Removal of perineuronal nets disrupts recall of a remote fear memory

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Throughout life animals learn to recognize cues that signal danger and instantaneously initiate an adequate threat response. Memories of such associations may last a lifetime and far outlast the intracellular molecules currently found to be important for memory processing. The memory engraving may be supported by other more stable molecular components, such as the extracellular matrix structure of perineuronal nets (PNNs). Here, we show that recall of remote, but not recent, visual fear memories in rats depend on intact PNNs in the secondary visual cortex (V2L). Supporting our behavioral findings, increased synchronized theta oscillations between V2L and basolateral amygdala, a physiological correlate of successful recall, was absent in rats with degraded PNNs in V2L. Together, our findings suggest a role for PNNs in remote memory processing by stabilizing the neural network of the engram.

The specialized extracellular matrix structure of perineuronal nets (PNNs) surround the cell body and proximal dendrites of subpopulations of neurons in the central nervous system in a lattice-like structure, in particular fast-spiking inhibitory interneurons that express parvalbumin (PV+) (1). The PNNs mature late in development in concert with the closure of so-called critical periods of heightened plasticity, when neuronal circuits are refined, and restrict neural plasticity in the adult brain (1). A central part of this refinement of neuronal circuits is the maturation of inhibitory neurons (2). This maturation, together with PNN development, contribute to restricting plasticity and stabilizing neuronal circuits (2–4). It has been established that the development of PNNs in the amygdala of adult animals contribute to the endurance of a memory after extinction as depletion of PNNs cause permanent erasure of the memory (5, 6). The PNNs facilitate the fast-spiking activity of PV+ neurons, and consequently the fine excitatory–inhibitory balance of neural networks necessary for cognitive functions (4, 7–10). Moreover, PV+ neurons are important for oscillatory activity, which is essential for consolidation and retrieval of memories (11–13). It has recently been hypothesized that PNNs may be a physical framework for remote memory storage (14). The meshlike structure of PNNs, tightly enwrapping the synaptic connections stabilizing their size and placement, in conjunction with their slow turnover rates, point in this direction; but the idea remains to be tested. We asked whether intact PNNs in the lateral secondary visual cortex (V2L), a cortical region important for remote memory (15–18), are required for the processing of remote visual fear memories.

Results

Intact PNNs in V2L Are Required for the Recall of Remote but Not Recent Visual Fear Memory. Rats were trained by pairing a white light (conditioned stimulus; CS) with a foot shock (unconditioned stimulus; US). Four weeks after training, we tested the animals for both light CS memory (Fig. 1C) and contextual memory (Fig. S4). One week before the memory test, we degraded the PNNs in V2L bilaterally with local injections of the bacterial enzyme chondroitinase ABC (chABC) (Fig. 1B and D). Strikingly, the chABC treatment disrupted recall of the remote visual fear memory (Fig. 1E) without influencing remote contextual memory (Fig. S4). In fact, visual fear memory expression in individual rats was correlated with the extent of chABC activity confined to V2L (Fig. 1G), with no similar correlations between memory expression and chABC activity in nearby brain regions. In a different group of animals with chABC injections purposely aimed at primary visual cortex (V1), chABC injections did not influence recall for memory (Fig. 1D and Fig. S4). To examine whether chABC treatment would influence recent memory in a similar manner, we injected chABC in V2L or V1 only 1 d after training, rather than 3 wk, and allowed the animals to recover for 6 d before memory testing (Fig. 1H). At this early time point, chABC treatment did not influence fear memory expression (Fig. 1I and J and Fig. S4) in either brain area, supporting the involvement of V2L in remote but not recent memories (15, 17, 19). These data suggest that PNNs in V1 have no role in either recent or remote memory recall.

Synchronized Oscillatory Neural Activity During Recall Is Disrupted After chABC Treatment. Synchronized oscillatory neural activity in the lower theta range (4–8 Hz) between brain regions is a physiological correlate of memory retrieval (13, 17, 20–22). To examine whether chABC treatment would affect the communication between V2L and the basolateral amygdala (BLA), we used chronically implanted electrodes and performed simultaneous local field potential recordings (LFP) from V2L and BLA during remote memory recall. Similar to our initial experiments, rats with chABC injected into V2L showed disrupted fear memory 30 d after fear conditioning (Fig. 1D and Fig. S4). In accordance with previous work in V1 (4, 23), the sensory response in V2L induced by the light stimulus (observed as a large current

Significance

Perineuronal nets (PNNs), a type of extracellular matrix only found in the central nervous system, wraps tightly around the cell soma and proximal dendrites of a subset of neurons. The PNNs are long-lasting structures that restrict plasticity, making them eligible candidates for memory processing. This work demonstrates that PNNs in the lateral secondary visual cortex (V2L) are essential for the recall of a remote visual fear memory. The results suggest a role of extracellular molecules in storage and retrieval of memories.


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Removal of PNNs in lateral secondary visual cortex disrupts recall of a remote fear memory. (A, Left) Coronal section of a rat brain, PNNs detected by Wisteria floribunda agglutinin (WFA; green) and neuronal cell bodies detected by Nissl staining (red). Au1, primary auditory cortex; V1, primary visual cortex; V2L, lateral secondary visual cortex; V2M, medial secondary visual cortex. (A, Right) Neuron expressing parvalbumin (Pvalb; red) enwrapped in a PNN (WFA; green). (B) Coronal section from a rat injected with chABC in V2L 1 wk before perfusion. PNNs detected by WFA staining (green). Activity by chABC causes reduced WFA staining (green) restricted to V2L. RSp, retrosplenial cortex; V1b, binocular V1; V1m, monocular V1. (C) Experimental timeline for remote fear conditioning (FC). (D) The extent of chABC digestion in gray superimposed on illustrations of brain sections (40) five different distances (mm) from bregma, red squares indicates V2L (Left), or V1 and V2L (Right). (E) Bilateral chABC injection in V2L (n = 7 rats) reduced freezing to light CS compared with sham controls (n = 12 rats). Each dot represents one animal, bars indicate population mean. Two-way ANOVA, treatment (acSF or chABC) × condition (baseline or light CS) followed by post hoc Sidak test revealed that chABC treatment disrupted CS memory retrieval (***P < 0.0001). (F) Bilateral chABC injection in V1 (n = 9 rats) did not influence freezing to light CS compared with sham controls (n = 13 rats). (G) The extent of chABC activity confined to V2L (mean from both hemispheres) was correlated with the amount of freezing during light cues; r = −0.67, P = 0.003, n = 17 rats. (H) Experimental timeline for recent FC. (I) Recent memory testing 1 wk after FC. Bilateral chABC injections in V2L (n = 8 rats) did not influence freezing to light CS compared with sham controls (n = 8 rats). (J) Recent memory testing 1 wk after FC. Bilateral chABC injections in V1 (n = 4 rats) did not influence freezing to light CS compared with sham controls (n = 8 rats). Detailed statistics are shown in Fig. S1.

Removing PNNs in V2L Has No Impact on Acquisition or Consolidation of Visual Fear Memory. Given the apparent involvement of V2L also during recent memory retrieval, we next looked at whether PNNs in V2L were important for acquisition and consolidation
Fig. 2.  Neural activity in BLA and V2L during remote memory testing is synchronized during successful visual fear memory retrieval of controls but not in chABC-treated animals. (A) Unpaired trained and chABC-treated animals had reduced freezing to light CS compared with sham controls. Each dot represents one animal, bars indicate population mean. Two-way ANOVA, treatment $\times$ condition treatment (aCSF or chABC) $\times$ condition (baseline or light CS) followed by post hoc Sidak test revealed that sham-operated controls showed higher levels of freezing during light CS compared with chABC-treated and unpaired trained animals ($***P = 0.0004$). (B) Representative LFP responses to light cue in V2L of sham controls (Left) and chABC treated (Right). (C) Synchronization of theta oscillations between V2L and BLA during successful memory retrieval in sham-operated animals (LFP coherency peak at 7 Hz, $n = 4$ rats; Left), compared with animals treated with chABC ($n = 4$ rats; Center) and in animals trained with unpaired light cue and foot shock ($n = 3$ rats; Right). Colored line indicates population mean, dotted line indicates SEM. (Right) LFP coherence from 5 to 8 Hz at baseline and during first 2 s of CS. One-way ANOVA with Tukey’s multiple comparison test: sham vs. chABC, $**P = 0.006$; sham vs. unpaired, $**P = 0.007$; chABC vs. unpaired, $P = 0.8$. ns, not significant. (D) Power spectral density of LFP activity during light CS in V2L (Upper) and BLA (Lower). Sham controls (Left), chABC treated (Center), and unpaired trained animals (Right). Detailed statistics are shown in Fig. S1.
of the visual fear memory. Injection of chABC 1 wk before fear conditioning in either V2L or V1 did not influence fear memory acquisition or recall after 4 d (Fig. 3A and B and Fig. S3). Given that PNNs gradually regenerate after chABC treatment (4, 24) (Fig. 3E), we next asked whether remote memory would be affected if we extended the time period between chABC injection and the memory test. Similar to the recent memory experiment, we injected chABC in V2L 1 wk before training, but waited 4 wk before memory testing (Fig. 3C). Using this extended protocol, the chABC treatment did not influence remote memory, suggesting that remote memory recall does not require intact PNNs in V2L during the first days subsequent to training, and that PNN regeneration at the time of remote memory testing was sufficient to allow normal memory retrieval (Fig. 3E). Finally, to test the effect of PNN removal on remote memory consolidation specifically, we injected chABC 1 wk after training and tested the memory when PNNs had regenerated 35 d after fear conditioning (Fig. 3D and E). Removing the PNNs at this time point did not affect memory recall (Fig. 3D). The activity of the enzyme and regeneration of PNNs were confirmed by histological staining for chondroitin sulfate 6 “stubs” (3B3 epitope) and WFA-positive PNNs at 10 and 38 d after enzymatic treatment (Fig. 3E). Taken together, the results suggest that intact PNNs in V2L are specifically important for storage and retrieval of remote visual fear memories.

**Discussion**

The brain faces the challenge of being plastic to form new memories and being stable to facilitate lifelong memory storage. The understanding of molecular processes needed for the transition from short-term memory to a consolidated long-term memory has come far, but those processes needed for a memory to persist across years remain unresolved. Proposed molecular candidates that may maintain long-term memory, e.g., protein kinase M zeta and calmodulin-dependent protein kinase II, are located in postsynaptic spines, have a short lifespan with a turnover of a few days, and are in need of constant replacement to support long-lasting memories (25–29). The PNNs, on the other hand, are highly stable structures relieved from constant renewal as they are not exposed to the catabolic intracellular environment. Therefore, it has been proposed that over time, PNNs might persist as a physical framework for stable remote memories (14). Our data show that intact extracellular matrix in V2L is indeed required for retrieval of remote visual fear memories.
memories. The stability that PNNs provide to a neuronal network seems essential for the ability to recall remote memories. Synchronized oscillations between brain areas are believed to facilitate a precise temporal pattern necessary for complex actions such as memory retrieval, where specific populations of neurons are recruited in a phase-locked spiking activity pattern (17, 30). The increased coherence between V2L and BLA in the theta range in control animals during remote and recent memory recall is in line with previous work from secondary auditory cortex (17). Strikingly, in chABC-treated animals that failed to retrieve the remote fear memory, this V2L–BLA coherence was absent. Theta synchronization between brain regions during defensive behavior depends on the synchronous activity of PV+ interneurons and their fast spiking GABAergic inputs (12, 17, 21, 31). We recently showed that chABC treatment in V1 result in decreased PV+ interneuron activity, which further affects the spiking patterns of excitatory neurons (4). Together with our present results including normal sensory responses in V2L after PNN removal, this suggests that PNN degradation impairs the capability of the V2L network to either sufficiently reactivate the memory engram, drive long-range synchronization between V2L and the BLA, or both. Our data indicates that the close interaction between PNNs and firing activity of PV+ neurons is of such importance that without PNNs, neuronal network activity is partly disrupted leading to a failure in memory retrieval. Rather than proposing that PNNs are a physical framework for remote memory storage, our data suggest that they play a role in ensuring correct firing patterns required during memory retrieval. Others have found that removing PNNs using chABC in other brain areas influence memory retrieval at recent time points (32–34), indicating that the role of PNNs could differ between brain areas. Our data clearly show that PNNs in V2L are exclusively important for recall of remote visual fear memories. Digesting PNNs with chABC has previously been shown to increase plasticity and promote learning (4–6, 23, 24, 35, 36). Comparable effects on plasticity have been observed in knockout mice lacking Ctrl1, an essential link protein specifically located in the PNNs (24, 37, 38). Hence, although chABC is not specific to PNNs, but instead cleaves all glycosaminoglycan chains, previous evidence strongly suggests that the functional effects caused by chABC treatment mainly arise from removal of PNNs.

Together, our findings show that intact PNNs in V2L are required for recall of remote fear memory, without influencing memory processing at early time points. Our findings support the emerging idea that memory processing is dependent not only on neurons and glia cells, but also on extracellular matrix molecules.

Materials and Methods

The laboratory work was done at the Department of Biosciences at the University of Oslo. All experiments were approved by the Norwegian Animal Research Committee (FDU) before initiation. Experiments were conducted with male Sprague–Dawley rats (Taconic Biosciences) housed in standard cages in groups of four. Food and water were available ad libitum throughout the period of experiments. Details on experimental procedures are provided in Supporting Information and briefly described below.

Surgical Procedures. Protease-free chABC was purchased from Amsbio and reconstituted in filtered PBS to a concentration of 61 U/ml. Craniotomies were made at two sites in each hemisphere over V2L or V1. The coordinates used for V2L were AP 5.8 mm and ML 6.0 mm, and AP 7.4 mm and ML 5.8 mm, relative to bregma. For V1 injections we used the same AP coordinates, and 4.0 mm and 3.8 mm ML, respectively. All injections were made at a depth of 0.7 mm relative to the surface of the brain. After surgery, the animals were allowed to recover for a minimum of 1 wk before testing.

Four tetradyes were assembled in a microdrive (Axona) and implanted in concomitance with chABC/cbSFI injections. Electrodes were implanted in V2L and BLA at the following coordinates: AP 6.7 mm and ML 5.9 mm (relative to bregma) and DV 0.6 mm (relative to the surface of the brain), and AP 3.1 mm and ML 5.35 mm and DV 8.1 mm.

Fear Conditioning. Fear conditioning was conducted in modular operant test chambers placed in sound attenuating chambers (MedAssociates). The floor in the chamber was made of stainless steel rods (4 mm diameter, spaced 1.5 cm apart) connected to an electric pulse generator that delivered the foot shock. All animals were familiarized with the chamber for 5 min the day before fear conditioning. The following day the animals were placed in the chamber, and explored it undisturbed for 5 min before a series of lights (the CS), each lasting 6 s, were administrated with a varying intertrial interval. The last 2 s of each CS was paired with a foot shock (intensity 0.30 mA). After these CSs and last CS, the animals were left in the chamber for 1 min before being returned to their home cage.

Recent and remote memory tests were conducted 4, 8, 30, or 35 d after memory acquisition, respectively. Two days before the light-cued fear memory test, the animals went through two contextual fear memory tests to examine generalization of contextual fear. The tests involved leaving the animals in the chamber for 5 min undisturbed. The first test was conducted in an altered version of the original training chamber. The next day the test was conducted in the original training chamber. The main difference between the two contexts was the placement of the walls (a diagonal wall divided the chamber in the training context), the smell (peppermint was added in the altered context), and the sound (the fan was turned off in the altered context). The light-cued fear memory test (CS test) was conducted in the altered context to avoid conditioned fear behavior to contextual cues. When inside the chamber, the animals were left undisturbed for 3 min before being exposed to six conditioned stimuli with varying intertrial intervals. CSs were paired with foot shocks (0.30 mA). Prior to each CS, the animals were allowed to explore the chambers for 1 min, during which infrared cameras and behavior was scored offline with a digital stopwatch. Freezing was defined as cessation of all movement except that caused by respiration. For CS memory testing, baseline freezing was determined by scoring the behavior during 6 s before the first CS, and light-cued freezing was determined by scoring the mean time spent freezing during the 6 s+CS presentations. Animals were randomly assigned to a treatment group (chABC or acSF) before or after conditioning, depending on the experimental setup (chABC before or after fear conditioning). We classified animals as chABC treated or not based on postmortem immunohistochemical staining for the chondroitin sulfate 6 stubs using the 3B3 epitope, an indicator of chABC activity. To be included in the behavioral analysis, a minimum of 40% of the chABC (detected by 3B3 staining) had to be recorded in BLA on five sections spanning the length of V2L. Animals below this threshold (5–40%) were excluded from the main behavioral analysis, but included in the correlation between freezing and chABC activity (Fig. 1G).

Histology and Immunohistochemistry. Rats were given an overdose of pentobarbital sodium (50 mg/kg) and transcardially perfused with 0.9% NaCl, followed by 4% paraformaldehyde in 0.01 M PBS. The tissue was left to postfixate overnight before being transferred to a cryoprotective 30% sucrose solution in 1X PBS for 3 d at 4 °C. The tissue was then flash frozen and cut into coronal sections (45 μm) using a cryostat (Omnidemicher). Nissl staining was done to determine the location of the walls in V2L or V1. The lectin WFA was used as a marker for PNNs as it labels aggrecan-based immunohistochemistry was also used for staining parvalbumin-positive interneurons (rabbit anti-parvalbumin (Swant); Alexa 594 goat anti-rabbit (Life A11037)) and for fluorescent labeling of the C6-5 stubs after chABC treatment (anti C6-5 clone MK302 (MAB 2035; Merck Millipore), Alexa 594 donkey anti-mouse (Life A21203)). The monoclonal anti-chondroitin-6-sulfate antibody (MAB 2035; Merck Milipore) recognizes the six inner monosaccharides at the chondroitin sulfate chain left on the core proteins after chABC cleavage, the so-called 3B3 epitope, thereby confirming the activity of chABC (39). Sections were photographed using a Zeiss Axioplan 2 microscope and Axioscam HRZ camera.

Local Field Potential Recordings. LFP signals were recorded from all 16 channels on each microdrive for all three test days (altered context, training context, and CS test). We only used data from the CS test for analysis. The recording system used was daqUSB (Axona). LFP signals were amplified 2,000–3,000 times, low-pass filtered at 500 Hz, and stored to disk at 4.8 kHz (16 bits/sample) for offline analysis. LFP traces for every stimulus period were extracted and aligned according stimulus onset. The latency of the visual responses in V2L was measured as time from stimulus onset to the first negative peak in every LFP trace. We recorded LFP during CS presentation in four sham-operated rats (12 trials), five chABC-treated rats (11 trials), and three rats (6 trials) trained with foot shock and light cue unpaired. Custom Matlab code was used to analyze oscillations of the LFP signal. The coherence between the LFP channels for BLA and V2 was estimated by the magnitude-squared cohercence, using the Matlab function mscohere.
Statistical Analysis. Statistical analysis was performed using Graphpad Prism (Graphpad Software). All fear-conditioning tests were analyzed using a two-way analysis of variance (ANOVA) with Holm–Sidak multiple comparisons test if a significant interaction effect was detected.


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