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Research Report

Protein synthesis and consolidation of memory-related synaptic changes

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ABSTRACT

Although sometimes disputed, it has been assumed for several decades that new proteins synthesized following a learning event are required for consolidation of subsequent memory. Published findings and new results described here challenge this idea. Protein synthesis inhibitors did not prevent Theta Bust Stimulation (TBS) from producing extremely stable long-term potentiation (LTP) in experiments using standard hippocampal slice protocols. However, the inhibitors were effective under conditions that likely depleted protein levels prior to attempts to induce the potentiation effect. Experiments showed that induction of LTP at one input, and thus a prior episode of protein synthesis, eliminated the effects of inhibitors on potentiation of a second input even in depleted slices. These observations suggest that a primary role of translation and transcription processes initiated by learning events is to prepare neurons to support future learning. Other work has provided support for an alternative theory of consolidation. Specifically, if the synaptic changes that support memory are to endure, learning events/TBS must engage a complex set of signaling processes that reorganize and re-stabilize the spine actin cytoskeleton. This is accomplished in fast (10 min) and slow (50 min) stages with the first requiring integrin activation and the second a recovery of integrin functioning. These results align with, and provide mechanisms for, the long-held view that memories are established and consolidated over a set of temporally distinct phases.

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1. Introduction

For over 50 years thinking about how memories are consolidated has been dominated by the hypothesis that the learning experience must initiate the synthesis of new proteins if the memory is to persist. Early support for this idea emerged from reports that protein synthesis inhibitors delivered around the time of the learning event impaired long-term retention of memories in a variety of behavioral tasks but had little or no effect when the retention interval was brief (Davis and Squire,

Abbreviations: ACSF, artificial cerebrospinal fluid; act-ß1, activated ß1 integrin; ANIS, anisomycin; BDNF, brain derived neurotrophic factor; bref., brefeldin A; LTP, long term potentiation; TBS, theta burst stimulation

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1984; Hernandez and Abel, 2008). Although agents that prevent transcription are highly toxic, their use also led to the related idea that behaviors resulting in enduring memories signal to the nucleus to initiate expression of plasticity-related genes and their protein products (Alberini, 2009; Kandel, 2001; Squire and Barondes, 1970).

The case for the protein synthesis hypothesis was further strengthened by evidence that:

- A learning experience can increase the expression of genes and proteins related to synaptic functioning (Gall et al., 1998; Ganguly et al., 2013; Guzowski et al., 2001; Robles et al., 2003).
- Suppression of these same genes or gene products via knock-outs or regionally targeted treatments (oligonucleotides, AAV transfection) impair retention (Guzowski et al., 2000; Minichiello et al., 1999; Nagy et al., 2006; Plath et al., 2006; Ploski et al., 2008).
- When applied locally to hippocampus or amygdala, highly selective manipulations of transcription have profound effects on long-term retention (Barrett et al., 2011; McQuown et al., 2011; Nonaka et al., 2014).

Growing evidence that long-term potentiation (LTP) is a substrate for many forms of memory prompted new investigations into the role of protein synthesis in the consolidation of learning-related synaptic plasticity. Consistent with the behavioral literature, neither protein-synthesis nor transcription inhibitors impaired the initial, early phase of LTP but both caused potentiation to gradually dissipate (Frey et al., 1996, 1988; Frey and Morris, 1997; Huang and Kandel, 1994; Sacktor, 2008; Tsokas et al., 2005). Evidence also emerged that the induction of LTP stimulates gene expression and translation events associated with learning (Kelleher et al., 2004; Miyashita et al., 2008; Park et al., 2006; Pevzner et al., 2012; Steward and Worley, 2002; Tao et al., 1998; Tsokas et al., 2005). Moreover, manipulations of gene expression and translation produced results that accord well with the above findings (Guzowski et al., 2000; Korte et al., 1998; Minichiello, 2009).

Given this large body of supporting evidence it is surprising that the protein synthesis hypothesis has not been universally accepted (Canal et al., 2007; Gold, 2008a, 2008b; Routtenberg and Rekart, 2005; Rudy, 2008). Opposition to the idea is based on two classes of evidence. One set indicates that at least some of the memory impairments produced by protein synthesis inhibitors may be the result of their off-target effects (e.g., Canal et al., 2007; Sharma et al., 2012). Another body of results indicates that memories and LTP can indeed persist even in the face of severe inhibition of protein synthesis (Abbas, 2013; Abbas et al., 2009, 2011; Abraham and Williams, 2008; Fonseca et al., 2006b; Martinez et al., 1981; Pang et al., 2004; Staubli et al., 1985; Villers et al., 2012).

The second data set is cause for concern because it not only challenges the central argument, it questions the relevance of the well-documented findings that both behavior and LTP-inducing stimulation induce changes in gene expression (Alberini, 2009; Bramham and Messaoudi, 2005; Chen et al., 2010; Guzowski et al., 2001; Taubenfeld et al., 2001). If neither long term memories nor LTP depend on the generation of new proteins induced by the initiating events, then what is the function of activity-regulated changes in gene and protein expression? Moreover, if memory consolidation does not depend on the initiation of new protein synthesis, then what events are critical for consolidation?

The present paper addresses the above issues. We report evidence that reinforces the conclusion that the consolidation of LTP is not blocked by protein synthesis inhibition, then describe circumstances in which the inhibitors are effective, and finally demonstrate that multiple LTP events obviate the negative actions of the inhibitors when such are present. These observations help explain some of the discrepant results in the literature and lead to the conclusion that induced synthesis is not, *under normal circumstances*, important to current encoding but instead paves the way for future memory formation. We also review studies demonstrating that early and delayed phases of LTP and memory consolidation dependent upon activation and subsequent recovery of signaling by integrin-class adhesion proteins, respectively, and that these events are protein synthesis independent. A final section will attempt to integrate the hypothesis that temporally distinct stages of integrin-driven cytoskeletal reorganization underly multiple stages of memory consolidation with evidence that learning and LTP induction trigger the production of proteins necessary for long term storage.

2. Reorganizing the spine actin cytoskeleton is a consolidating event

Our interest in the contribution of newly synthesized proteins to the consolidation of LTP emerged from research directed at understanding mechanisms that regulate the dendritic spine actin cytoskeleton and, thus, spine morphology. Results from our laboratories and elsewhere (Fukazawa et al., 2003; Kramar et al., 2006; Krucker et al., 2000; B. Lin et al., 2005; Okamoto et al., 2009; Wang et al., 2008) led to the conclusion that the enlargement and stabilization of the spine actin cytoskeleton initiated by Theta Burst Simulation (TBS) may be a critical consolidating event. Specifically, potentiation induced with either TBS or high frequency stimulation elicits, and depends upon, new actin polymerization in dendritic spines (Fig. 1). Further analyses demonstrated that these structural events are driven by separate signaling streams that control the assembly (polymerization) and subsequent stabilization of the new actin filaments (Chen et al., 2007; Fedulov et al., 2007; Kramar et al., 2006; Mantzur et al., 2009; Rehberg et al., 2010; Rex et al., 2009, 2010). Integrins, a group of transmembrane adhesion receptors that regulate the cytoskeleton at most types of cellular junctions (Brakebusch and Fassler, 2003), play a central role in these processes, as indicated by results of studies using toxins, small peptides, neutralizing antibodies, or genomic manipulations (Kramar et al., 2006; Nagy et al., 2006; Wang et al., 2008). Downstream intracellular signaling cascades (small GTPases and their effectors) initiated by integrins have also been linked to LTP stabilization (Rex et al., 2009). Notably,

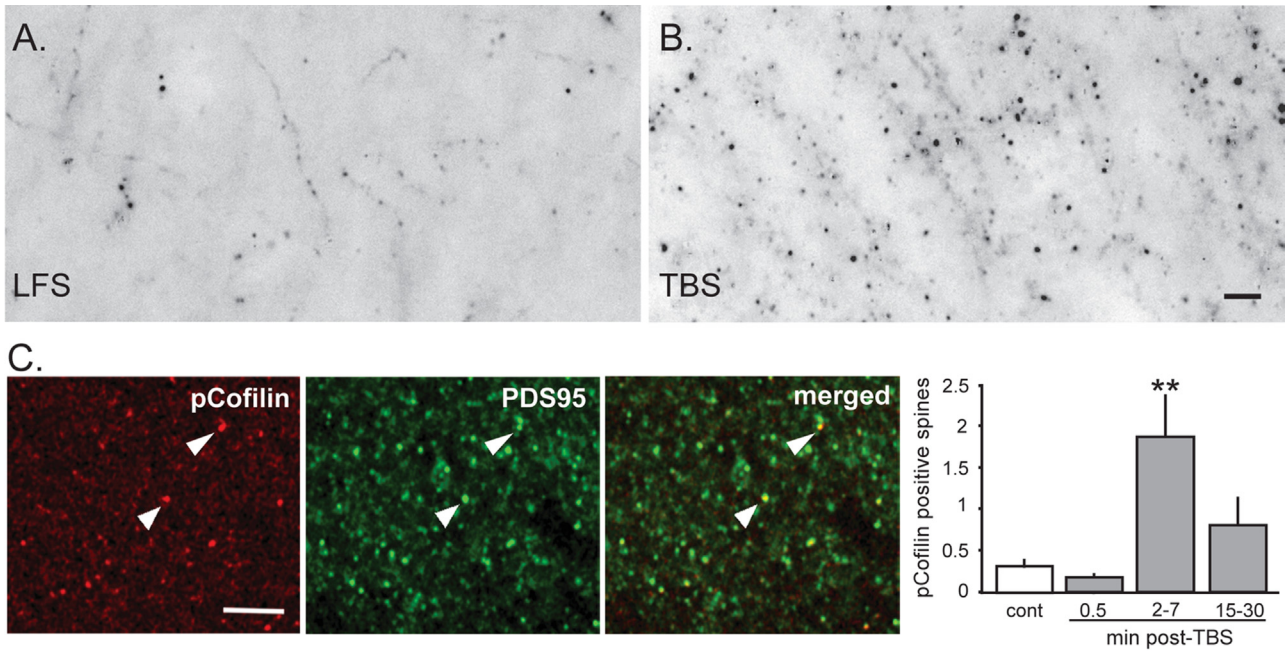

Fig. 1 – Theta burst stimulation (TBS) promotes actin polymerization and cofilin phosphorylation in adult hippocampal slices. Images were collected from proximal CA1b stratum radiatum following stimulation of Schaffer/commissural afferents. (A,B) In situ phalloidin labeling in slices that received (A) baseline low-frequency stimulation (LFS) or (B) TBS illustrates the increase in F-actin enriched spines (dark puncta) achieved with theta stimulation. (C) Deconvolved epifluorescence images show immunolabeling for phosphorylated (p) Cofilin, and excitatory synapse scaffold protein PSD95, and the merged image of the two for the same field; arrowheads indicate double-labeled synapses. Cofilin is downstream effector of RhoA: phosphorylation inactivates this F-actin severing protein thus allowing actin polymers to survive and elongate. Quantification of spine labeling (bar graph, right) shows that TBS transiently increases numbers of pCofilin+ enriched contacts indicating transient cofilin inactivation (mean ± SEM values for 8–9 slices/group; $^{**}p = 0.008$ vs controls). Scale bars 5 μm (in B for A and B). Modified from Baudry et al. (2012) and Chen et al. (2007).

these processes are modulated by receptors for estrogen, adenosine, and the neurotrophin BDNF (Lynch et al., 2013). Such findings suggest that reorganization and stabilization of the spine actin cytoskeleton is critical for the consolidation of LTP and memory, and behavioral studies have supported this conclusion (Lamprecht, 2011; Mantzur et al., 2009; Rex et al., 2010). A complementary set of findings revealed that the actin cytoskeleton is stabilized within a few minutes of TBS. In particular, treatments that disrupt the generation or stabilization of new F-actin prevent LTP consolidation if administered prior to or soon after TBS but have no effect on potentiation (or the remodeled spine actin network) when applied 15 min later (Rex et al., 2007, 2009, 2010). These results identified lasting changes to the spine actin cytoskeleton as a critical step for long term expression of LTP. Note, however, that these changes occur within 2–5 min and thus are likely too rapid to depend on the synthesis of new protein.

3. Protein synthesis inhibition fails to disrupt LTP

Although early actin remodeling is not likely to depend on new synthesis, it is possible that the maintenance of potentiation requires proteins that are generated following TBS. We describe here results of studies evaluating this possibility using the same hippocampal slice protocols employed in the actin studies. Specifically using adult rat hippocampal slices in an interface recording chamber, the synthesis inhibitors anisomycin (40 μM) or emetine (20 μM) were infused for 30 min before and 30 min after TBS; this schedule blocked incorporation of ^{35}S-methionine, bath applied at 2 μCi/ml as per Raymond et al. (2000), into protein by 88% (anisomycin) or 70% (emetine). However, neither compound detectably affected LTP: Initial potentiation, subsequent decay, and the degree of stable potentiation as assessed 30–40 min after TBS were about the same when induction occurred in the presence of the inhibitors as they were when LTP was induced more than hour of after washout (Fig. 2A and B). Moreover, LTP produced in the presence of anisomycin was stable for the remaining three hours of recording; there was no suggestion in the results for a late, protein synthesis dependent phase of LTP. These experiments point to the conclusion that structural changes generated in the minutes after induction are sufficient to produce long lasting potentiation and appear not to require newly translated proteins for their maintenance.

These results and other recent reports (Abbas, 2013; Abbas et al., 2009; Villers et al., 2012) are at odds with many published studies showing that enduring LTP was blocked by protein synthesis inhibitors (Fonseca et al., 2006a; Frey et al., 1988; Osten et al., 1996; Tsokas et al., 2005). Thus, we conducted additional experiments to determine potential sources for the discrepancy. One possibility is that the state

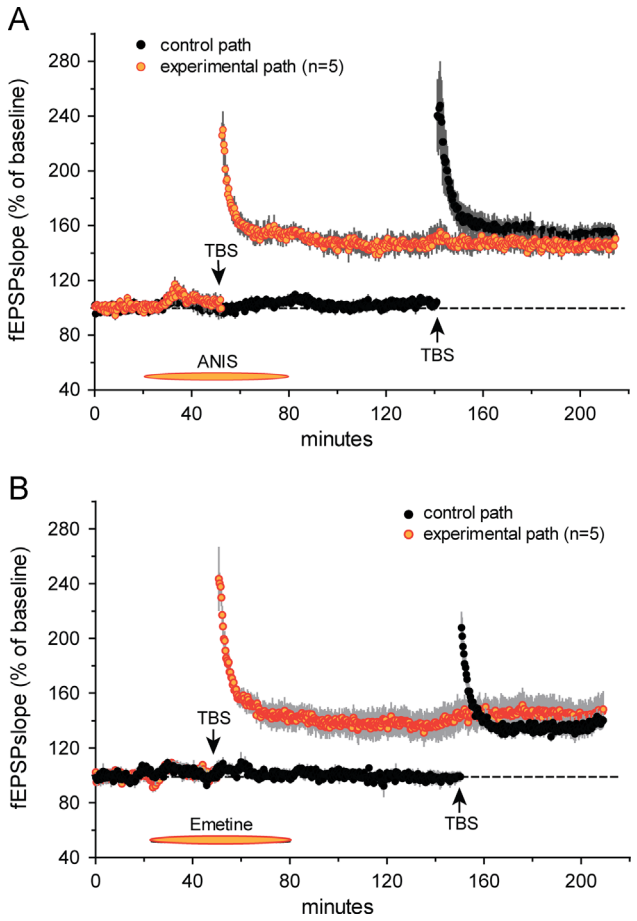

Fig. 2 – Protein synthesis inhibition does not block theta burst induced LTP. Adult hippocampal slices were prepared and placed in an interface chamber with as described (Kramar et al., 2012): two stimulation electrodes were used to drive separate but convergent populations (paths) of Schaffer/commissural afferents to the CA1b field of recording. In all experiments slices were allowed to equilibrate in the interface chamber, with constantly perfusing ACSF, for 1.5 h prior to testing. Following 20 min of stable baseline recording, 40 μM anisomycin (ANIS) (A) or 20 μM emetine (B) was infused into the slice bath beginning 30 min prior to application of a single train of theta burst stimulation (TBS, arrow) to the 'experimental path', and continuing for 30 min afterwards. After at least 60 min washout of the inhibitor, a second TBS train was applied to the 'control' path. As shown, neither anisomycin (A) or emetine (B) disrupted stable LTP in the experimental (inhibitor present) or control (inhibitor washout) path.

of the slice preparation at the time of the experiment is a critical variable. In some studies where protein synthesis inhibition impaired LTP, slices had been incubated in ACSF for several hours before the induction of LTP (Redondo et al., 2010; Sajikumar et al., 2007, 2005). Our slices were tested 2 h after preparation.

We therefore repeated the experiments using slices that had been maintained in the recording chamber for 5 h prior to the start of the experiment proper. Only cases in which baseline responses were stable throughout the prolonged interval prior to TBS were used. As shown in Fig. 3A, anisomycin had no effect on baseline responses (control path) but did block the induction of stable LTP in response to a single train of TBS. Note that LTP induced in a separate pathway more than four hours earlier was unaffected by anisomycin infusion (Fig. 3A, control path), a result which relates to the idea that delayed transcriptional events produce proteins needed for the maintenance of the potentiated state. The potency of the inhibitor in these longer standing preparations could not be attributed to a general decline in the viability of the slice or its capacity to maintain LTP because, as shown in a separate set of untreated slices, potentiation maintained in drug free ACSF for 6 h is robust and stable (Fig. 3B).

3.1. *Incubation time determines the effect of protein synthesis inhibitors*

Why should incubation time have such a dramatic effect on the efficacy of synthesis inhibitors? One possibility is suggested by evidence that the preparation of the slice causes transient increases in the activity of kinases involved in synaptic plasticity (Ho et al., 2004) followed by a return to conditions closer to those found in vivo. In parallel there is surge in the expression of transcriptional regulators (Taubenfeld et al., 2002; Zhou et al., 1995) and synaptic proteins. Thus, it is possible that at least some of the proteins necessary for consolidation are present at supra normal levels in freshly prepared slices, thereby creating an artificial condition in which inhibitors are ineffective. This argument predicts that slices incubated in protein synthesis inhibitors from the time of sectioning through the delivery of theta bursts will not exhibit LTP. The results did not support this hypothesis: slices incubated with anisomycin from cutting through testing exhibited normal and stable LTP (Fig. 4).

This outcome implies that the ability to sustain LTP in the face of synthesis inhibition is not due to proteins generated in response to preparation of the slices. Instead, it appears that the relevant proteins are already present, which suggests an alternative explanation for the time-dependent actions of protein synthesis inhibition. As suggested elsewhere (Sajikumar et al., 2005), it is possible that levels of memory-related proteins decrease with incubation time, reflecting their normal half lives and ongoing proteolytic activity. Under these depleted conditions, TBS could initiate changes in the actin cytoskeleton needed for rapid consolidation and perhaps also initiate the synthesis of proteins needed to sustain the potentiated state (Kelly et al., 2007; Ramachandran and Frey, 2009). To be clear, according to this account, relevant proteins are depleted during a prolonged incubation of slices creating a condition in which the production of enduring LTP will depend on proteins synthesized in response to afferent stimulation. So, blocking protein synthesis at the time of TBS will prevent LTP from enduring.

One way to test this hypothesis is to use a variation of the protocol that Frey and Morris (1997) developed to test their synaptic tag and capture hypothesis. The basic conclusion from their experiments was that weakly stimulated synapses that normally would not sustain LTP might capture proteins generated by earlier strong stimulation applied to other synapses on the same neuron. By doing so the weakly stimulated synapses would now express enduring LTP.

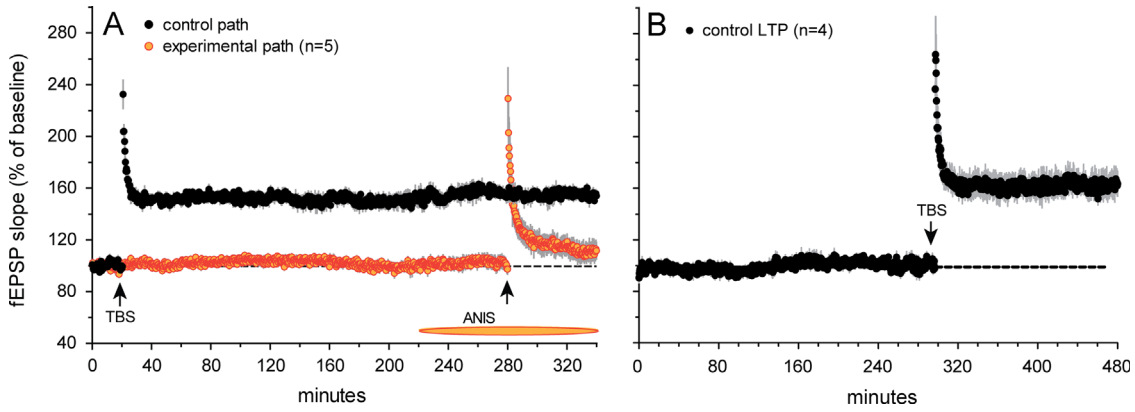

Fig. 3 – A protein synthesis inhibition blocks LTP in 'mature' slices. (A) Stable baseline recordings of Schaffer-commissural fEPSPs were collected from CA1b stratum radiatum for 4.5 h before applying ANIS to the slice bath 30 min prior to TBS (6 h after slice preparation). Under these conditions, TBS of the 'experimental' path induced an initial potentiation but the fEPSP slope gradually returned to baseline levels over a 1 h recording period. LTP induced 4.5 h earlier in a separate population of axons ('control path') was unaffected by the inhibitor. (B) In a separate set of slices, baseline recordings were collected for 4.5 prior to delivery of TBS (6 h after cutting) in the absence of the inhibitor. Robust LTP was obtained and remained stable during the 3 h post-TBS recording session.

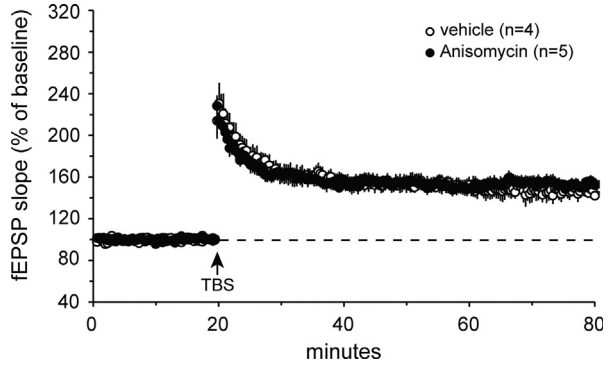

Fig. 4 – Incubation with anisomycin during slice preparation and through delivery of TBS fails to block LTP. Adult rat hippocampal slices were sectioned in the presence of 40 μM anisomycin and then continuously treated with inhibitor or vehicle (0.01% DMSO) in ACSF for the following 2.5 h. After 2 h of incubation and 20 min of stable baseline recording, TBS was applied to Schaffer/commissural afferents of CA1b stratum radiatum where fEPSPs were recorded. As shown, potentiation was robust, stable, and of comparable magnitude in slices incubated in anisomycin or vehicle. Note that the inhibitor incubation time for this study was much longer than that employed in the studies described in Fig. 3A.

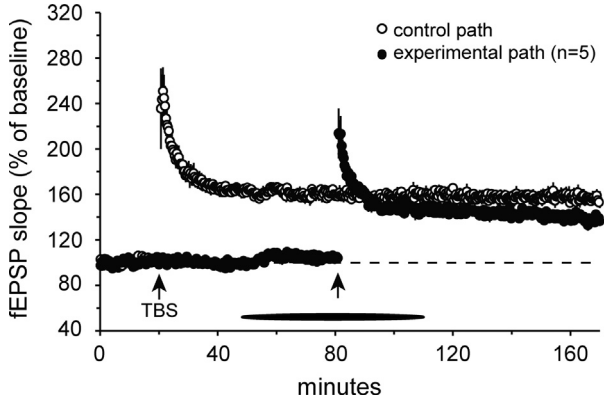

Fig. 5 – Prior induction of LTP in one pathway in mature slices greatly reduces the disruptive effects of anisomycin on LTP in a second input. Following a 5 h incubation in an interface chamber with constant normal ACSF perfusion, LTP was induced by TBS in one pathway ('control path') and potentiation of the fEPSP slope was recorded for the remainder of the experiment. Anisomycin (40 μM) was introduced into the bath (horizontal bar) prior to delivery of TBS to a second input ('experimental path') to the same dendritic field. The first LTP episode blocked the marked disruptive effects of anisomycin seen in the earlier study (Fig. 3A) using mature (~6 h) slices.

In this experiment we first incubated the slices for the duration needed to deplete the relevant proteins, creating a preparation in which anisomycin would normally prevent enduring LTP. We then applied TBS to one pathway with the intent of generating a new supply of relevant proteins; 45 min after this event, anisomycin was infused to prevent further protein synthesis that might be produced when a second TBS train was applied to a second input. Thus, if the second train of TBS produced stable LTP then it would have to have been supported by proteins generated by the first round of TBS.

As shown in Fig. 5, persistent LTP was established in the second pathway despite the presence of the protein synthesis inhibitor. These results support the motivating hypothesis and the argument that, in acute slice preparations, long incubations deplete proteins needed for LTP consolidation, making it necessary for the TBS train to replenish the pool in order to generate lasting potentiation.

It is worth noting that conceptually the above experiment is identical to that reported in Fig. 3. Yet the outcomes were quite different. As shown in Fig. 3, the first TBS train did not protect LTP produced by the second train from the effect of anisomycin, whereas in Fig. 5 it did. There is, however, one important difference between the two studies: the interval separating the two TBS trains was over 5 h in the first experiment (Fig. 3) but less than 60 min in the second (Fig. 5). Thus, consistent with experimental results reported by Frey and Morris (1997), one could have predicted these results from the intervals found to produce the synaptic tagging effect. In any event, the long delay in our experiments presumably provided sufficient time for degradation of the proteins generated by the first TBS episode.

3.2. *Summary of protein synthesis inhibition results*

Experiments described above have identified one potential source of the conflict between researchers examining the role of protein synthesis in LTP – the state of the slice at the time of LTP-inducing stimulation. Incubating slices for several hours prior to the induction of LTP depletes the pool of proteins that normally support the consolidation of potentiation and thereby create a condition in which induced synthesis is necessary to support new potentiation. Note that it is not unlikely that rate of depletion varies between different types of slice preparations: it may well be the case that several hours are not needed in some circumstances to instate a need for induced synthesis to maintain potentiation. Regarding this point, it would be instructive to measure the rate of breakdown for rapidly induced synaptic proteins likely to be involved in stabilizing the potentiated state.

Based on his recent experiments, Abbas (2013, p.301) reached the conclusion that '…. these results favor the notion that constitutive rather than triggered protein synthesis is important for LTP stabilization'. There remains, however, the possibility that constitutive synthesis is not sufficient; it could be the case that production of memory related proteins only occurs after LTP/learning episodes. This point is intriguing because it suggests that continuous learning, as undoubtedly happens in real world environments, not only encodes new information but is also required for the acquisition of future memories. It will be of interest in this regard to test if patterns of afferent stimulation that do not produce LTP (input 1) are as effective as TBS in preventing synthesis inhibitors from blocking LTP consolidation (input 2) in slices that have been maintained for several hours. In other words, is normal activity in brain networks sufficient to produce LTP-related proteins or are these only generated by discrete learning events?

4. Integrins support a late stage of consolidation

While our experiments do not support for the argument that new protein synthesis is required for consolidation outside of special circumstances, other work has suggested in an alternative explanation for both rapid and delayed stages of consolidation. Specifically, we find that TBS must engage an elaborate collection of signaling events leading to reorganization of the spine actin cytoskeleton in order to shift synapses into a stable potentiated state (Lynch et al., 2013, 2007). Disrupting any of several key steps in these parallel signaling streams has no effect on the induction and initial expression of LTP but blocks its stabilization. It takes only 1–2 min to mobilize the signaling which then rapidly dissipates although elevated levels of filamentous (F) actin remain (Babayan et al., 2012; Rex et al., 2009, 2010). These results are consistent with the long-held view that memories are constructed in stages (Davis and Squire, 1984; James, 1890; McGaugh, 2000). In the first stage, the memory trace is generated (initial expression); this does that depend on reorganization of the cytoskeleton. The later consolidating stage then requires stabilization of an expanded actin network.

We now describe experiments that uncovered a novel, delayed stage in the consolidation process (Babayan et al., 2012). This unexpected phenomenon was identified during recent analyses of integrins, synaptic membrane receptors that play a central role in the rapid phase of consolidation. We had previously shown that integrins containing the ß1 subunit are required for TBS-induced increases in spine F-actin and the expression of stable LTP. We then turned to the question of whether intermittent synaptic activity could essentially sustain ß1 integrins in the activated conformation (as detected using state-specific antisera and immunostaining). Specifically, after initially establishing that an initial round of TBS activated synaptic integrins and generated LTP, we attempted to reactivate the ß1 integrins by presenting a second TBS train (TBS2) to the same axons 10–40 min later. The results were surprising: the second TBS train did not reactivate integrins or their downstream actin management pathways. However, integrin activation was achieved when the interval separating the two TBS episodes was stretched to 50–60 min (Fig. 6A). Thus, there is an apparent refractory period of about 50 min during which a second TBS train cannot stimulate the synaptic integrins that are critical to structural remodeling and LTP (Chun et al., 2001; Huang et al., 2006; Kramar et al., 2006).

We then asked if the subsequent recovery of integrin reactivity was functionally significant to the maintenance of LTP (Babayan et al., 2012). To do this we infused function blocking ß1 integrin antisera prior to the recovery of integrin function (30 min post-TBS). Thus, if recovery of integrin reactivity is important to LTP it should be revealed by this treatment. Remarkably, neutralizing ß1 antisera infusion produced a rapid and complete elimination of LTP that had already passed through the first consolidation stage (Fig. 6B). However, the same antisera had no effect on the maintenance of LTP when it was infused after the refractory period, at 60 min post-TBS (Fig. 6C). Based on these results we conclude that integrin receptors not only contribute to an initial consolidation phase but upon re-entering the reactive state contribute to a second consolidating phase.

We then investigated the source of the integrin-refractory period. We suspected that this could be due to either (a) internalization of the receptors or (b) their degradation and replacement with new copies. The degradation hypothesis is consistent with evidence that theta bursts activate calpain (Vanderklish et al., 1995, 2000), a calcium

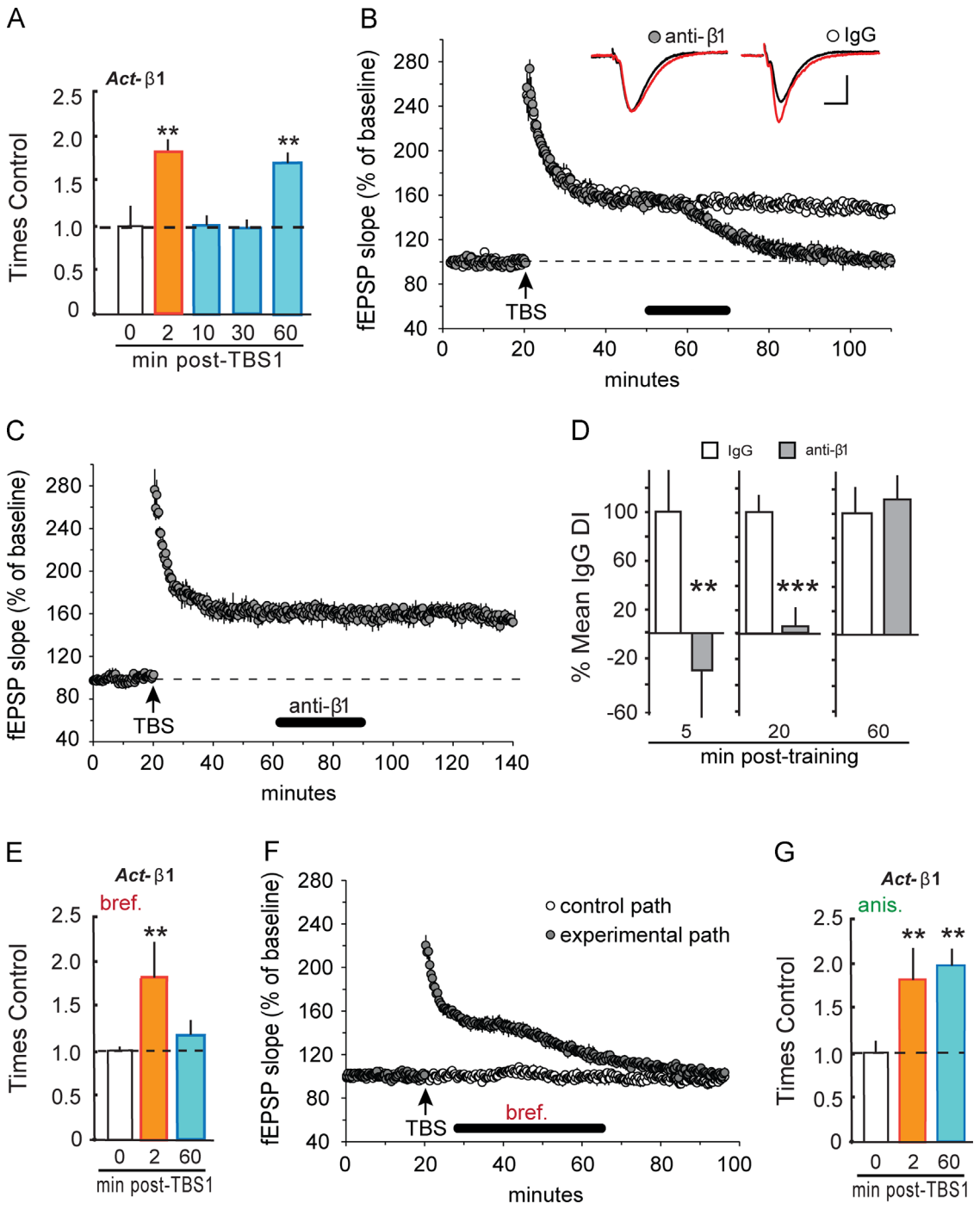

Fig. 6 – Integrin dynamics generate a delayed, second stage of consolidation for LTP and memory. (A) Immunoreactivity for the activated form of ß1 family integrins (Act-ß1) at synapses was significantly increased relative to controls two minutes following TBS (orange bar). In separate experiments, a second theta burst train (TBS2, blue bars) was delivered to the same synapses at different time points after TBS1 and the slices immunostained for activated-ß1 integrin at synapses. Note that TBS2 effectively increased Act-ß1 only after a 60 min delay following TBS1. (B) Local application of neutralizing antisera against ß1 integrins, starting 30 min after TBS1, caused a gradual decay of LTP while the control antibody (anti-IgG) did not. Inset: representative traces collected before (black) and 90 min after (red) TBS1. Scale bar = 1 mV, 5 ms. (C) Local perfusion of neutralizing anti-ß1 starting 60 min after delivery of TBS failed to disrupt LTP consolidation. (D) Object location memory scores ('discrimination index') on day 2 of testing for mice that received intrahippocampal infusion of neutralizing anti-ß1 integrin at 5, 20, or 60 min after training on day one. Infusions at 5 or 20 min blocked long term memory while those starting 60 min post-training did not. Anti-IgG infusion had no statistical effect at any time point. (E) Bar graph shows grouped data (quantification of Act-β1+ synapses) from slices that were perfused with brefeldin A (bref., 35 μM) for 40 min prior to delivery of TBS1 and collected 2 min afterwards (orange bar). The toxin had no detectable effect on integrin activation (compare to panel A). The same treatment applied before TBS2 blocked integrin activation (blue bar). (F) Plot shows that perfusion of brefeldin-A starting 10 min after TBS1 caused a gradual decay in field EPSP slope (i.e., a loss of potentiation) similar to effects of anti-ß1. (G) Using the same procedure as in E, perfusion of anisomycin (anis., 10 μM) failed to block activated-ß1

dependent protease that is found in spines (Perlmutter et al., 1988) and known to cleave synaptic integrins (Huttenlocher et al., 1997). To test the replacement hypothesis we used brefeldin A, a fungal antibiotic that prevents new surface expression (i.e., plasma membrane insertion) of integrins in slices (C.Y. Lin et al., 2005). In adult hippocampal slices a 40-min brefeldin A treatment prior to a single TBS train (when responsive integrins are in place) had no effect on integrin activation but it blocked integrin reactivation by a second TBS delivered 60 min later (Fig. 6E). Moreover, brefeldin A reversed LTP (Fig. 6F) when infused after induction in the manner seen with infusion of ß1 integrin neutralizing antisera (Fig. 6B).

The time course for receptor replacement via membrane insertion fits well with the delayed packaging and transport of select proteins from the Golgi apparatus (C.Y. Lin et al., 2005). This implies that the replacement function draws on existing pools of integrins to end the refractory period. However, it is also possible that recovery depends on the synthesis of new integrins. If this were the case then applying anisomycin beginning 40 min before a second TBS train should prevent integrin reactivation with that stimulation applied at 60 min. However, blocking protein synthesis did not interfere with integrin reactivation. Thus, the protein synthesis hypothesis again gains no support, and it is reasonable to infer that the refractory period ends as already existing integrins enter the synapse to replace degraded ones.

The discovery that LTP requires a second stage of integrin-dependent consolidation motivated us to determine if integrin activity also makes a delayed contribution to the maintenance of an established memory. To test this idea we trained mice in an object location memory task. They were given 10 min exposures to two identical objects placed at fixed locations in an open arena; 24 h later they were returned to the arena containing same objects but with one object moved to a novel location. As expected, during the retention test the mice spend greater time exploring the displaced object, indicating they remembered the original location (Babayan et al., 2012). Long-term object location memory is dependent on neurons in the CA1 field of rostral hippocampus (Barrett et al., 2011), the same region studied in the above hippocampal slice experiments. Thus, we determined if infusions of ß1 integrin neutralizing antisera into rostral CA1 would impair retention. When delivered 5 or 20 min after object location memory training, retention 24 h later was substantially impaired. Yet, when the neutralizing antisera were infused 60 min after training (after the integrin refractory period) the antisera had no effect of retention (Fig. 6D) (Babayan et al., 2012). The close correspondence between the outcomes of the LTP and behavioral experiments strongly suggest that ß1 integrins are supporting delayed stabilization in both cases.

4.1. *Summary*

Our previous research revealed that TBS activates a set of signaling events that consolidates the synaptic changes supporting LTP by rapidly remodeling and re-stabilizing the spine actin cytoskeleton. The experiments just described indicate that although these initial events are necessary for consolidation they are not sufficient. There is a later phase of integrin-dependent activity, which depends on processes that replace the likely degraded integrins that supported the initial consolidating events. It is important to emphasize that neither of these consolidation stages require new proteins to be generated by TBS.

It is not clear why the creation of a stable actin cytoskeleton requires two stages of integrin activity. However, one might speculate that during the first stage the adhesion receptors generate new actin networks and, via cross linking and capping, render them resistant to disruption. Notably, the actin depolymerizing protein cofilin is inactivated for about 10 min after TBS but then returns to its dephosphorylated, constitutively active configuration. There is therefore enough time to generate and stabilize the networks before the return of cofilin activity. Note however, that the synaptic integrins driving these events will no longer be signaling from about 10 min forward; it will not be possible during this period to anchor the reorganized cytoskeleton to the extracellular matrix. The return of functional integrins at 50–60 min post-TBS could thus produce the matrix–cytoskeletal linkages that underlie the extreme stability of cell junctions and, in the present case, the changes to spine and synapse morphology that maintain LTP.

5. Discussion

Both clinical and experimental evidence indicate that memories become more resistant to disruption as they age (McGaugh, 2003; Ribot, 1882). The term ‘consolidation’ emerged over 100 years ago to explain why new memories are especially vulnerable to interference (Muller and Pilzecker, 1900) – the reason is that the memory trace requires time to consolidate (see McGaugh, 2003, for a historical context). For several decades the idea that memory consolidation depends on new proteins synthesized in response to the learning event has dominated thinking about this problem. Even though there have always been skeptics, the modern literature contains dozens of reports of studies using protein synthesis inhibitors that provide support for this idea. Perhaps one reason why the protein synthesis hypothesis has continued to prevail is that no strong alternative explanation of how memories are consolidated has emerged.

The data assembled here should be considered in this context. First, our LTP experiments do not support for the protein synthesis hypothesis. We do not stand alone on this matter (Abbas, 2013; Abbas et al., 2009, 2011; Pang et al., 2004; Villers et al., 2012). However, it is important to note that we do not simply report that inhibiting protein synthesis failed to prevent the lasting form of LTP. Our experiments also provide insights into the conditions that determine when protein synthesis inhibition will produce impairment. Specifically, we argue that this can occur when the level of the relevant existing proteins is low. In this case TBS stimulation has to induce the synthesis of new proteins if LTP is to endure.

If the synthesis of new protein is not required to consolidate memories then what processes are critical? Our research provides a basis for offering a strong alternative explanation. Specifically, it revealed that critical consolidating processes include intracellular signaling events orchestrated by surface receptors belonging to transmitter (NMDA), modulatory (e.g.,

BDNF, adenosine), and adhesion (integrins) classes. Ultimately, these events produce recognizable changes to the substructures responsible for the anatomy of spines and their synapses. These actin-regulating processes operate in two stages. The first is completed in about 10 min while the second takes about 50 min. Neither stage is dependent upon TBS generating new proteins.

It is undeniable that the behavioral experiences that create memories, and the TBS that induces LTP, initiate signaling that produces proteins relevant to memory. Thus, it is important to address the significance of these results in the context of our findings. We reiterate that our experiments support the ideas that induced synthesis is only required when the supply of key proteins has been reduced and that replenishment can be had with earlier induction of LTP. Consider these observations in the context of an animal operating in a real world environment. Inputs that would initiate the synaptic events that support LTP and memory are surely occurring multiple times a minute, activities that would produce a constant supply of the synaptic proteins needed to support memory. But exceptions could occur: one could imagine circumstances both in everyday life and in the laboratory that lead to a depletion of the relevant proteins. Very little is known about how laboratory-housing conditions influence the basal level of memory-relevant proteins but it seems obvious that they constitute a kind of deprivation. Hence we predict that environmental enrichment during the hours preceding a training trial will greatly reduce the dependency of memories on induced synthesis. We should perhaps also be concerned with variability in protein availability due to the circadian regulation of transcription factors (Luo et al., 2013); might the influence of synthesis inhibitors on LTP and memory consolidation be dependent on when animals or slices are tested? The list of factors that may result in low levels of memory related proteins, and so relate to contradictory results with inhibitors, could go on. But the essence of our argument is simply this: encoding of new information via synaptic changes under what are likely normal conditions (prior learning or, its slice equivalent, prior LTP) does not require induced synthesis. We interpret the work on induced transcription and translation not in terms of how specific memories are formed but as an effort that led to discovery of 'memory proteins' that must be available if storage is to occur. By any measure, identification of these constitutes a major advance. As a substitute for the de novo synthesis hypothesis, we propose a three stage model: (a) formation of a trace, due to rapid accumulation of transmitter receptors, followed by (b) fast and delayed consolidation stages reflecting changes in the substructure governing architecture of synapses.

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