# Very long-term memories may be stored in the pattern of holes in the perineuronal net

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**A hypothesis and the experiments to test it propose that very long-term memories, such as fear conditioning, are stored as the pattern of holes in the perineuronal net (PNN), a specialized ECM that envelops mature neurons and restricts synapse formation. The 3D intertwining of PNN and synapses would be imaged by serial-section EM. Lifetimes of PNN vs. intrasynaptic components would be compared with pulse-chase $^{15}$N labeling in mice and $^{14}$C content in human cadaver brains. Genetically encoded indicators and antineoepitope antibodies should improve spatial and temporal resolution of the in vivo activity of proteases that locally erode PNN. Further techniques suggested include genetic KOs, better pharmacological inhibitors, and a genetically encoded snapshot reporter, which will capture the pattern of activity throughout a large ensemble of neurons at a time precisely defined by the triggering illumination, drive expression of effector genes to mark those cells, and allow selective excitation, inhibition, or ablation to test their functional importance. The snapshot reporter should enable more precise inhibition or potentiation of PNN erosion to compare with behavioral consequences. Finally, biosynthesis of PNN components and proteases would be imaged.**

memory | plasticity | extracellular matrix | genetically encoded reporters | protein turnover

A major problem in understanding memory is how it can be very long-lasting and stable from early childhood until death, despite massive interruptions in brain state as extreme as prolonged comas. Current prominent candidates for molecular substrates for long-term memory storage have focused on macromolecules such as calmodulin-dependent protein kinase II (CaMKII) coupled with the NMDA receptor (1) and protein phosphatase 2A (2), protein kinase M zeta (PKMζ) (3), and cytoplasmic polyadenylation element binding protein (CPEB) (4), all of which are inside postsynaptic spines. To retain information despite metabolic turnover, all such candidates need to have some sort of bistable switch (e.g., state of phosphorylation or prion conformation) and a mechanism by which older copies of the molecule pass on their status to newer copies to preserve the information. A major problem is that individual intracellular molecules typically last at most a few days before being turned over. Therefore, the information would have to survive being copied tens of thousands of times in a long-lived human, despite metabolic interruptions. Such robust fidelity would be extremely difficult to engineer. Even dynamic computer memory with sophisticated refresh and error correction circuits cannot cope with even a momentary hiccup in its power supply. Instead, long-term information storage in both computers and human civilizations requires writing the information onto physically stable storage media (e.g., magnetic disks, clay tablets, or acid-free paper), which do not require frequent energy-dependent recopying. Aside from some nuclear pore constituents, all of the known really long-lived proteins are insoluble ECM components such as crystallin, elastin, collagen, and proteoglycans (5), which gain stability by extensive cross-linkage and remoteness from intracellular degradative machinery, such as proteasomes, lysosomes, and autophagy.

A proteoglycan-rich matrix called the perineuronal net (PNN) has long been known to sheath mature CNS neurons (Figs. 1 and 2), with synapses forming through gaps in the PNN (6–12). The PNN is initially laid down at the end of critical periods in wiring of sensory inputs and may have contributions from both neurons and glia (13). Experimental global disruption of the PNN can reopen such critical periods, and therefore, the PNN is generally considered to restrict synaptic plasticity (14, 15). The endogenous enzymes that can digest the PNN, such as matrix metalloproteinases (MMPs; especially MMP-9), are known to be important in some way for synaptic plasticity (7, 16, 17). Despite the massive literature on PNN and the enzymes that degrade it, no clear mechanistic consensus has emerged to explain their important roles in synaptic plasticity and memory. Much of the problem is because the experiments showing effects on in vivo behavior rely on disruptions of the PNN or its degradative enzymes with low spatial and temporal resolution, leaving the possibility that these molecules are merely permissive and not carriers of detailed information. Much higher-resolution experiments have been done in synaptoneurosomes, slices, or cultures [for example, showing that MMP-9 is locally translated (18) and rapidly secreted at synapses in response to activity (19–21), that MMP inhibitors prevent late-phase synaptic potentiation (16–20), and that local puffing of MMP-9 onto spines can provoke spine enlargement and synaptic potentiation (19–22)], but such manipulations cannot yet be linked to specific behaviors or memories. New techniques will need to be developed to test the hypothesis that very long-term memories are stored in the pattern and size of holes in the PNN and that the holes are dynamically created or enlarged by the above-listed enzymes. In this view, the PNN is like a punched card, fantastically convoluted in 3D, in which the position and size of holes preserve the long-term location and strength of synapses (Fig. S1 and Movie S1).

The molecular and cellular bases for very long-term synaptic plasticity and memory are among the most central and controversial questions in neuroscience. Also, PNNs and MMPs have been heavily implicated in many neuropathologies ranging from traumatic injury, miswiring during critical periods, and epileptogenesis, addiction-related plasticity to Alzheimer's disease (6, 7, 9, 11). Fear conditioning is an important animal model for anxiety and posttraumatic stress disorder in humans (23, 24). The comparative roles of the PNN between species (e.g., *Drosophila* and *Caenorhabditis elegans*) have been neglected. As noted above, reviews on the PNN propose permissive, supportive roles, such as inhibiting neurite outgrowth, forming a physical barrier to new contacts, serving as a scaffold for other inhibitory molecules, binding integrins, limiting AMPA receptor mobility, reducing oxidative stress, and buffering ions (6, 8, 10, 11). Such

Author contributions: R.Y.T. wrote the paper.

The author declares no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1310158110/-/DCSupplemental.

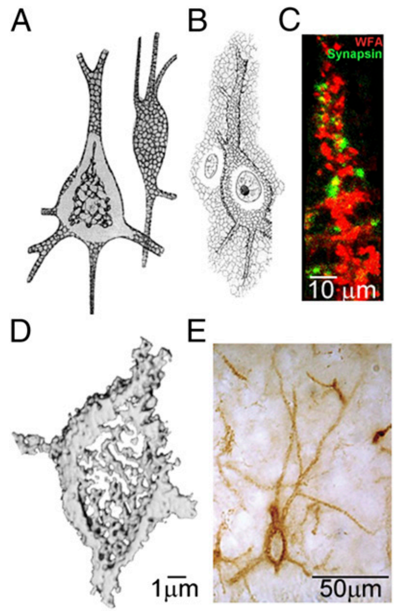


Fig. 1. Perineuronal nets. (*A*) Cat cerebral cells stained with reduced silver nitrate (75). (*B*) Dog cortical cells stained by the Bethes method (76). (*C*) WFA- and synapsin-stained cultured cortical neurons (77). (*D*) Confocal image of a rat somatosensory cortex interneuron labeled with fluorescent VVA (78). (*E*) WFA-stained somata and proximal dendrites of rat neurons (79).

roles are somewhat analogous to the importance of insulation on the wiring inside a computer: essential for function but not where bytes are dynamically stored. The closest previous statement to the current hypothesis was as follows: "[t]he extracellular matrix at synapses in the brain may have a similar function [as at the neuromuscular junction] and could well maintain overall connectivity despite the comings and goings of molecules inside neurons" (25). This statement from a chapter by Sejnowski (25) in a book entitled *What We Believe but Cannot Prove* was highly insightful but gave no mechanistic detail on how the ECM might store the information. The only experimental test proposed was that disruption of the ECM would interfere with memory, for which there is (and already was) much evidence (6, 12, 14–16), but the evidence is not incisive enough to be convincing. Meanwhile, reviews on MMPs in synaptic plasticity and learning conclude that the key substrates and downstream mechanisms remain unclear (7, 16, 26). In this article, I propose experiments to test this hypothesis and try to detail how they will improve on those hypotheses in the literature. A metaphor for PNN stably localizing a synapse is in Fig. S1.

## Determine the 3D Relationship Between PNN and Synapses by Serial Block Face Scanning EM

Important assumptions of my hypothesis are that the PNN forms a continuous barrier that encases relevant neurons during the critical period closure of each part of the brain and that the PNN permits synaptic connections between neurons through holes sized to fit the requirements of each synapse. Although published literature includes much optical imaging of the PNN, there are very few high-quality thin-section EM images (27) and no 3D reconstructions revealing the ultrastructure of the PNN from dendritic tips through the soma to the axonal end. I propose high-resolution EM reconstruction for 3D visualization using serial block face scanning EM (SBFSEM) (28) with several options of highlighting the PNN. The PNN has been labeled with biotinylated *Vicia villosa* agglutinin (VVA) and *Wisteria floribunda* agglutinin (WFA) (29–31) (Fig. 1). I propose to label the PNN in fixed brain slices with eosin-conjugated VVA and WFA, illuminate the eosin tags to photooxidize diaminobenzidine, and locally generate osmiophilic precipitates for SBFSEM (32). Because the PNN is extracellular, membrane permeabilization with detergents will not be required, and therefore, ultrastructure will be well-preserved. An alternative approach would be to create viral vectors encoding PNN proteins genetically fused to mini singlet oxygen generator (miniSOG) (33) or enhanced ascorbate peroxidase (APEX) (34) flanked with loxP sites. These viral vectors would then be injected before or during PNN deposition (35) into transgenic mice that express Cre recombinase in the amygdala (available from Jackson Laboratory), and therefore, miniSOG or APEX fusions would be incorporated into the developing PNN. Diaminobenzidine precipitates for SBFSEM would be generated in fixed sections by photooxidation or peroxidase reaction, respectively. The genetic tagging approach is more laborious than lectin staining of endogenous PNN, but it offers selectivity for specific protein components (whereas the lectins highlight the carbohydrate side chains), avoids concerns about diffusibility of lectins into fixed sections, and should also reveal nascent PNN proteins transiting through the secretory pathway in different cells (36).

## Determine Age of Proteins in PNN Vs. Synaptic Cleft

**Use Stable Isotope Labeling of Amino Acids in Mammals and MS Proteomics to Measure Lifetimes of Proteins Within Synapses Vs. the PNN and Find the Longest-Lived Proteins.** A key postulate of my hypothesis is that the PNN contains molecules that do not turn over after their initial deposition. The lifetime of PNN components relative to intrasynaptic proteins seems never to have been measured experimentally, although a long lifetime for

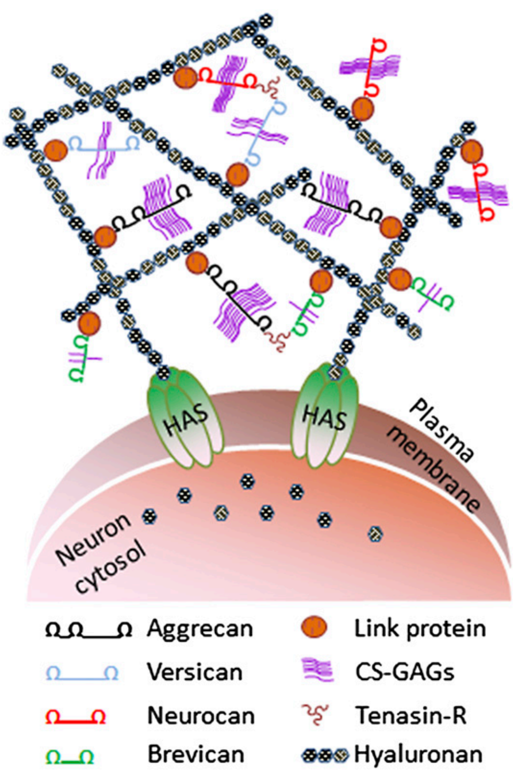


Fig. 2. The structure of the PNN. Hyaluronan, secreted by membrane-bound HA synthase (HAS), binds to members of the lectican family (aggrecan, brevican, versican, and neurocan) and is cross-linked by link proteins and tenascin-R to form supramolecular aggregates on the surface of neurons. Reproduced from ref. 80 with permission of John Wiley & Sons, Inc. CS-GAG, chondroitin sulfate – glycosaminoglycans.

the PNN is plausible by analogy to other long-lived ECMs (5, 25). Tritiated threonine was reported to turn over with biphasic half-lives of 13 and 38 d in total brain glycoproteins in adult rat brain, but no radioactivity incorporated into the proteins attached to chondroitin sulfate and heparin sulfate (37), implying very little turnover of those components of the PNN. I suggest an approach based on stable isotope labeling of amino acids in mammals followed by mass spectroscopic multidimensional protein identification technology (MudPIT). These methods have been used to show which synaptic proteins change significantly in abundance after deprivation of sensory input to the barrel cortex in mice (38) and that some nuclear pore proteins are unexpectedly very long-lived (39). Just as in these studies, mothers and pups are fed $^{15}N$ chow until the young mice are >95% $^{15}N$. At postnatal day 45, long enough for full maturation of the PNN in the amygdala (35), animals are killed, and PNNs and synaptosomes are isolated by established procedures (38, 40) from different portions of the brain for MudPIT. This sample serves as $t = 0$ reference for maximal $^{15}N$ labeling. The remaining animals would be switched to $^{14}N$ chow, killed 2 d or 1, 4, 18, or 78 wk later, and similarly processed. The final time point may have to be brought forward if the mice did not look like they would survive the full span. I expect the $^{14}N/^{15}N$ ratios to give turnover rates for tens of distinct, fully identified proteins in the PNN and hundreds in synapses as represented by synaptosomes. This information should be of tremendous long-term value, even if my hypothesis is incorrect or oversimplified. Pilot experiments verifying sample preparations and MudPIT could be done on control $^{14}N$ animals. For example, the PNN samples will need enzymatic deglycosylation before MudPIT. Synaptosomes may need exposure to chondroitinase to remove residual PNN. Also note that, unlike the PNN itself, the PNN-degrading enzymes are likely to be rapidly turning over, which was hinted by their acute up-regulation and local translation minutes after synaptic activation (18, 19, 21).

**Measurement of Turnover of PNN Vs. Synaptosomal Proteins in Humans Using $^{14}C$ Dating.** All humans born between 1955 and 1963 were subjected to a pulse of $^{14}C$ from atmospheric testing of nuclear weapons, and therefore, tissue samples containing >5 μg carbon can be analyzed for their date of biosynthesis with an accuracy of about ±2 y. This established technique (41–43) has given many important results, such as the fact that practically all of the DNA in the cerebral cortex is almost as old as the individual; therefore, postnatal cell division is quantitatively negligible. However, such dating has never been applied to PNN or synaptic proteins. I propose using the accelerator MS and dating of PNN and synaptosomal samples prepared from various regions of postmortem human brain, including amygdala. The advantages of these experiments are the multidecade time span retrospectively accessible and the direct relevance to human memory; the disadvantage is that I do not expect to resolve individual molecular species or lifetimes of less than several years. Again, pilot experiments will be needed to assess and minimize the extent of cross-contamination between PNN and synaptosomes, the amount of new carbon introduced by the isolation procedure, and the amount of input tissue required to yield the minimum of 5 μg carbon. If, for some reason, the $^{14}C$ experiments prove unfeasible, measurement of aspartate racemization provides another established form of birth-dating (5, 44).

## Image Protease Activation with Genetically Encoded Indicators After a Long-Term Potentiation or Learning Paradigm

An ideal test of the hypothesis would be to (*i*) continuously monitor protease activity with (*ii*) high time and space resolution throughout much of the relevant area of brain (e.g., amygdala) (*iii*) in the intact animal during an appropriate learning paradigm (e.g., fear conditioning). Although this triple crown is not yet feasible, genetically encoded indicators of protease activity should allow all possible pairs of criteria to be satisfied. Monitoring of MMP-2/-9 activity would require updating a reported FRET indicator (45) for MMP-14 by installing a better MMP-2/-9 substrate, probably the amino acid sequence PLGLAG, and replacing the cyan fluorescent proteins and YFPs with the best new green donor/red acceptor pair (46). To achieve *i* and *ii*, acute slices would be prepared and stimulated electrically or optogenetically while imaging the green/red FRET indicator with confocal or two-photon microscopy.

The most elegant variation would be to use a snapshot reporter (see below) to drive expression of a channel rhodopsin in vivo, and therefore, optical stimulation in the slice should recapitulate the population of neurons that had been active during a learning paradigm. To achieve *i* and *iii*, the fluorescence would be continuously monitored through a fiber optic probe or stick objective implanted in the amygdala during or just after a training paradigm, such as fear conditioning. To achieve *ii* and *iii*, the animal would be killed at a chosen time point during or just after training. Acute slices would be prepared from the amygdala and imaged with confocal or two-photon microscopy in the hope of finding synaptic punctae with a high green/red ratio indicating protease-induced cleavage and loss of FRET. One could also explore whether the degree of indicator cleavage in vivo can be preserved during tissue fixation to allow high-resolution ratio imaging in thin sections. Intact (high FRET) and cleaved (zero FRET) indicator molecules should retain those properties during fixation.

## Detect Newly Cut Holes in the PNN with Antibodies Against Neoepitopes

I think that there may be a way to image protease activation and fresh erosion of the PNN at a given time point without having to incorporate a genetically encoded protease reporter. Endoprotease attack creates two fragments with a new amino terminus and a new carboxyl terminus, which constitute neoepitopes against which specific antibodies can be raised (47). Such antibodies do not recognize the uncut sequence in the intact protein. Crucially, the two fragments have different retention times (Fig. S2): the left one containing the aggregating G1 domain is well-retained within the matrix, whereas the right one containing nonaggregating G3 diffuses away more readily (47). I predict that the ratio of labeling by the two antineoepitope Abs should provide a rough indication of how long ago the proteolysis took place. Old erosions should stain for only the longer-retained neoepitope, whereas fresh cuts should stain for both neoepitopes. The relationship between age and neoepitope ratio could be calibrated by standards in which widespread proteolysis was induced at known times (e.g., by injection of active enzyme or kainate) (21).

## Test MMP-2/-9 Double KO Mice for Behavioral Deficits

Although mice genetically deleted in MMP-2, MMP-9, or both have long been available, surprisingly little behavioral testing has been done on them. MMP-9 KO animals showed deficits in hippocampal late-phase long-term potentiation (LTP) and context-dependent fear conditioning (believed to require both hippocampus and amygdala) but not tone-cued fear conditioning (believed to require amygdala but not hippocampus) (20). At face value, this result would argue that MMP-9 is needed for hippocampal but not amygdalar plasticity. However, my own experience with these animals in cancer research is that MMP-2 and -9 have very similar substrate preferences and tend to compensate for each other after one is deleted; therefore, deletion of both is often necessary to get the full phenotype (48, 49). Therefore, I propose repeating the behavioral and associated electrophysiological tests similar to the tests by Nagy et al.

(20), especially tone-cued fear conditioning, with MMP-2/-9 double KO animals.

### Deliver Shorter Pulse of More Specific Protease Inhibitor and Interfere with Induction in Vivo

Several groups (reviewed in ref. 16) have shown that broad-spectrum MMP inhibitors can prevent late-phase LTP in various brain regions. Intracerebroventricular injection of such an inhibitor has been reported to attenuate water maze learning for many days after injection (50). To make this experiment more incisive and relevant to fear conditioning, I would replace the broad-spectrum inhibitor with a newer inhibitor, SB-3CT, which is commercially available and much more specific for MMP-2 and -9. Remarkably, after a single i.p. injection, SB-3CT rapidly crosses the blood–brain barrier and then washes out in just a few minutes (51). This convenient route of administration and rapid pharmacokinetics should permit more precise delineation of the interval, during which time MMP-2/-9 activity is required during acquisition of fear memories. Such time resolution should enable suppression of one learned association without interfering with previous or subsequent conditioning to different cues or contexts. Such evidence would strengthen the case that protease activity is instructive and encodes specific information and that it is not just globally permissive.

### Apply Snapshot Reporter to Mark Cells Activated During Amygdalar Fear Conditioning for High-Resolution Imaging, Optogenetic Manipulation, or Overexpression of Proteases or Protease Inhibitors

Here, I introduce the concept of snapshot reporters and focus on methods for the snapshot memorization of $Ca^{2+}$ in activated neurons with the retrospective ability to detect $Ca^{2+}$ changes with high spatiotemporal resolution. Snapshot reporters would be useful in neurobiology even independent of any perineuronal net hypothesis. Among the many obstacles to brain activity mapping are the enormous technical difficulty of simultaneously recording from thousands to millions of identified neurons at high speed in 3D in an intact, preferably behaving organism and the problem of identifying the neurons with firing that is actually important for the behavior. Recording activity can, at best, give correlations; targeted stimulation, inhibition, and ablation are necessary to establish causality. An imperfect partial solution to these problems is the use of promoters for immediate early genes, such as c-fos, Arc, and zif268 (52). Immunostaining for the expression of such genes can retrospectively highlight activated neurons throughout large sections of intact brain without requiring tissue transparency or sophisticated instrumentation. Effector proteins driven by these promoters can identify the activated neurons for subsequent electrophysiology, optically or pharmacologically stimulate or inhibit their firing, or ablate them. Unfortunately, the relationship between neuronal activity and induction of these immediate early genes is poorly defined, and therefore, the sensitivity, specificity, and temporal resolution of the response are often less than ideal. This powerful concept could be greatly improved by engineering a snapshot reporter system (a super-fos, so to speak) to drive expression of arbitrary reporter and effector proteins in response to defined elevations of $[Ca^{2+}]$ precisely coinciding with an external trigger, such as light. The light would only need to propagate diffusely throughout the brain region of interest without requiring image-quality resolution.

My approach to a snapshot reporter system should be constructed by tandemly fusing a DNA binding domain, a $Ca^{2+}$-triggered heterodimerization module, a light-triggered heterodimerization module, and a transcriptional activation domain (Fig. 3). When (and only when) high $[Ca^{2+}]$ and light are simultaneously present, the three chimeric proteins will join together into a three-hybrid unit to activate transcription of any reporter or effector

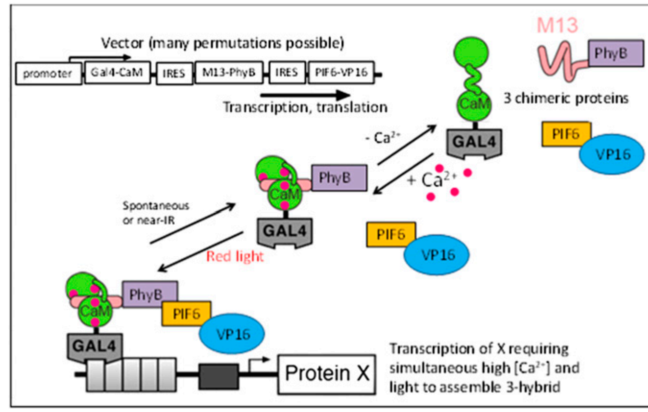


**Fig. 3.** Cartoon depicting one of the many ways to engineer a light- and $Ca^{2+}$-triggered transcriptional readout to serve as a snapshot reporter of neuronal activity. Many different permutations of the three fusions are possible, provided that no single fusion contains (Gal4 + VP16), (CaM + M13), or (PhyB + PIF6). PhyB, PIF6, and red light may be replaced by CRY2, CIBN, and blue light, respectively. Nuclear localization signals may have to be provided. For simplicity, binding of the Gal4 chimera to its cognate DNA is shown only after final assembly of the three-hybrid. IRES, internal ribosomal entry site.



gene appropriately placed downstream of the site on DNA for the DNA binding protein. The DNA binding and transcriptional activation domains need to be potent but with low background in mammalian cells, and therefore, the commonly used Gal4-VP16 pair should be suitable (53–55). The $Ca^{2+}$-triggered heterodimerization module will be one of the mutant calmodulin-M13 pairs that we previously engineered not to cross-react with endogenous calmodulin and that offer a range of $[Ca^{2+}]$ affinities (56). Such bioorthogonality will be essential here, because the calmodulin and M13 are not prefused with each other; therefore, the exogenous calmodulin fused to the DNA binding domain will not have any intramolecular advantage over endogenous free calmodulin.

Two light-triggered heterodimerization systems have been published (55–57). Robust light activation of the flavin-based cryptochrome 2/CRY2-binding domain (CRY2/CIBN) (55) and the phycocyanobilin-based phytochrome B/phytochrome interacting factor 6 (PIF6) system (57) has been reported, and both assayed by the ability of light to recruit a fused fluorescent protein to the membrane. Although the blue light CRY2/CIBN heterodimerization system will certainly suffice for a proof of principle and many biological applications, a phytochrome-based system may be ultimately preferable, because it should be activated by better-penetrating red light (650 nm) and inhibited by near-IR (750 nm). The availability of a turn-off wavelength could be useful to sharpen specificity and spatiotemporal resolution. Ideally, one could further engineer and improve the phytochrome B/PIF6 system to alter the chromophore of phytochrome B from exogenous phycocyanobilin to endogenous biliverdin and make the light-triggered binding of the two partners more robust. The large size of phytochrome B (908 aa encoded by 2,724 nt) is also incompatible with many viral vectors, probably requiring the trimming away of all nonessential domains. Mutations would be generated by structure-based intuition, DNA shuffling (58, 59), and random mutagenesis (PCR-based or somatic hypermutation) (60). High-throughput screening of phenotypes would be performed in mammalian cells in a simplified version of the snapshot reporter, in which the DNA binding domain will be directly fused to the phyB mutants so that transcription is dependent only on light-dependent heterodimerization independent of $Ca^{2+}$. The reporter gene would be β-lactamase (bla), because it is ideal for both positive and negative selection by FACS (61–64). One would stain and sort for bla expression [blue fluorescence with coumarin-cephalosporin-fluorescein 2/acetoxymethyl ester (CCF2/AM) live-cell substrate] 1 h or so after exposure to red light and no bla expression (green fluorescence with CCF2/AM) after near-IR or no light.

An interesting variation on the above scheme would be to replace the DNA binding and transcriptional activation domains by complementary fragments of Cre recombinase (55, 65); therefore, coincidence of high [$Ca^{2+}$] and light would reconstitute functional Cre and trigger excision of loxP-stop-loxP cassettes. The irreversibility of both of these steps would probably increase sensitivity at the potential cost of higher background and poorer temporal specificity. Also, a snapshot reporter based on Cre could not be targeted to specific cell types using Cre driver lines.

Fear conditioning in the amygdala is a particularly appropriate application for a snapshot reporter because of the advanced results already obtained with c-fos–driven expression of drug- or light-controlled channels (52). For example, the higher temporal resolution of a snapshot reporter should enable more precise delineation of which subsets of neurons are activated at what time by different contexts or cues, because the triggering light would be timed to coincide with (or follow with defined latency) one stimulus or a train of repetitions to achieve in situ response averaging. The reporter would be a fluorescent protein for postmortem optical reconstruction or a singlet oxygen generator for electron microscopic ultrastructure (33). Optogenetic or drug-controlled excitatory channels, hyperpolarizing pumps, optogenetic inhibition of synaptic release (66), or cell ablation with toxins or singlet oxygen generators (67) would test the sufficiency and necessity of the activated cells for the induction and expression of behavioral plasticity. The specific hypothesis that fear memories can be encoded in the pattern of holes in the perineuronal net could be tested by using the snapshot reporter to enhance expression of secreted proteases, such as MMP-9, aggrecanase, hyaluronidase, or chondroitinase. Whereas delocalized application of chondroitinase seems to render fear memories labile (35), judicious overexpression of hole-cutting proteases in just those neurons activated by a specific conditioning paradigm should specifically strengthen that association. A complementary experiment would be to express protease inhibitors such as tissue inhibitor of metalloproteinase-1 (TIMP-1) (68), which should block formation of memories of just those cue-shock associations with which the triggering light was paired. Finally, the snapshot reporter might be able to drive renewed expression of PNN components and thus, specifically weaken preestablished memories that were being refreshed (hence evoking neuronal activity and high [$Ca^{2+}$]) in coincidence with the triggering light.

## Use Time-Specific Tag for the Age Measurement of Proteins to Monitor de Novo Synthesis of Proteases and PNN Components

New protein synthesis, especially local synthesis at synapses (69, 70), is widely acknowledged to be crucial for plasticity. We recently introduced a time-specific tag for the age measurement of proteins (TimeSTAMP) tag, which allows drug-controlled labeling of newly synthesized copies of specific proteins (71). This tag was recently improved to incorporate a split YFP and the miniSOG tag for correlated light and EM, allowing live fluorescence and EM readouts (72). No other method currently offers such high spatiotemporal resolution and specificity for genetically designated proteins. Evidence has been presented that MMP-9 is locally translated in synaptoneurosomes (18) and up-regulated by gross pharmacological glutamatergic stimulation (18, 21). Such observations would be greatly improved by using TimeSTAMP (72) to image nascent MMP-9 with higher spatiotemporal resolution (72) in acute slices or intact brain as a function of electrical stimulation or behavioral conditioning, respectively. Such experiments would provide an alternative to map sites of PNN erosion. It would also be interesting to fuse TimeSTAMP to PNN constituents to see if and where the proteins are resynthesized after dissolution of carbohydrate side chains by injected chondroitinase or when extinction training is performed during the reconsolidation window (23). The latter procedure seems to erase fear in adults, whereas ordinary extinction overlays a new inhibitory memory (23).

## Conclusions

I have overemphasized techniques that my laboratory has been involved with because of personal familiarity and a wish to highlight their usefulness to a wider community. Actual techniques and experiments will be chosen by the scientists involved. The experiments may be based on partially or entirely wrong premises, in which case they will be forgotten. However, the importance of long-term memory is comparable with the significance of DNA. From 1943 to 1953, Avery et al. (73) and Watson and Crick (74) showed the importance of DNA in carrying genetic information from generation to generation through the double helix. However, the genetic code was still unknown. Watson and Crick (74) only brought brilliant insight without new experimental data. Franklin and Gosling (75) had the key X-ray diffraction pattern. The present paper has no fresh experimental results but many predictions. Perhaps in a few years, at least one prophecy can be vindicated.

**ACKNOWLEDGMENTS.** The author thanks Drs. Stephen Adams and Varda Lev-Ram for help with manuscript preparation. This work was supported by National Institutes of Health Grant NS27177.

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# Supporting Information

**Tsien 10.1073/pnas.1310158110**

**Fig. S1.** Damascus Gate, Jerusalem. The metaphor consists of the stable structure of the wall, which represents the PNN, whereas the gate represents an opening for a synapse. The masonry is stable while the people and the merchant stalls are transient, like other synaptic proteins that have a short presence and fast turnover at this site. Not shown here are rarer events remodeling the synapse, which would be represented by chiseling away the stonework.

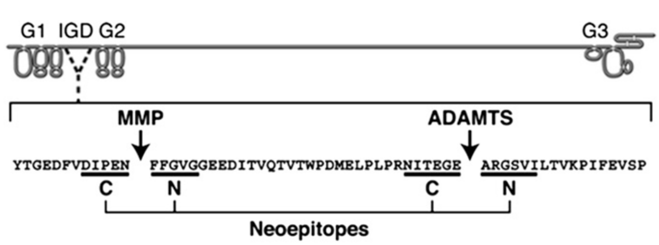


**Fig. S2.** Proteolytic cleavage sites within the aggrecan interglobular domain (IGD) (1). The structure of the aggrecan core protein is depicted with its three disulfide-bonded globular regions (G1, G2, and G3) and its IGD. The amino acid sequence of the IGD is shown together with the cleavage sites for matrix metalloproteinases (MMP) and aggrecanases (ADAMTS) and the C- and N-terminal neoepitopes generated by such cleavage.

1. Roughley PJ, Mort JS (2012) Analysis of aggrecan catabolism by immunoblotting and immunohistochemistry. *Methods Mol Biol* 836:219–237.

**Movie S1.** Damascus Gate, Jerusalem, Israel.

Movie S1