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.

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 (Fig. 1 D and F) 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. 1 I and J and Fig. S4) in either brain area, supporting the involvement of V2L in remote fear memory (15, 17, 19). These data suggest that PNNs in V1 have no role in either recent or remote memory recall.

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.

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. 1 B 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 remote fear memory (Fig. 1 D and F and Fig. S4). Strikingly, the chABC treatment disrupted 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.


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Removing 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) at five different distances (mm) from bregma, red squares indicate 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 test revealed that chABC treatment disrupted CS memory retrieval (**P < 0.001). (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 V2L (n = 4 rats) did not influence freezing to light CS compared with sham controls (n = 8 rats). Detailed statistics are shown in Fig. S1.

The coherency in LFP between the two brain areas at baseline, i.e., before the first CS onset, was not different between the groups (Fig. 2C, Upper), supporting the notion that PNN removal specifically affected memory processing. In addition, we investigated whether synchronized theta oscillations between V2L and BLA were present during recent memory retrieval. In accordance with previous work (17), we found increased theta coherence during CS presentation also at this time point, although with a higher frequency (coherence peak at 9 Hz) (Fig. S5). This indicates that V2L is involved at this initial stage of memory processing, in line with previous work from the secondary auditory cortex (16, 17).

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 × condition treatment (aCSF or chABC) × 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 coherency 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 resulted 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

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 0.1 M PBS. The tissue was left to postfixate overnight before being transferred to a cryoprotective 30% sucrose solution in 1× PBS for 3 d at 4 °C. The tissue was then flash frozen and cut into coronal sections (45 μm) using a cryostat (Ostrobotnicolor). Nissl staining was done to confirm the location of the injection sites. For immunohistochemical staining, a monoclonal anti-chondroitin sulfate 6 antibody was used for 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 also used for staining parvalbumin-positive interneurons (rabbit anti-parvalbumin; Swant; Alexa 594 goat anti-rabbit (Life A11037) and for parvalbumin-positive interneurons [rabbit anti-parvalbumin (Swant); Alexa 488; Life S-11223). This method for fluorescent immunohistochemistry was used for staining parvalbumin-positive interneurons (rabbit anti-parvalbumin (Swant); Alexa 594 goat anti-rabbit (Life A11037) and for fluorescent labeling of the C6-6 stubs after chABC treatment (anti C6-6 clone MK302 (MAB 2035; Merck Millipore), Alexa 594 donkey anti-mouse (Life A21203)). The monoclonal anti-chondroitin-6-sulfate antibody (MAB 2035; Merck Millipore) recognizes the six inner monosaccharides at the chondroitin sulfate chain left on the core proteins after chABC cleavage, the so-called C6-6 epitope, thereby confirming the activity of chABC (39). Sections were photographed using a Zeiss Axioplan 2 microscope and Axioacam 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 2,000,000 times, low-pass filtered at 500 Hz, and stored to disk at 4.8 k (16 bits/sample) for offline analysis. LFP traces for every stimulus period were extracted and aligned according to 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 also used to analyze oscilations of the LFP signal. The coherence between the LFP channels for BLA and V2 was estimated by the magnitude-squared coherence, using the Matlab function mscorre.
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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