

tion of PSA is of particular interest because this polyglycan seems important in regulating the adhesive properties of N-CAM during neuronal development (18). In larvae and adult of the fly *Calliphora vicina*, a nonsulfated, glucuronic acid moiety similar to the L2/HNK-1 carbohydrate epitope of several cell adhesion molecules, among them N-CAM, was recently detected (19). These data suggest a high degree of phylogenetic conservation of functionally important glycans.

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22. Supported by the Swiss National Science Foundation. We thank D. Wey for preparing the photographs and A. P. Corfield, I. J. Goldstein, D. van den Eijnden, and P. M. Lackie for comments.

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Modality-Specific Retrograde Amnesia of Fear

Jeansok J. Kim and Michael S. Fanselow

Emotional responses such as fear are rapidly acquired through classical conditioning. This report examines the neural substrate underlying memory of acquired fear. Rats were classically conditioned to fear both tone and context through the use of aversive foot shocks. Lesions were made in the hippocampus either 1, 7, 14, or 28 days after training. Contextual fear was abolished in the rats that received lesions 1 day after fear conditioning. However, rats for which the interval between learning and hippocampal lesions was longer retained significant contextual fear memory. In the same animals, lesions did not affect fear response to the tone at any time. These results indicate that fear memory is not a single process and that the hippocampus may have a time-limited role in associative fear memories evoked by polymodal (contextual) but not unimodal (tone) sensory stimuli.

The hippocampus is thought to serve a temporary function in the storage of memory because, when the hippocampus is damaged, recent but not remote memories are impaired (1). This memory syndrome is known as "retrograde amnesia," and it suggests that with the passage of time memo-

ries are stabilized (or consolidated) elsewhere in the brain (for example, in the neocortex). Because the hippocampus is not thought to be essential for learning and memory in most cases of simple classical conditioning (2), retrograde amnesia as a result of hippocampal damage has heretofore not been directly assessed in basic associative paradigms. Therefore, we tested whether retrograde amnesia occurs in rats

by using a simple classical fear-conditioning procedure.

Long-Evans female rats underwent 15 tone–foot shock pairings (tone: 2000 Hz, 90 dB, 30 s; foot shock: 1 mA, 2 s) in a distinctive chamber (3). A short (3-min), fixed intertrial interval was used to ensure reliable fear conditioning to both tone and chamber. After training, bilateral electrolytic lesions were made in the hippocampus either 1, 7, 14, or 28 days later ($n = 8$) (4). Figure 1 shows a transverse section from the brain of a typical rat in the hippocampus-lesioned group. For unlesioned controls the electrode was lowered to the hippocampus without passing current ($n = 6$). Additionally, control lesions were made 1 day after training in the area of neocortex overlying the hippocampus ($n = 8$). All animals were given 7 days to recover after surgery before testing.

To test fear conditioning associated with context (the chamber), each rat was placed back in the chamber for 8 min. The foot shock and tone were not given during this test. The amount of fear conditioned to the chamber was assessed by scoring freezing

Department of Psychology, University of California, Los Angeles, CA 90024.

Fig. 1. A transverse brain section stained with cresyl violet from a rat in the hippocampus-lesioned group. Lesions in this group included most of the dorsal hippocampal formation. Overall damage to the neocortex overlying the hippocampus was minor.

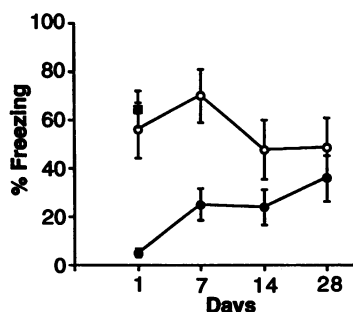
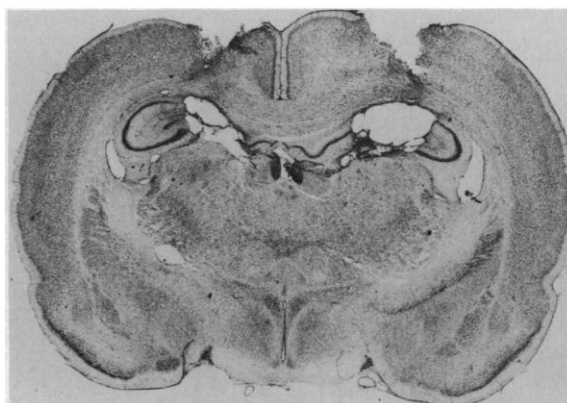


Fig. 2. Mean ($\pm$ SEM) freezing level during the context test. (○), Control; (●), hippocampus lesion; (■), cortex lesion. Freezing scores are expressed as the percentage of total observations during the 8-min test.

behavior. Freezing is a species-specific defensive reaction characterized by an immobile, crouching posture and has proven to be a reliable index of fear (5).

On the next day, animals were placed in a chamber that was entirely different in auditory, visual, olfactory, tactile, and spatial features from the one in which they were conditioned (3). After 3 min, the conditional tone was given for 8 min. Fear memory of the tone was assessed by scoring freezing.

Animals that received hippocampal lesions 1 day after conditioning failed to exhibit the freezing response during the context test (Fig. 2). In contrast, both day 1 control and cortex-lesioned animals

showed strong freezing behavior (56 and 64%, respectively). When lesions were made either 7, 14, or 28 days after conditioning, the rats with hippocampal lesions showed a significant retention of contextual fear [linear trend analysis for all time points, $F(1,48) = 5.82$; $P < 0.02$]. In fact, animals with hippocampal lesions made 28 days after conditioning exhibited freezing comparable to that of the control group. The long-term nature of contextual fear memory is indicated by the fact that the control rats did not forget; that is, the freezing response to the context did not decrease over time [$F(1,48) = 0.91$; $P > 0.10$].

Animals did not freeze in the new chamber during the 3 min preceding the tone presentation (Fig. 3A). Thus, the context fear conditioning was specific and did not generalize to the new chamber. In contrast to the response to the context, the hippocampal lesion did not affect freezing response to the tone at any time (Fig. 3B) [$F(3,48) = 0.72$; $P > 0.10$]. All groups exhibited robust freezing responses to the tone that was previously paired with foot shock. This fear response to the tone did not decrease over time for any group. Because lesions did not affect the tone-elicited freezing, the hippocampus is not necessary for the animal to show the freezing response.

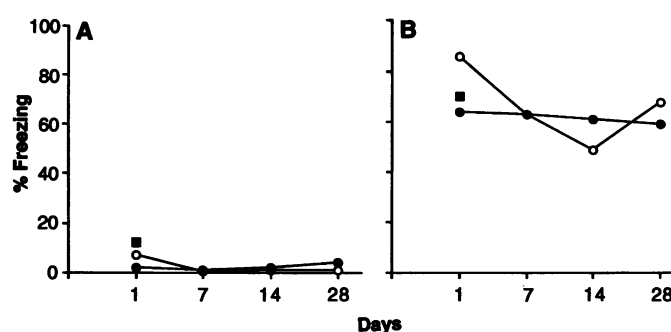
Our data imply that the hippocampus is crucial for the retention of some but not all

associative fear memories. Specifically, the fear memory related to a context-shock association requires an intact hippocampus. In contrast, the hippocampus is not crucial for a fear memory based on a tone-shock association. Similar dissociation between context and tone also was observed when the hippocampus was lesioned before conditioning (6). Therefore, it is likely that different anatomical structures mediate fear conditioning depending on the nature of the conditional stimulus on which the fear memory is based. Because animals retained the context-shock association when a long delay was imposed between the time of conditioning and the time of surgery, our finding supports the notion that the hippocampus has a transient function in the storage of memory (1). Importantly, this transient role occurs even when nonlesioned animals showed no evidence of forgetting over the retention period.

The hippocampus is thought to be required for some (for example, declarative), but not other (for example, procedural), types of memory (7). Several theories of hippocampal functioning in memory have suggested that this structure is essential in complex memory functioning, such as in spatial tasks (8), but not in basic associative processes (2). However, our data indicate that the hippocampus is critical for simple associative learning when contextual stimuli constitute a component of the associative structure. Obviously, the hippocampus must interact with other neural structures, such as the amygdala (9) and periaqueductal gray (10), that also have been implicated in the mediation of fear.

In conclusion, although the behavior generated by emotional memories is temporally stable, the neuroanatomical basis of those memories is dynamic. The hippocampus has a temporally restricted role in memory storage that is assumed by other structures over time. Additionally, because hippocampal damage causes retrograde amnesia of emotional memories, rapidly acquired fear conditioning may provide a profitable model for the analysis of retrograde amnesia and hippocampal functioning.

Fig. 3. Mean percentage freezing during the tone test (symbols as in Fig. 2). (A) All animals exhibited virtually no freezing response during the 3 min preceding the tone in a completely different chamber. (B) Hippocampus-lesioned animals showed robust freezing response to the tone during the 8 min of tone presentation. The amounts of freezing were not different between the groups at any given time.



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4. Lesions were produced by passing anodal constant current (1 mA, 10 s) through a stainless steel pin that was insulated with epoxy except for 0.5 mm at the tip. (Stereotaxic coordinates for the hippocampus were as follows: 2.8 mm posterior to bregma, 2.0 mm lateral to midline, and 4.0 mm ventral from the skull surface; 4.2 mm posterior to bregma, 3.0 mm lateral to midline, and 4.0 mm ventral from the skull.)
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Microdomains of High Calcium Concentration in a Presynaptic Terminal

R. Llinás,* M. Sugimori, R. B. Silver

Increases in intracellular calcium concentration are required for the release of neurotransmitter from presynaptic terminals in all neurons. However, the mechanism by which calcium exerts its effect is not known. A low-sensitivity calcium-dependent photoprotein (*n*-aequorin-J) was injected into the presynaptic terminal of the giant squid synapse to selectively detect high calcium concentration microdomains. During transmitter release, light emission occurred at specific points or quantum emission domains that remained in the same place during protracted stimulation. Intracellular calcium concentration microdomains on the order of 200 to 300 micromolar occur against the cytoplasmic surface of the plasmalemma during transmitter secretion, supporting the view that the synaptic vesicular fusion responsible for transmitter release is triggered by the activation of a low-affinity calcium-binding site at the active zone.

The role of intracellular free calcium as a trigger for initiating presynaptic transmitter release in chemical synapses was proposed as the "calcium hypothesis" several decades ago (1). The mechanism for this Ca^{2+} -dependent transmitter release remains unresolved, partly because the concentration of Ca^{2+} and the distribution of Ca^{2+} concentration within presynaptic terminals during transmission are unknown.

Presynaptic voltage-clamp studies in the giant squid synapse demonstrated a very short (200 μs) latency between Ca^{2+} entry and postsynaptic response, suggesting that Ca^{2+} channels are located at the site of vesicle accumulation and neurotransmitter

release (2). Moreover, direct measurement of presynaptic Ca^{2+} currents suggested that intracellular free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) near the Ca^{2+} channels could be on the order of several hundred micromolar (3). Computer models based on these data suggested that the $[\text{Ca}^{2+}]_i$ falls off steeply away from the cytoplasmic mouth of the Ca^{2+} channels (4–7). These small domes of increased $[\text{Ca}^{2+}]_i$ are called microdomains. Each Ca^{2+} channel opening is thought to produce a rapid (microseconds) increase in $[\text{Ca}^{2+}]_i$, which rapidly returns to its pre-opening value when the channel closes (6). Neurotransmitter release would thus be triggered by a large transient increase in $[\text{Ca}^{2+}]_i$ adjacent to the synaptic vesicles.

We have now tested directly the existence of such $[\text{Ca}^{2+}]_i$ microdomains by using aequorin (a protein that emits light in the presence of free Ca^{2+}) (8, 9) injected in the presynaptic terminal of the giant squid synapse (10). In the presence of aequorin, transient increases in $[\text{Ca}^{2+}]_i$ appear as

flashes of light localized in time and space.

We designed special methodology to identify, image, and characterize these flashes of light. Fluorescent *n*-aequorin-J (minimum Ca^{2+} sensitivity of 10^{-4} M) was injected into the presynaptic terminal (Fig. 1A) (11), and its distribution in the terminal was continually monitored by epifluorescence microscopy (12). Once the terminal was fully loaded with *n*-aequorin-J (Fig. 1B), the presynaptic fiber was continuously stimulated at 10 Hz (13), and a well-defined, stable set of quantum emission domains (QEDs) appeared as white spots (Fig. 1C) (14). The superposition of the fluorescent images of the terminal digit (Fig. 1B) and the QEDs (Fig. 1C) revealed that the distribution of QEDs coincided with the presynaptic terminal (Fig. 1D). QEDs were particularly evident at the center of the digit, where most synaptic contacts occur.

We also determined the distribution and number of QEDs in an unstimulated presynaptic terminal at high magnification (Fig. 1F). When QEDs were integrated for 30 s during tetanic stimulation (Fig. 1G), they were found to be organized in regions resembling semicircles or line segments and were approximately equally spaced over the presynaptic digit (Fig. 1, C and G).

Each QED fell within a contiguous rectangular juxtaposition of approximately 16 pixels (0.25 μm by 0.25 μm per pixel). The size distribution of the QEDs was determined after measuring more than 15,000 such events for both long-term image integrations (1,500 to 5,000 video frames); serially repeated shorter integrations [600 to 1,200 national television standard code (NTSC) video frames]; and sets of single video frames. QEDs fluctuated in size from 0.25 to 0.6 μm^2 (Fig. 2), with a mean of 0.313 μm^2 (range, ~ 0.25 μm^2 to ~ 0.375 μm^2). QED patterns such as those shown in Figs. 1 and 2 (areas 0.375 to 0.625 μm^2) represent individual QEDs occurring at nearly identical frame locations, with a small overlap. On average, these sites occupied 8.4% of the presynaptic-postsynaptic membrane contact area, which is close to the 5 to 10% determined by ultrastructural studies (15). The number of QEDs in a 70 μm by 40 μm contact area (15) was about 4500 (based on actual counting of QEDs in the contact area), quite close to the 4400 calculated for the number of active zones (range 3580 to 5400) from measurements and analysis of transmission electron micrographs (16).

The $[\text{Ca}^{2+}]_i$ reached during presynaptic activation (200 to 300 μM) was determined by sampling the number of QEDs over consecutive 10-, 15-, or 30-s periods. Many loci repeated periods of photon emission within consecutive sampling periods, as

R. Llinás and M. Sugimori, Department of Physiology and Biophysics, New York University Medical Center, 550 First Avenue, New York, NY 10016, and the Marine Biological Laboratory, Woods Hole, MA 02543. R. B. Silver, Section and Department of Physiology, Cornell University, Ithaca, NY 14853–6401, and the Marine Biological Laboratory, Woods Hole, MA 02543.

*To whom correspondence should be addressed, at the Department of Physiology and Biophysics.