectin-based nanoreflectors could be coupled with artificial photosynthetic membranes (*16*) or with bacteriorhodopsin-based bioelectronic devices (*17*) to enhance the power and efficiency of these systems.

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Transgenic RNAi Reveals Essential Function for CTCF in *H19* Gene Imprinting

Andrew M. Fedoriw,[1,2] Paula Stein,[3] Petr Svoboda,[3] Richard M. Schultz,[3] Marisa S. Bartolomei[1,2]*

The imprinted regulation of *H19* and *Insulin-like growth factor 2* expression involves binding of the vertebrate insulator protein, CCCTC binding factor (CTCF), to the maternally hypomethylated differentially methylated domain (DMD). How this hypomethylated state is maintained during oogenesis and the role of CTCF, if any, in this process are not understood. With the use of a transgenic RNA interference (RNAi)–based approach to generate oocytes with reduced amounts of CTCF protein, we found increased methylation of the *H19* DMD and decreased developmental competence of CTCF-deficient oocytes. Our results suggest that CTCF protects the *H19* DMD from de novo methylation during oocyte growth and is required for normal preimplantation development.

Genes that are subject to genomic imprinting in mammals exhibit differential expression of the parental alleles (*1*). The transcriptional machinery is thought to differentiate between the two alleles through heritable DNA and histone modifications. Although recent work has characterized epigenetic marks in somatic lineages, the mode by which this epigenetic control is established in the germ line remains poorly understood.

The maternally expressed *H19* gene lies about 100 kb from the paternally expressed *Insulin-like growth factor 2* gene (*Igf2*) (*2*). The imprinted expression of both genes depends on a 2-kb differentially methylated domain (DMD) located 2 kb upstream from the *H19* promoter (*3*, *4*) (Fig. 1A). The DMD is postulated to function as a methylation-sensitive insulator through the binding of the insulator protein CTCF to conserved upstream sequence elements (*5*–*9*). Binding of CTCF to the hypomethylated maternal DMD allows *H19* exclusive access to downstream enhancers. Mutation of CTCF binding sites causes acquisition of methylation during postimplantation development, suggesting that CTCF also maintains maternal allele–specific hypomethylation (*10*, *11*).

Most imprinted genes examined to date harbor maternal-specific hypermethylated imprinting control regions (*12*). In contrast, the *H19* DMD possesses one of the few maternal hypomethylation marks, implying that mechanisms may exist that not only confer germline methylation but also protect it from such modifications. It has been suggested that CTCF may be responsible for this protective role (*13*). To determine whether CTCF is required to maintain hypomethylation of the *H19* DMD during this crucial period, we selectively ablated CTCF mRNA in growing oocytes.

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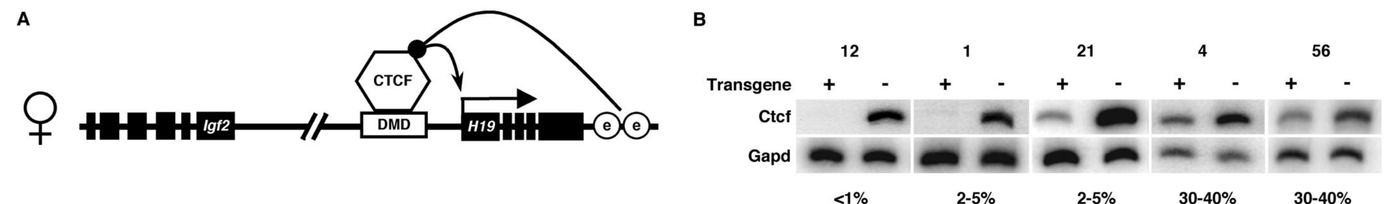

Fig. 1. Targeting of CTCF mRNA by transgenic RNA interference. (**A**) Model for imprinted regulation of *H19* and *Igf2*. Enhancer blocking is established on the maternal allele, allowing *H19* exclusive access to shared enhancers (indicated with an "e"). *Igf2* is solely expressed from the paternal chromosome, where hypermethylation of the DMD abrogates CTCF binding. (**B**) Typical reverse transcription PCR result for each transgenic line, comparing CTCF transcripts in oocytes from transgenic and nontransgenic littermates (*16*). GAPD was used to normalize expression levels between samples. Amounts of CTCF mRNA in transgenic oocytes, compared to that of nontransgenic oocytes, are shown below gel images.