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The substrate for long-lasting memory: if not protein synthesis, then what?

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Abstract

The prevailing textbook view that *de novo* protein synthesis is required for memory (e.g., Bear, 2006) is seriously flawed and the alternative hypothesis has been proposed in which post-translational modification (PTM) of proteins already synthesized and already present within the synapse is ‘the’ substrate for long-lasting memory (Routtenberg and Rekart, 2005). Protein synthesis serves a replenishment role. The first part of this review discusses how long-lasting memory can be achieved with ‘only’ PTM of existing synaptic proteins. The second part critically reviews a recent report published in *Neuron* 2007 that exemplifies the current view of protein synthesis and memory while also illustrating how these results can be understood within this new PTM framework.

A necessary yet unexpected conclusion to emerge from consideration of the consequences of a PTM mechanism as the necessary, sufficient and exclusive substrate for long-lasting memory (Routtenberg and Rekart, 2005), is that the central Hebbian dogma that cells that ‘fire together, wire together’ is an unlikely mechanism for long-lasting memory. Thus, a unique feature of the PTM model is that longevity of information storage is achieved not by stability of the synaptic mechanism, but by impermanent pseudoredundant circuits. This is so because PTM is a reversible process and thus any permanent connection, any ‘lasting effect’ cannot be in the form of stable synapse formation. We have therefore proposed a solution in which network level processes regulate cellular mechanisms, even as such mechanisms regulate the network. Thus, synapses are ‘meta-stabilized’ by regulated feedback mediated by the circuit in which the synapse is embedded. For example, spontaneous activity is proposed to be a substrate feedback mechanism we term ‘cryptic rehearsal’ to sustain for some period of time after learning an approximation to the state initially created by input. Additionally, because the duplication of these traces is ongoing, this provides a degenerate code (Routtenberg and Rekart, 2005) for the engram. Stability is thus achieved, not by stabilizing the synapse, but by implementing a pseudo-redundant yet malleable circuitry so that memory can be protected in the face of small catastrophes in network representation.

1. Introduction

Compromises are struck at every turn. Genetic determinants of synaptic specificity during development are counter-acted by ‘sensitive periods’ that allow postnatal input-dependent processes to modify these genetic programs. The overproduction of neurons early in development is balanced by programmed cell death, a Darwinian drama in the developing

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brain leading to survival of the fittest neurons. The synthesis of protein in the neuron must be ongoing and insensitive to neuronal activity yet it must also be responsive to their utilization, which is jointly determined by neuronal activity and the varying half-life of individual proteins. To fine-tune the level of protein precisely, a regulatory mechanism is required to achieve this balance, as too little or too much protein can, for different reasons interfere with function (Holahan et al., 2007).

In memory formation and its long-term storage a compromise is struck between the competing needs of flexibility required to encode and modify existing brain structure and the requirement to convert new memories to permanent, old ones (Fusi et al., 2005; Abraham and Robins, 2005). Another compromise that requires implementation is the need to persist memories for the long-term, yet not have the synapses so hardened that they cannot achieve 'updating' or incorporation of new learning within the framework of old learning. This issue, so critical to Hebb (1949) in developing cell assembly theory, has taken on an 'old-wine, new bottle' configuration in the study of memory schemata (Tse et al., 2007).

Current models of memory that rely on stabilization of synapses, consolidation of memories, activation of specific pathways as substrate do not achieve the balance that is required by the compromises just discussed. In its place the PTM hypothesis proposes the existence of a dynamic modification of already synthesized proteins and that this inherently reversible state is *the* molecular substrate for long-lasting memory. Because such modifications are labile they provide the necessary flexibility required of any theory sensitive to the compromises required of the memory system.

2. Long-lasting memories from metastable networks

The first attempt to address the flexibility – stability issue can be found in the proposal of Muller and Pilzecker (reviewed in McGaugh, 2000) who suggested a two-stage process: first, a perseverative neural one in which activity outlasted the presented stimulus. Hebb re-articulated this view as a 'dual trace' hypothesis suggesting that perseveration could be viewed as 'repeated and persistent activity' in co-active presynaptic and postsynaptic elements. Second, in the stabilizing, storage phase which Muller and Pilzecker termed consolidation, and Hebb saw as a 'growth process or metabolic change', perseveration was required to precede consolidation in order to achieve long term storage. The current view, essentially unchanged from these early articulations (but see Izquierdo, et al., 1998), is that memory is represented in the brain by a network of neurons composed of initially modifiable nodes called synapses ('articulation' in the French version of Cajal's treatise). These nodes are then rendered stable, leading to persistence of the memory trace. Substitute protein synthesis independent and protein synthesis dependent stages (e.g., Kandel, 2001) and one has a modern form of the dual trace proposal. In sum, Hebb's Neurophysiological Postulate stated that the memory trace is derived from two processes, first repeated and persistent activity and then growth or metabolic change: the initial protein synthesis *independent* stage is thought to last for hours and the *dependent* stage is thought to last for days, months, years, a lifetime. This long lasting memory is assumed to have a physical-structural change that is present within our brain tissue. This change, or plasticity, is represented by a morphological modification at synaptic nodes. Permanently stored information is thus consolidated in the stabilized synapse and the network so formed is referred to as an engram or 'memory trace.' The change is solidified by de novo synthesized proteins that are then targeted (tagged or marked) for the appropriate synaptic nodes to render the plastic change specific and permanent. This view of memory mechanism as a permanent structural entity represents the current neurobiological view of information storage (e.g., Kandel, 2001; Govindarajan et al. 2006).

Concerns have been raised with this prevailing theory and an alternative view, the PTM hypothesis has been proposed (Routtenberg and Rekart, 2005). Regardless of the correctness of the PTM hypothesis, the need for alternative models is clear, given their paucity. That this healthier scientific direction is in the process of developing can be appreciated by recent work from Paul Gold who has provided important new insights and empirically critical observations bearing on these issues (Gold, 2006; Canal et al., 2007).

3. The Alternative View: Long-lasting memory from frangible networks

The authenticity of information storage is so strong in our own minds that a different perspective, beginning with the view that memory is in fact, a metaphor, may be hard to imagine. Two major departures from current dogma are proposed. First, memory storage, as currently conceived, does not occur. Rather it is here viewed as a reification of a metaphor, a powerful myth perpetuated by our own experience of information permanently stored in our brains. Our own name is a good example. While our *subjective experience* tells us that memory is long-lasting, the biological facts to be reviewed here may be more consistent with a different metaphor: a dynamic network, evanescent, impermanent, self-organizing 'blooming, buzzing, confusion' in Sherrington's felicitous phrase, that is capable of serving two different masters with opposing goals: to always keep matters in a state of instability, poised for modifiability; the other, to maintain quasi-stability in the dynamic network representing the information. The requirement for impermanence of synaptic change advocated here disagrees with Hebb's original formulation that sought a more or less permanent change, a 'lasting effect.'

The second major departure from current thought is that protein synthesis is viewed here as a permissive replenishment process, and thus is not the instructive mechanism for memory formation that is currently thought. This issue will be discussed in the latter part of this paper.

4. The Structure of Long-lasting Memories: Dynamic Selection from Pseudo-redundant Traces Maintained by Regulated Positive Feedback

We propose a view of memory that is counter-intuitive: that our subjective sense of having stored memories in our brain does not reflect the cellular mechanism by which this occurs: stable memories do not require stable synapses. In brief, there is no long-term storage of memory, no dual traces, but rather the multiple representation by dynamic, self-reinforcing but impermanent networks of prior events.

To preserve the idea that long-term memories exist without instruction from new protein synthesis we proposed that the core mediator of memory storage is the post-translational modification of proteins already present at the synapse. There is little doubt given the abundant evidence that this mechanism is recruited to modify synapses in the initial stages of information storage. What is new here is that in addition to being necessary to promote early stages of memory it is sufficient to maintain long-lasting memories.

In order for this mechanism to implement, positive feedback mechanisms are in place to maintain the memory. *At the molecular level* it is well known that kinases implicated in memory formation have autophosphorylation mechanisms that would cause the kinase to be active well beyond the event itself. Indeed, this may last for several hours in the case of the role of protein kinase C and learning. Negative controls such as increased proteolytic susceptibility of activated kinases are in place to negatively regulate this positive feedback.

At the cellular level there is abundant indirect evidence that after transmitter is released so-called retrograde factors can enhance or dampen presynaptic transmitter release mechanisms. Thus, in one scenario, summarized in the dialogue model of synaptic change (e.g., Colley and Routtenberg, 1993), retrograde factors may be cis fatty acids that would directly stimulate presynaptic kinases leading to enhancement of transmitter release which in turn would enhance retrograde signaling, leading to ongoing dialogue, and so on. Again, negative control mechanisms, e.g., phosphoprotein phosphatases, would be in place to fine tune this enhancement and prevent catastrophic activation leading to epilepsy.

At the network level, the so-called ‘spontaneous’ activity of the brain has been proposed to act as an implicit physiological rehearsal mechanism preferentially activating those networks that had been previously been engaged by external input-dependent processes (Routtenberg and Rekart, 2005). To prevent either the formation of a thernuclear engram, or persistent recurrence of the same memory, dampening mechanisms beginning with inhibitory interneurons, would serve to allow for switching among different networks. My ability to remember the singer of Volare, a popular song of the 50s, without speaking his name (Domenico Meduno) for 20 years would benefit by the repeated trips to Dominicks for groceries, smoking a Macanudo and talking to my colleague Doug Medin. What is being said, then, is that no long-term storage bin exists for Mr Meduno, but rather, current events continually reactivate circuits close enough to the original to keep the networks initially recruited alive and well.

This illustration will require a more formal statement of a key proposal of the present model: that the longevity of a memory and its resistance to disruption is proportional to the extent of its duplication by traces that approximate the memory. Thus, the greater the number of different network representations involved in the process the less likely will it be forgotten; the fewer the number the more likely disrupted. Consolidation theories that argue for the two-process form of memory base their view on the time-dependency of disruptive manipulations (e.g., McGaugh, 2000). But the disruption is of memories recently formed, hence not well duplicated. The reason that disruption does not work hours after learning, according to the PTM hypothesis is that at a later time point trace duplication has proceeded so that it is represented in brain locations where the disruption is ineffective. [One wishes to know the rate and distribution of these traces as they multiply; it is well known that an intense learning experience, i.e., one which would rapidly reduplicate itself, can overcome the effect of an agent which is disruptive under normal learning circumstances.]

Here it is proposed that the only type of trace that is ever formed is a metastable one and as such is impermanent. Indeed, in order for the networks representing a memory to incorporate new information it is obligatory that the original network be poised for demolition or dispersal. Because of pseudo-redundancy memory is not lost when a subset of metastable traces are disassembled. Phenomena such as PTSD may be understood as the rapid recruitment of trace duplicating mechanisms probably driven by extremes in hormonal levels that are known to regulate memory. The resistance to its forgetting is well known; the prediction is that it may only be attenuated if the lesions or disruption occurs simultaneously in a widely distributed group of brain locations.

To summarize to this point: Information storage is a delicate balancing act. The nervous system is required to be flexible to encode new information but it must also be stable to store that memory. And at the same time the storage system must allow concurrent retrieval processes to co-exist with both encoding and storage (Routtenberg, 1972). As proposed over 36 years ago, information storage and retrieval emerges from a reciprocal, rapidly oscillating, interaction among competing processes to permit the semblance of co-occurrence.

A. Some glimpses of how the network might be organized: The r(eye)t stuff

The sound RIT, with the I pronounced 'eye' is used as a word with many different meanings in the English language. A short preliminary list of phrases are: No, not a left, a right turn; Write a book; right the canoe; right of Attila the Hun; Isle of Wright; right on, brother; you are more right than wrong; take a right turn [did you, dear reader, immediately recall that this was already used ...how did you do that?] rite of passage or last rites; advertising (rite guard; dry rite).

This is a preliminary list of the ways RIT is used, and even when the meaning is similar its use is different, as for example in conversation, as a substitute for nodding the head to show agreement, but possibly only to indicate that one is listening.

The problem is that in the auditory system that processes and decodes the sound there is no difference in the sound itself, though the meaning is vastly different. The issue here then is how to conceptualize the discrimination that we make when we hear the word. Those who have read Lashley will know (no) this reprises his stimulus equivalence discussion 'In search of the engram.' But it may be worthwhile to think briefly about how this problem for the nervous system is solved within the context of the recently proposed PTM theory. Essentially one (won) thinks of the formation of separate networks representing the major uses of the sound, with in all (awl) cases, the presence of overlap in these networks given the sound encoding requirement. In some sense (cents) it is remarkable how little interference or misunderstanding arises. This must be seen (scene) in the context of the 'set' of the recipient, defined as the integrated operation of the network at that instant in (inn) time (thyme). Indeed, one might conclude that the 'set' is so (sew) powerfully represented that the network is adjusting to the nuances of the monologue on a moment – to – moment basis. Sherrington's 'enchanted loom' is an integrated whole, that crests and falls, ebbs and flows, twists and turns, but at all times does so as a unity. The speaker about to discuss a summer canoe trip provides a 'setting' for the listener such that when he discusses his dunking when the canoe tips (don't think waiter) over, the listener knows exactly what is involved when he rights (don't think pen) the vessel (don't think blood)...but do think Lashley (1950).

So the precise meaning is not found in the representation of the sound in auditory cortex, for example, but rather in the network extending beyond the boundaries of this cortex. It is this flexible, open-ended architecture that provides for the subtle shifting, sorting and essentially infinite combinations among networks so that the same sound can represent quite different meanings and differentiated in the millisecond time scale.

This same principle of pseudo – redundant networks representing the same sound but different meanings, can be implemented so as to include brain locales where the meanings of these words is appreciated and thus differentiated. For example, the representation of the sound of RIT can mean different things to the dorsolateral frontal or superior temporal gyrus in the temporal cortex. The same sound, a nuanced variant of the original meaning, but represented by different networks.

Even one's own name is represented by a metastable, degenerate network. Because one's name is used in many different contexts, it is represented by different neural networks with a considerable amount of overlap, with a multiply represented network in the auditory cortex. Why multiply represented? So that this core auditory representation can reach out and touch, yes interface, with those other networks that represent the shades of meaning of the name. This last example may illustrate why in AD patient the loss of one's own name is last to go; there are so many representations of one's name, that as some of these networks fail, there still remain a significant number of others.

These considerations suggest what may seem an initially surprising perhaps novel concept: when someone asks what your name is and you answer correctly, and then 5 sec later the same request is made and the same answer given, it is asserted here that though the output is the same, the same neural network that retrieved that information was not. The percent overlap of the two networks, in this example, will range from a value greater than zero to a value less than 100.

B. The fragmentary nature of memory

A mnemonic fable: I am in the Bayerele Museum in Basel and see down the hall a sculpture that is once disproportionately tall and thin and yet curiously affecting. The sculptor's name is? It's on the tip of my tongue. How does one know one knows yet one clearly cannot retrieve the information. It begins with a 'G.' It has an Italian-like sound. Fragments are correct, yet memory is not there. I saw a similar sculpture three years ago in another place, the Maeght Foundation. I even can time-stamp that memory, but again the sculptor's name is not articulated. Five minutes later, while sipping a Pernod, it comes to me: Giacometti. How do I know that this is correct? What changed from 2 seconds ago, when I did not know the answer? These facts of memory indicate that *the memory* is composed of fragments, or mnemonic atoms, that when put together gives us the chemical compound we refer to as the memory. Clearly, the construction of a network is required; one that represents different 'facets of memory' in different modules. When the coordinated and synchronized theta and gamma activity of the network (e.g., Fries et al., 2007) does not function then fragments appear. The partial recovery is sufficient to allow fragment retrieval.

So how explain consolidation of memories? Resistance to manipulation when established, easily disrupted when not. The sculptor's name appears to be a permanent memory, which with disuse becomes 'fragmentable' and more difficult to retrieve. Yet it has the appearance of permanence. This introspection it is argued here is simply misleading. What may underlie the *appearance* of permanence is the extensive distribution of representations in multiple locations, the networks maintained by endogenous brain activity and a former commissioner of Major League Baseball. This redundancy protects against memory loss as only a proportion of the networks fade away, the others are protected by the brain's rehearsal mechanism, endogenous activity. As the number of traces increase the ease of retrieval, viewed as selection from the total network, increases in difficulty. A useful metaphor here may be defragging the hard drive. Defragmentation then is viewed as the substrate for retrieval, if one views this process as putting closer together formerly disparate items (see earlier how Dominicks, Macanudo, Medin help retrieve Domenico Meduno).

Duplication along with cryptic rehearsal (Routtenberg and Rekart, 2005) can well explain a) the propensity for old memories to resist the ravages of time, b) the newer memories to be more readily lost, especially in the aged. 'Reminiscence' is a desperate attempt to stave off the stampede of trace diminution by internal rehearsal mechanisms triggered by endogenous activity. Because older memories are represented more extensively, the probability is that it is those that will be recovered. 'Shrinkage of amnesia' occurs after a traumatic head injury and events prior to trauma begin to recover, beginning from the most distant, then up to a point close to the event itself. Presumably the most distant memories also have more duplicated connections to more elaborate networks hence as the non-specific trauma ('diaschisis') subsides, the most mature networks, revived by endogenous activity, begin to function once again.

5. Origins of the PTM hypothesis

One proximal cause for this special issue may have been stimulated by our proposal that *de novo* protein synthesis as the key process essential for long-lasting memory was seriously

flawed *and* that post-translational modification (PTM) of proteins already synthesized and already present at the synapse is sufficient as the substrate mechanism (Routtenberg and Rekart, 2005). But both the critical view of protein synthesis and the role of PTM were, in fact, articulated decades ago. As discussed in our 2005 review concerns about protein synthesis were articulated by one of its discoverers, Louis Flexner, in the early 1970s. And concerning PTM, I had reviewed early evidence to say that PTM could be the substrate for long-lasting memory (Routtenberg, 1982). This paper comes from a symposium organized by Hans-Jurgen Matthies in East Germany 8 years before the Berlin wall fell (no parallels intended). It was a more modest proposal then, and contrasts with our 2005 review which now claims sufficiency of the PTM mechanism for long-lasting memory.

At a Gordon Conference on Neural Plasticity which took place circa 1980 by the tranquil blue-green waters of Lake Winnepesaukee, I vividly recall a particular session which discussed how amphetamine rescued the memory loss caused by protein synthesis inhibitors (PSIs) in the presence of greater than 90% synthesis inhibition. This was a replication of the Flexner finding. I turned to Harold Gainer and said, "There goes the mechanism for long-lasting memory." He and I thought that this mechanism was dead in the water, but we were wrong. At a recent Irvine Memory Conference in 2005 I asked Larry Squire for a copy of his venerable, and hence pre-Web, review of protein synthesis inhibitors and memory (Davis and Squire, 1984) to provide the backdrop for a discussion of alternative mechanisms. To my surprise, I found a brief discussion of the precursor PTM hypothesis discussed in the Matthies review. I had then suggested that the synthesis inhibitors used would likely affect post-translational modification of proteins, and reflecting back on it, the idea that PTM could play a sufficient role was born. Davis and Squire were skeptical of this speculation in 1984, noting that there was little evidence for such a claim. But it is now clear in 2007 that PSIs do in fact alter PTM of a variety of different systems. A particular favorite of mine, given its pre-eminent position as a key step in the cascade of events associated with the instructive *de novo* protein synthesis model (Kandel, 2001), is the demonstration that anisomycin increases the phosphorylation of CREB (Shafer and Slice (sic), 2005). Despite the fact that the agent of change distorts, as a side effect, the very signaling sequence proposed as part of the formation of long-lasting memory, the view persists that protein synthesis is critical for long-lasting memory. To illustrate the persistence of this view I have chosen a paper from the work of Ivan Izquierdo and Jorge Medina, scientists for whom I have the utmost respect, but with respect, disagree completely with their point of view, despite its current popularity. Perhaps in my idealism I believe that truth and democracy are orthogonal.

To summarize this section: our hypothesis rejects the current widely held view that memory has two phases. The current dogma is that the first dynamic perseverative phase is disruptable by a variety of treatments but not protein synthesis inhibitors, hence protein synthesis independent, the second consolidation phase is only disrupted by such inhibitors, hence protein synthesis dependent. But, as reviewed by Routtenberg and Rekart (2005) and by Gold (2006), shortly after protein synthesis inhibitors were shown to block memory, several reports in the 1970s and 80s demonstrated that amnesic effects could be reversed by amphetamine and noradrenergic agonists, nicotine, caffeine, although protein synthesis inhibition was greater than 90%. Although it was concluded that protein synthesis could not be instructive in the process of consolidating short-term memories, it is my historical thought that these studies were powerless to overcome the 'molecular imperative' that was beginning to assert itself, and thus the role of the gene in brain function, a new and important direction in neurobiology, resurrected the *de novo* synthesis model.

6. Review of Beckenschtein et al. (Neuron, 2007)

To illustrate both this author's critical view of the role of protein synthesis inhibitors and the re-interpretation by the PTM hypothesis, I have chosen to review a recent article, which rates high marks for clarity, logical sequence of an extensive series experiments, as well as accurately reflecting current views. Thus, this is a 'strong paper' on the basis of the experiments carried out. Nonetheless, I find that I disagree with its most fundamental assumptions. What follows, then, is a detailed analysis of certain findings and discussion of alternative ways to look at their data.

The authors begin by introducing on p. 261 the mantra of the protein synthesis credo: "Memory can be divided into at least two phases: a protein and RNA synthesis-independent phase that lasts minutes to 1–3 hr (short-term memory) and a protein and mRNA synthesis-dependent phase that lasts several hours to days, weeks, or even longer (long-term memory, or LTM)." And then without even a perfunctory bow to conflicting data over the past 40 years, and without any editorial oversight or apparent reviewer critique they assert that "protein synthesis inhibitors are still the best tools to examine the "when" and the "where" of protein synthesis requirements for learning and memory (p. 262)." If this is true, then as pointed out recently by several authors, this is a very shaky foundation upon which to rest their prevailing theory of memory (Routtenberg and Rekart, 2005; Rudy, et al., 2006; Gold, 2006; Canal et al., 2007).

It has long been known that PSIs inhibit or alter adrenergic function. Indeed, as reviewed in the Rekart paper, and affirmed at the Gordon conference 30 years ago, amphetamine can reverse the effects of PSIs. The reader is referred to Gold (2006) who has neatly dealt with the objections raised by Davis and Squire (1984) of these rescue results. Thus, it is of special interest that Canal et al. (2007) showed massive release of adrenergic transmitter following intracranial application of anisomycin, precisely the method used by Bekinschtein et al. (2007). Although the latter authors may not have been aware of this paper, there were enough concerns raised prior to this to acknowledge alternative interpretations. From the outset, therefore, questions can be raised concerning the mechanism of action of the applied PSI. This is unlikely to be a nonfunctional side effect, as adrenergic blockers rescue the memory impairing effect of the anisomycin, again without altering level of protein synthesis inhibition. Clearly too much or too little transmitter will have an impact on learning, following the Yerkes-Dodson law. This inverted-U function in this context has been recently reviewed by Gold (2006).

So how explain the results of the myriad studies showing impairment with protein synthesis inhibitors? We did not exhaust the possibilities, only ourselves, when we got to 8 alternative mechanisms. Rudy et al. (2006) reminded us of the well-known anisomycin-induced apoptosis and Canal et al. (2007) provided the transmitter release outcome in support of our view of protein synthesis inhibitors as a dirty drug. Moreover, as we pointed out in our 2005 review and re-affirmed in Canal et al. (2007) testing the protein synthesis hypothesis using inhibitors is the least satisfactory approach, contrary to that articulated by Bekinschtein et al. Why so unsatisfactory? We have already mentioned the myriad consequences of the inhibitor, which can only lead one to hang the moniker of dirty drug on this agent. Increased CREB phosphorylation and enhanced transmitter release are just the tip of the iceberg.

7. Concatenating Temporal Cascades of Molecular Events

An interesting result reported by Bekinschtein et al is that "a delayed wave of protein synthesis in the hippocampus is required for persistence, but not formation, of LTM." This idea of waves of protein synthesis was probably first suggested in a report, later withdrawn, by Nottebohm and co-workers (Chew et al., 1996; Vicario and Nottebohm, 1998). These

data and our evidence for such waves of synthesis were reviewed in a chapter entitled 'It's about time' as part of a McGaugh Festschrift (Routtenberg, 2003). In that review discussion of alterations in genome – associated events over time after learning were organized over the first 72 hr after a learning experience. In addition it was proposed that these set of signaling systems are concatenated over time into a continuous cascade and integrated smoothly by the cell so that at a subjective level there is no 'hitch' in our memory system. Indeed, as reviewed in the 2003 paper, we had reported on alterations in E-box transcription factor binding only 6 hr after treatment (Kinney et al., 1996), and increased GAP-43 synthesis only after 24 hr after kainate – induced seizures (McNamara and Routtenberg, 1995) and transcriptional activity only 48 hr after long-term potentiation (LTP) induction (Namgung et al., 1997). Note that none of these alterations were observed before that time point indicated. Our own studies, therefore, and at least part of those from Nottebohm are perfectly consistent with the idea of Bekinschtein et al. that such synthesis does occur in waves. The difference is that I do not think these synthetic/genomic mechanisms are instructive, but rather permissive cell biological activities dedicated to the nourishment of synapses and recruited to regulate the levels of protein as they are utilized by PTM mechanisms.

It is attractive to think, therefore, that such waves are the consequence of replenishment mechanisms that are recruited following a learning experience. The 12 hr time point that appears to Bekinschtein et al. as necessary for long-lasting memory, may reflect a blockade of synthesis of replenishing proteins that has no direct linkage to the storage of information, the alteration of the synapse. Rather *the inhibitor could be viewed as depriving the synapse of the 'food' it needs to maintain the PTM of that synapse*; the PTM represents the synaptic modification that is part of the network that represents the memory. There is also nothing magical about the 12 hr time point as Chew et al. (1996) suggested that the 89 hr was a key time point for protein synthesis and maintaining persistent memories. This discussion is reminiscent of the views discussed in Gold (2006) of the lack of precise timing of consolidation processes.

My discussion above is flawed because it completely ignores the fact that anisomycin does much more than inhibit protein synthesis and so one would be on thin ice concluding that the effect is related to synthesis inhibition when there are multiple other consequences that in some combination play the determining role in altering retention. The application of a protein synthesis inhibitor 12 hr after learning that causes an impairment 7 days, but not 2 days, later, is of interest. But the inhibitor sets into motion the alteration of so many different processes it is difficult to know what combination of effects produces the observed result.

It is well to remember that high levels of release of biogenic amine transmitters (norepinephrine, dopamine, serotonin) shown by Canal et al. (2007), would lead to hyper-activation of transmembrane signaling cascades. We have previously shown that hyper-activation of one such cascade, the protein kinase C system, can block perforant path LTP (Colley and Routtenberg, 1989). This is once again the classic inverted-U function. It is therefore entirely possible that anisomycin is having a cascading effect on signal transducing mechanisms, disrupting normal PTM processes, and reasonable to presume that this hyper-activation and thus-hyper-phosphorylation of substrate proteins, contributes to the amnesia observed by Bekinschtein et al. (2007).

8. The 'Then What': Post-translational modification (PTM) as 'the' substrate for long-lasting memory

Theories are supplanted by other theories, not data. In our view a satisfactory alternative to a de novo protein synthesis model is the 'PTM hypothesis.' Indeed, Rekart and I proposed that

PTM is the only mechanism required for long-lasting memory, and that synthesis is important for replenishment, a permissive process,

a. Tests of the theory: can it be falsified?

A unique prediction of our 'PTM hypothesis' is that inhibition of PTM weeks after learning should attenuate the memory for the event. We have recently reported, in fact, that the broad spectrum serine/threonine kinase inhibitor H-7 (1-(5- isoquinolineulphonyl) 2-methylpiperazine) is capable of such a feat when given to the anterior cingulate cortex (Holahan and Routtenberg, 2007) 3 weeks after learning. What is critical about this initial test is that the result could have falsified our hypothesis by showing that this treatment was without effect. Because there was an effect, the concept remains viable, though not proven. An especially important concern is that the H-7 may simply reduce the level of neuronal activity, acting essentially as a local anesthetic (I am grateful to Greg Quirk for pointing this out). It would be necessary to show, for example, that while local anesthetics reduce activity, H-7 does not, for the hypothesis to remain viable. Some evidence for this comes from our earlier studies on LTP and kinase inhibition, wherein the H-7 by itself has no effect on baseline activity nor on the initial potentiated response, but does dramatically prevent any long-lasting persistent enhancement of the response (Colley et al., 1990). A recent report by Shema et al. (2007) is entirely supportive of this long-lasting memory involvement of the PTM mechanism.

Back to Beckinschtein: Our PTM hypothesis as supported by the H-7 data just discussed raises the question: if we were to use a protein kinase inhibitor at 12 hr would we observe a decrement in long-lasting memory. The Holahan and Routtenberg (2007) study shows that even 21 days after learning kinase inhibition can have an effect. Thus, one might be surprised that 12 hr later there would not be an effect. If this result obtained one could argue that PTM is the mechanism promoting long-lasting memory.

Beckenshtein et al. then go on to show that one target of anisomycin is BDNF as there is an increase in the level of this growth factor at 12 hr and anisomycin blocks this increment. I choose not to use the phrase increase in synthesis, because a major regulatory step in BDNF function is its proteolytic conversion from proBDNF, the inactive form, to matureBDNF or mBDNF, the active form (e.g., Pang et al., 2004). Although not addressed by Beckenshtein et al. one needs to know how this important regulatory mechanism plays into their view that it is its role as a synthesized molecule at 12 hr that is important for long-lasting memory. Moreover, anisomycin can alter protease activity directly (Kageyama et al., 2002) or by inhibition of the synthesis of proteases. Thus, if the proteases that regulate the conversion of proBDNF to mBDNF are inhibited by whatever mechanism then a reduced level of mBDNF would be observed. But this does would mean that BDNF synthesis is not an instructional mechanism for memory persistence. Rather, a PTM mechanism, protease activation may be compromised. In support of this view, Pang et al. (2004) indeed focused on the proteolytic cleavage of proBDNF to mBDNF as critical for the persistence of LTP.

As briefly addressed in Routtenberg and Rekart (2005), the protein synthesis model requires complex trafficking of molecules that target particular synapses that are 'tagged' or 'marked.' In the case of the Beckenshtein et al. report it is relevant to ask: how does a molecule synthesized 12 hr after learning 'know' where to go to specifically enhance or be the substrate of the memory 7 days later. It strains one's credulity to imagine that the synapses activated during learning are then somehow the specific and selective target for the BDNF synthesized 12 hr later. Moreover, how would one differentially target items learned 9 hr later vs those learned 12 hr later (as their results would imply). In our view these synthesis cascades arise not to instruct the synapse to change but as replenishment signals.

Note that the PTM model by restricting change to proteins already present at the synapse avoids the issues of complex trafficking.

The BDNF results from Bai Lu's group (Pang et al., 2004) shows that the protein synthesis inhibitor effects on LTP can be reversed by addition of mBDNF to the bath, while leaving protein synthesis inhibition intact. This is a clear demonstration that persistent LTP can be generated in the presence of high levels of protein synthesis inhibition, a result taken by Beckenshtein et al to mean that BDNF is sufficient for persistent plasticity. Another interpretation is that protein synthesis is not required for persistent LTP. These results would thus seem to call into question the plethora of LTP and anisomycin findings (e.g., Frey and Morris, 1997; Fonseca et al., 2006) that claim that LTP persistence requires intact synthesis. Once again the conclusion seems inescapable that protein synthesis cannot be the mechanism of memory persistence. The only way to 'rescue' the *de novo* synthesis model would be to assume that BDNF is the only synthetic event that is critical for memory, a highly unlikely proposition (which also leaves unanswered the problem of targeting). On the other hand, BDNF sets into motion a range of transmembrane PTM-dependent signaling cascades which could likely play a role in the rescue. It might be useful to consider the possibility that the instructive mechanism for memory recruits the proteolytic regulation of BDNF, a PTM mechanism, while the permissive mechanism requires synthesis of proBDNF.

9. Time course of long-term memory: is the formation – persistence distinction useful?

Beckinshtein et al show that when anisomycin is given 12 hr, but not 9 hr, after learning that 2 days later there is no effect, while 7 days later there is a deficit. They interpret their results to mean that a synthetic step at 12 hr is important for persistence of LTM (7 days) but not its formation (2 days). Curiously, a current series of studies on learning-induced mossy fiber growth from our laboratory appears relevant to the time course which they describe (Holahan et al., 2006; Rekart et al., 2007). We found that after 5 days of water maze learning in which the animals are now well trained, there is no apparent alteration in mossy fibers. However, if the animals are housed in their home cages for 7 days after this 5 day training, there is a prominent 'sprouting' or 'growth' of mossy fibers from the stratum lucidum into the stratum oriens. As the animals on the last days of training have a good 24 hr memory of the task, it is clear that the memory is formed in the absence of the growth of the mossy fibers. We suggest several potential functions for this growth, but here it is worth emphasizing the curious coincidence in time frames with the Beckinshtein et al. results.

10. Coda: Post-translational modification as substrate for long-lasting memory in evanescent memory networks

Current theories of memory fall into roughly two categories. In one the system is pre-eminent and molecular reagents are used to manipulate the system. In the other, the molecular and cellular events are pre-eminent and some nod is made toward the fact that this probably has to do with memory. One sub-text of the PTM hypothesis is that it attempts to construct a 'mobius strip' mechanism in which molecular and network events are at once on both sides, oscillating rapidly. These are mutually self-regulating processes so that the network is determined by the instantaneous PTM state of proteins on both sides of the synapse, and the PTM state of these proteins is determined by the activity of the network. This inextricability is a key feature of the PTM hypothesis, and it is within this interstice that the compromises necessary for memory formation are achieved.

A necessary yet unexpected conclusion to emerge from consideration of the consequences of a PTM mechanism as the necessary, sufficient and exclusive substrate for long-lasting memory (Routtenberg and Rekart, 2005), is that the central Hebbian dogma that cells that ‘fire together, wire together’ is an unlikely mechanism for long-lasting memory. Thus, a unique feature of the PTM model is that longevity of information storage is achieved not by stability of the synaptic mechanism, but by impermanent pseudoredundant circuits. This is so because PTM is a reversible process and thus any permanent connection, any ‘lasting effect’ cannot be in the form of stable synapse formation. We have therefore proposed a solution in which network level processes regulate cellular mechanisms, even as such mechanisms regulate the network. Thus, synapses are ‘meta-stabilized’ by regulated feedback mediated by the circuit in which the synapse is embedded. For example, spontaneous activity is proposed to be a substrate feedback mechanism we term ‘cryptic rehearsal’ to sustain for some period of time after learning an approximation to the state initially created by input. Additionally, because the duplication of these traces is ongoing, this provides a degenerate code (Routtenberg and Rekart, 2005) for the engram. Stability is thus achieved, not by stabilizing the synapse, but by implementing a pseudo-redundant yet malleable circuitry so that memory can be protected in the face of small catastrophes in network representation.

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