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On the research of time past: the hunt for the substrate of memory

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Abstract

The search for memory is one of the oldest quests in written human history. For at least two millennia, we have tried to understand how we learn and remember. We have gradually converged on the brain and looked inside it to find the basis of knowledge, the trace of memory. The search for memory has been conducted on multiple levels, from the organ to the cell to the synapse, and has been distributed across disciplines with less chronological or intellectual overlap than one might hope. Frequently, the study of the mind and its memories has been severely restricted by technological or philosophical limitations. However, in the last few years, certain technologies have emerged, offering new routes of inquiry into the basis of memory. The 2016 Kavli Futures Symposium was devoted to the past and future of memory studies. At the workshop, participants evaluated the logic and data underlying the existing and emerging theories of memory. In this paper, written in the spirit of the workshop, we briefly review the history of the hunt for memory, summarizing some of the key debates at each level of spatial resolution. We then discuss the exciting new opportunities to unravel the mystery of memory.

Keywords

memory; engram; memory encoding; memory localization; optogenetics; neural labeling; engram cell labeling; neural plasticity; synaptic plasticity; history of memory

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Introduction

How are experiences stored in the brain? The search for memory is considerably older than cognitive science, neuroscience, or psychology. It predates modern medicine and anatomy, the theory of evolution, and our belief in a heliocentric universe. For at least 2400 years, humans have wondered what enables perception and thought and what accounts for learning and memory. The search for the mind has been led by priests and physicians, philosophers and physicists. The lines of inquiry have diverged and the theories have differed, but the central goal has remained the same: to understand the world and how we understand it.

How has the hunt for memory changed in the last 2000 years? The modern scientific search for memory is critically dependent on two fundamental assumptions: (1) that the memory trace is physical and (2) that the trace will be found in the brain. A modern reader may find both of these issues trivial. If someone proposed to look for your memories in your bone marrow, most would consider them a lunatic. However, the search for memory originated in philosophy and divinity, not in science and certainly not in neuroanatomy. Our memories were not always considered a physical entity, nor were they suspected to lie in the brain.

“Of something or of nothing:”^b the physical basis of memory

Modern science is less than 500 years old, and almost all of our modern discussions of the mind show vestiges of these prescientific theories. Three theories of human knowledge, all generated before the first century AD, have been particularly influential over the last two millennia: (1) knowledge originates in a foreign entity—humans can accept knowledge in the form of food or light created by this external source;^c (2) knowledge is an innate property of the human mind—we know and remember things that are pre-embedded and accessed through intelligence;^d and (3) knowledge is built up systematically through iterations of the mind experiencing the world. The model of accumulated sensory experience dominates today, having been resuscitated at the beginning of the most recent scientific revolution.^e

These theories have profound implications for where and how we would look for memory. However, when each theory was first articulated, no experiments would have been designed at all. Historically, the idea of peering inside you to find memory or the mind was generally considered ridiculous. Many have maintained that the physical material of the body could

^bPlato's *Theaetetus* (369 BC) is a dialogue on the nature of knowledge in which Socrates coaxes a theory of epistemology from the hapless Theaetetus. It contains the following exchange: “*Socrates*: And you would admit that there is such a thing as memory? *Theaetetus*: Yes. *Socrates*: And is memory of something or of nothing? *Theaetetus*: Of something, surely.”

^cOne of the most beloved theories maintains that knowledge is found in the fruit trees of the Garden of Eden: “In the day ye eat thereof, then your eyes shall be opened, and ye shall be as gods, knowing good and evil” (*King James Bible* (1611), Genesis 3:5). Augustine of Hippo was a notable proponent of the theory of divine illumination. Just as the earth cannot light itself, but requires the sun, “The mind needs to be enlightened by light from outside itself, so that it can participate in truth, because it is not itself the nature of truth” (*Confessions* (397–400), IV.xv)

^dPlato favored the theory that truth was an inborn property of the human soul (see *Theaetetus* (369 BC)), as did René Descartes: “[W]e come to know [truths] by the power of our own native intelligence, without any sensory experience” (1643 letter, AT 8b: 166–167).

^eThe theory was popularized by Aristotle, who maintained that information was written onto the substance of the mind: “What the mind thinks must be in it in the same sense as letters are on a tablet which bears no actual writing; that is just what happens in the case of the mind” (*On the Soul* (350 BC), 3.4 430a1). John Locke and David Hume are widely credited with disinterring Aristotle's notions and overthrowing Descartes' regime.

never give rise to the processes of the mind.^f Others have considered the mind and body as interdependent physical entities.^g These concerns are referred to as the “mind–body problem,” the (unsolved) mystery of how the physical material of our bodies could generate our mental states.

Only in the last 500 years have investigations into the basis of human knowledge and memory migrated into the realm of science. With the transition comes the demand for theories to be tested with evidence acquired through experimentation. If the mind and its memories have no physical basis, then there cannot be a scientific discipline to study them. We can relegate the conversations forever to the territory of philosophy. So the question is: do we believe we will ever find the physical evidence of memory?

At the beginning of the 20th century, Richard Semon^{1,2} invented the term *engram* to signify “the enduring though primarily latent modification in the irritable substance produced by a stimulus (from an experience).”³ In this theory, interaction with the world leaves a physical residue within the excitable substance of the nervous system. Some internal modifications may manifest directly as a behavioral change or memory; others accumulated over time into a learned behavior.^{4,5} The first internal modification will not necessarily produce the full behavioral readout of memory (the first handful of sand does not a castle make), but the residues will, past some threshold, result in a robust, behaviorally observable behavior that signifies the internal memory. Semon’s theory of a physical form of memory was in direct opposition to the “psychical” form successfully advocated well into the 20th century by William McDougall, William James’s successor as chair of psychology at Harvard, among others.^{6,7}

The stomach contains evidence of food, and the liver certainly contains evidence of drink. Why wouldn’t the brain contain evidence of thoughts? Why are we still so mystical about the physical basis of memory?

“I am a brain, Watson. The rest of me is a mere appendix:”^h discovering the brain

The putative organ of thought has migrated quite a lot over the centuries. Numerous structures or cavities were proposed as the seat of knowledge. In eras where hydraulics were the height of technological sophistication, the suitable organ of thought was a hydraulic pump, the most obvious being the heart circulating blood. Later, the ventricles were observed to be circulating cerebrospinal fluid and the pineal was theorized to be the source of the mechanical propulsion. In light of these technological metaphors, the ancient Egyptians and Aristotle (335 BCE) hailed the heart as the organ of intelligence, Leonardo da Vinci voted for the ventricles (1506), and Descartes preferred the pineal (1649). Once the era

^fDescartes is widely credited with the formalized theory of mind-body dualism (*Meditations* (1641), II.6–8), though he is generally misinterpreted (see Noam Chomsky’s 2014 talk at the Vatican for discussion¹⁸⁴). Descartes maintained that the material of the body (*res extensa*), subject to purely mechanical laws, could not account for the mind. The mind must therefore have a different substrate (*res cogitans*), a fairly legitimate scientific theory before the discovery of electromagnetism and electrochemical transmission.

^gSupporters of this theory include the Roman physician Galen, the Buddha, David Hume, Charles Darwin, Francis Crick, and Cristof Koch.

^hArthur Conan Doyle, *The Adventure of the Mazarin Stone* (1921).

of electricity began, the most suitable organ of thought would obviously have to be electrical. In our current era, the height of technological sophistication is the computer and, unsurprisingly, the brain is now almost exclusively described as such.

Metaphors can occasionally prove useful, but the natural world is not obliged to abide by them. As is so often the case in science, things progress when people use their eyes. The latest scientific revolution, precipitated by Nicolaus Copernicus and Andreas Vesalius, placed the sun and brain at the center of their respective universes.ⁱ Both realignments occurred as a consequence of a suddenly enhanced ability to see. The creation of the telescope let people peer into the heavens, while the decriminalization of human dissection allowed people to peer into the body. Beginning in the Renaissance, artists, scientists, and surgeons began to systematically look for the physical basis of perception. How does the body extract information from the world? First and foremost, the eye was established as the organ of sight. Other senses followed and, suspiciously, all the sensory organs were in direct communication with the brain. The most reasonable conclusion was that the nerve hub was, somehow, important.

“The Hollow Men:”^j discovering brain areas

By the mid-19th century, thought and memory had been localized to the brain, and many were content to stay at organ-level resolution. Those supporting the “holist” view of brain function maintained that brain functions were performed by the brain as a whole, an organ which could not be divided into components. The holist theory of whole-brain function ended not with a whimper but with a bang, or rather a series of them. Throughout the 19th century, the pioneers of cognitive psychology, including Paul Broca and Carl Wernicke, used the damages of stroke, seizure, and head injury to pinpoint distinct brain functions. Through this careful mapping, the misfortunes of a select few were used to create a theory of many. At the beginning of the 20th century, the Great War supplied the many unfortunates to paint a high-resolution picture of brain function.^k Localized brain damage did not indiscriminately destroy all cognitive capabilities as one might expect under the holist theory. Instead, specific damages produced specific and remarkably reproducible deficits. Brain functions were not all distributed broadly throughout the entire organ, but instead resided in distinct locations.

ⁱCopernicus’ *De revolutionibus orbium coelestium* (1543) and Vesalius’ *De humani corporis fabrica* (1543) are widely considered the beginning of the modern scientific era.

^jT.S. Eliot, “The Hollow Men” (1925)

^kThe ancient Egyptians and Greeks, most notably Hippocrates (see *On Injuries of the Head* (400 BC)), had also noticed the connection between head trauma and specific behavioral deficits. However, at the beginning of the 20th century, the scale of slaughter during World War I, the Victorian obsession with categorization, and the advancements in septic technique in the late 1800s combined to yield an unprecedentedly large population of individuals who survived head injury and could be catalogued. In 1919 alone, the British Journal of Surgery published G. Horrax, “Observations on a series of gunshot wounds of the head” 7(25): 10–54; A. Newton & A. Brown, “A study of gunshot wounds of the brain” 7(25): 72–94; and G. Jefferson, “The physiological pathology of gunshot wounds of the head” 7(26): 262–289. However, the injuries are still generally classified according to type (e.g., scalp versus penetrating versus perforating versus fracture), complication (e.g., fungal infection, embedded shrapnel) and/or general symptoms (e.g., headache versus paralysis versus ability to work) as the preoccupation was with reducing mortality, not conducting science. Gordon Holmes emerged as arguably the greatest neurological hero of the Great War, carefully piecing together delicate maps of brain function while he treated the horrific brain injuries of his fellow soldiers. One hundred years later, the modern researchers of the Human Connectome Project are continuing this work, creating maps from the thankfully intact human brain.

By the mid-20th century, localism had long overthrown holism: the brain had identifiable areas that performed distinct functions, and some were even being removed therapeutically.¹ However, in the field of memory research, holism was alive and well. After a systematic search for memory through a series of cortical lesion studies in rats, Karl Lashley famously concluded: “It is not possible to demonstrate the isolated localization of a memory trace anywhere within the nervous system... The engram is represented throughout the region.”⁸

Loss-of-function lesion studies allow brain functions to be inferred by subtraction. However, complementary evidence of positive functions is also needed. Intractable epilepsy, to this day, is one of the only conditions that justifies invasive recording from or stimulation of the human brain. While evaluating an epileptic patient for surgery, Wilder Penfield and Theodore Rasmussen were allowed to stimulate the temporal cortex of a living human. In so doing, they happened to elicit the recall of random episodic memories.⁹ These findings were tantalizing, but it was the temporal cortex of Henry Molaison that altered memory research most profoundly. Having identified the medial temporal lobe as the source of Molaison’s seizures, the neurosurgeon William Scoville removed the anterior hippocampus, the amygdala, and the surrounding cortex from both sides of the brain. The consequences were severe memory loss in the form of both anterograde and retrograde amnesia for episodic memories.¹⁰ Molaison’s overall intelligence was not negatively affected, and his ability to learn motor tasks was intact. Brenda Milner’s and Suzanne Corkin’s subsequent work with “patient HM” launched the modern era of human memory research.^{10–12} New memories could not be formed without the medial temporal lobe, and the recall of previously stored episodic memories was heavily impaired (for review, see Refs. 13–15). In subsequent years, distinct memory systems have been described and localized in humans.^{16,17} Not all memories are created equal, nor are they created in the same place. Different types of learning occur in different brain areas. But what material actually learns?

One by one blossomed the stars:^m creating the neuron doctrine

By the beginning of the 20th century, the holist–localist debate had been settled at the organ level: the brain had distinct regions with distinct functions. The cortex *alone* could be reproducibly grouped into 52 territories.^{18,19}ⁿ However, while losing on the organ front, the holist theory had retreated to a different level of resolution, down to the cell.

By the end of the 19th century, cellular theory^{20,21} was widely accepted. However, many scholars of the brain maintained that it was exempt from such rules. Joseph von Gerlach described the brain as an indivisible “protoplasmic network” acting as a functional whole.²² Early supporters of this “reticular theory” included Franz Nissl and the highly respected Camillo Golgi, who devoted his Nobel acceptance speech to a refutation of the neuron doctrine. In a cruel twist of fate, the father of modern neuroscience, Santiago Ramon y Cajal, used Golgi’s own invention (silver staining) to create the images that convinced the

¹Antonio Egas Moniz won the 1949 Nobel Prize in Physiology & Medicine for the “discovery of the therapeutic value of leucotomy [lobotomy] in certain psychoses.” See <http://www.nobelprize.org>.

^m“Silently, one by one, in the infinite meadows of heaven, blossomed the lovely stars, the forget-me-nots of the angels.” – Henry Wadsworth Longfellow, *Evangeline, A Tale of Acadie* (1847).

ⁿThis year, David van Essen and colleagues, as part of the Human Connectome Project, provided evidence for 100 new cortical areas. See Ref. 105.

world that the brain was made of cells. The resulting neuron doctrine formally extended cell theory into the nervous system. The brain was built from discrete areas built from discrete cells. Localism won this hand, too.

The next logical step would be to extend the holist–localist debate to perception and memory, to information processing and storage. The brain perceives the world, creates and deploys concepts, and orchestrates behavior. Are these internal representations located in one place or throughout the brain? Again, we find a split decision. In the localist corner, we have the intellectual powerhouse William James, who theorized that “every brain-cell has its own individual consciousness, which no other cell knows anything about.”²³ The theory was later developed into the notion of the “grandmother cell,” the idea that an individual neuron can represent a concept^{24,25} and will be activated by multimodal stimuli associated with that concept. In the holist corner are proponents of the distributed representation theory, the notion that each stimulus, concept, or behavior is represented in a pattern of neural activity across neurons.^{26,27}

Once again, epilepsy provides the only substantial insights to date. The most spectacular observations come from Fried and colleagues, who performed recordings on epileptic patients and found brain cells that reliably responded to pictures of popular actors, athletes, and movie characters, among others.²⁸ The results have been interpreted to support both the localist (grandmother) and holist (distributed representation) theories. The debate has not been settled.²⁹ The distributed representation theory cannot be tested, because we cannot see. We cannot collect the patterns of individual neural firing from the brains of living humans. However, the scientific community just recently developed the ability to do this in mice.

“She would give them order. She would create constellations.”^o discovering network engrams

When the technology does not exist to extract the requisite evidence, the hypothesis cannot be conclusively tested. If information is encoded in patterns of neural firing across neurons, we have to examine the firing of individual neurons during information encoding. In healthy humans, we cannot currently do this. A 1-mm³ voxel in a functional magnetic resonance imaging (fMRI) scan contains thousands to tens of thousands of neurons. We can observe none of their individual spiking behaviors. From a depth recording in an epileptic patient, we can extract the spiking of a random subset of a few hundred neurons. Neither method is sufficient to conclusively determine the mechanism of neuronal information encoding.

Recently, new technical developments have made it possible to examine the neural mechanisms of memory encoding in mice.³⁰ The tremendous body of work can be concisely summarized in the following four technical strategies.

^oThomas Pynchon, *The Crying of Lot 49* (1965).

Technical strategies

Transgene expression—It is now possible to induce the artificial expression of proteins in mammals through transgenic technology^{31–34} (for review, see Ref. 35). Genes can be artificially introduced into or deleted from cells, including brain cells.^{36–38} Using the template of the transplanted gene (transgene), the host cell will create the desired protein capable of performing a function or labeling the cell for visualization purposes.

Activity-dependent expression—It is possible to limit the expression of the transgene to specific populations of cells. For example, the transgene can be expressed only in neurons that are active. Immediate early genes (IEGs) naturally turn on immediately after neuronal activity^{39–43} (for review, see Ref. 44). The IEG promoters can be used to control an introduced transgene.^{30,45–47} The protein of interest will only show up in neurons that fired, producing activity-dependent labeling of cells.

Time-dependent expression—It is possible to limit the expression of transgenes to specific time windows. A transgene attached directly to an IEG promoter would be promiscuously expressed any time the cell fired. However, in a two-component transgenic system, the time window of transgene expression can be controlled, with the aid of a third, exogenous molecule (often an ingested antibiotic (e.g., doxycycline)).^{48,49} An IEG promoter is attached to a transgene that can itself induce gene expression (a transactivator). Neuronal activity produces the transactivator protein, which binds the promoter of a second transgene. Crucially, the transactivator protein is engineered to be either repressed or induced by a systemically administered molecule (e.g., rendered inactive by doxycycline (DOX)). When the subject is fed a DOX-rich diet, neuronal activity will cause expression of the first but not the second transgene. Only when the subject is taken off DOX is the second transgene expressed, thus labeling the target cells. This method permits labeling to be restricted to a 24-h period during which learning occurs.³⁰

Non-electrical control of neural firing—It is now possible to activate neurons with light, a technique referred to as *optogenetics*.^{50,51} Neuronal firing is controlled by ion channels. These ion gates admit charged molecules (mostly sodium, potassium, and chloride) under the direction of certain amino acids and their derivatives (usually glutamate or GABA) acting on synaptic receptors. Other species, including algae, have ion channels that open and close based not on amino acids but on certain wavelengths of light. It is now quite common to express these light-controlled ion channels in neurons.^{52,53} Specific colors of light can then turn the neurons on or off as needed.^{54,55} Critically, a non-electrical strategy provides a way of targeting only certain populations. All cells will be affected by electrical stimulation in a distance-dependent manner. The restricted expression of light-sensitive molecules allows for optical stimulation of neurons selected by activity status, cell type, or timeframe.^P

These four strategies can be combined in the following way to reveal the neurons participating in a memory.⁵⁶ An IEG promoter is used to express a transactivator protein in

^PNote: It is also possible to remotely control neurons with chemicals and engineered receptors.¹⁸⁶

an activity-dependent manner. The transactivator causes expression of a light-sensitive ion channel (e.g., channelrhodopsin⁵³) tethered to a visual marker (e.g., green fluorescent protein (GFP)). The animal is fed a DOX-rich diet to keep the expression of the opsin turned off, even if the host neuron is active. For a brief period of time, the animal no longer receives the antibiotic. During this time window, the animal learns something (for example, the features of a novel context and/or that the context is pleasant or dangerous). The animal is then fed the antibiotic to stop the artificial expression of the transgenes. Within a few hours of training, the light-sensitive ion channel (tethered to the cell marker) will be expressed, but only in neurons that were active while the animal was learning.^{2,30,57} It is therefore possible to see which neurons participated in the learning.^{56,58–61} Importantly, light can be used to reversibly turn neural ensembles on^{56,58,59,62–65} (with the excitatory channelrhodopsin) or off^{60,61,66} (with the inhibitory halorhodopsin). In this way, it can be empirically determined whether those particular neural ensembles encoded the memory.

What has been learned from mapping memory in mice? Firstly, when a mouse learns a task, a subset of neurons is activated, not the whole brain, nor a whole brain area.^{30,58,59} If these neurons are directly reactivated (i.e., with light), the memory, or more specifically the behavioral readout of the target memory, can be elicited.^{56,59} If the cells are inhibited, the natural cue-induced recall of the target memory is impaired.^{60,61,66–68} Control experiments have shown that activation and inhibition of these cell ensembles affects the targeted memory specifically, not other similar but non-labelled memories.^{56,58–60} If the labelled neural ensembles are destroyed, so too is the memory.⁶⁹ However, if the cells are not destroyed, they can be manipulated to update an existing memory,^{58,59,62} including to switch the emotional valence of a negative experience to a positive one.⁶² These findings, arising independently from a number of research groups, have established that the hippocampal cells that are active during a defined training episode are both sufficient and necessary for the recall of a particular contextual memory, including social memory.⁷⁰ The labelled cellular ensembles represent a component of a bona fide, specific memory engram. It is now possible to search for the mechanism of memory storage.

The persistence of memory and plasticity of synapses

Engram-labeling studies have shown that certain populations of neurons encode specific memories in mice. How, exactly, do the cells encode the memory? Does each neuron contain a memory? Is the memory in the location of the neurons, the firing order, or the relative spike rates? The answer might be none of the above. Ramon y Cajal speculated that structural changes within neurons were the mechanism of learning: “The organ of thought is, within certain limits, malleable and capable of perfection... The cerebral cortex is similar to a garden filled with trees, the pyramidal cells, which, thanks to an intelligent culture, can multiply their branches, sending their roots deeper and producing more and more varied and exquisite flowers and fruits.”^{71,72}

Synapses, the remarkably dynamic connections between neurons, have always seemed particularly suspicious in the study of memory.^{73,74} Each neuron has thousands of connections, and they are not static. Almost 70 years ago, Donald Hebb theorized that synaptic connections between co-activated cells change in an activity-dependent manner,⁷⁵

leading to the postulate that neurons that “fire together, wire together”⁷⁶ into cellular assemblies. Early studies in sea slugs demonstrated that synapses could be directly conditioned,⁷⁷ bringing synaptic plasticity to the forefront as a plausible subcellular learning mechanism. Since the original Hebbian postulate, an enormous amount of evidence has been compiled to suggest that neurons do, in fact, alter synaptic connectivity in an activity-dependent manner. Neurons can reinforce synaptic connections following rules of spike timing-dependent plasticity (STDP).^{78–80} When an action potential is fired, synapses that were active just before the action potential (i.e., relevant to the generation of the action potential) are reinforced.⁸¹ Meanwhile, synapses that were active after the action potential (i.e., irrelevant to its generation) are weakened.⁸¹ Synapses can become transiently depressed or overly enthusiastic, through processes known as short-term plasticity.⁸² The effectiveness of synaptic transmission can also be enhanced or diminished in a semipermanent fashion through processes known as long-term potentiation (LTP) and long-term depression (LTD), respectively.⁸³ These persistent forms of synaptic strengthening and weakening, known as associative (or Hebbian) synaptic plasticity, establish patterns of connectivity that are generally thought to encode information.^{84–86}

However, decades later, the coding scheme remains elusive.⁸⁷ At the cellular level, neuronal codes have been observed: neuronal firing patterns can signal various components of space and time.⁹ At the subcellular level, the code has not yet been cracked. How exactly is acquired information (e.g., the location of a food source or the duration of a wait interval) mapped to a pattern of synaptic alterations? How is the encoded information entered into the neuronal operations that underlie goal-directed behavior?⁸⁷ Until recently, the problem has been intractable. There are thousands of synapses per neuron, and each can be changed through a wide variety of mechanisms: more neurotransmitter can be released,^{88,89} more receptors can be expressed,^{90–94} and the properties of the receptors^{95,96} or the release machinery^{97–99} can be altered. Additionally, new synaptic junctions can be formed,^{100,101} and structurally intact but functionally silent synapses can be revealed.^{97,102–104} It has been difficult to pinpoint which of these mechanisms, if any, results in which “memory.” Confounding the problem is the manner in which synaptic plasticity is often induced. Electrical stimulation is widely used to mimic the natural learning experience and induce synaptic change.^{105,106} Comparatively few studies have examined the consequences of ethologically relevant learning on synaptic function.^{107–110} Plasticity studies have focused on artificial stimulation protocols, which recapitulate some but not all aspects of learning-induced plasticity,⁸⁷ rather than actual learning-induced changes, because it was not previously possible to identify the cells allocated to a particular experience. With these new technologies, we now know where to look.

By comparing the physiological properties of engram and non-engram cells *ex vivo*, it was found that both synaptic strength and specificity of connectivity increased between certain neurons during learning, forming functional coalitions of cells across brain areas that

⁹Place cells,¹⁸⁷ grid cells,¹⁸⁸ head-direction cells,¹⁸⁹ boundary-vector cells,¹⁹⁰ and time cells¹⁹¹ have all been proposed to represent components of space and time through neuronal firing patterns. Neuronal firing patterns within the motor cortices also signal the temporal and spatial components of motor movement of a limb reliably enough that these signals can be decoded and used to guide prosthetic limb movement after injury.^{192–194}

manifest the memory.⁵⁹ These findings are compelling, but alone they are correlates of actual learning. Importantly, it is also now possible to test candidate plasticity mechanisms by interfering with plasticity and determining the consequences on both engram cells and memory function in a unitary experimental preparation. For example, it has been established for decades that long-term memory formation requires new gene expression^{111,112} (for review, see Refs. 113 and 114): when a protein synthesis inhibitor (e.g., puromycin or anisomycin) is delivered to mice immediately after a training experience, amnesia for the target memory results.^{59,111} Only recently has it become possible to test the mechanisms underlying amnesia, induced either by protein synthesis inhibitors or mouse models of early-stage Alzheimer's disease. Anisomycin treatment and early Alzheimer's produced amnesia that correlated with reduced synaptic strength and number.^{59,65} However, the specific connectivity pattern between engram cells were altered, and the resulting neural ensembles survived the amnesia.^{59,65} Importantly, when the amnesic engram cells were directly stimulated with light, memory retrieval occurred, demonstrating the persistence of memory in a latent but reactivatable state.^{59,65} Thus, a plasticity program including new gene expression is activated specifically in cells undergoing learning, and disruption of this process impairs both synaptic strengthening and the manifestation of the long-term memory as a behavior.⁵⁹ Alzheimer's pathology appears to attack these synaptic targets,⁶⁵ as has long been hypothesized.¹¹⁵ However, the memory remained in the amnesic brain, presumably in the altered pattern of neural connectivity, and could be reactivated by direct stimulation of the engram cells.^{59,65}

Using these new techniques, it is for the first time possible to dissociate the mechanisms underlying memory retrieval and those underlying memory storage. When enhanced synaptic strength between engram cells is abolished, the memory is not.^{59,65} Gross synaptic strengthening can be excluded as a candidate mechanism for memory storage, though it seems essential for natural memory retrieval.^{1,59} Indeed, a corroborating study showed that amnesia could be induced in rats by depressing amygdala synapses *in vivo*.¹¹⁶ Remarkably, the artificial re-potentialization of those synapses restored the behavioral response, though such stimulation did not create the conditioned behavioral response in naive animals because no relevant memory was present.¹¹⁶ Similar findings have been reported in sea slugs, where reminder training seems to reverse amnesia through the restoration of the learned synaptic structure.^{117,118}

These findings collectively implicate synaptic strength as a crucial determinant of memory accessibility or retrievability. When synapses are not operational, information—however stored—cannot be extracted from cells. Permanent or widespread disruption of synaptic structure and function would be devastating, producing cognitive deficits from autism¹¹⁹ to late-stage Alzheimer's^{120,121} to clinical depression.¹²² However, *subtle* disruption of synaptic transmission may be necessary for brain function: recent work has shown that excitatory neurotransmitter receptors are removed and modified during sleep,¹²³ while much more widespread dendritic changes have been found in hibernating animals.¹²⁴ These results suggest that the brain may adaptively modulate the accessibility of stored information by altering the strength of transmission at synapses. Indeed, several types of systematic synaptic alteration have been extensively studied and characterized, including associative plasticity (mechanisms that strengthen or weaken individual synapses during learning¹²⁵), homeostatic

plasticity (negative feedback mechanisms that act more globally across dendritic trees to ensure stability^{126,127}), and meta-plasticity (mechanisms that alter the threshold for subsequent plasticity¹²⁸). These processes of synaptic regulation, operational in excitatory and inhibitory^{129,130} networks, may be part of a sophisticated and dynamic control system by which information is extracted from or hidden within neurons according to the electrical, chemical, or metabolic state of the network. Determining how the various forms of synaptic control are deployed across synapses, dendrites, neurons, and brain regions may reveal how the brain rapidly and transiently accesses information.

However, the empirical findings clearly show that permanently enhanced synaptic transmission does not and cannot store the memory information itself.¹ How then is the memory stored?

“To see a World in a Grain of Sand:”^r is there a memory molecule?

The substrate of memory has been at least partially localized within the brain, to certain brain areas, and now to constellations of neurons. We can now address the long-neglected question: how do the residues of past experience encode that experience? Where within those constellations are the memories? Conventionally, the answer has been: at the synapse itself.¹³¹ The proteins forming the neurotransmitter receptors change with learning, thus encoding information in their enhanced density, function, and affiliation with synaptic scaffolds, all regulated by the receptor’s phosphorylation status.^{132–134} Synaptic weight changes can now be excluded as a means of information storage, though they appear to dictate engram retrieval.^{59,65} What alternate mechanisms could store the information itself?

The most intuitive answer would be long-lived molecules. Candidates for molecular substrates of information storage include the particularly long-lived proteins associated with DNA (i.e., nucleoporins and histones¹³⁵). These proteins could make permanent changes in how often or under what circumstances a protein is expressed. Alternately, some have suggested that DNA (or the epigenetic modifications on it^{136,137}) is the most suitable candidate for memory, being the cellular storage mechanism for other (lifelong) information.⁵ A more radical speculation is that information might be stored in genetic material itself,¹³⁸ though how that could happen at a sufficiently fast rate remains to be described.

The physical connectivity within neural ensembles is a plausible new candidate substrate for memory information storage, with many merits, including robustness to insult,^{59,65} bioenergetic efficiency, stability of information storage in a potentially binary format,^{139,140} and a high capacity for informational content (see Refs. 1 and 141 for review). If physical connectivity mediates memory storage, how is it maintained? The late Roger Tsien recently proposed the notion that perineuronal nets, the extracellular matrices around neurons and synapses, provide the architecture for information.¹⁴² Structural changes

^r“To see a World in a Grain of Sand/And Heaven in a Wild Flower/Hold Infinity in the palm of your hand/And Eternity in an hour.” – William Blake, *Auguries of Innocence* (1803).

⁵It should be noted that many of these theories work within the more conservative paradigm whereby genetic changes operate solely through the modulation of gene expression and its slow effect on late-phase synaptic plasticity.

in the extracellular matrix, mediated by matrix metalloproteases and other enzymes, could provide long-lasting information storage,¹⁴² delineating the neural constellations that are formed during learning and maintained as connectivity patterns even under amnesia.^{59,65} This hypothesis addresses a fundamental problem with any theory of channel- or receptor-based encoding: proteins are not long for this world.^{142,143} Most mammalian proteins live for only a few days^{144,145} while synaptic proteins can manage up to a week.¹⁴⁶ Individual synaptic signaling proteins simply cannot “remember” for very long, on either side of the synapse, making them a suitable substrate for short- but not long-term memory. If the engram requires a protein, it should arguably be a fairly long-lived one. The longest-lived proteins are generally structural,¹⁴³ especially those in the extracellular matrix.¹⁴² Despite these theoretical advantages, little is currently known about how neural ensembles are formed and maintained. Even less is known about the code that maps the elements of experience to the pattern of cellular and synaptic connectivity assumed to encode those facts.

An alternative to storage supported by very long-lasting molecules is engram maintenance in short-lived molecules, or molecular states, which exists as part of a long-lived arrangement. Antoine Danchin has famously used the metaphor of the Delphic boat to describe how life emerges from dynamic cellular components. The metaphor comes from a riddle posed by the oracle at Delphi: “If we consider a boat made of planks, what is it that makes a boat a boat?” The question seems simple enough: looks like a boat, acts like a boat. However, “as time passes, some of the planks begin to rot and have to be replaced. There comes a time when not one of the original planks is left. Is it still the same boat? The owner would certainly say yes, this is my boat. Yet none of the material it was originally built from is still there.”^t Structures composed of short-lived components could constitute a long-term memory if the configurations were preserved by normal homeostatic replenishment of steady-state components. For example, synapses are built from actin polymers, which restructure with learning.^{147,148} Embedded within the actin network are scaffolding proteins,¹⁴⁹ the sites into which neurotransmitter receptors are inserted. Synaptic proteins could hop in and out of the synaptic lattices just like electrons move within semiconductors. In this model, consistent with the engram connectivity model, the structure of the actin–scaffold lattice (semiconductor) would dictate the behavior of the synapses,^{84,150–152} not the receptors (electrons) that occupy them with some probability. Other theories involve information storage or processing in microtubules,¹⁵³ the long polarized helices of tubulin subunits that compose the cellular skeleton.^{154,155} New microtubule tracks are laid directly toward the synapse during activity,^{156–158} possibly encoding a change in status. The tubulin subunits or helical structure could be modified directly,^{159,160} or the pairing between microtubules and actin structures could be altered by cytoskeletal binding proteins,^{161,162} changing the likelihood of synaptic proteins being recruited to the synapse.¹⁶³ Posttranslational modification of particular synaptic proteins has also been suggested to mediate memory storage by modifying the trafficking and behavior of the molecules.¹⁶⁴ The short-term modification could be maintained in the long term by active positive feedback that preserves the altered state. While such a mechanism would certainly be rapid and plastic, it also carries the weakness of vulnerability to disruption and the thermodynamic cost of any necessarily

^tAntoine Danchin, *The Delphic Boat* (2003).

active mechanism of maintenance. Substantial changes in synaptic behavior could also be achieved by altering the biophysical properties of membranes (e.g., the relative compositions of fatty acids and cholesterol would alter the behavior of membrane-embedded receptors) or the dimensions of the synapse itself. Importantly, many synaptic processes are controlled by glia,¹⁶⁵ making non-neuronal brain cells potential mediators of memory encoding, maintenance, or extraction.

None of the above potential mechanisms need to function exclusively in isolation, and indeed some of them may represent different manifestations of the same substrate. Early studies of memory engram cells has shown that much work remains to be done in order to discriminate between the enabling mechanism of memory retrieval and the essential mechanisms of information storage. Recent technological advances, including multiphoton brain imaging,^{166,167} may permit us to look inside cells *during* learning to see the cellular events as they occur. Indeed, a tantalizing new model of cellular learning has recently been provided. Behavioral results long ago established that animals learn to tell time, specifically the durations of intervals.^{168,169} A simple instance of interval-duration learning occurs in Pavlovian conditioning paradigms, where a neutral conditioned stimulus (CS) predicts an aversive unconditioned stimulus (US), which occurs at some delay. Regardless of species, subjects do not learn to simply respond to the CS alone, they learn to respond at the right latency. In eye-blink conditioning, the encoding of the CS–US interval has been tracked to Purkinje cells in the cerebellum. When developing an appropriately timed response, the cerebellar neurons establish new sequences of neural firing^{170,171} by learning to pause for particular time intervals,^{172–174} just as we learn to play the piano by perfecting the timing between individual finger strikes. Recently, these cells have been artificially conditioned by direct stimulation of their immediate inputs to learn the CS–US interval, so that the entirety of the paradigm (encoding of CS, association to US, timed response, etc.) has been reduced to a manipulatable experiential fact—the CS–US interval. Using this experimental paradigm, it was revealed that the learned firing pause is initiated by metabotropic signaling cascades that terminate by acting on inhibitory ion channels.¹⁷⁵ The results provide a means of dissecting the components of the cascade between the metabotropic receptor and the inhibitory ion channels to determine if and how the previously experienced CS–US interval is encoded and decoded.^{174,175}

Intracellular neurochemical processes are attractive as bioenergetically efficient, local, and flexible tools for neural computation (eloquently discussed in Ref. 176). Cheap, dynamic, and local chemical computation could provide a perfect complement to a one-time expensive investment in infrastructural change (for example, in reorganization of extracellular or cytoskeletal components) across brain areas. By examining the intra- and extracellular processes that create and maintain neural ensembles, it may be possible to unite the molecular, neuronal, and circuit mechanisms of neural learning. However, at the behavioral level, learning differs significantly across brain regions,¹⁶ so it will be important to determine whether conditioned or procedural learning within the cerebellum and striatum, for example, proceed via distinct cellular or molecular mechanisms from learning within the cortex and hippocampus. Indeed, plasticity processes that seem to be crucial for information storage have been characterized in mammalian visual cortex cells and in perceptual regions as early as olfactory sensory neurons.^{177,178} Moreover, synaptic dysfunction in peripheral

nerves has been shown to produce learning deficits.¹⁷⁹ Tracing the engram ensembles from their peripheral origins may reveal new mechanisms of information storage distributed throughout the nervous system.

These are speculations, but they are suddenly testable speculations. After centuries of suspecting, doubting, hoping, and wishing for a trace of past thoughts, modern neuroscientists have been able to map the constellations of neurons forming a memory. It is now possible to look inside and between those cells for residues of past experience.

“There is no easy way from the earth to the stars:” the future of memory research

In the practice of science, we demand empirical evidence to distinguish facts from speculations and to draw meaningful conclusions. We can now see more clearly into the brains of mice and, accordingly, we can now see where their memories are housed. If memories are encoded in cellular-resolution patterns of connectivity, we may soon be able to decode them. If they are not, we will have to look more closely, inventing new ways of noninvasively seeing dynamic synapses, organelles, or subcellular structures at finer resolutions or inventing new computational approaches to handle the behavior of these networks. Many proposed substrates of memory will remain in the realm of speculation until technologies capable of testing their suitability emerge. Hippocrates once speculated on the future of epilepsy, the “sacred disease:” “It appears to me to be nowise [sic] more divine nor more sacred than other [things], but has a natural cause from which it originates. Men regard its nature and cause as divine from ignorance and wonder... And this notion of its divinity is kept up by their inability to comprehend it.”^u Over the last several millennia, the symptoms of epilepsy have remained roughly constant. What has changed is the conviction that causes can be determined, mechanisms mapped, and solutions engineered.

Because the mind is part of the natural world, there is no reason to believe that it will be any less tangible and ultimately comprehensible than other components. However, there is ample reason to suspect that the answers (or the interpretations) lie outside the current radius of neuroscience. There are three major sources of progress available. The first is the expansion of knowledge through basic research. Perhaps the critical discoveries have simply not been made yet. For example, the formulation of gravitation and later electromagnetism as fundamental interactions crystallized centuries of observations that had previously lacked a certain coherence. The second is the infusion of existing knowledge from other fields. For example, the discovery of rhodopsin is not recent,^v and the rhodopsins are quite commonplace, found in algae, bacteria, and viruses, as well as the retina of mammals, including humans. However, when channelrhodopsin was strategically imported from single-celled algae into mammalian neurons,^{50,52,53} neuroscience was endowed with a new power to manipulate individual neurons at will. The third is the reorientation of existing knowledge. Certain answers may be sitting right in front of us, in the territory of

^uHippocrates, *On the Sacred Disease* (400 BC).

^vBeginning in the 1850s, early investigators of the retina, including Heinrich Müller, Franz Boll, and Willy Kühne, pieced together an understanding of the light-sensitive molecules of the visual system, identifying “visual purple,” which later became rhodopsin.¹⁹⁵

conventional biochemistry and physiology. However, a paradigm shift may be required to look at the existing data from a more productive angle.

Neuroscience is currently dominated by the metaphor of the mind as a computer. Under this regime, we implicitly look for “bits” that “encode” “information,” terms and concepts that were invented or formalized within the last century.^w It may be that an organ that arose through 200 million years of cephalization, composed of specialized cells with synaptic connections that originated at least a billion years ago,¹⁸⁰ behaves in a manner similar to our human-made machines. However, the brain is not obliged to compute in a digital fashion. The representations that mediate basic perception and motor action may be stored and transmitted in analog format,¹⁸¹ or the “computations” may be non-algorithmic.¹⁸² The brain may be a nonlinear system that bears no resemblance to a computer at all. The mind has previously been conceptualized as other fashionable technologies—it has been propelled by hydraulics, driven by mechanical gears, and powered by combustion; it has been imagined as a system of telegraph wires and a GPS system. It will be reimagined as other popular tools and toys that capture our imaginations. Our minds will presumably continue to function regardless of which technological transformation we demand next.

According to Thomas Kuhn, “To be accepted as a paradigm, a theory must seem better than its competitors, but it need not, and in fact never does, explain all the facts with which it can be confronted.”¹⁸³ By adhering too strictly to any dominant model, we severely limit the questions that can be asked and the acceptable answers that can be produced. Any anomalies that arise—observations that do not fit the theory—will be ignored or suppressed, subconsciously as well as systematically. This is because “assimilating a new sort of fact demands a more than additive adjustment of theory, and until that adjustment is completed—until the scientist has learned to see nature in a different way—the new fact is not quite a scientific fact at all.”¹⁸³

If past progress is any indication, future developments will happen not by subscribing to a theory and pursuing data to support it but by pursuing the central question and respecting anomalies as they arise. The invention of novel experimental techniques and methodologies is allowing researchers to pursue anomalies in the neuroscience of a memory in a way not previously possible, dissecting mechanisms of memory retrieval from those of memory encoding. These investigations will generate useful predictions on the nature of memory storage and novel therapeutic tactics. Sustained progress may soon result in a new theory of memory with enough explanatory value to temporarily satisfy the original question: How are experiences stored in the brain?

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^w“Information” has only recently become a precise term rather than a hazy concept. In 1928, Hartley attempted a “quantitative measure of ‘information’...which is based on physical as contrasted with psychological considerations.”¹⁹⁶ Two decades later, Claude Shannon produced “A Mathematical Theory of Communication,”¹⁹⁷ which coined the term “bit” and formalized information theory as a field of study, rather than an offshoot of electrical engineering. See James Gleick’s *The Information* for a full history.¹⁹⁸

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