INVESTIGATION OF THE MOLECULAR MECHANISMS OF SYNAPTIC TAGGING AND CAPTURE

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ABSTRACT

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Jung Whan Park

Ted Abel

Memory formation is continuously influenced by past, present, and future experiences. Memories linked to events that require more attention or involve emotional arousal are more persistent than ordinary memories. Information from multiple inputs that consist of memory is integrated in the hippocampus, a brain region responsible for memory storage. As a form of hippocampal long-term potentiation, pathway-specific synaptic tagging and capture (STC) has been proposed as a synaptic model of memory because it illustrates the interaction of two independent sets of synapses. This pathway-specificity is a remarkable property of neuronal signaling because it requires highly coordinated cellular signaling only at the activated synapses. However, elucidating the mechanism that is responsible for this specificity is a big challenge in the field. In my dissertation, I focused on PKA anchoring and RNA-binding proteins because they can contribute to STC through compartmentalization of PKA signaling and regulation of dendritic expression of RNAs, respectively. In **Chapter 1**, I review the mechanism of STC and discuss how compartmentalized PKA signaling contributes to STC. PKA is involved in the process of STC by orchestrating the activity of synaptic molecules and by mediating gene expression. In **Chapter 2**, I combine genetic and pharmacological approaches to determine the role of PKA anchoring in STC and memory. The results from electrophysiological, biochemical and behavioral experiments suggest that presynaptically anchored PKA contributes to STC and memory by regulating the size

of the readily releasable pool of synaptic vesicles. In **Chapter 3**, I perform genetic and viral approaches to define whether an RNA-binding protein translin (also known as testes-brain RNA-binding protein, TBRBP) is involved in STC and memory. The data from electrophysiological, behavioral and gene expression studies suggest that translin mediates STC and memory via RNA processing. Taken together, my thesis work provides evidence that presynaptic PKA anchoring-mediated synaptic vesicle release and postsynaptic processing of specific RNAs by translin are critical for STC and memory.

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Abstract

Synaptic tagging and capture (STC) hypothesis has been receiving increasing attention because it reflects heterosynaptic association of information processing during memory formation in the brain. Indeed, electrophysiological and behavioral studies suggest that STC is a better cellular model for memory formation than the conventional homosynaptic experiment. In STC, a short-lasting potentiation in one pathway becomes persistent when it is paired with a long-lasting potentiation in the other independent pathway. It has been proposed that the setting of synapse-specific tag and the capture of non-synapse-specific diffusible gene products by the tag determine the fate of each pathway. However, the mechanism of STC is still elusive and three major questions should be answered: 1. What is the tag and how does it modulate synapse-specific plasticity? 2. How does the tag capture gene products? 3. What are the gene products and how are they produced? Although several molecules and processes have been suggested to answer these questions, they only provide partial explanations about the phenomenon. This chapter will discuss how PKA modulates synapse-specific neuronal processing by coordinating signaling molecules and processes through PKA anchoring proteins, and how anchored PKA is involved in the generation and capture of plasticity related gene products.

1.1 Introduction

Synaptic plasticity, the activity-dependent change in synaptic strength, has been extensively studied as a cellular/physiological correlate of memory storage (Mark Mayford, Siegelbaum, & Kandel, 2012). Memory is stored in the hippocampus, and physiological and behavioral studies have been conducted to unravel the mechanism of memory processes in this brain area. Hippocampal long-term potentiation (LTP) has been studied as the primary model for memory storage because of its long duration, input specificity and associativity (Bliss & Gardner-Medwin, 1973; Bliss & Lomo, 1973). The hippocampal Schaffer collateral - CA1 region is an output area of the hippocampus to the cortex, and damage to CA1 impairs memory formation (Auer, Jensen, & Whishaw, 1989; Zola-Morgan, Squire, & Amaral, 1986). Various forms of synaptic plasticity in CA1 have distinct molecular requirements. Early-LTP (E-LTP), the neural correlate of short-term memory (STM), lasts 1 to 2 hours and requires NMDA receptor and Ca^{2+}/calmodulin-dependent protein kinases II (CaMKII) activation, but does not require PKA activation, transcription, and protein synthesis (Y. Y. Huang & Kandel, 1994; Tsien, Huerta, & Tonegawa, 1996). In contrast, late-LTP (L-LTP), the neural correlate of long-term memory (LTM), lasts several hours and requires PKA activation, transcription, and protein synthesis in addition to NMDA receptor and CaMKII activation (Abel et al., 1997; Frey, Frey, Schollmeier, & Krug, 1996; Frey, Krug, Reymann, & Matthies, 1988; Y. Y. Huang & Kandel, 1994; Matsushita et al., 2001; P V Nguyen & Kandel, 1997; Peter V Nguyen, Abel, & Kandel, 1994; Otmakhov et al., 2004; Tsien et al., 1996). As with L-LTP, studies of hippocampus-dependent behavioral tasks also demonstrate that PKA activation, transcription and protein synthesis are critical determinants discriminating STM from LTM formation (Abel et al., 1997; Barondes & Jarvik, 1964; Meiri & Rosenblum, 1998; Pittenger & Kandel, 1998).

These findings provide critical groundwork to understand the mechanisms underlying L-LTP and LTM. However, studies on homosynaptic LTP recordings and LTM from a single behavioral experience using naïve animals have substantial limitations because they do not reflect the complex nature of memory processing that requires integration of multiple synaptic

inputs from several interacting experiences. Memory formation is continuously influenced by past, present, and future experiences. Memories linked to events that require more attention or involve emotional arousal are more persistent than ordinary memories (Richter-Levin, Akirav, 2003). To account for this complex memory processing, a synaptic model addressing integration of multiple inputs is needed. Synaptic tagging and capture (STC), first described in rodent hippocampal CA1 and *Aplysia* neurons in 1997 (Frey and Morris 1997; Martin et al. 1997), demonstrates the association and integration of synaptic activities of two independent sets of synapses. Frey and Morris placed two stimulating electrodes on either side of a recording electrode in the hippocampal area CA1 in order to stimulate two independent sets of synapses that were converging onto the same population of CA1 neurons (**Fig. 1.1*A***). In this experiment, they showed that weak stimulation-induced E-LTP in one pathway became persistent when the weak pathway was paired with strong stimulation-induced L-LTP in the other pathway. According to the STC hypothesis, strong stimulation (S1) not only *tags* the activated synapses, but also *induces* production of gene products (Plasticity Related Products, PRPs). These tagged synapses then *capture* the gene products that migrate within the neurons. The capture of PRPs by the tag allows L-LTP expression in the S1 pathway (**Fig. 1.1*B***). On the contrary, weak stimulation (S2) only generates tags, and therefore L-LTP is not induced because PRPs are not available for the tag to capture (**Fig. 1.1*C***). However, once this S2 pathway is paired with the S1 pathway, the tag in the S2 pathway can capture PRPs produced from the S1 pathway and thereby transform E-LTP to L-LTP in the S2 pathway (**Fig 1.1*D***). Input specificity described by STC is efficient in that the tagged synapses can use the same pool of PRPs produced by the strong stimulation. The time window or the duration of the tag, which allows for successful L-LTP capture, is about 1-2 hours (Frey & Morris, 1998). Behaviorally, weak task-induced STM can also become long-lasting by a strong independent task that produces protein synthesis-dependent LTM (Ballarini, Moncada, Martinez, Alen, & Viola, 2009; Moncada & Viola, 2007; S.-H. Wang, Redondo, & Morris, 2010). Hence, heterosynaptic capture and the longer period of associativity described by STC provide a better representation of the complex integrative nature of memory processing.

Although STC has been studied by many researchers over the last decade, the identity of the tag remains elusive. The tagging process is the most critical component of STC because PRP production alone is not sufficient for L-LTP expression (Barco, Alarcon, & Kandel, 2002). Lines of evidence suggest multiple requirements for being a tag. A tag should be 1) spatially restricted to activated synapses, 2) transient and reversible, 3) interacting with PRPs, and 4) independent of protein synthesis (Kelleher, Govindarajan, & Tonegawa, 2004; Kelsey C Martin & Kosik, 2002). As many molecules and processes (*e.g.* NMDA receptor, PKA, CaMKII, TrkB, actin polymerization, *etc.*) have been independently proposed as tags (Kelsey C Martin & Kosik, 2002; Redondo & Morris, 2011), tagging likely involves the coordination of multiple molecules or processes rather than a single molecule or a single process. Kinase-mediated processes are the strongest candidate tagging mechanisms. By reversible phosphorylation of their targets, kinases provide a history of activated synapses. The most convincing evidence supporting this idea is that low frequency stimulation (LFS) resets tags and this tag-reset is mediated by increased phosphatase activities. Also, LFS-induced detagging does not affect already established STC and gene expression (Barco et al., 2002; S Sajikumar & Frey, 2004; Young, Isiegas, Abel, & Nguyen, 2006; Young & Nguyen, 2005). More importantly, increased phosphatase activities by LFS interfere with PKA activity (Young et al., 2006). If PKA is the central molecule coordinating tagging processes, the next question that arises is how such a diffusible molecule can modulate spatially restricted STC processes. In fact, PKA signaling is highly localized by a family of scaffold proteins known as A-kinase-anchoring-proteins (AKAPs) (Michel & Scott, 2002). In this article, we will discuss PKA as a key tagging molecule that modulates proposed tagging mechanisms and how AKAPs compartmentalize PKA and its targets to ensure synapse-specific tagging processes.

1.2 The role of PKA in synaptic plasticity and memory formation

In the brain, the heterosynaptic neuromodulatory system is critical for information processing, and likely a deterministic factor making memory long-lasting. Major neuromodulators in the brain are the dopaminergic, adrenergic, and serotonergic systems. These neuromodulators project to

various brain regions, including the hippocampus. At the synaptic level, they may modulate L-LTP by lowering the threshold for tag setting or PRP production (Richter-Levin & Akirav, 2003). Dopaminergic and noradrenergic signaling not only modulate electrically induced L-LTP but also facilitate L-LTP and STC expression (Connor, Wang, & Nguyen, 2011; Frey, Matthies, & Reymann, 1991; Gelinas & Nguyen, 2005; Gelinas, Tenorio, Lemon, Abel, & Nguyen, 2008; Havekes et al., 2012). Interestingly, these modulatory innervations are linked to G-protein coupled receptors (GPCRs). Upon ligand binding, GPCRs activate adenylate cyclases (ACs) to produce the ubiquitous secondary messenger, cAMP. Targets of cAMP are PKA, exchange proteins directly activated by cAMP (Epac), and the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. Although Epac and HCN channels are involved in regulation of the neuronal function, their roles in synaptic plasticity and memory are less well understood (Benarroch, 2013; Laurent, Breckler, Berthouze, & Lezoualc'h, 2012). As a major target of cAMP, the role of PKA in L-LTP and LTM has been extensively studied. Mice expressing a dominant negative PKA regulatory subunit have significantly reduced L-LTP in the area CA1 and exhibit deficits in hippocampus-dependent memory (Abel et al., 1997). The PKA inhibitors Rp-cAMP and KT5720 block L-LTP (Frey, Huang, & Kandel, 1993; Matthies & Reymann, 1993), and PKA activation mediates a form of L-LTP facilitated by β-adrenergic receptor activation in the area CA1 (Gelinas et al., 2008). Additionally, PKA activation by dopaminergic innervations is involved in LTM formation, and pharmacological activation of PKA enhances LTM formation as well as L-LTP expression (Barad, Bourtchouladze, Winder, Golan, & Kandel, 1998; Bernabeu et al., 1997). In the next section, we will expand our knowledge on the contribution of PKA to synaptic plasticity by providing evidence that supports the role of PKA in STC.

1.3 The requirement for PKA in synaptic tagging and capture

The most compelling evidence for PKA as a critical molecule for L-LTP is that direct activation of PKA by pharmacological agents such as Sp-cAMP (a cAMP analog), forskolin (FSK, adenylate cyclase activator that enhances cAMP production), and inhibitors of phosphodiesterases (PDE,

cAMP degrading enzyme) successfully induces transcription- and protein synthesis-dependent long-lasting potentiation which occludes electrically induced L-LTP (Abel et al., 1997; Frey et al., 1993; Y. Y. Huang & Kandel, 1994; Selbach, Brown, & Haas, 1998; Slack & Walsh, 1995; Woo, Abel, & Nguyen, 2002). The fact that PKA activation itself is sufficient for L-LTP expression strongly suggests that PKA mediates tagging and capture processes. Indeed, PKA is required for STC. PKA inhibitor KT5720 treatment, and expression of a dominant negative PKA regulatory subunit impair STC (Young et al., 2006). PKA activation by a PDE inhibitor, caffeine facilitates STC (Sreedharan Sajikumar, Li, Abraham, & Xiao, 2009). Also, PDE4 inhibitor, rolipram augments protein synthesis-dependent L-LTP and STC (Navakkode, Sajikumar, & Frey, 2004). Moreover, LFS activates phosphatases that resets tagging by dephosphorylation of PKA targets (S Sajikumar & Frey, 2004; Young et al., 2006). In line with this, inhibition of protein phosphatase 1 (PP1) by PKA is critical for L-LTP expression, and PP1 inhibitors rescues L-LTP deficits in mice expressing a dominant negative PKA regulatory subunit (Blitzer et al., 1998; Woo et al., 2002). Importantly, PKA inhibitors are effective only when they are treated during the induction phase of STC (Barco et al., 2002; Young et al., 2006). This transient involvement of PKA in STC complies with the requirement for being a tag molecule (see sect. 1.1). However, PKA is not the only tag molecule that has been proposed, and we will discuss how PKA interacts with other candidate tagging mechanisms in the next section.

1.4 Other potential tagging mechanisms possibly mediated by PKA

NMDA Receptors (NMDARs) are required for L-LTP and LTM (Morris, Anderson, Lynch, & Baudry, 1986; P V Nguyen & Woo, 2003). The NMDAR has been suggested as a tagging molecule because the NMDAR inhibitor AP5 blocks STC, although PRPs are still available for being captured (Barco et al., 2002). It is worth noting that Ca^{2+}-influx through NMDARs activates Ca^{2+}-dependent ACs, which results in the activation of PKA. Also, increased Ca^{2+} permeability of the NMDAR by PKA phosphorylation facilitates LTP (Skeberdis et al., 2006). Therefore PKA, at least in part, mediates tagging processes involving NMDARs.

CaMKII is activated by Ca^{2+}-influx through NMDARs after LTP induction, and is another strong candidate tag molecule. Pharmacological inhibition of CaMKII blocks STC, providing evidence that STC requires CaMKII. Also, inhibition of CaMKII does not affect PRP production and subsequent capture of PRPs (Redondo et al., 2010). Following NMDAR stimulation, an active form of CaMKII moves to the post synaptic density (PSD) of activated dendritic spines, then phosphorylates its targets (Shen & Meyer, 1999). Because PKA modulates NMDAR-mediated Ca^{2+} signaling, it is likely that PKA and CaMKII act in concert in the process of tagging. Indeed, it has been reported that inhibition of PP1 by PKA gates CaMKII signaling by preventing dephosphorylation of CaMKII during L-LTP expression (Blitzer et al., 1998). However, the role of CaMKII as a tag is not clear. Unlike PKA phosphorylation of AMPA receptor (AMPAR) subunit GluR1 at S845, CaMKII mediated phosphorylation of GluR1 at S831 does not increase open probability of the receptor (Banke et al., 2000). In addition, a GFP reporter flanked by the 5'- and 3'-UTR of CaMKII shows local dendritic synthesis after stimulation, and the protein level of CaMKII increases in dendrites within 5 minutes after tetanization (Aakalu, Smith, Nguyen, Jiang, & Schuman, 2001; Ouyang, Rosenstein, Kreiman, Schuman, & Kennedy, 1999). Dendritic local synthesis of CaMKII does not comply with the criteria for being a tag, since the tagging process is independent of protein synthesis. Therefore, CaMKII is possibly a component of PRPs.

TrkB is a tyrosine kinase that has been suggested as a potential tag. Its ligand is brain derived neurotrophic factor (BDNF), which will be discussed later as a strong candidate PRP (see sect. 5.5). Bath application of BDNF induces protein synthesis-dependent long-lasting potentiation, suggesting that BDNF-TrkB signaling is sufficient to elicit tagging and capture processes as PKA activation does (Kang, Jia, Suh, Tang, & Schuman, 1996; Messaoudi, Ying, Kanhema, Croll, & Bramham, 2002). Inhibition of TrkB blocks STC and a behavioral version of tagging, and TrkB activation after stimulation lasts about 1-2 hours. Also, TrkB activation does not require protein synthesis (Lu et al., 2011). Therefore, TrkB satisfies the requirements for a tag. In fact, PKA activation gates BDNF induced TrkB phosphorylation (Ji, Pang, Feng, & Lu, 2005). In addition, TrkB phosphorylation is increased after PKA activation by forskolin (Patterson et al., 2001). This

suggests that PKA is upstream of BDNF-TrkB signaling and that the described roles of TrkB as a tag are likely modulated by PKA.

Actin Dynamics are important for the structural modification of synapses and memory formation (Chen, Rex, Casale, Gall, & Lynch, 2007; Y.-Y. Hou et al., 2009; Krucker, Siggins, & Halpain, 2000; Lisman, 2003). NMDAR-dependent actin polymerization in dendritic spines is critical for L-LTP (B. Lin et al., 2005), and BDNF signaling modulates this process (Rex et al., 2007). LFS reverses LTP by depolymerizing actin (Kramár, Lin, Rex, Gall, & Lynch, 2006). Moreover, inhibition of actin polymerization impairs tagging process but does not affect PRP production (Ramachandran & Frey, 2009). Actin remodeling is controlled by cofilin, an actin depolymerizing factor. Cofilin-mediated actin dynamics regulate spine morphology and AMPAR trafficking during synaptic plasticity (Chen et al., 2007; J. Gu et al., 2010). Phosphorylation of cofilin by LIM kinase inhibits its activity, which allows actin polymerization. In fact, the activity of LIM kinase is modulated by PKA (Lamprecht & LeDoux, 2004; Nadella et al., 2009).

1.5 Plasticity related products

As a tag, PKA interacts with plasticity related products (PRPs) by regulating the synthesis and function of these gene products. PRPs are produced from gene transcription and protein synthesis after neuronal activity. While it was initially believed that only proteins produced in the soma serve as PRPs, it is now widely accepted that mRNAs also serve as PRPs by its dendritic targeting and subsequent local dendritic translation. mRNAs packaged in RNA granules are transported close to synapses in a translationaly silent state (Krichevsky & Kosik, 2001; M Mayford, Baranes, Podsypanina, & Kandel, 1996; D. O. Wang, Martin, & Zukin, 2010). Upon LTP induction, polyribosomes and local translation machinery at spine necks are activated to translate these locally targeted mRNAs (Kelleher, Govindarajan, & Tonegawa, 2004; Ostroff, Fiala, Allwardt, & Harris, 2002; Oswald Steward & Schuman, 2001).

PKA activates the transcription factor cAMP response element (CRE)-binding protein (CREB) to promote CRE-driven gene expression critical for both L-LTP and LTM (Impey et al., 1996; Impey, Smith, et al., 1998). In addition, CREB-dependent gene expression facilitates synaptic plasticity including STC in both *Aplysia* and mice (Barco et al., 2002; Casadio et al., 1999; K C Martin et al., 1997). Thus, CREB mediated CRE-driven gene expression provides a pool of PRPs critical for both L-LTP and LTM. Infusion of PKA inhibitor into the nucleus blocks CREB phosphorylation and impairs L-LTP, but not E-LTP (Matsushita et al., 2001). PKA facilitates nuclear translocation of extracellular-signal-regulated protein kinase (ERK), which leads to CREB phosphorylation (Impey, Obrietan, et al., 1998; Patterson et al., 2001; Roberson et al., 1999). Therefore, PKA directly and indirectly activates CREB. In addition to nuclear gene transcription, translation of mRNAs contributes to PRP production. Dendritic protein synthesis is mainly controlled by mitogen-activated protein kinase (MAPK), BDNF-TrkB signaling pathways, and actin dynamics (Kelleher, Govindarajan, Jung, Kang, & Tonegawa, 2004; Kuczewski, Porcher, & Gaiarsa, 2010; Santos, Comprido, & Duarte, 2010). PKA cross-talks with the MAPK pathway at multiple levels (Gerits, Kostenko, Shiryaev, Johannessen, & Moens, 2008) and modulates BDNF-TrkB signaling and actin dynamics as described earlier (see sect. 5.4). Collectively, PKA attributes to PRP production both at the level of transcription and protein synthesis.

As a CREB target gene, BDNF has been proposed as the strongest candidate PRP (Barco et al., 2005; Tao, Finkbeiner, Arnold, Shaywitz, & Greenberg, 1998). Activity-dependent dendritic targeting and expression of BDNF and TrkB support this idea (Tongiorgi, Righi, & Cattaneo, 1997). BDNF promotes synaptic remodeling through actin dynamics, PSD reconstitution, and local translation during L-LTP (Kang & Schuman, 1996; Liao et al., 2007; Rex et al., 2007; Yoshii & Constantine-Paton, 2007). Additionally, BDNF deletion in the hippocampus impairs STC (Barco et al., 2005). These observations suggest multiple roles of BDNF such that it is not only produced as a PRP but also induces production of other PRPs and aids tag setting, all of which are modulated by PKA (also see sect. 5.4 TrkB).

Another well-known candidate PRP is the AMPAR. AMPAR trafficking and incorporation into synapses that are regulated by both actin and PKA are critical for L-LTP expression (Esteban et al., 2003; Malinow, Mainen, & Hayashi, 2000; Sheng & Lee, 2001). PKA phosphorylation at S845 of GluR1 promotes an increase in open probability, frequency, and duration of the receptor that leads to stable LTP expression (Banke et al., 2000; Esteban et al., 2003; Greengard, Jen, Nairn, & Stevens, 1991). Although AMPARs can be considered as tags, since their activity and incorporation make synapses more excitable, they are more likely PRPs because their requirement for synaptic plasticity is not transient and their synthesis is required for their rapid turnover. Indeed, the maintenance phase of LTP requires a PKA-mediated increase in AMPAR synthesis 3 hours after LTP induction (Nayak, Zastrow, Lickteig, Zahniser, & Browning, 1998; Yao et al., 2008). Taken together, PKA regulates the property, trafficking, as well as synthesis of AMPARs to ensure L-LTP expression.

1.6 The role of PKA anchoring in synaptic plasticity and memory

In the previous sections, we discussed PKA as a key molecule mediating the processes of STC, in which synaptic inputs from the two independent pathways (S1 and S2) are integrated. This pathway-specificity is a unique property of neuronal communication that can only be achieved by highly compartmentalized and spatially restricted cellular signaling. This is surprising because secondary messengers including cAMP, kinases such as PKA, mRNAs, and many proteins are diffusible throughout the cell. This suggests that there is a way to localize signaling molecules together to ensure spatially restricted signaling. Spatially compartmentalized PKA signaling is achieved by PKA anchoring proteins, or AKAPs. There are more than 50 AKAPs that are localized to specific intracellular regions. By binding PKA regulatory subunits as well as other signaling molecules, AKAPs provide a compartmentalized pool of PKA signaling (Marcie Colledge & Scott, 1999; Michel & Scott, 2002). The importance of the compartmentalized PKA signaling in synaptic plasticity and memory formation is confirmed by pharmacological and genetic disruption of PKA anchoring by the PKA anchoring disrupting peptide Ht31. This peptide is derived from the

human thyroid anchoring protein that binds PKA and has been used to block anchoring of PKA without affecting PKA activity (Marcie Colledge & Scott, 1999). Conditional expression of Ht31 in neurons within the hippocampus impairs hippocampal L-LTP and hippocampus-dependent spatial memory, and reduces GluR1 S845 phosphorylation (Myungsook Kim et al., 2011; Nie, McDonough, Huang, Nguyen, & Abel, 2007). Similar to PKA, the application of Ht31 peptide at different time point reveals that PKA anchoring is transiently required for L-LTP, not E-LTP, and STC during the induction phase (Havekes et al., 2012; T. Huang, McDonough, & Abel, 2006). In addition, PKA anchoring disruption by Ht31 reduces synaptic AMPARs and AMPAR currents and occludes long-term depression (LTD). (Rosenmund et al., 1994; E. M. Snyder et al., 2005). It should be noted that the effect of Ht31 directly matches the effect of PKA inhibition in synaptic plasticity and memory formation (see sect. 5.2 and 5.3). This suggests that PKA exerts its activity through anchored signaling complexes controlled by AKAPs.

1.7 Examples of AKAPs modulating neuronal function

In the brain, several AKAPs have been identified as scaffold proteins that tie PKA signaling to Ca^{2+} signaling, MAPK signaling, cytoskeletal dynamics, and gene expression mechanisms. Therefore, AKAPs contribute to the formation of highly coordinated signalosomes that are critical for synaptic plasticity and neuronal information processing.

AKAP5/79/150 is targeted to the plasma membrane and associated with PSD-95. It recruits NMDARs, AMPARs, $GABA_A$ receptors, L-type Ca^{2+} channels, K^+ channels, synapse-associated protein (SAP)-97, PKC, protein phosphatase 2B (PP2B or calcineurin), β-adrenergic receptors (β-ARs), as well as PKA (Brandon et al., 2003; Bregman, Bhattacharyya, & Rubin, 1989; Carr, Stofko-Hahn, Fraser, Cone, & Scott, 1992; Coghlan et al., 1995; M Colledge et al., 2000; Fraser et al., 2000; T. Gao et al., 1997; Gomez, Alam, Smith, Horne, & Dell'Acqua, 2002; Hoshi et al., 2003). Deletion of AKAP5/79/150 leads to impaired synaptic plasticity, altered AMPAR currents, and disrupted hippocampus-dependent spatial memory. AKAP5/79/150 also mediates nuclear

PKA signaling, and perturbation of PKA anchoring reduces nuclear CREB phosphorylation (Feliciello et al., 1996; Feliciello, Li, Avvedimento, Gottesman, & Rubin, 1997).

Gravin (AKAP12/250), also known as Src-suppressed C kinase substrate (SSecks) in mice, binds not only PKA but also other signaling molecules including PKC, calmodulin, PP2B, β-ARs, actin, and PDE4D (X Lin, Tombler, Nelson, Ross, & Gelman, 1996; Xueying Lin & Gelman, 2002; Nauert, Klauck, Langeberg, & Scott, 1997; Shih, Lin, Scott, Wang, & Malbon, 1999; Willoughby, Wong, Schaack, Scott, & Cooper, 2006). By bringing PDEs close to ACs, Gravin provides cAMP gradients to shape compartmentalized PKA signaling. Gravin is also localized to the actin cytoskeleton and regulates actin remodeling (Gelman, Lee, Tombler, Gordon, & Lin, 1998; X Lin et al., 1996). Its localization to the plasma membrane, the endoplasmic reticulum, and the perinuclear region has also been reported (Streb, Kitchen, Gelman, & Miano, 2004). Along with AKAP 5/79/150, it mediates PKA phosphorylation of β-ARs that leads to desensitization of the receptor and activation of the MAPK pathway (Baillie et al., 2003; Daaka, Luttrell, & Lefkowitz, 1997). Mice lacking the α-isoform of Gravin show impaired PKA-dependent L-LTP, β-AR-mediated metaplasticity, and hippocampus-dependent contextual fear memory, possibly due to reduced phosphorylation of β-ARs and MAPK (Havekes et al., 2012). Interestingly, FSK-mediated long-lasting potentiation is not affected in these mice, suggesting that the cell-wide activation of PKA overcomes compartmentalization barriers or that the presence of other AKAPs is sufficient to support this form of potentiation (Havekes et al., 2012).

Microtubule-Associated Protein 2 (MAP2) is the first identified AKAP. As a predominantly expressed AKAP in the brain, MAP2 binds a third of neuronal PKA, and regulates microtubule stabilization and long-distance transport along dendrites and axons (Sánchez, Díaz-Nido, & Avila, 2000; Theurkauf & Vallee, 1982). As a dominant AKAP, MAP2 establishes a pool of PKA along dendritic shafts so that, upon cAMP elevation, catalytic subunits of PKA can rapidly translocate to dendritic spines for synaptic plasticity (Zhong et al., 2009). In addition, loss of MAP2 results in reduction of the total amount of PKA and CREB phosphorylation (Harada, Teng, Takei, Oguchi, &

Hirokawa, 2002). The latter suggests MAP2-mediated synapse-to-nucleus signaling of PKA. Deletion of the PKA binding site of MAP2 results in abnormal CA1 architecture and disruption of contextual fear memory (Khuchua et al., 2003). Also, MAP2 mRNA is the first mRNA found to be targeted to dendrites for subsequent local synthesis of the protein (Garner, Tucker, & Matus, 1988; O Steward & Halpain, 1999).

1.8 PKA-centric unified model of synaptic tagging and capture

Since first being described in 1997, a large number of studies have proposed various molecules and processes as the mechanisms of synaptic tagging and capture. Although the identity of tagging and capture processes is still elusive, it is likely the collective interaction of molecules, rather than a single molecule, that accounts for these processes. Considering the crucial role of PKA in synaptic plasticity and memory formation, a AKAP-mediated compartmentalized pool of signaling complexes could contribute to the heterosynaptic nature of information processing in the brain, here represented as STC.

When a set of synapses receives supra-threshold stimulation, Ca^{2+}-influx through NMDARs and activation of neuromodulatory GPCRs trigger a large increase of cAMP production by ACs. Following the cAMP wave, a large amount of PKA is activated from both the reserve pool in the dendritic shafts maintained by MAP2 and the local pool maintained by AKAPs in the spine. Activated dendritic PKA then enters the activated spine and interacts with NMDARs, AMPARs, TrkB, and Ca^{2+} signaling cascades in concert with locally activated PKA in the spine (tagging). Having a reserve pool in dendritic shafts is an efficient way to supply PKA to activated synapses on demand. PKA from this reserve pool promotes gene transcription by activating CREB in the nucleus. Also, the cross-talk between PKA and MAPK signaling initiates protein synthesis to produce PRPs such as BDNF. PKA gates subsequent BDNF-TrkB signaling to augment protein synthesis and synaptic remodeling. In addition, PKA regulates AMPAR trafficking via actin

dynamics (capture). AKAPs tightly regulate all of these processes by clustering signaling components so that stable L-LTP in this set of synapses is ensured (**Fig. 1.2*A***).

When a set of synapses receives sub-threshold stimulation, the amount of activated PKA is not sufficient to induce PRP production, and E-LTP is induced. PKA bound to only local AKAPs tags the set of synapses by priming synaptic proteins such as NMDARs, AMPARs, and TrkB (**Fig. 1.2*B***). If this E-LTP pathway is paired with the L-LTP pathway, PRPs produced by the L-LTP pathway can be captured by the E-LTP pathway, so that E-LTP is transformed to L-LTP. For example, BDNF produced by the L-LTP pathway can strengthen synaptic connection of the weak pathway by interacting with the *primed* signaling molecules such as TrkB (**Fig. 1.2*C***).

In summary, PKA activation through NMDAR activity alone can only set tags at a subset of synapses by priming local targets such as NMDARs, AMPARs and TrkB. PKA phosphorylation of these targets fades over time, which creates a limited time window of tagging. However, PKA activation through both NMDARs and neuromodulatory GPCRs triggers PRP production as well as local tag setting. PKA-mediated capture processes involve the interaction of PRPs with their signaling partners that have already been primed by PKA (*e.g.* BDNF-TrkB signaling gated by PKA). Finally, AKAP supervises the heterosynaptic coordination of complex signaling by tethering signaling participants together at the synapse.

1.9 Remaining challenges to elucidate the mechanism of synaptic tagging and capture

Most of the literature included in this chapter focuses on the postsynaptic mechanisms of STC. Due to technical challenges, it is hard to assess potential presynaptic components of STC. However, there is evidence supporting the presynaptic role of PKA activity and PKA anchoring in L-LTP and memory formation. Long-lasting potentiations induced by Sp-cAMP or FSK relies on increased presynaptic transmitter release (Bolshakov, Golan, Kandel, & Siegelbaum, 1997; Chavez-Noriega & Stevens, 1994). Additionally, studies using transgenic mice expressing Ht31

suggest that presynaptic CA3-PKA anchoring is required for theta-burst L-LTP and spatial memory (Nie et al., 2007). Moreover, the induction of theta-burst L-LTP increases the release of BDNF as well as synaptic vesicles from presynaptic terminals (Zakharenko et al., 2003). Also, BDNF deletion in both CA3 and CA1 results in complete impairment of STC, while BDNF deletion in only postsynaptic CA1 has a delayed impairment suggesting that BDNF in presynaptic CA3 has a role in the early phase of STC (Barco et al., 2005). In **Chapter 2**, I will investigate the presynaptic role of PKA anchoring in STC.

As briefly discussed in section 1.5, dendritically transported mRNAs and their local translation provides a dendritic pool of PRPs for STC. However, molecules responsible for dendritic transport and translation of mRNAs are not well described. RNA-binding proteins potentially mediate these processes because they regulate stability, movement, and expression of bound mRNAs (Ule & Darnell, 2006). The most extensively studied RNA-binding protein is fragile X mental retardation protein (FMRP). FMRP mediates dendritic transport of certain mRNAs and controls their translation activity-dependent manner (Sidorov, Auerbach, & Bear, 2013). However, intact STC in FMRP KO mice (Connor, Hoeffer, Klann, & Nguyen, 2011) suggests that other RNA-binding proteins can mediate these processes. In **Chapter 3**, I will examine the role of an RNA-binding protein translin, also known as testis-brain RNA-binding protein (TBRBP), in STC.

Figure Legends

Figure 1.1. Synaptic tagging and capture.

A*.** Schematic diagram of the two-pathway experiment. Two stimulating electrodes are positioned to stimulate two independent pathways (S1 and S2) converging onto the same CA1 neurons. ***B. A strong stimulation activates local tags and induces PRP production. The capture of PRPs by the activated tags allows L-LTP expression in the S1 pathway. ***C*.** A weak stimulation only activates local tags and induces E-LTP in the S2 pathway. ***D*.** Pairing of S1 and S2 pathways results in capture of PRPs from the S1 pathway by activated tags in the S2 pathway which transforms E-LTP to L-LTP in the S2 pathway. Left lower insets are representative traces (black: baseline, red: hours after stimulation), and right lower insets are representative slope recordings of field excitatory postsynaptic potentials (fEPSP) over hours. (Modified with permission from Ted Huang Ph.D.)

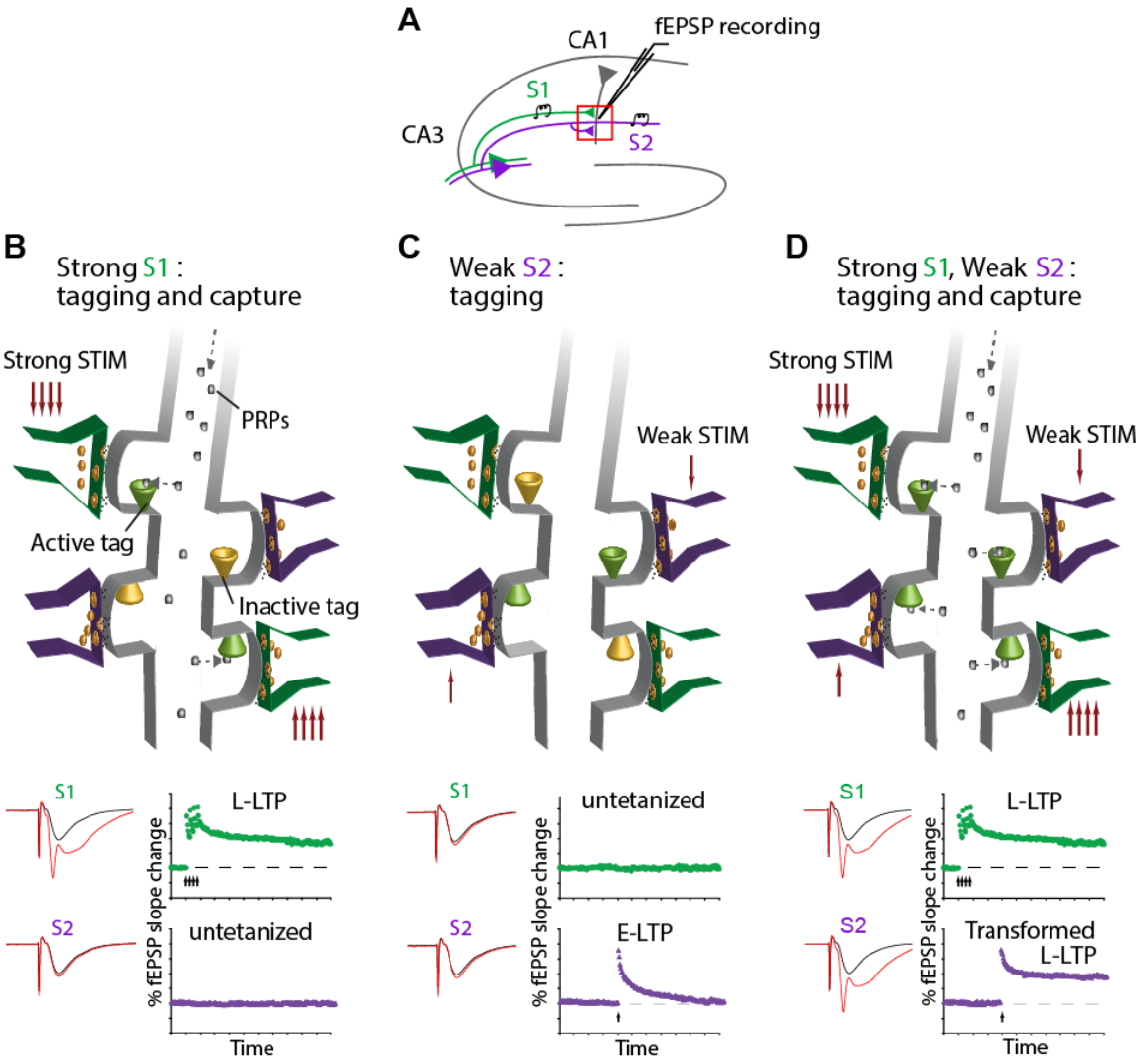
Figure 1.2. A PKA-centric model of synaptic tagging and capture.

***A*.** In strong pathway, a large amount of cAMP is produced by adenylyl cyclases (ACs) through NMDAR- and GPCR-mediated processes, which in turn activates PKA from both the local AKAP pool in the spine and the dendritic MAP2 pool. Once activated, PKA catalytic subunits are liberated from PKA regulatory subunits to induce CREB-mediated gene expression and somatic/dendritic protein synthesis to produce PRPs, as well as tagging the activated synapses by priming molecules in the spine (e.g. NMDARs, AMPARs, and TrkB). Upon arrival of PRPs into the spine, only the tagged synapses interact with/capture PRPs (possibly through AMPAR trafficking and BDNF-TrkB signaling) to strengthen the pathway.

***B*.** Weak pathway has a sub-threshold level of cAMP produced by NMDAR-mediated Ca^{2+}-dependent AC activation resulting in local activation of PKA only in the spine that establishes tags in the synapses, but does not induce PRP production. ***C*.** The weak pathway is strengthened if PRPs from the strong pathway are captured by the tags when the two pathways are paired.

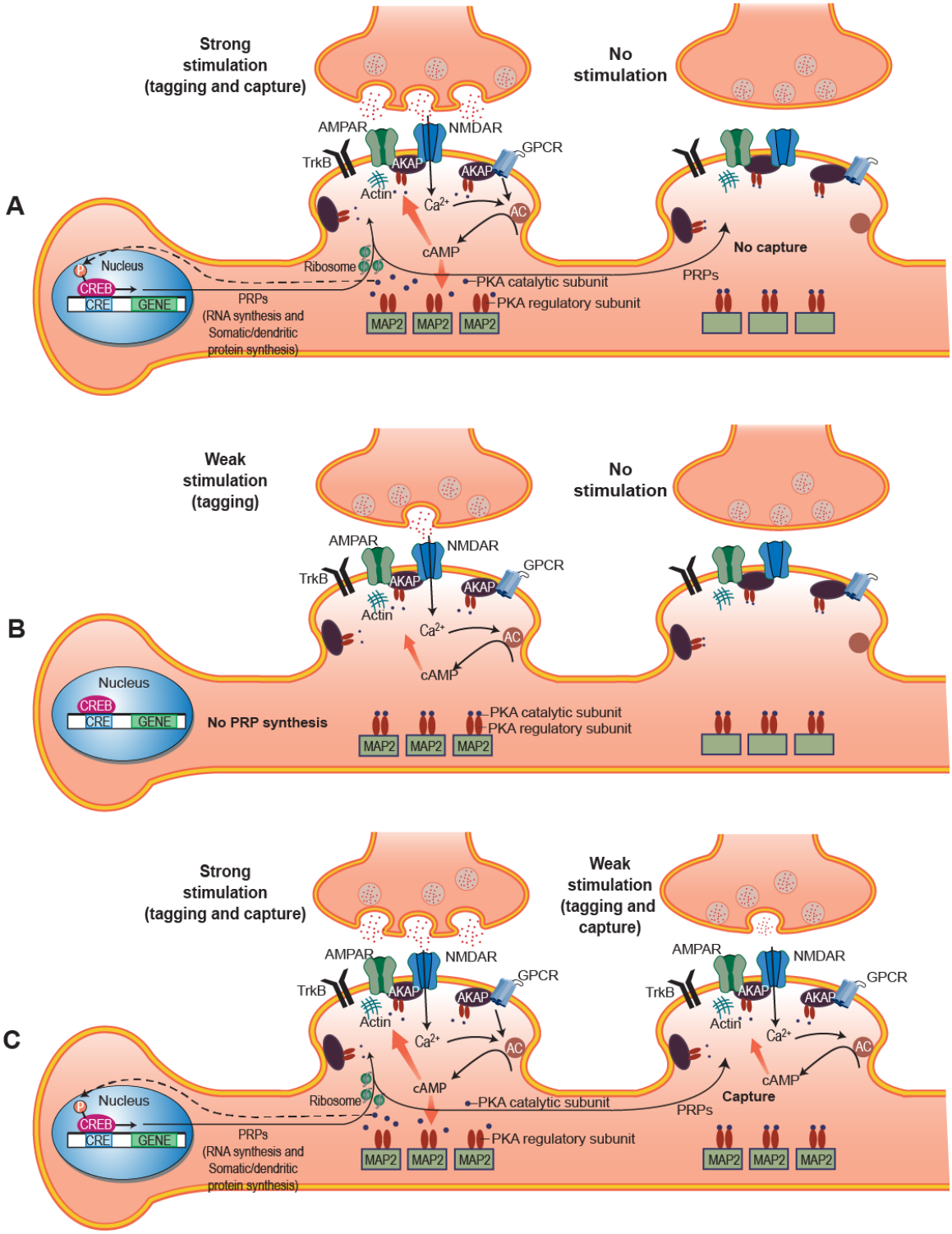
Figures

Figure 1.1



Modified with permission from Ted Huang Ph.D

Figure 1.2


A
Strong stimulation (tagging and capture)
No stimulation
AMPAR
NMDAR
TrkB
AKAP
GPCR
Actin
Ca2+
AC
cAMP
Ribosome
Nucleus
CREB
CRE
GENE
PRPs
(RNA synthesis and Somatic/dendritic protein synthesis)
PKA catalytic subunit
PKA regulatory subunit
MAP2
No capture
PRPs
B
Weak stimulation (tagging)
No stimulation
No PRP synthesis
C
Strong stimulation (tagging and capture)
Weak stimulation (tagging and capture)
Capture

Author Contributions

Jung Whan (Alan Jung) Park wrote this chapter with comments and suggestions by Morgan Bridi, Vincent Luczak, and Ted Abel.

CHAPTER 2: A presynaptic role for PKA in synaptic tagging and memory

Abstract

Protein kinase A (PKA) and other signaling molecules are spatially restricted within neurons by A-kinase anchoring proteins (AKAPs). Although studies on compartmentalized PKA signaling have focused on postsynaptic mechanisms, presynaptically anchored PKA may contribute to synaptic plasticity and memory because PKA also regulates presynaptic transmitter release. Here, we examine this issue using genetic and pharmacological application of Ht31, a PKA anchoring disrupting peptide. At the hippocampal Schaffer collateral CA3-CA1 synapse, Ht31 treatment elicits a rapid decay of synaptic responses to repetitive stimuli, indicating a fast depletion of the readily releasable pool of synaptic vesicles. The interaction between PKA and proteins involved in producing this pool of synaptic vesicles is supported by biochemical assays showing that synaptic vesicle protein 2 (SV2), Rim1, and SNAP25 are components of a complex that interacts with cAMP. Moreover, acute treatment with Ht31 reduces the levels of SV2. Finally, transgenic mouse lines expressing Ht31 in excitatory neurons at the Schaffer collateral CA3-CA1 synapse highlight a requirement for presynaptically anchored PKA in pathway-specific synaptic tagging and long-term contextual fear memory. These results suggest that a presynaptically compartmentalized PKA is critical for synaptic plasticity and memory by regulating the readily releasable pool of synaptic vesicles.

Introduction

Diffusible PKA signaling molecules are spatially restricted at synaptic loci by A-kinase anchoring proteins (AKAPs), which allow input-specific synaptic plasticity, such as synaptic tagging (T. Huang et al., 2006; J. L. Sanderson & Dell'Acqua, 2011). Synaptic tagging is a pathway-specific form of hippocampal long-term potentiation (LTP) in which short-lasting LTP following weak stimulation in one pathway is stabilized into long-lasting LTP by subsequent strong stimulation in a separate pathway (Frey & Morris, 1997, 1998). Because of the pathway-specific interaction between two independent sets of synapses, synaptic tagging has been studied as a physiological model for memory that requires the integration of independent, but related, information (Clopath, 2012; Redondo & Morris, 2011). However, it is not clear how compartmentalized PKA signaling contributes to synaptic tagging and memory.

A major question in understanding the physiological role of PKA anchoring is whether spatially restricted PKA exerts its action presynaptically or postsynaptically. Studies of compartmentalized PKA signaling have largely focused on AKAP signaling complexes. For example, AKAP150 (AKAP5) and Gravin (AKAP12) postsynaptically coordinate AMPA receptor trafficking or β-adrenergic receptor signaling to mediated synaptic plasticity and memory (Havekes et al., 2012; Robertson, Gibson, Benke, & Dell'Acqua, 2009; J. L. Sanderson et al., 2012; M. Zhang et al., 2013). However, the genetic disruption of PKA binding to AKAP150 does not cause deficits in hippocampus-dependent spatial memory (Weisenhaus et al., 2010), and postsynaptic blockade of PKA anchoring is insufficient to impair certain forms of LTP and spatial memory (Nie et al., 2007). These findings suggest a presynaptic role for compartmentalized PKA signaling in synaptic plasticity and memory, but how presynaptically anchored PKA is involved in these processes is unknown.

At the presynaptic terminal, activation of cAMP-PKA signaling enhances synaptic vesicle release by increasing the size of the readily releasable pool (Moulder et al., 2008; Trudeau, Emery, & Haydon, 1996). PKA substrates such as Rim1 and SNAP25 are involved in this

process, and deletion of Rim1 impairs memory (Jahn & Fasshauer, 2012; Powell et al., 2004). Although it is not a direct PKA substrate, synaptic vesicle protein 2 (SV2) is postulated to interact with cAMP-PKA signaling and also modulates the size of the readily releasable pool of synaptic vesicles (Custer, Austin, Sullivan, & Bajjalieh, 2006; Luo et al., 2009; Pyle, Schivell, Hidaka, & Bajjalieh, 2000; T. Xu & Bajjalieh, 2001). Therefore, compartmentalized PKA signaling may contribute to neuronal function by regulating the readily releasable pool of synaptic vesicles. To investigate the presynaptic role for compartmentalized PKA in synaptic plasticity and memory, we used genetic and pharmacological application of Ht31, a peptide that blocks PKA anchoring (Carr, Hausken, Fraser, Stofko-Hahn, & Scott, 1992; T. Huang et al., 2006; Nie et al., 2007). Our data suggest that presynaptic PKA anchoring maintains the size of the readily releasable pool of synaptic vesicles via SV2, thereby mediating synaptic tagging and long-term memory.

Materials and Methods

Ht31 transgenic mice

We used two transgenic mouse lines expressing Ht31, a PKA anchoring blocking peptide that contains the PKA binding site of AKAP-Lbc (Klussmann et al., 2001), in forebrain regions (Nie et al., 2007). Briefly, tetO-Ht31 line 1 and line 16 mice were crossed with CaMKIIα-tTA line B mice (M Mayford et al., 1996). Resulting bigenic offsprings carrying both the tetO-Ht31 transgene and the CaMKII-tTA transgene are referred to as Ht31 transgenic mice. Bigenic tetO-Ht31 line 1 mice are referred to as Ht31(1) mice, and bigenic tetO-Ht31 line 16 mice are referred to as Ht31(16) mice. Littermates that do not carry any of these transgenes or carry a single transgene are referred to as wild-type littermates. Mice were backcrossed to C57BL/6J for more than 15 generations. All experiments were performed according to the National Institutes of Health guidelines and were fully approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Reagents

Stearated-Ht31 (stHt31) and -Ht31P (stHt31P, a peptide identical to stHt31 except for proline substitutions to prevent binding to PKA) peptides were produced by American Peptide Company, Inc. (Sunnyvale, CA). The sequences of the peptides are: stHt31, St-N-DLIEEAASRIVDAVIEQVKAAGAY-C; stH31P, St-N-DLIEEAASRPVDAVPEQVKAAGAY-C (Huang et al., 2006). The peptides were delivered as lyophilized powder, resuspended at a stock concentration of 10 mM in 50 mM Tris–HCl (pH 7.0) and were used at 100 µM final concentration in artificial cerebrospinal fluid (aCSF). Forskolin (FSK, Molecular grade, Sigma), an adenylyl cyclase activator, was freshly prepared as a 50 mM solution in 100% ethanol and delivered at 50 µM final concentration in aCSF as previously described (Havekes et al., 2012).

In situ hybridization

Ht31 mRNA expression patterns of both transgenic mice were confirmed by *in situ* hybridization using an [α - S^{35}] dATP-labeled oligonucleotide probe (5'-CTCGGTTTATCGCCTGGGTCATTGGGCCTTGC-3') specific to the Ht31 transgene sequence as previously described (Nie et al., 2007). Three animals (2-5-month-old) per group were used, and films were exposed for 2 weeks in the cold room. The films were then developed and scanned for analysis.

Electrophysiology

Experiments were performed in the hippocampal Schaffer collateral pathway as previously described (Bridi & Abel, 2013; Havekes et al., 2012; Vecsey et al., 2009). Briefly, both male and female 2-5-month-old mice were sacrificed by cervical dislocation, and hippocampi were quickly collected in chilled, oxygenated aCSF containing 124 mM NaCl, 4.4 mM KCl, 1.3 mM $MgSO_4 \cdot 7H_2O$, 1 mM $NaH_2PO_4 \cdot H_2O$, 26.2 mM $NaHCO_3$, 2.5 mM $CaCl_2 \cdot 2H_2O$, and 10 mM D-glucose bubbled with 95% O_2 / 5% CO_2. 400 µm thick transverse hippocampal slices were placed in an interface recording chamber at 28ºC (Fine Science Tools, Foster City, CA). Slices were equilibrated for at least 2 hours in aCSF (pH 7.4). The stimulus strength was set to elicit 40% of the maximum field excitatory postsynaptic potential (fEPSP) amplitude. The average of the baseline initial fEPSP slope values over the first 20 min was used to normalize each initial fEPSP slope.

Synaptic tagging experiments were performed as previously described (T. Huang et al., 2006). Briefly, weak stimulation of one train (one 1 s 100 Hz train) was delivered to elicit short-lasting LTP in one pathway. Strong stimulation of massed 4-train (four 1 s 100 Hz trains delivered 5 seconds apart) was delivered to elicit long-lasting LTP in the other pathway. After the 20-min baseline recordings, one train was delivered first to one pathway (S1) and massed four-train was followed in the other pathway (S2) 30 min after the S1 stimulation (**Fig. 5B**). Paired pulse facilitation (PPF) at 50 ms interval was used to confirm the independence of the two inputs. For FSK and stHt31 peptide experiments, 50 µM of FSK was perfused over slices for 15 minutes after

20-min baseline recordings. stHt31 or the control stHt31P peptide was delivered for a total of 45 minutes starting 15 min prior to the FSK application.

To examine how PKA anchoring disruption physiologically affects FSK-induced long-lasting potentiation, slices from 2-month-old male C57BL/6J mice were co-treated with FSK and either stHt31 or the control stHt31P peptide as described above. Synaptic properties were measured 5 minutes after the FSK treatment. The time point was selected based on the observation that both PKA activity and PKA anchoring are required only during the induction phase for long-lasting LTP and synaptic tagging (Barco et al., 2002; T. Huang et al., 2006; Young et al., 2006), and we found that FSK treatment starts to potentiate fEPSPs at this time point (Havekes et al., 2012; Myungsook Kim et al., 2011). The input-output curve was examined by measuring initial fEPSP slopes in response to increasing stimulation intensities. Following measurement of the input-output curve, the stimulus strength was set to elicit 40% of the maximum fEPSP amplitude, and PPF at 50 ms was measured. To measure the size of the readily releasable pool of synaptic vesicles, 10 Hz repetitive stimulations were delivered for 3 seconds.

Western blotting

Hippocampal slices from 2-month-old male C57BL/6J mice were co-treated with FSK and either stHt31 or the control stHt31P peptide as described above. 15 sections of stratum radiatum of CA1 were collected 5 minutes after the FSK treatment and pooled as one sample. Frozen sections were homogenized in lysis buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris pH 8, 10% glycerol, 1% NP40, 0.1% SDS, 0.1% TritonX-100, 10 mM Na_4VO_3) with protease and phosphatase inhibitors. Proteins were separated by 4-20% Tris-Glycine SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 5% BSA-TBST or 5% milk + 0.5% BSA-TBST and incubated overnight at 4°C with primary antibodies (SV2, 1:10,000, Buckley and Kelly, 1985; Rim1, 1:1000, Synaptic Systems; synapsin1, 1:1000, Cell Signaling; phospho-synapsin1, 1:2000, Cell Signaling; synaptophysin, 1:500, Millipore; synaptotagmin1, 1:500, Millipore; SNAP25, 1:2000, Abcam). The membranes were washed and incubated with

appropriate horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (1:5000, Santa Cruz) for 1 hour at room temperature. Blots were exposed using ImageQuant LAS 4000 digital imaging system, quantified using ImageJ, and the density of signal was normalized to β-tubulin levels (1:20,000, Sigma).

Contextual fear conditioning

Two to five-month-old male and female Ht31 transgenic mice and wild-type littermates were single housed one week before behavioral experiments under a 12 hour light/12 hour dark cycle with lights on at 7 am. Food and water were available *ad libitum*. Sexes were balanced across groups. All experiments were conducted between 2-6 hours after lights on. Mice were handled for 3 minutes per day for 6 consecutive days prior to the experiments. The experiments were performed as previously described (Havekes et al., 2012). Briefly, mice were allowed to explore the conditioning box for 2 minutes and 28 seconds followed by a 2 second 1.5 mA foot shock. Either 1 h (short-term memory test) or 24 h (long-term memory test) later, mice were returned to the same chamber for 5 minutes to assess contextual fear memory formation. Naïve mice were used for each behavioral experiment. Mice behavior was recorded and freezing levels (the complete lack of movement except for respiration and heart beat) were scored.

cAMP affinity-precipitation

Two hippocampi from a C57BL/6J mouse (8-10 weeks of age) were freshly dissected and added to 2 mL ice cold lysis buffer (20 mM HEPES pH 7.4, 20 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 1X protease inhibitor cocktail (Sigma, P8340), 50 mM NaF, 100 µM Na_4VO_3). Tissue was homogenized for 30 seconds by rotor-stator (TissueRuptor; Qiagen), adjusted to 1% IGEPAL CA-630 (Sigma; NP-40 equivalent) and incubated at 4°C for 45 minutes on a nutator before centrifugation at 1,000 *g* for 5 min at 4°C. The resulting supernatant was transferred to a new tube, and protein concentration was determined by Bradford assay (Bio-Rad). 500 µg of protein was adjusted to a final volume of 500 µl with lysis buffer + 1 % IGEPAL. Control samples included

50 mM cAMP and maintained a 500 µl volume. Rp-8-AHA-cAMP-agarose beads (Axxora; BLG-A012) were washed four times in lysis buffer + 1% IGEPAL, and 60 µl of a 1:1 suspension was added to all samples before incubation at 4°C for 5 hours on a nutator. Beads were centrifuged at 1,000 *g* for 30 seconds at 4°C and resuspended in 200 µl of wash buffer (10 mM HEPES pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1X protease inhibitor cocktail, 50 mM NaF, 100 µM Na_4VO_3, 0.1% IGEPAL). After three washes, bound protein was eluted from the beads by resuspension in 50 µl of 1X SDS sample buffer. The experiment was repeated with 5 mice.

Data analysis

Data were analyzed using Statistica 10 and SPSS V10. The maintenance of LTP was analyzed using a two-way repeated-measures ANOVA test on the last 20-min of the recordings (Vecsey et al., 2009). Dunnett's post hoc test was performed if applicable. The average of the normalized slopes over the last 20-min was evaluated by a t-test. Input–output data were analyzed using a t-test comparing the average linear regression slopes between each group. PPF data were analyzed using a t-test to compare the ratio of the initial fEPSP slopes elicited by the two stimuli. To evaluate changes of the PFV amplitude or initial fEPSP slopes with increasing stimulation intensities, a two-way repeated-measures ANOVA test was performed with group and stimulation intensity as factors, and the presynaptic fiber volley (PFV) amplitude or initial fEPSP slopes as variables. Short-term depression induced by 10 Hz stimulation was analyzed using a two-way repeated-measures ANOVA test with group and pulse number as factors, and initial fEPSP slope as the variable. For evaluation of biochemical and behavioral data, t-tests were performed. Differences were considered statistically significant when $p < 0.05$. Data were plotted as mean ± S.E.M.

Results

Disruption of PKA anchoring impairs forskolin-induced long-lasting potentiation and causes faster depletion of a pool of synaptic vesicles

Activation of cAMP-PKA signaling by the adenylyl cyclase activator forskolin (FSK) induces long-lasting potentiation in the hippocampal Schaffer collateral pathway (Chavez-Noriega & Stevens, 1992). This form of long-lasting potentiation has been widely used to study the role of PKA signaling in synaptic plasticity (e.g., Harada et al., 2002; Menegon et al., 2006; Vecsey et al., 2009; Havekes et al., 2012). To investigate whether PKA anchoring is critical for FSK-induced long-lasting potentiation, we blocked PKA anchoring by delivering stearated-Ht31 (stHt31) peptide to hippocampal slices. As a control, we used stHt31P peptide, which is identical to stHt31 peptide except for proline substitutions that prevent binding to PKA (Carr, Hausken, et al., 1992). We first tested whether stHt31P peptide treatment affects FSK-induced potentiation and found no significant effects of this control peptide. (FSK only vs. FSK with stHt31P peptide, n = 4 for both groups, two-way repeated-measures ANOVA, $F_{(1,6)} = 0.053$, $p = 0.826$; the average of the last 20 minutes of the initial fEPSP slope, FSK only: 153.8 ± 9.5%, n = 4, FSK with stHt31P peptide: 157.4 ± 10.7%, n = 4; t-test, $p = 0.826$). This suggests that the peptide treatment itself does not exert any non-specific physiological effects, so we directly compared stHt31 peptide treatment to stHt31P peptide treatment in subsequent studies. Acute treatment with stHt31 peptide resulted in impaired FSK-induced potentiation compared to treatment with the control stHt31P peptide (**Fig. 1A**; n = 4 for both groups; two-way repeated-measures ANOVA, $F_{(1,6)} = 15.045$, $p = 0.008$). The average of the initial fEPSP slope over the last 20 min of the recordings was reduced in hippocampal slices treated with stHt31 peptide relative to slices treated with the control stHt31P peptide (stHt31 peptide: 108.3 ± 6.8%, n = 4, stHt31P peptide: 157.4 ± 10.7%, n = 4; t-test, $p = 0.008$).

Long-lasting potentiation induced by FSK or cAMP analogs involves enhanced presynaptic transmitter release that is mediated by a PKA-dependent increase in the size of the readily releasable pool of synaptic vesicles (Baba, Sakisaka, Mochida, & Takai, 2005; Bolshakov et al., 1997; Chavez-Noriega & Stevens, 1992, 1994; Ma, Zablow, Kandel, & Siegelbaum, 1999; Moulder et al., 2008). Short-term depression induced by 10 Hz stimulation reflects the depletion of the readily releasable pool of synaptic vesicles (Dobrunz & Stevens, 1997; Zucker & Regehr, 2002). Using this approach, we examined the effect of disrupting PKA anchoring on the size of the readily releasable pool. Synaptic responses to 10 Hz stimulation showed initial rapid decay followed by steady decrease in slices treated with stHt31 peptide compared to slices treated with stHt31P peptide (**Fig. 1*B***; n = 8 for both groups; two-way repeated-measures ANOVA, $F_{(1,14)} = 8.377$, $p = 0.012$). These results suggest that the disruption of PKA anchoring reduces the size of the readily releasable pool of synaptic vesicles.

To assess whether the loss of compartmentalized PKA signaling alters other synaptic properties, the impact of stHt31 peptide treatment on synaptic transmission was analyzed. We first measured the input-output relationship and found no significant effects of blocking PKA anchoring (**Fig. 1*C***; stHt31 peptide: 5.6 ± 1, n = 9; stHt31P peptide: 5.8 ± 1, n = 8; t-test, $p = 0.902$). Moreover, the maximum initial fEPSP slope size was similar between the two groups (stHt31 peptide: 7.5 ± 0.3 mV/ms, n = 9; stHt31P peptide: 7.4 ± 0.6 mV/ms, n = 8; t-test, $p = 0.823$). We then measured paired-pulse facilitation (PPF), a presynaptic form of short-term synaptic plasticity in which the synaptic response to the second pulse is facilitated when two synaptic pulses are evoked in close succession (Katz & Miledi, 1968; Zucker & Regehr, 2002). PPF after FSK application was similar between slices treated with stHt31 peptide and slices treated with stHt31P peptide (**Fig. 1*D***; stHt31 peptide: 1.3 ± 0.1, n = 9; stHt31P peptide: 1.4 ± 0.02, n = 8; t-test, $p = 0.46$). These results extend our previous findings that slices treated with either stHt31 or stHt31P peptide treatment show similar input-output relationship or PPF under basal conditions (T. Huang et al., 2006). Additionally, we determined the effect of stHt31 peptide treatment on the presynaptic fiber volley (PFV) and evoked synaptic responses. Changes in PFV

with increasing stimulation intensities were not significantly different between slices treated with stHt31 or stHt31P peptide (**Fig. 1*E***; n = 9 for stHt31 peptide, n = 8 for stHt31P peptide; two-way repeated-measures ANOVA, $F_{(1,15)} = 1.575$, $p = 0.606$). Changes in initial fEPSP slopes with increasing stimulation intensities were also not altered in slices treated with stHt31 peptide compared to slices treated with stHt31P peptide (**Fig. 1*F***; n = 9 for stHt31 peptide, n = 8 for stHt31P peptide; two-way repeated-measures ANOVA, $F_{(1,15)} = 1.118$, $p = 0.295$). Overall, our findings suggest that the disruption of PKA anchoring impairs FSK-induced long-lasting potentiation by reducing the size of the readily releasable pool of synaptic vesicles.

Disruption of PKA anchoring reduces the levels of synaptic vesicle protein 2 (SV2)

Because our data reveal that stHt31 peptide treatment impairs FSK-induced long-lasting potentiation by reducing the size of the readily releasable pool of synaptic vesicles, we examined the effects of stHt31 peptide treatment on presynaptic proteins involved in the maintenance of the readily releasable pool size (**Fig. 2**). Synaptic vesicle protein 2 (SV2) modulates the size of the readily releasable pool without affecting synaptic structure or the Ca^{2+} sensitivity of synaptic vesicle release (Custer et al., 2006; Iezzi, Theander, Janz, Loze, & Wollheim, 2005; Janz, Goda, Geppert, Missler, & Südhof, 1999; Lynch et al., 2004; T. Xu & Bajjalieh, 2001). Relative to stHt31P peptide treatment, stHt31 peptide treatment reduced SV2 protein levels (**Fig. 2*A***; stHt31 peptide: 64 ± 6.9%, n = 9; stHt31P peptide: 100 ± 8.7%, n = 9; t-test, $p = 0.003$). PKA-dependent phosphorylation of synapsin1, another synaptic vesicle protein, modulates the size of the readily releasable pool of synaptic vesicles (Bonanomi et al., 2005; Chi, Greengard, & Ryan, 2001; Hosaka, Hammer, & Südhof, 1999; Ryan, Li, Chin, Greengard, & Smith, 1996). We expected that the disruption of PKA anchoring would reduce the phosphorylation level of synapsin1, but slices treated with stHt31 peptide did not show a reduction in the ratio of phospho-synapsin1 to total synapsin1 (**Fig. 2*B***; stHt31 peptide: 89.9 ± 13.8%, n = 9; stHt31P peptide: 100 ± 14.8%, n = 9; t-test, $p = 0.6$). Also, stHt31 peptide treatment did not affect the total protein levels of synapsin1

relative to stHt31P peptide treatment (stHt31 peptide: 111.2 ± 9.6%, n = 9; stHt31P peptide: 100 ± 7%, n = 9; t-test, p = 0.329). Rim1 and SNAP25 are involved in making synaptic vesicles competent for release (Deng, Kaeser, Xu, & Südhof, 2011; Kaeser, 2011; Nagy et al., 2004). Compared to stHt31P peptide treatment, stHt31 peptide treatment showed unaltered levels of both proteins (**Fig. 2*C***; stHt31 peptide: 97.9 ± 22.2%, n = 9; stHt31P peptide: 100 ± 18.3%, n = 9; t-test, p = 0.94, **Fig. 2*D***; stHt31 peptide: 105.9 ± 10.3%, n = 9; stHt31P peptide: 100 ± 9.8%, n = 9; t-test, p = 0.71). Additionally, we measured protein levels of synaptophysin, a synaptic vesicle marker, and found no significant effect of stHt31 peptide treatment (**Fig. 2*E***; stHt31 peptide: 107.9 ± 12.4%, n = 9; stHt31P peptide: 100 ± 13.8%, n = 9, t-test, p = 0.66). The lack of an effect of stHt31 treatment on synaptophysin levels suggests that the number of synaptic vesicles is not affected by the mislocalization of PKA. Finally, protein levels of the Ca^{2+} sensor synaptotagmin1 were not affected by stHt31 peptide treatment (**Fig. 2*F***; stHt31 peptide: 103.4 ± 7.3%, n = 9; stHt31P peptide: 100 ± 8.8%, n = 9; t-test, p = 0.76). These synaptotagmin1 data likely suggest that the disruption of PKA anchoring does not alter Ca^{2+}-dependence of synaptic vesicle release, an idea that is supported by the lack of an effect of stHt31 peptide treatment on PPF (**Fig. 1*D***). To determine if any of the presynaptic proteins are in complexes associated with cAMP, we carried out cAMP affinity-precipitation assays (M Colledge et al., 2000; Lygren et al., 2007) on hippocampal extracts. SV2, Rim1 and SNAP25, but not synaptophysin, were enriched in cAMP affinity precipitates (**Fig. 3*A***), suggesting an interaction of PKA with SV2, RIM1, and SNAP25.

Taken together, our electrophysiological and biochemical data lead to a model in which compartmentalized cAMP-PKA signaling regulates the size of the readily releasable pool of synaptic vesicles. Through a cAMP-bound protein complex that links synaptic vesicles to the vesicle release site (the active zone), localized cAMP-PKA signaling orchestrates the interactions between vesicular protein SV2 and the active zone-bound proteins Rim1 and SNAP25 (**Fig. 3*B***). Loss of compartmentalized PKA signaling results in reduced levels of SV2 protein, which in turn attenuates the processes (priming) that make synaptic vesicles release competent (**Fig. 3*C***).

Long-term contextual fear memory is impaired only in a transgenic mouse line in which PKA anchoring is disrupted in both CA3 and CA1 neurons

Our electrophysiological and biochemical data suggest a role for PKA anchoring in synaptic vesicle release from presynaptic CA3 neurons in the hippocampal Schaffer collateral pathway. The input from CA3 to CA1 is critical for the formation of contextual fear memory (Cravens, Vargas-Pinto, Christian, & Nakazawa, 2006; Nakashiba, Buhl, McHugh, & Tonegawa, 2009; Nakashiba, Young, McHugh, Buhl, & Tonegawa, 2008). To restrict Ht31 expression to pre- or postsynaptic compartments, we used transgenic mouse lines (Ht31(1) and Ht31(16) mice) that show the functional significance of the differential expression of Ht31 in excitatory neurons at the hippocampal Schaffer collateral CA3-CA1 synapses (Nie et al., 2007, **Fig. 4*A***). Previous behavioral studies showed that spatial memory assessed by the Morris water maze was impaired in Ht31(1) mice that express Ht31 in both CA3 and CA1 neurons, but not in Ht31(16) mice that express Ht31 only in CA1 neurons (Nie et al., 2007). To investigate whether PKA anchoring in presynaptic CA3 neurons is required for hippocampus-dependent contextual fear memory formation, we performed contextual fear conditioning with Ht31(1) and Ht31(16) mice. A short-term contextual fear memory test conducted 1 hour after training revealed that both Ht31(1) and Ht31(16) mice showed freezing levels similar to their wild-type littermates (**Fig. 4*B***; Ht31(1), preshock, Wild-type: 5.9 ± 1.6%, n = 6; Ht31(1): 10.6 ± 2.3%, n = 8; t-test, $p = 0.135$, 1 hr test, Wild-type: 30.4 ± 5.7%, n = 6; Ht31(1): 42.6 ± 4.1%, n = 8; t-test, $p = 0.076$; Ht31(16), preshock, Wild-type: 7.02 ± 1.3%, n = 10; Ht31(16): 12.9 ± 1.7%, n = 8; t-test, $p = 0.008$, 1 hr test, Wild-type: 35.5 ± 2.8%, n = 10; Ht31(16): 31.6 ± 3.6%, n = 8; t-test, $p = 0.37$). Next, we tested long-term contextual fear memory 24 hours after training in a new cohort of mice. Ht31(1) mice, in which PKA anchoring is disrupted in both CA3 and CA1 neurons, showed significantly reduced contextual freezing levels compared to wild-type littermates (**Fig. 4*C***; preshock, Wild-type: 3.7 ± 0.8%, n = 11; Ht31(1): 4.5 ± 0.6%, n = 9; t-test, $p = 0.648$, 24 hr test, Wild-type: 39.8 ± 3.4%, n = 11; Ht31(1): 28.53 ± 3.45%, n = 9; t-test, $p = 0.033$). In contrast, Ht31(16) mice, in which PKA anchoring is spared in CA3 neurons but disrupted in CA1 neurons, showed similar freezing levels

to wild-type littermates 24 hours after training (**Fig. 4*C***; preshock, Wild-type: 2.6 ± 1.1%, n = 12; Ht31(16): 5.3 ± 1.1%, n = 10; t-test, p = 0.078, 24 hr test, Wild-type: 44.6 ± 5.6%, n = 12; Ht31(16): 39.7 ± 3.1%, n = 10; t-test, p = 0.452). In addition, both Ht31(1) and Ht31(16) mice showed unaltered anxiety-related behavior or exploratory behavior as measured in the elevated zero maze and open field tasks (data not shown). Ht31 expression is not restricted to the hippocampus in Ht31(1) and Ht31(16) mice (**Fig. 4*A***). However, it is worth noting that although Ht31(16) mice express Ht31 in extra-hippocampal areas more robustly than Ht31(1) mice (**Fig. 4*A***), long-term contextual fear memory is not impaired in Ht31(16) mice. This suggests that the extra-hippocampal expression of Ht31 in both transgenic lines does not impact contextual fear memory. Overall, these findings indicate that PKA anchoring in presynaptic CA3 neurons is critical for hippocampus-dependent long-term memory.

Forskolin-induced long-lasting potentiation and synaptic tagging are impaired only in a transgenic mouse line in which PKA anchoring is disrupted in both CA3 and CA1 neurons

To examine whether stHt31 peptide treatment impairs FSK-induced long-lasting potentiation by perturbing excitatory glutamatergic presynaptic function, we examined this form of synaptic plasticity in hippocampal slices from Ht31(1) and Ht31(16) mice. Electrophysiological recordings from wild-type littermates of each mouse line were combined for data analyses as no differences were observed between the two groups (data not shown). Basal synaptic properties including the input-output relationship and PPF are unaltered in both mouse lines (Nie et al., 2007). Slices from Ht31(1) mice showed impaired FSK-induced potentiation compared to wild-type littermates, confirming our previous observations (Myungsook Kim et al., 2011). In contrast, slices from Ht31(16) mice showed robust FSK-induced long-lasting potentiation similar to wild-type littermates (**Fig. 5*A***; n = 7 for wild-type littermates, n = 5 for Ht31(1) mice, n = 5 for Ht31(16) mice; two-way repeated-measures ANOVA, $F_{(2,14)}$ = 11.373, p = 0.001, Dunnett's post hoc test, Ht31(1) vs. wild-type p = 0.001, Ht31(16) vs. wild-type p = 0.99). The average of the initial fEPSP

slope over the last 20 min of the recordings was reduced in slices from Ht31(1) mice, but was similar between slices from Ht31(16) mice and from wild-type littermates (wild-type: 168.6 ± 9.1%, n = 7; Ht31(1): 116 ± 4.5%, n = 5; Ht31(16): 169 ± 13.1%, n = 5; one-way ANOVA , $F_{(2,14)}$ = 11.373, p = 0.001, Dunnett's post hoc test, Ht31(1) vs. wild-type p = 0.001, Ht31(16) vs. wild-type p = 0.99). These data indicate that compartmentalized PKA in presynaptic glutamatergic CA3 neurons is critical for long-lasting potentiation induced by the activation of adenylyl cyclases.

Compartmentalized PKA signaling is required for input-specific synaptic plasticity including synaptic tagging (T. Huang et al., 2006). Synaptic tagging measures the pathway-specific interactions between two independent pathways (**Fig.5*B***): Short-lasting LTP in a weak pathway (S1) can be stabilized into long-lasting LTP by subsequent strong stimulation in a separate pathway (S2) (Frey & Morris, 1997, 1998). We extended our findings on the presynaptic requirement for PKA anchoring in synaptic plasticity by examining synaptic tagging in these two Ht31 transgenic mouse lines. We previously showed that weak 1 train stimulation (one 1 s 100 Hz train) induced only a short-term form of LTP that lasted less than 2 hours in both Ht31(1) and Ht31(16) mice (Nie et al., 2007), and stHt31 peptide treatment did not affect this form of short-lasting LTP (T. Huang et al., 2006). However, pairing this weak stimulation in S1 with strong stimulation in S2 resulted in a long-lasting strengthening of LTP in S1 in slices from Ht31(16) mice, in which PKA anchoring is spared in CA3 neurons but disrupted in CA1 neurons. In contrast, this form of synaptic strengthening in S1 was absent in slices from Ht31(1) mice, in which PKA anchoring is disrupted in both CA3 and CA1 neurons, compared to slices from wild-type littermates (**Fig.5*C*** *left panel*; n = 7 for wild-type littermates, n = 5 for Ht31(1), n = 5 for Ht31(16); two-way repeated-measures ANOVA, $F_{(2,14)}$ = 26.139, p = 0.00002, Dunnett's post hoc test, Ht31(1) vs. wild-type p = 0.00011, Ht31(16) vs. wild-type p = 0.195). The average of the initial fEPSP slopes over the last 20 min of the recordings was reduced in slices from Ht31(1) mice, but was similar between slices from Ht31(16) mice and from wild-type littermates (wild-type: 145.9 ± 4.5%, n = 7; Ht31(1): 105.8 ± 4.5%, n = 5; Ht31(16): 153.8 ± 6.6%, n = 5; one-way ANOVA, $F_{(2,14)}$ = 26.139, p = 0.00002, Dunnett's post hoc test, Ht31(1) vs. wild-type p = 0.00011,

Ht31(16) vs. wild-type $p = 0.195$). In S2, a strong massed 4-train stimulation (four 1s 100 Hz trains, 5 seconds intertrain interval) successfully induced long-lasting LTP in hippocampal slices from both Ht31 mouse lines and wild-type littermates (**Fig.5*C*** *right panel*; n = 7 for wild-type littermates, n = 5 for Ht31(1), n = 5 for Ht31(16); two-way repeated-measures ANOVA, $F_{(2,14)} = 0.2$, $p = 0.821$). These results are consistent with the findings that massed 4-train–induced long-lasting LTP is independent of PKA or PKA anchoring (Havekes et al., 2012; MyungSook Kim, Huang, Abel, & Blackwell, 2010; Woo, Duffy, Abel, & Nguyen, 2003). The average of the initial fEPSP slope over the last 20 min of the recordings was similar in all groups (wild-type: 156.9 ± 7%, n = 7; Ht31(1): 157.7 ± 6.2%, n = 5; Ht31(16): 151.9 ± 8.9%, n = 5; one-way ANOVA, $F_{(2,14)} = 0.2$, $p = 0.821$). These results indicate that synaptic tagging requires PKA anchoring in both CA3 and CA1 neurons and that restricted disruption of PKA anchoring only in CA1 neurons is not sufficient to block synaptic tagging. This highlights a role for presynaptic PKA anchoring in synaptic tagging.

Discussion

Compartmentalization of PKA and other signaling molecules by A-kinase anchoring proteins (AKAPs) is critical for synaptic plasticity and memory (Havekes et al., 2012; T. Huang et al., 2006; Lu et al., 2007; Nie et al., 2007; J. L. Sanderson & Dell'Acqua, 2011; Tunquist et al., 2008). Studies using genetic manipulation of specific AKAP complexes have focused on postsynaptic mechanisms (Havekes et al., 2012; J. L. Sanderson et al., 2012; M. Zhang et al., 2013). However, genetic blockade of PKA anchoring reveals that postsynaptic PKA anchoring is insufficient for physiologically relevant LTP induced by theta-burst stimulation and for spatial memory (Nie et al., 2007). Here, our data suggest that PKA anchoring in presynaptic CA3 neurons in the hippocampal Schaffer collateral pathway is critical for a form of long-lasting potentiation induced by the activation of adenylyl cyclases, synaptic tagging, and long-term contextual fear memory. Furthermore, the pharmacological disruption of PKA anchoring reduces the size of the readily releasable pool of synaptic vesicles. Biochemical assays demonstrate that the disruption of PKA anchoring leads to decreased levels of SV2, a component of a cAMP-bound complex containing Rim1 and SNAP25. In summary, our study suggests that presynaptically compartmentalized PKA maintains the readily releasable pool of synaptic vesicles to support prolonged enhancement of synaptic vesicle release, which is critical for long-lasting synaptic plasticity, synaptic tagging, and long-term memory.

In synaptic tagging, two independent afferents converge onto the same population of postsynaptic neurons. Because the interaction between the two afferents occurs in the same postsynaptic neurons, the models for synaptic tagging have focused on postsynaptic mechanisms (Redondo et al., 2010). Therefore, we anticipated that disrupting PKA anchoring in postsynaptic CA1 neurons would be sufficient to impair synaptic tagging. Surprisingly, however, we find that synaptic tagging is impaired when PKA anchoring is disrupted in both presynaptic CA3 and postsynaptic CA1 neurons, but is intact when PKA anchoring is disrupted only in postsynaptic CA1 neurons. These findings suggest a presynaptic requirement for compartmentalized PKA

signaling in synaptic tagging. The presynaptic and postsynaptic components for synaptic tagging could be linked by retrograde signaling molecules. In postsynaptic neurons, strong stimulation produces retrograde messengers that travel to the presynaptic terminals of the weak pathway to enhance synaptic vesicle release, thereby strengthening the weak pathway. For example, BDNF released from postsynaptic neurons is one factor that acts on presynaptic terminals to facilitate synaptic vesicle release. During this process, BDNF promotes cAMP-PKA signaling at presynaptic terminals by increasing presynaptic Ca^{2+} levels which activate Ca^{2+}-dependent adenylyl cyclases (Y. X. Li, Zhang, Lester, Schuman, & Davidson, 1998; Magby, Bi, Chen, Lee, & Plummer, 2006; Tyler & Pozzo-Miller, 2001; Villacres, Wong, Chavkin, & Storm, 1998; H. Wang et al., 2003). Indeed, BDNF deletion in both presynaptic CA3 and postsynaptic CA1 neurons or only in postsynaptic CA1 neurons impairs synaptic tagging, suggesting that BDNF produced in postsynaptic CA1 neurons is critical for synaptic tagging (Barco et al., 2005). Future approaches that genetically block PKA anchoring selectively in CA3 neurons, that reverse the order of strong and weak stimulation, and that apply stHt31 peptide at various time points during synaptic tagging will be necessary to define the mechanism by which presynaptic PKA anchoring mediates synaptic tagging.

We show that the disruption of PKA anchoring decreases the size of the readily releasable pool of synaptic vesicles, but does not affect the presynaptic action potential generation, Ca^{2+}-dependent synaptic vesicle release probability, or postsynaptic response mechanisms. We estimated the size of the readily releasable pool of synaptic vesicles with a widely used method: a short-term depression induced by 10 Hz stimulation (Dobrunz & Stevens, 1997; Zucker & Regehr, 2002). This form of short-term plasticity is not due to desensitization of postsynaptic receptors (such as AMPA receptors) or activation of metabotropic glutamate receptors (Dobrunz & Stevens, 1997; Hagler & Goda, 2001; L. Y. Wang & Kaczmarek, 1998). Also, the observed depression is not a simple rundown because the disruption of PKA anchoring allows stable synaptic responses to even higher frequency theta-burst stimulation (Nie et al., 2007). Interestingly, the observed pattern of short-term depression under conditions of disrupted

PKA anchoring is also found when PKA phosphorylation of snapin, a presynaptic PKA substrate, is genetically ablated (Thakur, Stevens, Sheng, & Rettig, 2004). Therefore, spatially restricted PKA signaling in presynaptic terminals plays an important role in maintaining the readily releasable pool of synaptic vesicles. Future experiments assessing the readily releasable pool using other methods, such as fluorescence imaging, will strengthen our findings that compartmentalized PKA signaling regulates the readily releasable pool (Denker & Rizzoli, 2010; Rizzoli & Betz, 2005).

Both SV2 and FSK modulates the size of the readily releasable pool without affecting action potential generation, Ca^{2+} sensitivity of release or the number of morphologically docked vesicles (Custer et al., 2006; Iezzi et al., 2005; Janz et al., 1999; D. Kim & Thayer, 2001; Moulder et al., 2008; Trudeau et al., 1996; T. Xu & Bajjalieh, 2001). Our data suggest that PKA anchoring regulates SV2 protein levels to maintain the size of the readily releasable pool of synaptic vesicles. It should be noted that cultured hippocampal neurons from SV2 knockout mice exhibit enhanced PPF and alleviated short-term depression induced by 10 Hz stimulation (Custer et al., 2006; Janz et al., 1999; Nowack, Yao, Custer, & Bajjalieh, 2010). These data contradict our findings that the reduced levels of SV2 after the disruption of PKA anchoring do not lead to alterations in PPF, but instead lead to facilitation of short-term depression induced by 10 Hz stimulation. This discrepancy could be attributed to the fact that our recordings were performed on synapses between CA3 and CA1 neurons in adult hippocampal slices, while the SV2 knockout recordings were performed on autapses from hippocampal neuronal cultures prepared from embryos or newborns. Developmental effects may also explain the discrepancy because the pre- and postsynaptic threshold for LTP changes during the first 3 weeks of development and hippocampus-dependent memory appears after 3 weeks of age (Dumas, 2012). Given that two isoforms of SV2 (SV2A and SV2B) are present in the hippocampus (Bajjalieh, Frantz, Weimann, McConnell, & Scheller, 1994; Bajjalieh, Peterson, Linial, & Scheller, 1993), siRNA-mediated approaches to knock down a specific isoform of SV2 in adult hippocampal neurons will be

necessary to better understand the role of SV2 in PKA anchoring-mediated synaptic vesicle exocytosis.

Readily releasable synaptic vesicles are primed for fast Ca^{2+}-dependent vesicle exocytosis, but the mechanism of priming is a matter of debate. Rim1 has been suggested as a key component of a protein complex that mediates priming (Kaeser et al., 2011; Powell, 2006; Südhof, 2013). The role of Rim1 in priming, however, is challenged by the findings that PKA phosphorylation of Rim1 is dispensable for long-lasting LTP and memory (Kaeser et al., 2008). Here, we propose that a cAMP-bound complex containing Rim1, SV2, and SNAP25 is critical for priming, and that localized PKA signaling modulates the size of the readily releasable pool by selective regulation of SV2 protein levels (**Fig. 3*B* and 3*C***). SV2 is likely a central component of a PKA anchoring-mediated priming complex, allowing fast coupling of primed vesicles to Ca^{2+}-dependent vesicle exocytosis. Indeed, SV2 colocalizes with Ca^{2+} channels and regulates assembly of the SNARE complex, which is critical for the vesicle exocytosis (Ahmari, Buchanan, & Smith, 2000; T. Xu & Bajjalieh, 2001).

An important open question is how altering PKA localization leads to reduced SV2 protein levels. One possibility is that the disruption of PKA anchoring leads to alterations in protein complexes that stabilize SV2 protein. As our cAMP affinity precipitation data suggests, synaptic vesicle exocytosis is tightly regulated by myriad of interacting molecules (Jahn & Fasshauer, 2012). Therefore, the observed reduction in SV2 levels after the disruption of PKA anchoring could be an outcome of altered phosphorylation of multiple PKA substrates interacting with SV2. Although the availability and sources for antibodies against phospho-PKA substrates involved in vesicle exocytosis is limited, future studies using genetic and biochemical approaches could reveal how presynaptically compartmentalized PKA regulates the stability of SV2 and other components of the priming complex.

Little is known about what specific AKAPs regulate synaptic vesicle release. Studies of AKAP150 and Gravin revealed that these commonly studied AKAPs mediate synaptic plasticity

and memory via postsynaptic mechanisms (Havekes et al., 2012; Lu et al., 2007; Tunquist et al., 2008). A potential presynaptic AKAP is AKAP220 that binds glycogen synthase kinase-3β (GSK-3β) and mediates PKA phosphorylation of GSK-3β to inactivate it (Tanji et al., 2002). Activation of GSK-3β decreases presynaptic transmitter release and reduces the size of the presynaptic active zone, which leads to impaired hippocampal LTP and spatial memory (S. J. Liu et al., 2003; Zhu et al., 2007). Genetic manipulation of AKAP220 in CA3 would determine if AKAP220 orchestrates PKA-mediated synaptic vesicle exocytosis.

Here, we demonstrate that presynaptically compartmentalized PKA is critical for synaptic tagging and memory by modulating the size of the readily releasable pool of synaptic vesicles. Moreover, our data suggest that the interaction between PKA and a cAMP-bound protein complex composed of SV2, SNAP25, and Rim1 is involved in regulating the readily releasable pool of synaptic vesicles. Overall, our study paves a way to elucidate the role of presynaptic effects of PKA signaling on synaptic plasticity, synaptic tagging and memory.

Figure Legends

Figure 2.1. Pharmacological disruption of PKA anchoring impairs forskolin (FSK)-induced long-lasting potentiation and facilitates short-term depression in the hippocampal Schaffer collateral pathway.

A. Compared to the control stearated-Ht31P (stHt31P) peptide treatment (100 µM), stearated-Ht31 (stHt31) peptide treatment (100 µM) impairs a form of long-lasting potentiation induced by 15 minutes of bath application of 50 µM FSK (two-way repeated-measures ANOVA, $F_{(1,6)}$ = 15.045, p = 0.008). Representative traces acquired at the baseline (black) and at the last 20 minutes of the recordings (red) are shown on top of the graph. ***B***. In FSK-treated slices, application of stHt31 peptide facilitated short-term depression induced by 10 Hz stimulation relative to application of stHt31P peptide (two-way repeated-measures ANOVA, $F_{(1,14)}$ = 8.377, p = 0.012). Values were normalized to the initial response. ***C.*** Slices co-treated with FSK and stHt31 peptide showed input-output relationships similar to slices co-treated with FSK and stHt31P peptide (t-test, p = 0.902). ***D***. Paired-pulse facilitation (PPF) at 50 ms interval was not significantly different between FSK-treated slices perfused with stHt31 or stHt31P peptide (t-test, p = 0.46). ***E*** and ***F***. Slices treated with stHt31 or stHt31P peptide along with FSK showed comparable changes in the presynaptic fiber volley (PFV) amplitude and initial fEPSP slope with increasing stimulation intensities (two-way repeated-measures ANOVA, for PFV amplitude: $F_{(1,15)}$ = 1.575, p = 0.606; for initial fEPSP slope: $F_{(1,15)}$ = 1.118, p = 0.295). Values were normalized to the maximum response. Error bars reflect S.E.M.

Figure 2.2. Pharmacological disruption of PKA anchoring selectively reduces the levels of synaptic vesicle protein 2 (SV2) in forskolin-treated hippocampal slices.

A. stHt31 peptide treatment (100 µM) reduced the protein levels of SV2 relative to stHt31P peptide treatment (100 µM, t-test, $p = 0.003$). ***B***. The ratio of phospho-synapsin1 to total synapsin1 was similar between slices treated with stHt31 or stHt31P (t-test, $p = 0.6$). The protein levels of other presynaptic proteins were not altered in slices treated with stHt31 peptide compared to slices treated with stHt31P peptide (***C***; Rim1: t-test, $p = 0.94$, ***D***; SNAP25: t-test, $p = 0.71$, ***E***; synaptophysin: t-test, $p = 0.66$, ***F***; synaptotagmin1: t-test, $p = 0.76$). β-tubulin was used as the loading control and the expression level was normalized to the level of the control stHt31P peptide treatment group. Representative blots are shown on the left of each graph. * indicates $p = 0.003$. Error bars reflect S.E.M.

Figure 2.3. SV2, Rim1, and SNAP25 co-affinity precipitate with cAMP, and presynaptically compartmentalized PKA may regulate the readily releasable pool of synaptic vesicles through SV2.

A. cAMP affinity-precipitation (AP) was performed using Rp-8-AHA-cAMP-agarose beads. Under control conditions, excessive amount of cAMP was added to compete against Rp-8-AHA-cAMP-agarose beads. cAMP affinity-precipitates of hippocampal extracts showed enrichment of Rim1, SV2, and SNAP25. The synaptophysin band in the AP lane was non-specific. ***B***. A-kinase anchoring proteins (AKAPs) maintain a pool of PKA in close proximity to a cAMP-bound protein complex that bridges between a synaptic vesicle protein SV2 and active zone proteins, including Rim1 and SNAP25. This spatial compartmentalization allows fast coupling of cAMP-PKA signaling with the priming of the docked vesicles, which increases the size of the readily releasable pool of synaptic vesicles. Ca^{2+}-influx following neuronal activation triggers fast fusion

of the primed synaptic vesicles, and the large size of the readily releasable pool of synaptic vesicles sustains long-lasting synaptic plasticity. ***C***. When PKA anchoring is disrupted by Ht31, PKA is sequestered away from the active zone. The free PKA is unable to coordinate interactions among proteins involved in vesicle exocytosis, which results in the reduction of SV2 protein levels. In addition, AKAPs may serve as the cAMP-bound complex, and Ht31 treatment disrupts the complex, thereby reducing SV2 levels. The reduced protein levels of SV2 destabilize the link between the docked synaptic vesicles and the active zone, thus the priming of the docked vesicles is attenuated. The resulting smaller size of the readily releasable pool of synaptic vesicles causes faster depletion of the vesicle pool during neuronal activity. Therefore, long-lasting synaptic plasticity is prevented.

Figure 2.4. Disruption of PKA anchoring in both CA3 and CA1 neurons, but not in CA1 neurons alone, impairs long-term contextual fear memory.

A. Representative *in situ* hybridization images from sagittal brain sections demonstrated that both transgenic mouse lines expressed Ht31 in the major forebrain regions (upper panels). In the hippocampus, Ht31(1) mice expressed Ht31 throughout hippocampal subregions including the dentate gyrus, CA3, and CA1. Ht31(16) mice expressed Ht31 sparsely in CA3, but robustly in the dentate gyrus and CA1 (lower panels). ***B.*** Ht31(1) mice and wild-type littermates showed similar freezing levels during the retention test 1 hour after training (t-test, $p = 0.076$). Compared to wild-type littermates, Ht31(16) mice exhibited comparable freezing levels during the retention test 1 hour after training (t-test, $p = 0.37$). ***C.*** Ht31(1) mice displayed significantly decreased freezing levels during the retention test 24 hours after training relative to wild-type littermates (t-test, $p = 0.033$). However, Ht31(16) mice and wild-type littermates exhibited similar freezing levels during the retention test 24 hours after training (t-test, $p = 0.452$). * indicates $p = 0.033$. Error bars reflect S.E.M.

Figure 2.5. Disruption of PKA anchoring in both presynaptic CA3 and postsynaptic CA1 neurons, but not in postsynaptic CA1 neurons alone, impairs forskolin (FSK)-induced long-lasting potentiation and synaptic tagging.

A. Ht31(16) mice and wild-type littermates, but not Ht31(1) mice, showed a form of long-lasting potentiation induced by 15 minutes of bath application of 50 μM FSK at the hippocampal Schaffer collateral CA3-CA1 synapses (two-way repeated-measures ANOVA, $F_{(2,14)} = 11.373$, $p = 0.001$, Dunnett's post hoc test, Ht31(1) vs. wild-type $p = 0.001$, Ht31(16) vs. wild-type $p = 0.99$). ***B***. A schematic diagram of the two-pathway experiment. Two stimulating electrodes were placed on either side of the recording electrode at the Schaffer collateral CA3-CA1 synapses to activate two independent sets of inputs (S1 and S2) onto the same postsynaptic population of neurons in CA1. Weak one train stimulation (one 1s 100 Hz train) was delivered to S1 first, and 30 minutes later, strong massed 4-train stimulation (four 1s 100 Hz trains with 5 seconds intertrain interval) was delivered to S2. ***C.*** Synaptic tagging is impaired in hippocampal slices from Ht31(1), but not in Ht31(16) mice. In S1, weak one train stimulation induced long-lasting potentiation in hippocampal slices from both wild-type littermates and Ht31(16) mice, but not in slices from Ht31(1) mice (***Left panel***; two-way repeated-measures ANOVA, $F_{(2,14)} = 26.139$, $p = 0.00002$, Dunnett's post hoc test, Ht31(1) vs. wild-type $p = 0.00011$, Ht31(16) vs. wild-type $p = 0.195$). In S2, strong massed 4-train stimulation elicited similar levels of long-lasting LTP in slices from Ht31(1), Ht31(16) mice, and wild-type littermates (***Right panel***; two-way repeated-measures ANOVA, $F_{(2,14)} = 0.2$, $p = 0.821$). Representative traces acquired at the baseline (black) and at the last 20 minutes of the recordings (red) are shown on top of the graph. Error bars reflect S.E.M.

Author Contributions

Experiments were designed by Jung Whan (Alan Jung) Park, Robbert Havekes, Vincent Luczak and Ted Abel. Electrophysiological recordings were conducted by A.J.P. Western blotting was performed by Jennifer Choi. cAMP-affinity precipitation assay was carried out by V.L. Behavioral experiments were conducted by A.J.P., R.H., Ting Nie, and Ted Huang. A.J.P wrote this chapter with comments and suggestions by R.H., Morgan Bridi, Shane Poplawski, Kyle Krainock and Ted Abel.

Figures

Figure 2.1

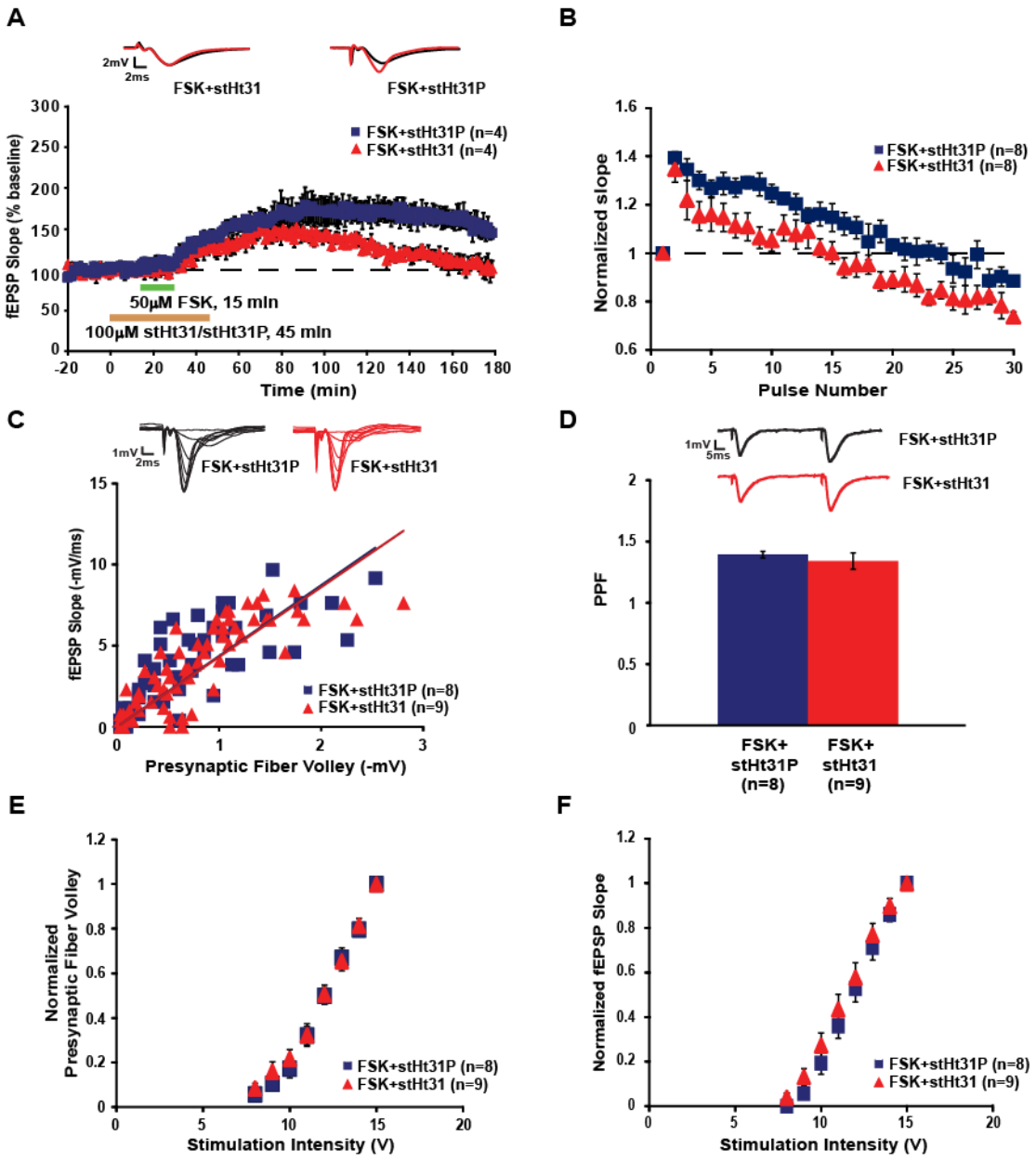
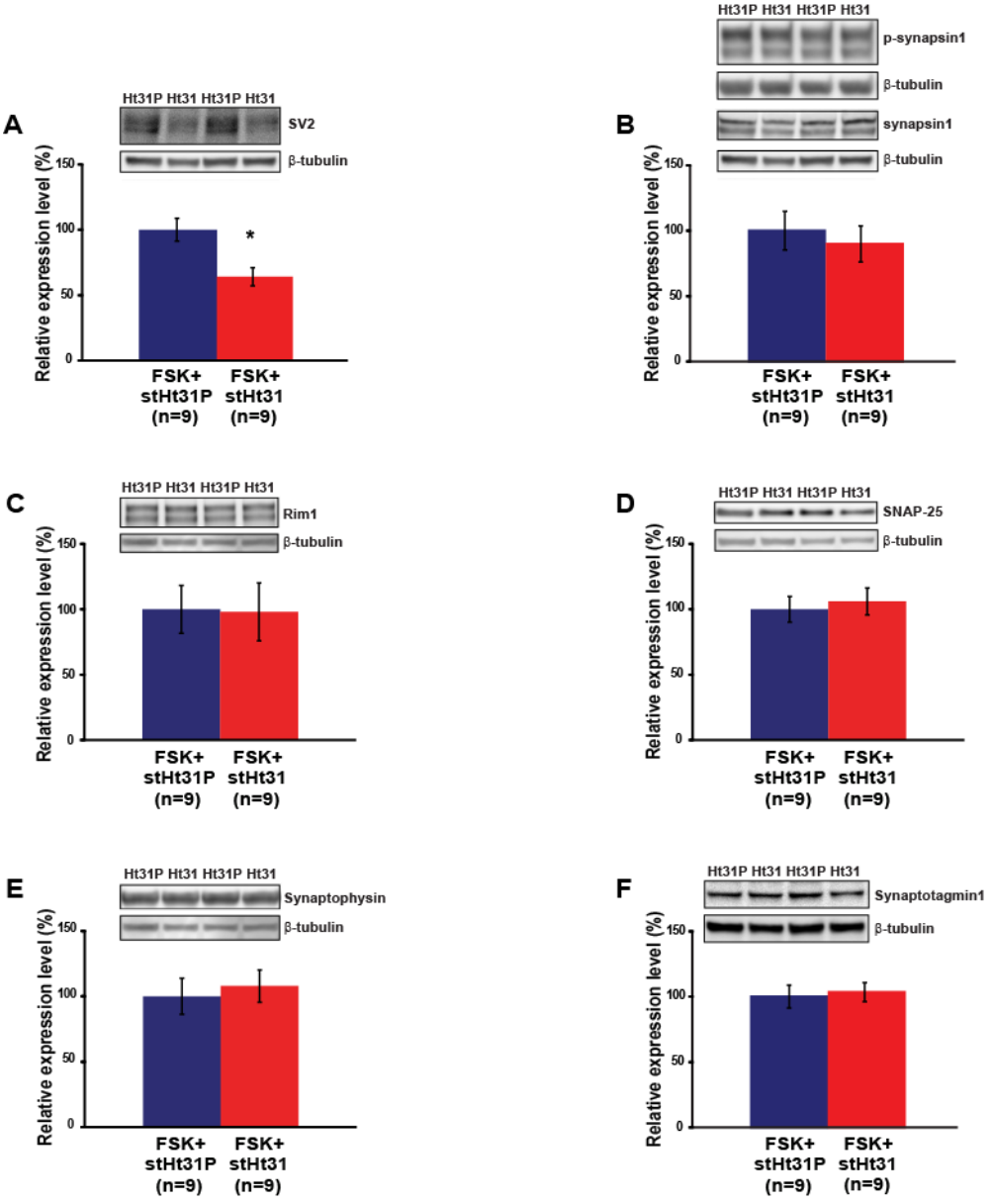


Figure 2.2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Relative expression level (%)
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100
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0
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synapsin1
β-tubulin
Relative expression level (%)
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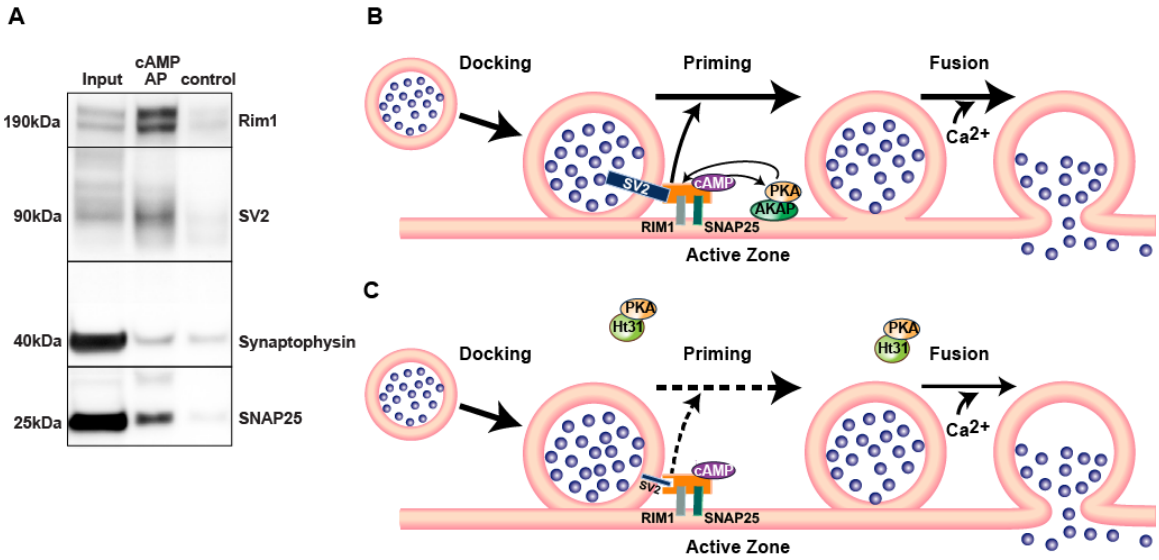
Figure 2.3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Active Zone
C
PKA
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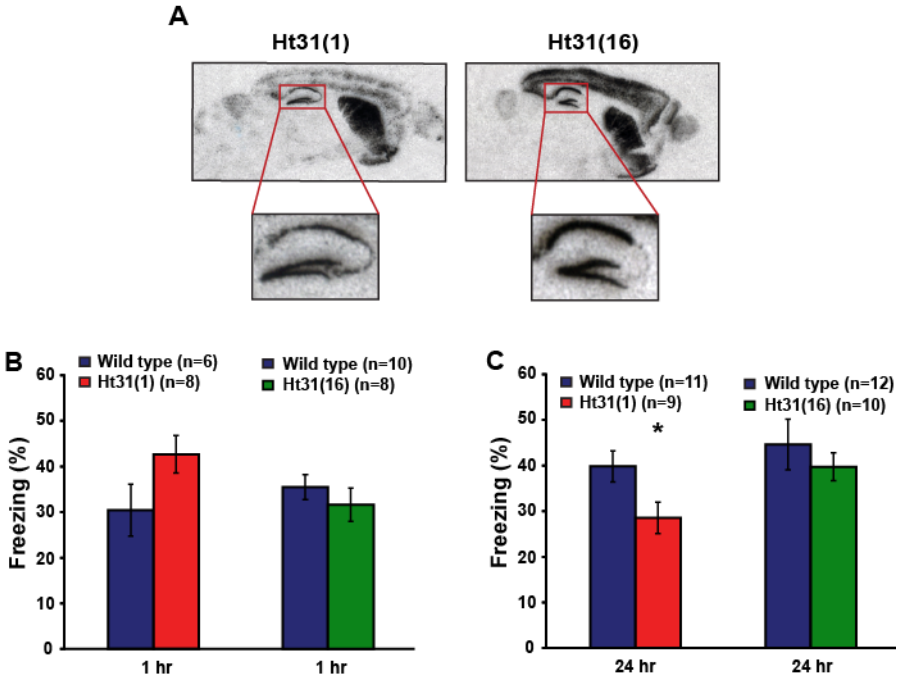
Figure 2.4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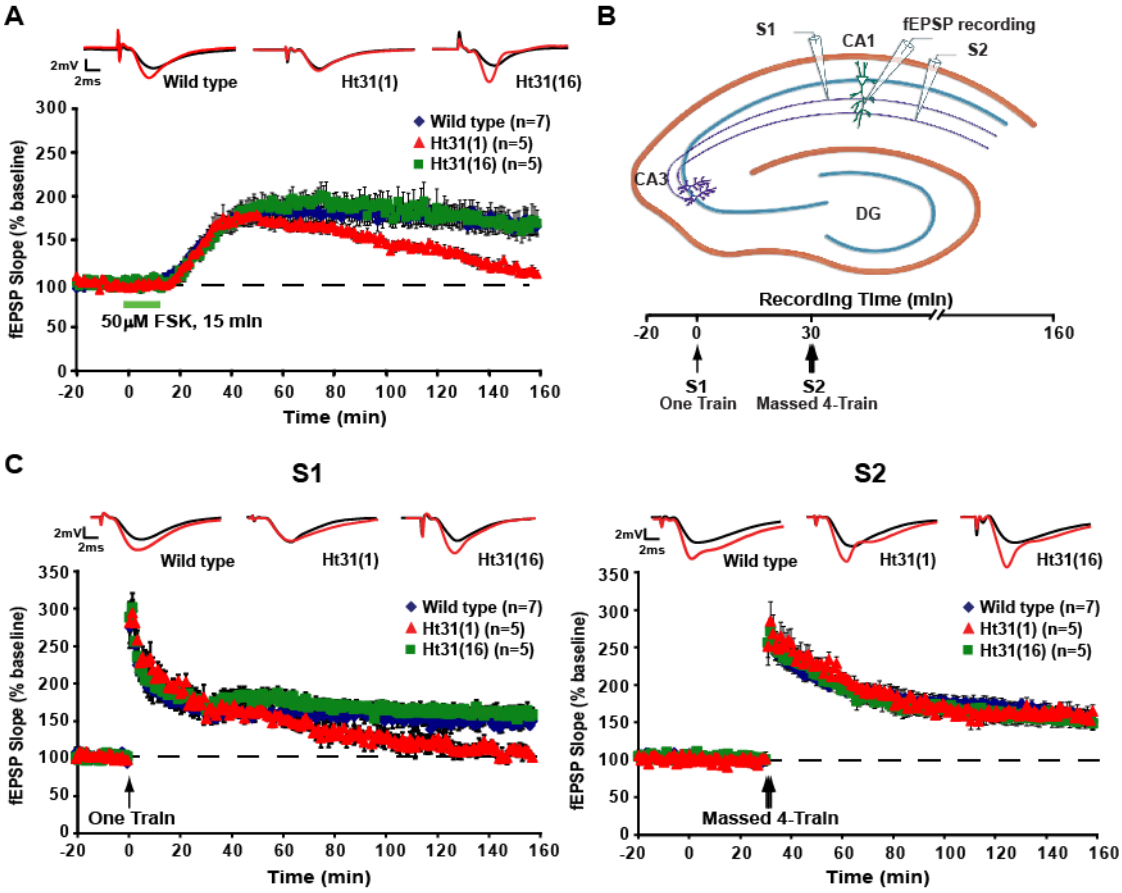
Ht31(1)
Ht31(16)
B
Wild type (n=6)
Ht31(1) (n=8)
Wild type (n=10)
Ht31(16) (n=8)
Freezing (%)
1 hr
1 hr
C
Wild type (n=11)
Ht31(1) (n=9)
Wild type (n=12)
Ht31(16) (n=10)
*
Freezing (%)
24 hr
24 hr

Figure 2.5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2mV
2ms
Wild type
Ht31(1)
Ht31(16)
Wild type (n=7)
Ht31(1) (n=5)
Ht31(16) (n=5)
fEPSP Slope (% baseline)
50μM FSK, 15 min
Time (min)
B
S1
CA1
fEPSP recording
S2
CA3
DG
Recording Time (min)
S1
One Train
S2
Massed 4-Train
C
S1
S2
One Train
Massed 4-Train

CHAPTER 3: The RNA-binding protein translin mediates synaptic tagging and memory via RNA processing

Abstract

Pathway-specific synaptic tagging and capture illustrates the interaction of two independent sets of synapses and has been studied as a cellular model for memory storage that requires integration of independent, but related information. Translin (also known as testes-brain RNA-binding protein, TBRBP) is associated with the miRNA pathway and can contribute to this pathway-specific plasticity by regulating RNA processing at activated synapses. Here, we investigate the role of translin in synaptic tagging and capture as well as long-term memory. Translin KO mice display deficits in synaptic tagging and capture and have impairments in certain forms of long-lasting long-term potentiation (LTP). Translin KO mice also exhibit disrupted hippocampus-dependent long-term object-location memory. Viral expression of translin in the hippocampus of adult translin KO mice rescued the deficits in synaptic tagging and capture and long-term memory. Additionally, translin KO mice show upregulation of specific miRNAs that are implicated in neurological disorders after trainings for the object-location memory task. Hence, our results suggest that translin mediates activity-dependent long-lasting LTP and long-term memory by regulating RNA processing.

Introduction

Hippocampal long-term potentiation (LTP) has been studied as a cellular correlate of memory storage (Mark Mayford et al., 2012). Synaptic tagging and capture is a pathway-specific form of LTP, in which protein synthesis-independent short-lasting LTP in one pathway becomes long-lasting when it is paired with protein synthesis-dependent long-lasting LTP in a separate pathway (Frey & Morris, 1997, 1998). Because of the interaction between two independent sets of synapses, synaptic tagging and capture has been studied as a physiological model for memory that requires integration of independent, but related, information (Clopath, 2012; Redondo & Morris, 2011). This pathway-specificity is believed to be provided, in part, by the regulation of dendritic processing of mRNAs (Barco, Lopez de Armentia, & Alarcon, 2008; Kelsey C Martin & Kosik, 2002; Smalheiser & Lugli, 2009). RNA-binding proteins are suggested to play important roles in synaptic tagging and capture because they regulate stability, movement, and processing of bound mRNAs (Doyle & Kiebler, 2011; Ule & Darnell, 2006).

Translin, also known as testis-brain RNA-binding protein (TBRBP), is evolutionarily conserved from yeast to mammals, implicating its critical role in cellular function (Devon, Taylor, Millar, & Porteous, 2000). This RNA-binding protein is mostly expressed in testes and the brain (W. Gu et al., 1998; Han, Gu, & Hecht, 1995) and are present somatodendritically in neurons (Finkenstadt et al., 2000; Kobayashi, Takashima, & Anzai, 1998; Muramatsu, Ohmae, & Anzai, 1998; X. Q. Wu, Petrusz, & Hecht, 1999). Additionally, translin and its target mRNAs show activity-dependent dendritic trafficking along microtubules (Han, Yiu, & Hecht, 1995; Y.-C. Wu et al., 2011). Moreover, translin forms a hetero-octamer with its partner protein translin-associated factor X (TRAX), a catalytic subunit with endoRNase activity (Finkenstadt et al., 2000; Y. Liu et al., 2009; Tian et al., 2011; Ye et al., 2011). This translin-TRAX hetero-octamer enhances the activity of RNA-induced silencing complex (RISC), which mediates the miRNA-mediated processing of mRNAs (Y. Liu et al., 2009). Thus, the miRNA-mediated regulation of mRNAs at activated synapses by translin can contribute to pathway-specific synaptic plasticity and memory.

Genetic, electrophysiological, biochemical and behavioral approaches using translin knockout (KO) mice reveal that translin is required for synaptic tagging and capture, certain forms of long-lasting LTP and long-term memory storage. Finally, we provide evidence that translin regulates these processes by activity-dependent regulation of the levels of specific miRNAs.

Materials and Methods

Translin knockout (KO) mice

The generation and maintenance of translin KO mice (MGI:2677496) were described previously (Chennathukuzhi et al., 2003; Stein et al., 2006). Mice were backcrossed to C57BL/6J for more than 15 generations. Heterozygous male and heterozygous female mice were mated to produce homozygous translin KO mice and wildtype littermates. Mice were maintained on a 12 hour light/12 hour dark cycle with lights on at 7 am (ZT0). Food and water were available *ad libitum*. All experiments were performed during the light cycle using translin KO mice and wildtype littermates as controls. 2- to 5-month-old mice were used for all experiments except for the LTD experiment in which 4- to 6-week-old mice were used. All experiments were performed according to the National Institutes of Health guidelines, and were fully approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Drugs

Forskolin (FSK, Molecular grade, Sigma), an adenylyl cyclase activator, was freshly prepared as a 50 mM solution in 100% ethanol and delivered at 50 μM final concentration in artificial cerebrospinal fluid (aCSF, pH 7.4) as described before (Havekes et al., 2012). (*RS*)-3,5-Dihydroxyphenylglycine (DHPG, Tocris), a potent agonist of group I metabotropic glutamate receptors (mGluRs), was freshly prepared as a 10 mM solution in milliQ water and delivered at 100 μM final concentration in aCSF as previously described (Citri, Soler-Llavina, Bhattacharyya, & Malenka, 2009).

Antibody production

Translin and TRAX antibodies were produced (New England Peptide, Inc.) based on the sequences provided previously (Finkenstadt et al., 2000). The antibody synthesis was based on

the C-terminal sequences of the human translin and TRAX. The peptide sequence for translin is CKYDLSIRGFNKETA, and for TRAX is CDVFSVKTEMIDQEEGIS.

DNA manipulation and adeno-associated virus (AAV) constructs

The $pAAV_9$-CaMKIIα0.4-myc-translin and $pAAV_9$-CaMKIIα0.4-eGFP were produced through standard methods and packaged by the University of Pennsylvania viral core. Titers ranged from 1.06×10^{13} to 2.02×10^{13} genome copy numbers. The 0.4kb CaMKIIα promoter fragment was used to drive expression selectively in excitatory neurons.

Electrophysiology

Experiments were performed as described before (Bridi & Abel, 2013; Havekes et al., 2012; Vecsey et al., 2009). Briefly, both male and female 2-5-month-old mice were sacrificed by cervical dislocation and hippocampi were quickly collected in chilled, oxygenated aCSF (124 mM NaCl, 4.4 mM KCl, 1.3 mM $MgSO_4 \cdot 7H_2O$, 1 mM $NaH_2PO_4 \cdot H_2O$, 26.2 mM $NaHCO_3$, 2.5 mM $CaCl_2 \cdot 2H_2O$ and 10 mM D-glucose) bubbled with 95% O_2 / 5% CO_2. 400 µm thick transverse hippocampal slices were placed in an interface recording chamber at 28ºC (Fine Science Tools, Foster City, CA). aCSF was constantly perfused over slices at 1 ml/min (or 2.5 ml/min for the mGluR-LTD experiment). Slices were equilibrated for at least 2 hours in aCSF. The stimulus strength was set to elicit 40% of the maximum fEPSP amplitude. The first 20-min baseline values were averaged and the average was used to normalize each initial fEPSP slope. The input–output relationship and paired-pulse facilitation (PPF) were measured as previously described (Vecsey et al., 2009, 2013).

To electrically induce LTP, spaced 4-train (four 1 s 100 Hz trains delivered 5 minutes apart), massed 4-train (four 1 s 100 Hz trains delivered 5 seconds apart), theta-burst stimulation (TBS, 40 ms of 15 bursts of four 100 Hz pulses delivered for a total of 3 s at 5 Hz), and one-train (one 1 s 100 Hz train) stimulation were delivered after 20 min baseline recordings. To chemically induce LTP, 50 µM of FSK in aCSF was perfused over slices for 15 minutes following 20-min

baseline recordings. To chemically induce LTD, 100 μM of DHPG in aCSF was perfused over slices for 10 minutes following 20-min baseline recordings. Two-pathway synaptic tagging and capture experiments were performed as previously described (T. Huang et al., 2006). Briefly, strong massed 4-train stimulation that elicits a long-lasting form of LTP (L-LTP) was delivered to one pathway (S1) after the 20-min baseline recordings. Thirty minutes later, weak one-train stimulation that induces a short-lasting early form of LTP (E-LTP) was given to the other pathway (S2). PPF at 50 ms interval was used to confirm the independence of the two inputs.

Western blot analyses

Hippocampal tissue homogenization, protein separation and transfer to polyvinylidene difluoride membranes were performed as previously described (Vecsey et al., 2012). Membranes were blocked in 5% BSA or 5% non-fat milk in TBST and incubated with primary antibodies (translin, 1:100,000; TRAX, 1:1,000; FMRP, 1:10,000, Millipore; myc, 1:5,000, Cell Signaling) overnight at 4°C. They were washed and incubated with appropriate horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (1:10,000, Santa Cruz) for 1 h in room temperature. Blots were exposed on a film by ECL and quantified using ImageJ. The density of signal was normalized to β-tubulin levels (1:50,000, Sigma).

Object-place recognition task

All animals were single housed one week before behavioral experiments. Sexes were balanced across groups, and mice from the same litter were used. Mice were handled for 3 minutes per day for 6 consecutive days prior to the experiment. All experiments were conducted between ZT0 and ZT2 as previously described (Havekes et al., 2012). Briefly, mice underwent 6 min habituation session in an empty box followed by three 6 min training sessions in the same box containing three different objects. The inter-session interval was 3 min. An internal cue was attached on one wall of the box so that mice can locate each object relative to the cue when they were freely exploring the environment. Twenty-four hours later, mice were placed back in the box

where the location of one of the objects was displaced. Time spent on the displaced and non-displaced objects was measured during the 6 min exploration period. The identity and location of each object were balanced between subjects. Exploration on an object was determined by sniffing, touching, and facing in a close proximity. The spatial memory for the displaced object was examined by preference that was measured by time spent on the displaced object over total exploration time.

Viral injections

The AAVs were injected using a nanofil 33G beveled needles (WPI) attached to a 10 µl Hamilton syringe controlled by a microsyringe pump (UMP3; WPI). The coordinates were: A/P -1.9 mm, D/V +/- 1.5 mm, and 1.5 mm below bregma. The needle was slowly lowered to the target site over the course of 3 minutes and remained at the target site for 1 minute before beginning of the injection (0.2 µl per minute). Approximately 1 µl (corrected for genome copy number between constructs) was injected per hippocampus. After the injection, the needle was remained at the target site for 1 minute and then was slowly removed over a 5 minute period.

Immunohistochemistry

Transcardial perfusions and immunohistochemical stainings were conducted as previously described (Vecsey et al., 2009). Brain sections were made from translin KO mice injected with myc-translin virus. The sections were permeabilized with 0.3% Triton X-100 for 1 h in PBS at room temperature and incubated with anti-GFAP (mouse, 1:100, Millipore) and anti-myc (rabbit, 1:200, Cell Signaling) antibodies overnight at 4 °C. After washing in PBS, sections were incubated for 3 hours with biotinylated goat-anti-rabbit (1:1000, Jackson ImmunoResearch Laboratories) and goat-anti-mouse Alexa Fluor 488 (1:1000, Invitrogen) secondary antibodies at room temperature. Following washes in PBS, sections were incubated with the avidin-biotin-horseradish peroxidase complex (1:500 ABC kit, Vector Laboratories) in PBS for 1.5 hour at room temperature. After washing in PBS, myc signal was amlplified with TSA-Plus kit (PerkinElmer) for

10 min. Then, sections were stained with DAPI (Molecular probes, Life technologies). Sections were mounted with gelatin (0.7%) and dried for 24 hours. The mounted sections were coverslipped with PermaFluor (Thermoscientific) and dried for 24 hours. Imaging was conducted on a Leica confocal microscope.

cDNA synthesis and Quantitative real-time reverse transcription (RT)-PCR

Total RNA extraction was performed as previously described (Vecsey et al., 2007). Hippocampal tissue was collected from translin KO mice and wildtype littermates that either remained in the homecage or underwent the object-place recognition task. For miRNA assays, 250 ng RNA was used in each miScript miRNA PCR Arrays (Qiagen). cDNA synthesis with miScript HiSpec Buffer was performed according to the manufacturer's protocol. cDNA reactions were diluted in 200 µl of RNase-free water. A neurological development and disease miRNA PCR array (MIMM-107ZE-1, Qiagen) was used to probe for candidate miRNAs that are affected in translin KO mice underwent the object-place recognition task. The experiment was performed in duplicate according to the manufacturer's protocol with cDNA samples from a pair of translin KO mouse and wildtype littermates. The candidate miRNAs were subject to real-time RT-PCR reactions that were prepared in 384-well optical reaction plates with optical adhesive covers (ABI, Foster City, CA). Each reaction was composed of 1 µl cDNA, 5 µl 2x Quantitect SYBERGreen Master Mix (Qiagen), 1 µl Universal primer, 2 µl RNase-free water, and 1 µl of one of the following miScript Primers (Qiagen): let 7b-5p – MS00001225 , let 7c-5p – MS00005852, let 7d-5p – MS00001232, let 7e-5p – MS00032186, miR 124-3p – MS00029211, miR 125b-5p – MS00005992, miR128-3p – MS00011116, miR 9-5p – MS00012873, miR 9-3p – MS00005887, miR 409-3p – MS00011970, SNORD68 – MS00033712. Reactions were performed in duplicate on the Viia7 Real-Time PCR system (Life technologies, Carlsbad, CA). The ΔΔCt method was used for relative quantification of gene expression between the handling-only and the trained mice as described previously (Vecsey 2007).

Data analysis

Data analyses were performed using Statistica 10 and SPSS V10. The maintenance of LTP or LTD was analyzed using a two-way repeated-measures ANOVA test on the last 20-min of the recordings (Vecsey et al., 2009). The average of the normalized slopes over the last 20-min was evaluated by a t-test. Input–output data were analyzed using a t-test comparing the average linear regression slopes between each group. PPF data were analyzed using a two-way repeated-measures ANOVA with group and inter-stimulus interval as factors, and PPF as the variable. For evaluation of biochemical, behavioral, and gene expression data, a t-test or a one-way ANOVA was performed. Dunnett's post hoc test was performed if applicable. Differences were considered statistically significant when $p < 0.05$. Data were plotted as mean ± S.E.M.

Results

Translin KO mice show impairments in synaptic tagging and capture

Biochemical and behavioral studies suggest that translin plays important roles in neuronal function (Chiaruttini et al., 2009; Stein et al., 2006), but its significance in neuronal physiology has not been examined. To investigate the role of translin in synaptic plasticity, basal synaptic properties in translin KO mice were first examined. Electrophysiological recordings at the hippocampal Schaffer collateral CA3-CA1 synapse showed no difference in the input-output relationship between hippocampal slices from translin KO mice and wildtype littermates (**Fig. 3.1*A***; translin KO mice: 5 ± 1.9, n = 6; wildtype littermates: 3.8 ± 0.6, n = 5, t-test, $p = 0.56$). The maximum fEPSP slope was also not altered in slices from translin KO mice (translin KO mice: 7.4 ± 1.3 mV/ms, n = 8; wildtype littermates: 7.2 ± 0.8 mV/ms, n = 8, t-test, $p = 0.88$). We then measured paired-pulse facilitation (PPF), a presynaptic form of short-term plasticity in which the synaptic response is facilitated by two closely spaced synaptic pulses (Katz & Miledi, 1968; Zucker & Regehr, 2002). Slices from translin KO mice exhibited PPF at various interpulse intervals similar to slices from wildtype littermates (**Fig. 3.1*B***; n = 8 for each group, two-way repeated measures ANOVA, $F_{(1,14)} = 0.07$, $p = 0.8$). Taken together, these data suggest that translin is dispensable for basal synaptic properties.

In synaptic tagging and capture, protein synthesis-independent E- LTP in one pathway becomes persistent by protein synthesis-dependent L- LTP in a separate pathway (Frey & Morris, 1997, 1998). This process requires pathway-specific transport of plasticity-related proteins (Redondo et al., 2010). Because translin mediates dendritic transport of mRNAs and controls translation of its bound mRNAs (Chennathukuzhi et al., 2003; Kwon & Hecht, 1993), we investigated whether translin is required for this pathway-specific plasticity. Strong massed 4-train stimulation was delivered to induce L-LTP in a strong pathway (S1), and one-train stimulation was delivered to induce E-LTP in a weak pathway (S2) (**Fig. 3.1*C***). One-train stimulation elicited E-LTP that lasted less than 2 hours in slices from both translin KO mice and wildtype littermates

(**Fig. 3.1*D***; n = 5 for each group, two-way repeated-measures ANOVA, $F_{(1,8)} = 0.07$, $p = 0.8$). The average of the initial fEPSP slope over the last 20 min of the recordings was not significantly different between slices from translin KO mice and wildtype littermates (wildtype littermates: 104.7 ± 5.04%, n = 5; translin KO mice: 107.7 ± 12.2%, n = 5, t-test, $p = 0.801$). In S1, strong stimulation induced L-LTP in slices from both translin KO mice and wildtype littermates at a similar level (**Fig. 3.1*E*** *left panel*; n = 5 for each group, two-way repeated-measures ANOVA, $F_{(1,8)} = 0.03$, $p = 0.88$). The average of the initial fEPSP slope over the last 20 min of the recordings was similar between slices from translin KO mice and wildtype littermates (wildtype littermates: 149 ± 11.2%, n = 5; translin KO mice: 150.8 ± 5.4%, n = 5, t-test, $p = 0.875$). In S2, however, E-LTP elicited by one-train stimulation following S1 stimulation became long-lasting only in slices from wildtype littermates, not in slices from translin KO mice (**Fig. 3.*1E*** *right panel*; n = 5 for each group, two-way repeated-measures ANOVA, $F_{(1,8)} = 36.5$, $p = 0.0003$). The average of the initial fEPSP slope over the last 20 min of the recordings was reduced in slices from translin KO mice (wildtype littermates: 148.1 ± 5.8%, n = 5; translin KO mice: 102.6 ± 6.1%, n = 5, t-test, $p = 0.0003$). Our results suggest that synaptic tagging and capture requires translin.

Translin KO mice have deficits in certain forms of L-LTP

We found that lack of translin impairs synaptic tagging and capture, while it does not impact L-LTP induced by massed 4-train stimulation. This suggests that translin is involved only in certain forms of synaptic plasticity. To understand the physiological role of translin, we examined different forms LTP that have distinct molecular requirements at the hippocampal Schaffer collateral CA3-CA1 synapse. A form of L-LTP induced by spaced 4-train stimulation requires compartmentalized PKA activity in postsynaptic CA1 neurons (Duffy & Nguyen, 2003; Nie et al., 2007). Hippocampal slices from translin KO mice showed impairments in this form of L-LTP (**Fig. 3.2*A***; n = 5 for each group, two-way repeated-measures ANOVA, $F_{(1,8)} = 34.43$, $p = 0.00038$). The average of the initial fEPSP slope over the last 20 min of the recordings was reduced in slices

from translin KO mice compared to slices from wildtype littermates (wildtype littermates: 176.6 ± 13.5%, n = 5; translin KO mice: 89.5 ± 9.6%, n = 5, t-test, p = 0.00037). Theta-bust stimulation (TBS) induces a different form of L-LTP which relies on the increased transmitter release or compartmentalized PKA activity in presynaptic CA3 neurons (Bayazitov, Richardson, Fricke, & Zakharenko, 2007; Nie et al., 2007; Zakharenko et al., 2003). This form of L-LTP was unaffected in slices from translin KO mice (**Fig. 3.2*B***; n = 5 for each group, two-way repeated-measures ANOVA, $F_{(1,8)} = 0.007$, $p = 0.94$). The average of the initial fEPSP slope over the last 20 min of the recordings was similar between slices from translin KO mice and wildtype littermates (wildtype littermates: 150.03 ± 7.8%, n = 5; translin KO mice: 151.2 ± 13.4%, n = 5, t-test, p = 0.936). Furthermore, slices from translin KO mice and wildtype littermates showed similar forskolin-induced long-lasting potentiation, which also requires the enhancement of presynaptic transmitter release (Bolshakov et al., 1997; Bouron, 1999; Bozdagi, Shan, Tanaka, Benson, & Huntley, 2000; Chavez-Noriega & Stevens, 1994; D. Kim & Thayer, 2001; Ma et al., 1999; Moulder et al., 2008; Trudeau et al., 1996) (**Fig. 3.2*C***; n = 5 for translin KO mice, n = 6 for wildtype littermates, two-way repeated-measures ANOVA, $F_{(1,9)} = 0.07$, $p = 0.79$). The average of the initial fEPSP slope over the last 20 min of the recordings was comparable between slices from translin KO mice and wildtype littermates (wildtype littermates: 180 ± 14.3%, n = 5; translin KO mice: 180.4 ± 11.8%, n = 5, t-test, p = 0.982). Finally, L-LTP elicited by massed 4-train stimulation was unaltered in slices from translin KO mice as shown in **Fig. 3.1*E*** (**Fig. 3.2*D***; n = 5 for translin KO mice, n = 5 for wildtype littermates, two-way repeated-measures ANOVA, $F_{(1,8)} = 0.923$, $p = 0.365$). The average of the initial fEPSP slope over the last 20 min of the recordings was similar between slices from translin KO mice and wildtype littermates (wildtype littermates: 143.6 ± 8.2%, n = 5; translin KO mice: 154.6 ± 9.9%, n = 5, t-test, p = 0.364). The unaltered one-train-induced E-LTP (**Fig. 3.1*D***) and massed 4-train-induced L-LTP (**Fig. 3.2*D***) in translin KO mice suggest the specificity of the synaptic tagging and capture deficits in these mice (**Fig. 3.1*E***). Collectively, these data suggest that translin is selectively required for certain forms of L-LTP.

Translin KO mice exhibit unaltered mGluR-LTD and protein levels of hippocampal FMRP

The most well studied RNA-binding protein is fragile X mental retardation protein (FMRP). Interestingly, FMRP KO mice generally do not show deficits in hippocampal LTP including synaptic tagging and capture as well as spaced 4-train-induced L-LTP (Connor, Hoeffer, et al., 2011; Godfraind et al., 1996; Lauterborn et al., 2007; J. Li, Pelletier, Perez Velazquez, & Carlen, 2002; Paradee et al., 1999; J. Zhang, Hou, Klann, & Nelson, 2009). This indicates the possibility that translin and FMRP are differentially involved in synaptic plasticity, although they have similar properties as RNA-binding proteins. To address this question, we tested metabotropic glutamate receptor-mediated LTD (mGluR-LTD) in hippocampal slices from translin KO mice. Exaggerated mGluR-LTD is a well characterized phenotype of FMRP KO mice and has been proposed as an underlying mechanism of fragile X syndrome (L. Hou et al., 2006; Huber, Gallagher, Warren, & Bear, 2002; Nosyreva & Huber, 2006). In contrast to the findings from FMRP KO mice, mGluR-LTD was unaffected in slices from translin KO mice (**Fig. 3.3*A***; n = 5 for each group, two-way repeated measures ANOVA, $F_{(1,8)} = 0.08$, $p = 0.79$). The average of the initial fEPSP slope over the last 20 min of the recordings was comparable between slices from translin KO mice and wildtype littermates (wildtype littermates: 75.9 ± 3.2%, n = 5; translin KO mice: 78.9 ± 3.1%, n = 5, t-test, $p = 0.473$). If translin and FMRP are functionally independent regardless of their similarities as RNA-binding proteins, loss of translin would not cause compensatory increase in the protein levels of FMRP. Indeed, Western blot analyses showed no changes in the protein levels of hippocampal FMRP in translin KO mice relative to wildtype littermates (**Fig. 3.3*B***; translin KO mice: 102.5 ± 0.4%, n = 6; wildtype littermates: 100 ± 4.3%, n = 6, t-test, $p = 0.36$). Despite their functional similarities, our data indicate that translin and FMRP play distinct roles in hippocampal synaptic plasticity.

Translin and TRAX levels are restored 20 days after the viral injection of translin in the adult translin KO hippocampus

We used a viral approach to express translin in the adult hippocampus of translin KO mice to test whether the observed LTP deficits in translin KO mice are specifically due to the loss of translin. Because translin is primarily expressed in neurons (X. Q. Wu et al., 1999), we generated an adeno-associated virus (AAV) in which the CaMKIIα promoter fragment drives neuronal expression of myc-tagged-translin. The eGFP construct was used as a control (**Fig. 3.4*A***). The expression time course experiment showed that the virally-expressed translin reached the expression level of wildtype translin 15 to 20 days after the viral injection into the adult translin KO hippocampus (**Fig. 3.4*B***; one-way ANOVA, $F_{(4,11)} = 15.41$, $p = 0.0002$, for 15 day post-injection, translin KO mice: 105.6 ± 30.3%, n = 3; wildtype littermates: 100 ± 15.9%, n = 3, Dunnett's post hoc test, wildtype vs. 15 day $p = 0.998$, for 20 day post-injection, translin KO mice: 159.6 ± 24.4%, n = 4; wildtype littermates: 100 ± 15.9%, n = 3, Dunnett's post hoc test, wildtype vs. 20 day $p = 0.083$). A linear relationship between the expression level of the myc tag and myc-translin was also found (linear regression, R^2=0.99). The expression of translin-associated factor X (TRAX) is post-transcriptionally controlled by translin such that TRAX is not translated from its mRNA when translin is absent (Chennathukuzhi et al., 2003; Claussen, Koch, Jin, & Suter, 2006; Jaendling, Ramayah, Pryce, & McFarlane, 2008; Yang et al., 2004). Indeed, TRAX protein was absent in adult translin KO hippocampus, but reinstated to the wildtype expression levels 20 days after the viral injection of translin (**Fig. 3.4*C***; one-way ANOVA, $F_{(4,11)} = 14.69$, $p = 0.0002$, for 20 day post-injection, translin KO mice: 93.9 ± 12.4%, n = 4; wildtype littermates: 100 ± 14%, n = 3, Dunnett's post hoc test, wildtype vs. 20 day $p = 0.985$). These data suggest that the function of translin in the KO hippocampus is restored to the wildtype level 20 days after the viral injection of translin, and we performed all subsequent viral experiments at 20 day post-injection. The expression pattern of the viral translin was examined to see whether it matches the wildtype translin expression pattern. Using a myc tag antibody, we found that the viral translin was expressed in the nucleus, cell body and Schafer collateral, but not in nucleoli and glia (**Fig. 3.4*D***).

This expression pattern matches the wildtype translin expression pattern (Kobayashi et al., 1998; X. Q. Wu et al., 1999), suggesting that the virally expressed translin functions as endogenous translin.

Virally expressing translin in the adult translin KO hippocampus rescues synaptic tagging and capture deficits

To examine the physiological effects of the viral expression of translin, we injected either translin or eGFP virus into the adult translin KO hippocampus and performed electrophysiological recordings 20 days after the injection. The input-output relationship was not significantly different between slices from the hippocampi injected with the translin virus or with the eGFP virus (**Fig. 3.5*A***; translin virus group: 3.6 ± 0.5, n = 6; eGFP virus group: 3.5 ± 0.5, n = 6, t-test, $p = 0.85$). Also, PPF was similar between slices from the hippocampi injected with the translin virus or with the eGFP virus (**Fig. 3.5*B***; n = 6 for each group, two-way repeated measures ANOVA, $F_{(1,10)} = 0.224$, $p = 0.646$). In addition, we compared these input-output and PPF data with those obtained from wildtype and translin KO hippocampal slices shown in **Figure 3.1*A* and *B***. No significant differences across groups were found for both properties (for the input-output, one-way ANOVA, $F_{(3,19)} = 0.479$, $p = 0.701$; for PPF, two-way repeated measures ANOVA, $F_{(3,24)} = 0.469$, $p = 0.707$). These data further suggest that translin is dispensable for basal synaptic properties and the expression of either virus does not cause any non-specific alterations in basal synaptic function.

We next examined synaptic tagging and capture from translin KO hippocampal slices that virally express either translin or eGFP. In S1, strong massed 4-train stimulation induced similar levels of L-LTP in slices expressing either virus (**Fig. 3.5*C*** ***left***; n = 4 for each group, two-way repeated-measures ANOVA, $F_{(1,6)} = 2.53$, $p = 0.16$). In S2, weak one-train stimulation elicited long-lasting LTP only in slices expressing translin (**Fig. 3.5*C*** ***right***; n = 4 for each group, two-way

repeated-measures ANOVA, $F_{(1,6)} = 29.33$, $p = 0.002$). The average of the initial fEPSP slope over the last 20 min of the recordings in S1 was similar between translin KO hippocampal slices expressing either translin or eGFP (eGFP virus group: 140.4 ± 2.8%, n = 4; translin virus group: 160 ± 13.9%, n = 4, t-test, $p = 0.163$). The average of the initial fEPSP slope over the last 20 min of the recordings in S2 was higher in translin KO hippocampal slices expressing translin compared to the KO slices expressing eGFP (eGFP virus group: 104.8 ± 6.4%, n = 4; translin virus group: 141.8 ± 4.7%, n = 4, t-test, $p = 0.0016$). Taken together, these data suggest that synaptic tagging and capture requires translin and the observed deficits in translin KO mice were not due to non-specific effects of developmental deletion of translin.

Viral expression of translin in the adult translin KO hippocampus rescues impaired long-term object-place memory in translin KO mice

To investigate whether translin is required for memory, we performed a hippocampus-dependent object-place recognition task (Havekes et al., 2012). Mice explore the displaced object more if they remember the original location of each object, and memory was measured as preference for the displaced object. Translin KO mice showed significantly less preference for the displaced object 24 hours after training compared to wildtype littermates (**Fig. 3.6*A***; translin KO mice: 44.7 ± 3.2%, n = 9; wildtype littermates: 61 ± 3.6%, n = 9, t-test, $p = 0.0027$). This suggests that translin KO mice have impaired long-term memory for this spatial task. To determine whether this memory deficit is specifically due to the lack of translin in the hippocampus, we adopted the viral approach. Either translin or eGFP virus was injected into the adult hippocampus of translin KO mice or wildtype littermates. Twenty days after the injection, the preference for the displaced object 24 hours after training was compared between mice injected with either virus and wildtype littermates not injected with any of these viruses (**Fig. 3.6*B***; one-way ANOVA, $F_{(3,29)} = 13.89$, $p = 0.00001$, non-injected wildtype littermates: 51.7 ± 2.1%, n = 8; translin-injected translin KO mice: 54.7 ± 2.4%, n = 8; eGFP-injected translin KO mice: 41.9 ± 2 %, n = 8; eGFP-injected wildtype

littermates: 52.1 ± 2 %, n = 9). Translin KO mice virally expressing translin and non-injected wildtype littermates showed similar preference for the displaced object 24 hours after training (**Fig. 3.6*B***; Dunnett's post hoc test, $p = 0.354$). Translin KO mice expressing eGFP in the hippocampus, however, displayed significantly less preference for the displaced object compared to non-injected wildtype littermates 24 hours after training (**Fig. 3.6*B***; Dunnett's post hoc test, $p = 0.0003$). We also found that eGFP expression in the wildtype hippocampus did not affect the level of preference compared to the non-injected wildtype littermates. This suggests that the viral expression itself did not have any non-specific effects on the memory formation (**Fig. 3.6*B***; Dunnett's post hoc test, $p = 0.992$). Therefore, viral expression of translin within the adult translin KO hippocampus rescued the memory deficits in translin KO mice, indicating that hippocampal translin is indeed required for hippocampus-dependent long-term memory storage.

Translin KO mice exhibit altered levels of specific miRNAs

Translin is associated with the miRNA pathway and controls dendritic transport as well as expression of specific mRNAs (Chennathukuzhi et al., 2003; Kobayashi et al., 1998; Severt et al., 1999; X. Q. Wu & Hecht, 2000). Therefore, it is likely that translin mediates synaptic plasticity and memory through activity-dependent regulation of RNA metabolism. To investigate activity-dependent changes in miRNA levels mediated by translin, hippocampal tissue from translin KO mice and wildtype littermates was collected 30 minutes after training for the object-place recognition task. We used a neuronal disease-related miRNA PCR array (MIMM-107ZE-1, Qiagen) to probe for candidate miRNAs that are affected in translin KO mice after the training. The candidate miRNAs were confirmed using a real-time RT-PCR. Compared to wildtype littermates, translin KO mice displayed significantly elevated levels of let 7c-5p (translin KO mice: 1.5 ± 0.1, n = 6; wildtype littermates: 1 ± 0.1, n = 7, t-test, $p = 0.003$), miR125b-5p (translin KO mice: 1.3 ± 0.1, n = 6; wildtype littermates: 1 ± 0.1, n = 7, t-test, $p = 0.045$), miR128-3p (translin KO mice: 1.4 ± 0.1, n = 6; wildtype littermates: 1 ± 0.1, n = 7, t-test, $p = 0.001$), miR9-3p (translin

KO mice: 1.4 ± 0.1, n = 6; wildtype littermates: 1 ± 0.1, n = 7, t-test, $p = 0.03$), and miR409-3p (translin KO mice: 1.9 ± 0.2, n = 6; wildtype littermates: 1 ± 0.03, n = 7, t-test, $p = 0.00008$) (**Fig. 3.7*A***). However, most of these miRNA levels were similar between translin KO mice and wildtype littermates that remained in the home cage (**Fig. 3.7*B***; t-test, $p > 0.1$), except for miR409-3p that was upregulated in translin KO mice relative to wildtype littermates (**Fig. 3.7*B***; translin KO mice: 1.4 ± 0.1, n = 6; wildtype littermates: 1 ± 0.1, n = 7, t-test, $p = 0.03$). These data suggest that translin mediates synaptic plasticity and memory through activity-dependent regulation of specific miRNAs.

Discussion

RNA-binding proteins have been suggested to play important roles in input-specific synaptic plasticity and memory because they not only stabilize RNAs bound to them but also mediate dendritic processing of their RNA targets (Doyle & Kiebler, 2011; Ule & Darnell, 2006). However, it is not clear what specific RNA-binding proteins and their target RNAs are crucial for synaptic plasticity and memory. Loss of FMRP does not affect hippocampal LTP including synaptic tagging and capture, but enhances LTD (Connor, Hoeffer, et al., 2011; Godfraind et al., 1996; L. Hou et al., 2006; Huber et al., 2002; Lauterborn et al., 2007; J. Li et al., 2002; Nosyreva & Huber, 2006; Paradee et al., 1999; J. Zhang et al., 2009). Additionally, a KO mouse model for another RNA-binding protein CPEB-1 shows deficits only in a certain form of LTP and has a mild influence on synaptic tagging and capture. Indeed, several RNA-binding proteins involved in specific aspects of neuronal plasticity have been identified (Tolino, Köhrmann, & Kiebler, 2012; Ule & Darnell, 2006). Here, we demonstrate that translin is dispensable for mGluR-LTD, but rather required for synaptic tagging and capture, specific forms of L-LTP and long-term memory. Our gene expression data suggest that translin mediates these processes by regulating levels of specific miRNAs.

To understand the role of translin in synaptic plasticity and memory, it is important to know its targets. We show that virally expressed translin is present in the nucleus, soma and dendrites in hippocampal CA1 neurons. This is consistent with the localization of translin (Kobayashi et al., 1998; X. Q. Wu et al., 1999), and this expression pattern brings a concern whether the major target of translin is DNA or RNA. Initially, translin is found as a DNA-binding protein in lymphoid cell lines, and is suggested to be involved in chromosomal translocation and DNA damage repair (Aoki et al., 1995; Aoki, Suzuki, Ishida, & Kasai, 1999; Kasai et al., 1997). However, the deletion of translin in mice, *Drosophila*, and yeast does not show alterations in these processes (Chennathukuzhi et al., 2003; Claussen et al., 2006; Jaendling et al., 2008). It seems that the primary localization of translin switches developmentally from the nucleus to the

cytoplasm in male germ cells in testes (Cho, Chennathukuzhi, Handel, Eppig, & Hecht, 2004) and in adult rat brains (Finkenstadt et al., 2000). Moreover, translin has RNase, but not DNase, activities (J. Wang, Boja, Oubrahim, & Chock, 2004) and preferentially binds RNA (Lluis, Hoe, Schleit, & Robertus, 2010). Therefore, translin likely mediates synaptic plasticity and memory by primarily regulating RNA metabolism in the cytoplasm. Imaging approaches to track the movement of translin between the dendrites and the nucleus after synaptic activation or learning will help to address this question.

We found that translin regulates learning-induced changes in specific miRNAs, but the mechanism by which translin mediates synaptic tagging and memory is not determined. A miRNA target gene search (miRbase or Targetscan) reveals that the miRNAs specifically upregulated after learning in translin KO mice target genes involved in the spatiotemporal regulation of PKA signaling, synaptic plasticity, and memory (Gerits et al., 2008; O'Dell, Connor, Gelinas, & Nguyen, 2010; Sadana & Dessauer, 2009; J. L. Sanderson & Dell'Acqua, 2011; T. M. Sanderson & Sher, 2013; D. O. Wang et al., 2010). These targets include G-protein coupled β-adrenergic receptors, various kinds of cAMP producing enzyme adenylyl cyclase, isoforms of cAMP degrading enzyme phosphodiesterase, several PKA anchoring proteins (also known as A-kinase anchoring proteins, AKAPs), mitogen-activated protein kinases (MAPKs), and local translational machineries. These findings suggest that translin suppresses specific miRNAs to promote compartmentalized cellular signaling and local translation during neuronal activity. Gene expression and biochemical studies should be followed to reveal the consequences of the miRNA regulation by translin.

An important question is how translin is involved in synaptic tagging and capture processes. In the model for synaptic tagging and capture, kinases transiently mark activated synapses (tagging), and the synaptic mark interacts with newly synthesized proteins (capture) in postsynaptic compartments for sustained synaptic potentiation (Redondo & Morris, 2011). As a candidate tagging mechanism, compartmentalized PKA activity through AKAPs is required for synaptic tagging and capture (T. Huang et al., 2006). AKAP5 mRNA, for example, is targeted by

miRNAs upregulated in translin KO mice after learning (miRbase or Targetscan). AKAP5 makes a postsynaptic complex with β-adrenergic receptors and adenylyl cyclases, and this protein complex facilitates local PKA signaling and downstream MAPK pathway activity (Fraser et al., 2000; M. Zhang et al., 2013). Additionally, those miRNAs upregulated in translin KO mice also suppress the expression of local translational machineries (miRbase or Targetscan), and our electrophysiology data imply a postsynaptic role for translin in protein synthesis-dependent forms of synaptic plasticity. Therefore, translin may orchestrate dendritic cAMP-PKA signaling with the local synthesis of proteins to coordinate tagging with capture processes (**Fig. 8**). This possibility is supported by our data that translin KO mice have impairments in synaptic tagging and capture as well as spaced 4-train-induced L-LTP, both of which require PKA activity and PKA anchoring (Barco et al., 2002; Duffy & Nguyen, 2003; T. Huang et al., 2006; Nie et al., 2007; Young et al., 2006).

It is worth noting that translin KO mice exhibit delayed growth, altered anxiety-related behavior and changes in noradrenergic and serotonergic levels (Chennathukuzhi et al., 2003; Stein et al., 2006). These alterations may contribute to the mixed results on the significance of translin in long-term memory assessed by aversive watermaze or fear conditioning tasks (Stein et al., 2006). Based on the findings that the behavior of translin KO mice is unaltered in a non-aversive neutral open field (Stein et al., 2006), we performed object-place recognition tasks where mice explore in an open field with different objects. Moreover, the viral expression of translin in the hippocampus of adult translin KO mice rescued the impaired synaptic tagging and capture and object-place memory in these mice. This suggests that the observed deficits in translin KO mice are not caused by constitutive deletion of translin and that translin within the hippocampus plays a critical role in synaptic plasticity and memory. Conditional KO approaches or siRNA-mediated deletion of translin will augment our results.

Dendritic localization of RNAs and protein synthesis at activated synapses are critical for input-specific neuronal plasticity. Several RNA-binding proteins are involved in these processes,

and our data provides an insight into the mechanism by which translin mediates synaptic plasticity and memory. Because translin regulates expression of miRNAs that are associated with neurodegenerative diseases such as Alzheimer's disease and schizophrenia, our study can lead to development of new therapeutic approaches for neuronal disorders.

Figure Legends

Figure 3.1. Translin KO mice exhibit impairments in synaptic tagging and capture in the hippocampal Schaffer collateral pathway.

A. The input-output relationship was similar between hippocampal slices from translin KO mice and wildtype littermates (t-test, $p = 0.56$). ***B.*** Slices from translin KO mice and wildtype littermates displayed similar PPF at different interpulse intervals (two-way repeated measures ANOVA, $F_{(1,14)} = 0.07$, $p = 0.8$). ***C***. A schematic diagram of the two-pathway experiment. Two stimulating electrodes were placed on either side of the recording electrode at the Schaffer collateral CA3-CA1 synapses to activate two independent sets of inputs (S1 and S2) onto the same postsynaptic population of neurons in CA1. Strong massed 4-train stimulation (four 1 s 100 Hz trains delivered 5 seconds apart) was delivered to S1 first, and 30 minutes later, weak one-train stimulation (one 1s 100 Hz train) was delivered to S2. ***D***. Translin KO mice showed unaltered E-LTP induced by one-train stimulation compared to wildtype littermates (two-way repeated-measures ANOVA, $F_{(1,8)} = 0.07$, $p = 0.8$). ***E***. In S1, massed 4-train stimulation elicited similar levels of L-LTP in slices from translin KO mice or wildtype littermates (*left panel*, two-way repeated-measures ANOVA, $F_{(1,8)} = 0.03$, $p = 0.88$). In S2, one-train stimulation induced long-lasting LTP in slices from wildtype littermates, but not in slices from translin KO mice (*right panel*, two-way repeated-measures ANOVA, $F_{(1,8)} = 36.49$, $p = 0.0003$). Representative traces before (black) and after (red) stimulation are shown on top of each graph, and n refers to the number of mice used. Error bars reflect S.E.M.

Figure 3.2. Translin KO mice exhibit deficits in certain forms of LTP.

A. Hippocampal slices from translin KO mice showed impaired L-LTP induced by spaced 4-train stimulation (four 1s 100 Hz stimuli delivered 5 minutes apart) compared to slices from wildtype littermates (two-way repeated-measures ANOVA, $F_{(1,8)} = 34.43$, $p = 0.00038$). ***B***. Theta-burst stimulation (four 100 Hz pulses delivered in bursts that are 200 ms apart for a total of 3 s) induced similar levels of L-LTP in slices from translin KO mice or wildtype littermates (two-way repeated-measures ANOVA, $F_{(1,8)} = 0.007$, $p = 0.94$). ***C***. Slices from translin KO mice and wildtype littermates displayed similar levels of forskolin (FSK)-induced long-lasting potentiation (two-way repeated-measures ANOVA, $F_{(1,9)} = 0.07$, $p = 0.79$). ***D***. Massed 4-train stimulation (four 1 s 100 Hz trains delivered 5 seconds apart) elicited L-LTP that was not significantly different between slices from translin KO mice and wildtype littermates (two-way repeated-measures ANOVA, $F_{(1,8)} = 0.923$, $p = 0.365$). Representative traces before (black) and after (red) stimulation are shown on top of each graph, and n refers to the number of mice used. Error bars reflect S.E.M.

Figure 3.3. Translin KO mice show unaltered mGluR-LTD and unchanged hippocampal protein levels of FMRP.

A. Hippocampal slices from translin KO mice and wildtype littermates displayed similar mGluR-LTD induced by bath application of 100 mM of DHPG for 10 minutes (two-way repeated measures ANOVA, $F_{(1,8)} = 0.08$, $p = 0.79$). Lower insets are linear regressions of the first (left) and the last (right) 20 minutes of fEPSP slope values. Representative traces before (black) and after (red) stimulation are shown on top of the graph. ***B***. Hippocampal extracts from translin KO mice and wildtype littermates had similar protein levels of FMRP (t-test, $p = 0.36$). □-tubulin was used as the loading control and the expression level was normalized to the level of wildtype littermates. Representative blots are shown on top of the graph. n refers to the number of mice used. Error bars reflect S.E.M.

Figure 3.4. Viral injection of myc-tagged-translin in the hippocampus of adult translin KO mice restores the endogenous expression levels of translin and TRAX at 20 days post-injection.

A. Viral construct designs (top panel), and the representative expression of eGFP and myc-translin in the hippocampus of adult translin KO mice (bottom panel). The neuron-specific expression of eGFP or myc-translin was driven by the CaMKIIα-promoter fragment. Representative images were taken from coronal brain sections of the translin KO mice. ***B***. The time course of myc-translin expression in the adult translin KO hippocampus. The expression level of myc-translin reached the endogenous expression level of wildtype translin 15 to 20 days after the viral injection (one-way ANOVA, $F_{(4,11)} = 15.41$, $p = 0.0002$, Dunnett's post hoc test, wildtype vs. 15 day post-injection $p = 0.998$, wildtype vs. 20 day post-injection $p = 0.083$). ***C***. The time course of TRAX expression in the adult translin KO hippocampus. The expression level of TRAX reached the endogenous expression level of wildtype TRAX 20 days after the viral injection (one-way ANOVA, $F_{(4,11)} = 14.69$, $p = 0.0002$, Dunnett's post hoc test, wildtype vs. 20 day post-injection $p = 0.985$). ***D***. Representative immunostaining in hippocampal CA1 showed that myc-translin is present in the nucleus, cell body and Schaffer collateral, but not in nucleoli and glia (red: myc, green, GFAP, blue: DAPI). n refers to the number of hippocampus injected with the virus. ITR: Inverted Terminal Repeat

Figure 3.5. Viral expression of translin in the hippocampus of adult translin KO mice reverses the deficits in synaptic tagging and capture.

All recordings were performed 20 days after the injection of either translin or eGFP virus into the hippocampus of adult translin KO mice. ***A***. The input-output relationship was similar between translin KO hippocampal slices virally expressing either translin or eGFP (t-test, $p = 0.85$).

Comparing these viral data with the wildtype and the KO data from Fig. 1*A* revealed that there were no significant differences across groups (one-way ANOVA, $F_{(3,19)} = 0.479$, $p = 0.701$) ***B***. Translin KO hippocampal slices virally expressing either translin or eGFP showed similar PPF (two-way repeated measures ANOVA, $F_{(1,10)} = 0.224$, $p = 0.646$). Comparing these viral data with the wildtype and the KO data from Fig. 1*B* showed no significant differences across groups (two-way repeated measures ANOVA, $F_{(3,24)} = 0.469$, $p = 0.707$). ***C*** *left panel*. In S1, L-LTP induced by massed 4-train stimulation was comparable between translin KO hippocampal slices virally expressing either translin or eGFP (two-way repeated-measures ANOVA, $F_{(1,6)} = 2.53$, $p = 0.16$). ***C*** *right panel*. In S2, one-train stimulation induced long-lasting LTP in translin KO hippocampal slices virally expressing translin, but not eGFP (two-way repeated-measures ANOVA, $F_{(1,6)} = 29.33$, $p = 0.002$). Representative traces before (black) and after (red) stimulation are shown on top of each graph, and n refers to the number of mice used. Data adopted from **Fig.1** is shaded. Error bars reflect S.E.M.

Figure 3.6. Viral expression of translin in the hippocampus of adult translin KO reverses the deficits in long-term memory for the displaced object in translin KO mice.

A. Translin KO mice exhibited significantly less preference for the displaced object 24 hours after training compared to wildtype littermates (t-test, $p = 0.0027$). ***B***. Viral expression of eGFP in the hippocampus of wildtype littermates had no influence on their preference for the displaced object 24 hours after training (one-way ANOVA, $F_{(3,29)} = 13.89$, $p = 0$ .00001, Dunnett's post hoc test, $p = 0.992$). Compared to non-injected wildtype littermates, adult translin KO mice injected with eGFP virus into the hippocampus showed significantly less preference for the displaced object 24 hours after training (Dunnett's post hoc test, $p = 0.0003$). However, adult translin KO mice injected with translin virus into the hippocampus and non-injected wildtype littermates displayed similar preference for the displaced object 24 hours after training (Dunnett's post hoc test, $p = 0.354$).

DO: displaced object. n refers to the number of mice used. * indicates $p < 0.003$. Error bars reflect S.E.M.

Figure 3.7. Translin KO mice exhibit altered hippocampal expression of specific miRNAs after object-place recognition task.

A. Thirty minutes after the training for the object-place recognition task, translin KO mice, relative to wildtype littermates, showed significantly increased levels of let 7c-5p (t-test, $p = 0.003$), miR125b-5p (t-test, $p = 0.045$), miR128-3p (t-test, $p = 0.001$), miR9-3p (t-test, $p = 0.03$) and miR409-3p (t-test, $p = 0.00008$), and unaltered levels of other miRNAs tested (t-test, $p > 0.09$). ***B***. Under homecage conditions, translin KO mice had increased levels of miR409-3p (t-test, $p = 0.03$), but showed similar levels of other tested miRNAs (t-test, $p > 0.1$) compared to wildtype littermates. The values of translin KO mice were plotted relative to that of wildtype littermates. n refers to the number of mice used. * indicates $p < 0.05$, ** indicates $p < 0.005$, *** indicates $p < 0.0005$. Error bars reflect S.E.M.

Figure 3.8. A model describing a role for translin in synaptic plasticity.

Translin mediates dendritic transport of mRNAs along microtubules. Translin controls the activity-dependent dendritic expression of its target mRNAs via miRNA-mediated RNA processing. Neuronal activity liberates mRNAs from the translin-mediated suppression of their expression. The released mRNAs are translated by polyribosomes at the activated synapses, and this local translation allows pathway-specific synaptic plasticity.

Author Contributions

Experiments were designed by Jung Whan (Alan Jung) Park and Ted Abel. Electrophysiological, biochemical, behavioral, gene expression, and imaging experiments were conducted by A.J.P. myc-translin construct was provided by Jay Baraban, and viral injections were carried out by Robbert Havekes. A.J.P wrote this chapter with comments and suggestions by R.H., Morgan Bridi, Shane Poplawski, Kyle Krainock and Ted Abel.

Figures

Figure 3.1

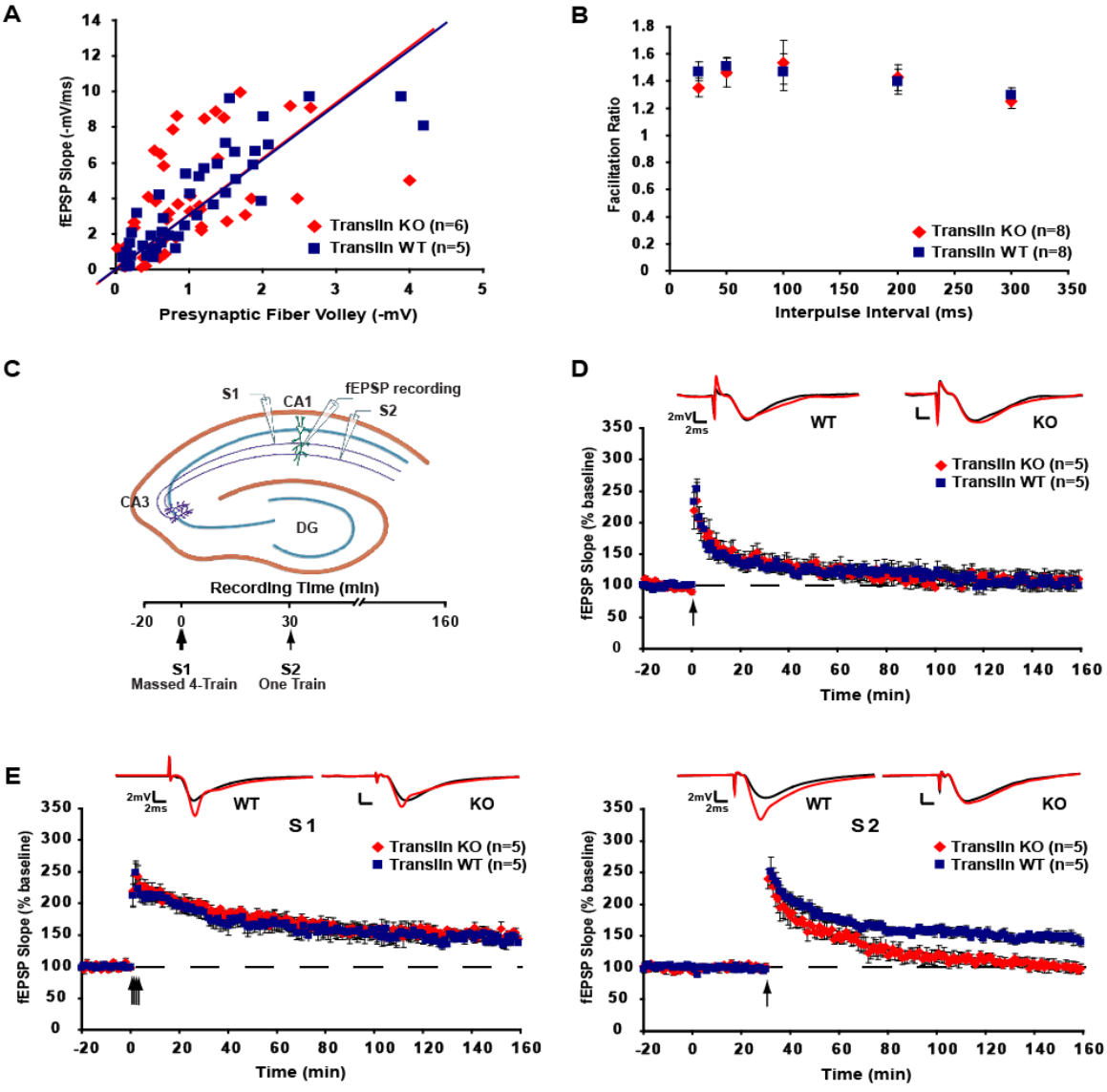
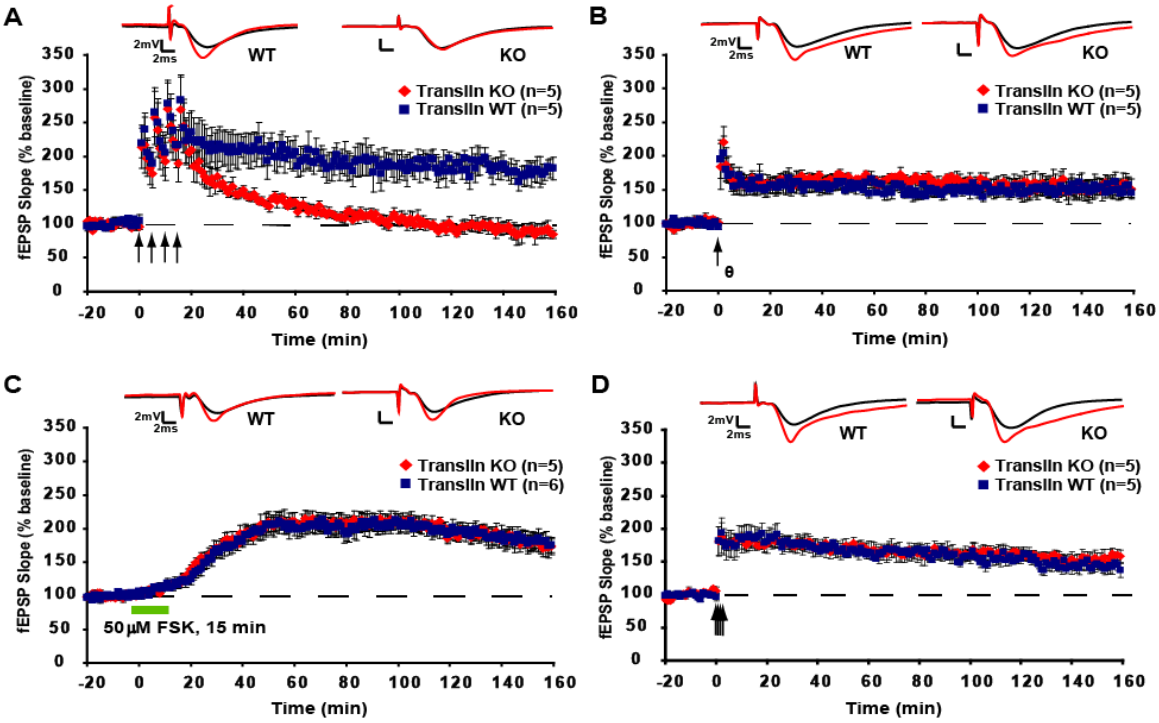


Figure 3.2



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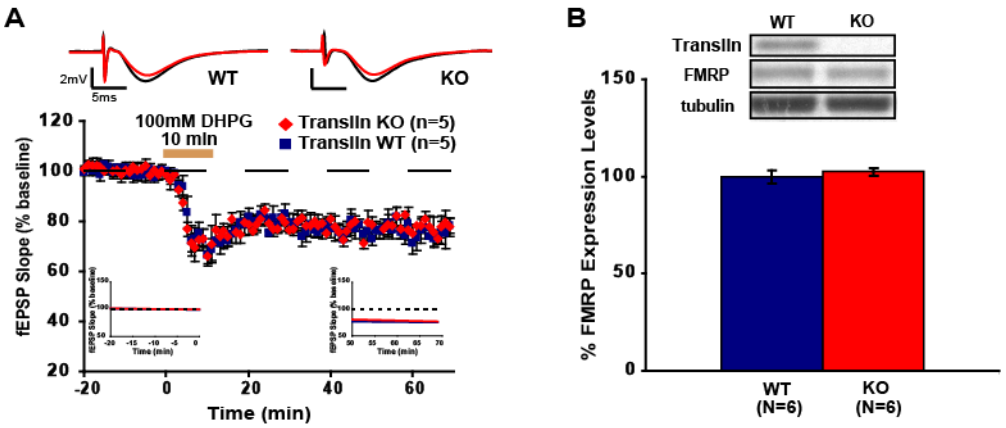

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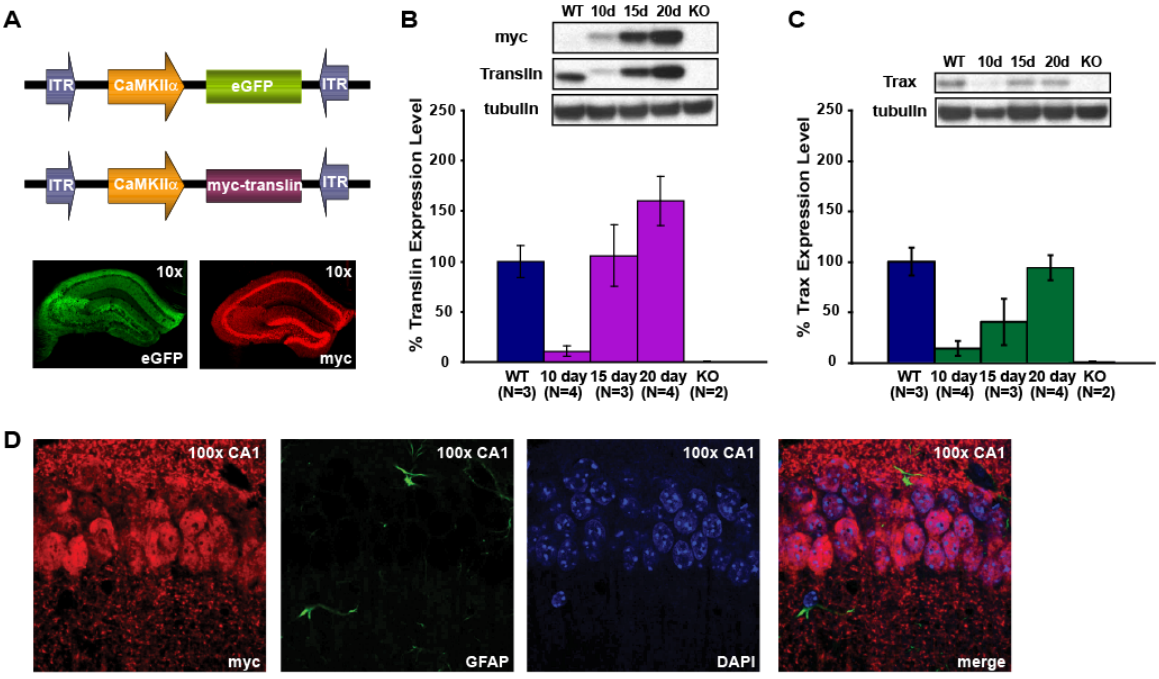
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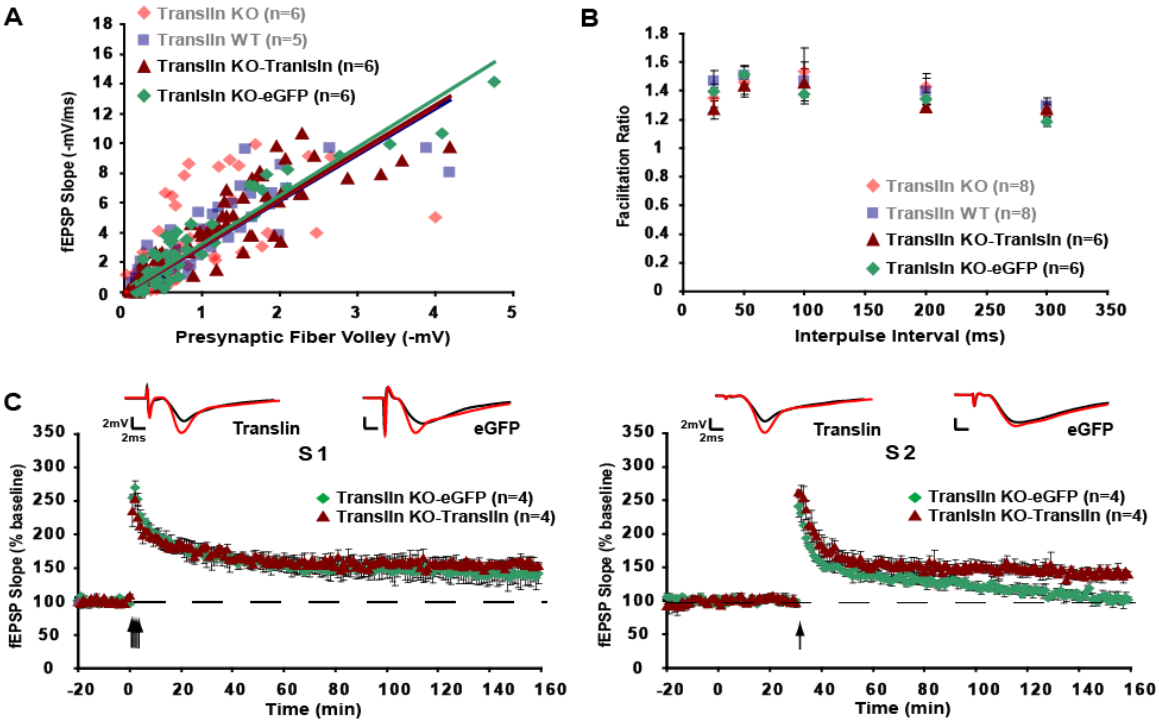
Figure 3.5



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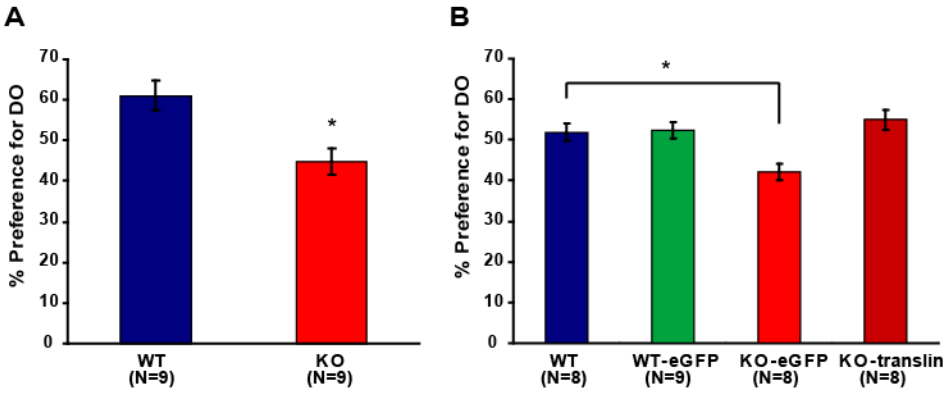
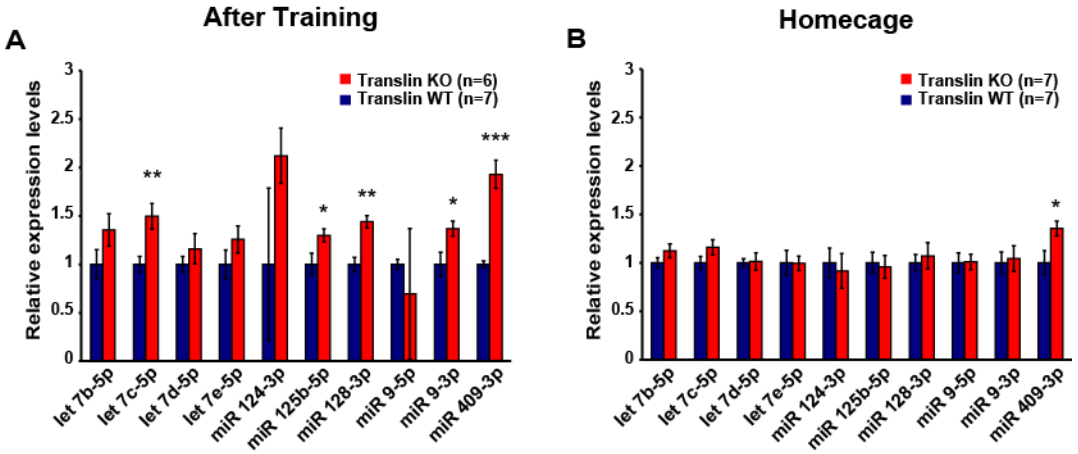


Figure 3.7



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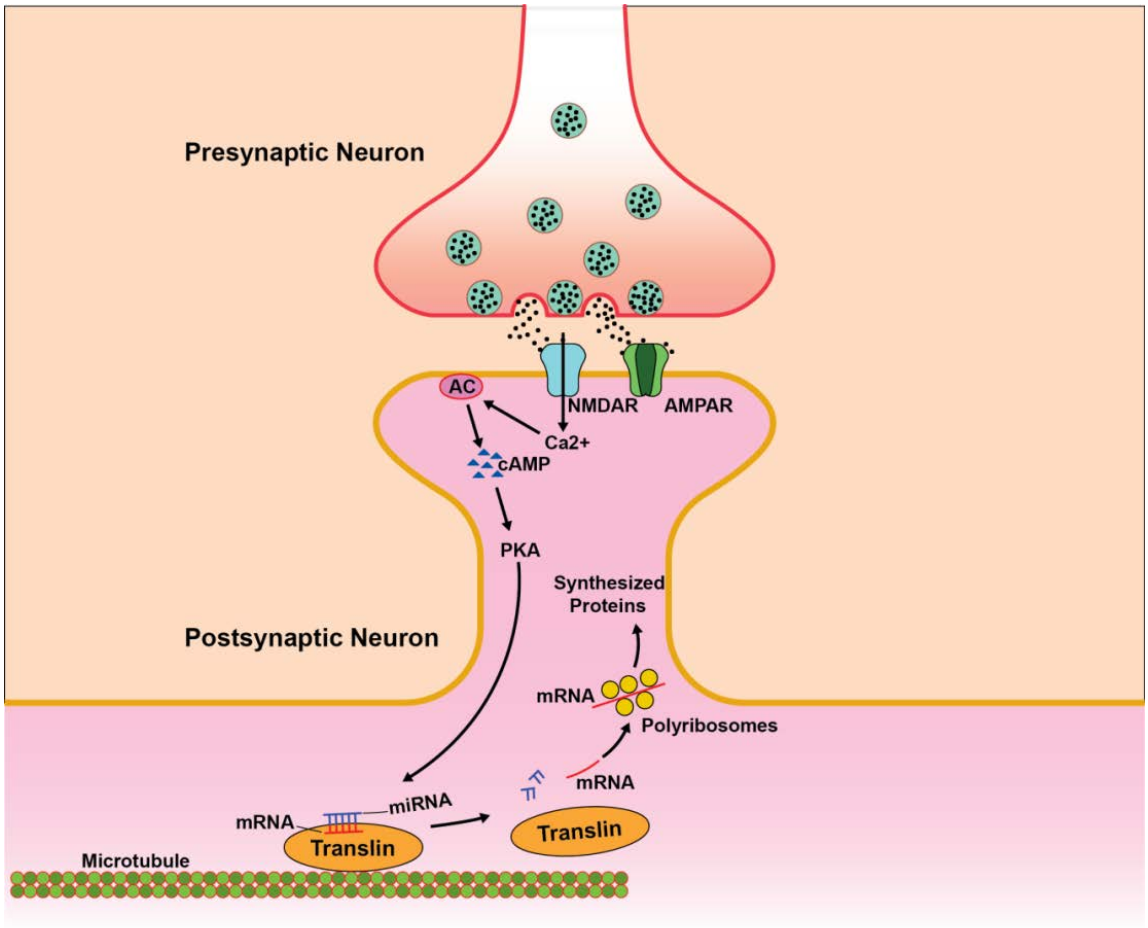


CHAPTER 4: Remaining questions and future approaches

My thesis work has focused on determining the molecular mechanisms underlying pathway-specific synaptic tagging and capture (STC) and memory. Although hippocampal long-term potentiation (LTP) has been studied extensively as a cellular correlate of memory, conventional one pathway LTP does not reflect the nature of memory, which involves the integration of related, but independent, information. Because STC illustrates the interaction of two independent sets of synapses, it may be a better model for memory. In **Chapter 1**, I introduced the concept of STC and the molecules that have been suggested to mediate STC. I also provided a model of STC with PKA as a key signaling molecule underlying this form of plasticity. In **Chapter 2**, I demonstrated that PKA anchoring regulates the size of the readily releasable pool of synaptic vesicles and is required presynaptically for STC and memory. In **Chapter 3**, I determined that translin postsynaptically mediates activity-dependent RNA processing and is critical for STC and memory. Overall, my thesis research provides insights for both presynaptic and postsynaptic mechanisms of STC and memory. In **Chapter 4,** I will briefly review my primary findings and outline future directions to delineate remaining questions to determine the molecular mechanism of STC and memory. Additionally, I will suggest a model of STC based on my findings and discussion (**Fig. 4.1**).

The role of presynaptic PKA anchoring in STC and memory

In **Chapter 2**, I showed that the disruption of PKA anchoring in presynaptic neurons impairs STC and long-term contextual fear memory. Moreover, pharmacological disruption of compartmentalized PKA signaling reduces the size of the readily releasable pool of synaptic vesicles. The interaction between PKA and molecules regulating the size of the readily releasable pool is suggested by the finding that synaptic vesicle protein 2 (SV2), Rim1, and SNAP25 are

components of a cAMP-bound protein complex. Additionally, the disruption of PKA anchoring decreases the protein levels of SV2. Together, our findings suggest that PKA anchoring presynaptically mediates STC and memory by regulating the size of the readily releasable pool of synaptic vesicles through SV2. These findings lead to four questions about the role of presynaptic PKA anchoring in STC: (1) How does presynaptic PKA anchoring mediate STC? (2) How does PKA anchoring regulate SV2 protein levels? (3) What are the direct presynaptic targets of anchored PKA? (4) What is the specific PKA anchoring protein (A-kinase anchoring protein, AKAP) that localizes PKA in the presynaptic compartment?

Compartmentalized PKA activity is a candidate tagging mechanism, as discussed in **Chapter 1**. In **Chapter 2**, we showed that the disruption of PKA anchoring does not affect protein synthesis-dependent long-lasting LTP induced by strong stimulation. This finding suggests that PKA anchoring is not required for the synthesis of plasticity related proteins (PRPs). Instead, presynaptically compartmentalized PKA can tag presynaptic terminals by maintaining the readily releasable pool of synaptic vesicles for a sustained enhancement of synaptic vesicle release (**Fig. 4.1**). Genetic disruption of PKA anchoring only in the presynaptic compartment is necessary for an in-depth investigation on this presynaptic tagging mechanism. Vincent Luczak in our lab has recently developed a Cre-mediated recombination system that expresses a PKA anchoring blocker (superAKAPis) specifically in presynaptic CA3 neurons at the hippocampal Schaffer collateral CA3-CA1 synapse (Gold et al., 2006; Schnütgen et al., 2003). Findings in **Chapter 2** suggest that forskolin-induced long-lasting potentiation requires presynaptic PKA anchoring, and our preliminary data show that selective presynaptic disruption of PKA anchoring using this recombination system impairs this form of synaptic plasticity. The combination of this genetic approach with electron microscopy and mass spectrometry will allow us to assess how presynaptic PKA anchoring affects the synaptic vesicle pool and presynaptic PKA substrates after synaptic activity. I expect that the disruption of PKA anchoring will reduce the number of synaptic vesicles fused to the active zone and the phosphorylation levels of a couple of PKA substrates.

To determine the molecular mechanisms by which presynaptic PKA anchoring mediates STC, it is necessary to look at presynaptic PKA substrates. PKA regulates synaptic vesicle release through its targets such as rabphilin, Rim1α, synapsin1, SNAP25, and snapin (Chheda, Ashery, Thakur, Rettig, & Sheng, 2001; Fykse, Li, & Südhof, 1995; György Lonart et al., 2003; Menegon et al., 2006; Nagy et al., 2004). These proteins are parts of the synaptic vesicle exocytotic machinery and are implicated in neuronal diseases such as Huntington's (Smith et al., 2007; Smith, Petersén, Bates, Brundin, & Li, 2005; Q. Xu et al., 2013). However, the role of PKA phosphorylation of rabphilin and Rim1α in LTP at the Schaffer collateral synapse and memory is unclear (Kaeser et al., 2008; G Lonart & Südhof, 1998). Also, synapsin knockout mice exhibit normal LTP (Spillane, Rosahl, Südhof, & Malenka, 1995), and we found that the disruption of PKA anchoring does not affect PKA phosphorylation of synapsin. It is worth noting that these presynaptic PKA targets work in concert during synaptic vesicle exocytosis. Rabphilin interacts with Rim1α and SNAP25 during synaptic vesicle exocytosis (J.-D. Lee et al., 2008; C. Li et al., 1994; Shirataki et al., 1993; Y. Wang, Okamoto, Schmitz, Hofmann, & Südhof, 1997; Y. Wang, Sugita, & Sudhof, 2000). In addition, the interaction of SNAP25 with snapin is involved in synaptic homeostasis (Dickman, Tong, & Davis, 2012). Moreover, our data suggest that Rim1, SNAP25, and SV2 are components of a cAMP-bound complex. Therefore, presynaptic PKA substrates likely form a functional exocytotic complex, and the integrity of this complex may govern the vesicle release process. In this regard, the deletion or blocking PKA phosphorylation of one of the components of the complex may not affect synaptic plasticity and memory unless it is detrimental to the integrity of the exocytotic machinery. The disruption of PKA anchoring can destabilize the integrity of the complex regulating synaptic vesicle exocytosis by affecting phosphorylation of certain populations of PKA substrates, thus reducing the levels of SV2. Future experiments using biochemical assays and mass spectrometry will be necessary to investigate the interaction between compartmentalized PKA signaling and the proteins involved in synaptic vesicle exocytosis.

There are more than 50 AKAPs that provide spatiotemporal control of PKA activity in distinct subcellular domains (Michel & Scott, 2002; J. L. Sanderson & Dell'Acqua, 2011). Although little is known about the specific presynaptic AKAPs that regulate synaptic vesicle release, some AKAPs are implicated in the presynaptic function. AKAP2/10 regulates vesicle recycling through its interaction with small GTPases Rab11 and Rab4 (Eggers, Schafer, Goldenring, & Taylor, 2009). AKAP7/15/18 is associated with the voltage-dependent Na^+ channel that is responsible for the action potential generation (Catterall, 2000) and modulates the channel activity by mediating PKA phosphorylation of $Na_v1.2\alpha$ subunit (a R. Cantrell, Tibbs, Westenbroek, Scheuer, & Catterall, 1999; a Cantrell, 2002). Another presynaptic AKAP candidate is AKAP220. By making a complex with PKA, glycogen synthase kinase-3β (GSK-3β) and protein phosphatase1 (PP1), AKAP220 tightly regulates the phosphorylation state of GSK-3β (Tanji et al., 2002). Deactivation of GSK-3β by PKA phosphorylation promotes LTP and increases presynaptic transmitter release and the size of the active zone (Tanji et al., 2002; Zhu et al., 2007). However, activation of GSK-3β by dephosphorylation impairs memory and is implicated in Alzheimer's disease (Hooper, Killick, & Lovestone, 2008; S. J. Liu et al., 2003). Conventional reagents that universally disrupt PKA anchoring, such as Ht31, are unable to specify individual AKAPs that are involved in the presynaptic plasticity. Recently, new PKA anchoring blockers selective for AKAP2/10 and AKAP7/15/18 have been developed (Gold et al., 2013; Schäfer et al., 2013). These pharmacological agents are engineered based on structures of each AKAP. Genetic approaches to express these AKAP-specific PKA anchoring blockers in presynaptic neurons will elucidate the mechanism by which specific AKAPs control the presynaptic function.

The role of translin in synaptic plasticity and memory

In **Chapter 3**, I demonstrated that translin is required for STC and long-term object-location memory. Additionally, I showed that translin is critical for certain forms of LTP, but not for mGluR-LTD. Our data suggest that translin mediates these processes by postsynaptically regulating the

expression of specific miRNAs including let 7c-5p, miR 125b-5p, miR 128-3p, miR 9-3p, and miR 409-3p. This section will discuss future approaches to determine the mechanism by which translin mediates STC.

The critical future experiment is to determine the targets of the miRNAs that are upregulated after learning in translin KO mice. A bioinformatics-based target search (miRBase or Targetscan) reveals a list of candidate target mRNAs of the miRNAs. Interestingly, the proteins coded by the target mRNAs include components of the mitogen-activated protein kinase (MAPK) pathway and local translation machineries. Moreover, various isoforms of cAMP degrading enzyme phosphodiesterase (PDE), AKAP and adenylyl cyclase are predicted as targets of the miRNAs. These targets are critical for spatiotemporal regulation of PKA signaling, synaptic plasticity, and memory (Gerits et al., 2008; Sadana & Dessauer, 2009; J. L. Sanderson & Dell'Acqua, 2011; T. M. Sanderson & Sher, 2013). Thus, translin can be responsible for the activity-dependent regulation of dendritic signaling through miRNA-mediated RNA processing (**Fig. 4.1**), and the target validation at the level of both mRNA and protein is necessary. It is also possible that translin regulates the expression of other miRNAs that are not tested in our study, and future experiments need to examine a greater set of translin targets. miR 206, for example, regulates BDNF expression and affects memory in an Alzheimer mouse model (S.-T. Lee et al., 2012), and translin may control the expression of miR 206 given that translin mediates activity-dependent dendritic transport of BDNF mRNAs (Chiaruttini et al., 2009; Y.-C. Wu et al., 2011). It is also of interest to determine if translin regulates the expression of its target RNAs through a direct interaction. Immunoprecipitaion assays using the myc tag of virally expressed translin can show whether translin directly binds its target RNAs.

During STC, synaptic activity tags the activated synapses and the tag captures PRPs for long-lasting LTP. Our study in **Chapter 3** raises a question about how translin mediates these processes. Given that strong stimulation induces protein synthesis-dependent long-lasting LTP and the activation of mGluRs elicits protein synthesis-dependent LTD in translin KO mice, translin

may be dispensable for PRP synthesis. As described in **Chapter 1**, PKA is a strong candidate tag molecule. If translin indeed controls spatiotemporal regulation of PKA signaling as suggested by the miRNA target search, translin can play important roles in the tagging process. cAMP or PKA activity assays in synaptosomes prepared from translin KO mice after synaptic activity will help to address this notion, and I expect that the level of cAMP or PKA activity will be reduced in the KO synaptosomes. Another possibility is that translin mediates local translation of PRPs that are specifically required for STC, not for other forms of long-lasting LTP. The translating ribosome affinity purification (TRAP) approach (Heiman et al., 2008) will define translin-sensitive PRP profiles by comparing translating mRNAs after synaptic activity between translin KO mice and wildtype littermates.

There are several RBPs that are similar to or different from translin, and many of them generally share similar properties, have overlapping mRNA targets and in some cases, interact with each other (Tolino et al., 2012). It is likely that a specific form of neuronal activity recruits a particular RBP or a certain group of RBPs for synaptic plasticity and memory. Our data in **Chapter** 3 suggest that translin is preferentially involved in LTP mechanisms while fragile X mental retardation protein (FMRP) is more likely involved in LTD mechanisms. The requirement for translin, not FMRP, in STC suggests a specific role for translin in conveying genomic information to synapses via RNA processing (**Fig. 4.1**). To understand spatiotemporal regulation of RNA processing by translin, parallel approaches using various techniques will be necessary. For example, a fluorescent reporter system can be used in cultured neurons to visualize the movement of RNA granules containing translin under certain neuronal activity (Rackham & Brown, 2004). High-throughput sequencing together with UV-crosslinking and immunoprecipitation (HITS-CLIP) is able to globally map translin-binding footprint regions of RNAs at a resolution of ~30-60 nucleotides in living tissues (C. Zhang & Darnell, 2011). Furthermore, *in vitro* reconstitution of translin and RNAs can test the physiological relevance of the translin-RNA interaction (Elvira et al., 2006).

Potential contribution of retrograde messengers to synaptic tagging and capture

My work suggests that STC requires presynaptic PKA anchoring to facilitate transmitter release and postsynaptic translin to express proteins required for STC. However, the link between presynaptic- and postsynaptic processes of STC remains to be defined. Retrograde messengers can be the link because they are originated from postsynaptic neurons, but regulate presynaptic transmitter release (Alger, 2002). BDNF is a candidate retrograde messenger. BDNF is released from postsynaptic neurons upon neuronal activities and activates its receptor TrkB in presynaptic neurons, thus increasing the number of docked vesicles and enhancing transmitter release (Hartmann, Heumann, & Lessmann, 2001; Tyler & Pozzo-Miller, 2001; B. Xu et al., 2000). Presynaptic PKA anchoring and postsynaptic translin in STC can be tied together via BDNF, given that PKA gates BDNF-TrkB signaling (Ji et al., 2005) and translin mediates BDNF1 expression (preliminary data). Another well-known retrograde messenger that is involved in LTP is nitric oxide (NO). Produced by NO synthase in postsynaptic neurons, NO is presynaptically required for LTP (Haul, Gödecke, Schrader, Haas, & Luhmann, 1999; S. H. Snyder, Jaffrey, & Zakhary, 1998). The treatment of scavengers of candidate retrograde messengers or blockers for their synthesis during STC can identify retrograde messengers involved in STC. Additionally, a target search for the miRNAs regulated by translin may reveal molecules connecting the postsynaptic component to the presynaptic component of STC. Based on my findings and discussion, here I provide a model that can help decades of work in the field to understand the mechanism of STC (**Fig. 4.1**). The proposed future approaches will test this model and broaden our knowledge on STC.

Figure legends

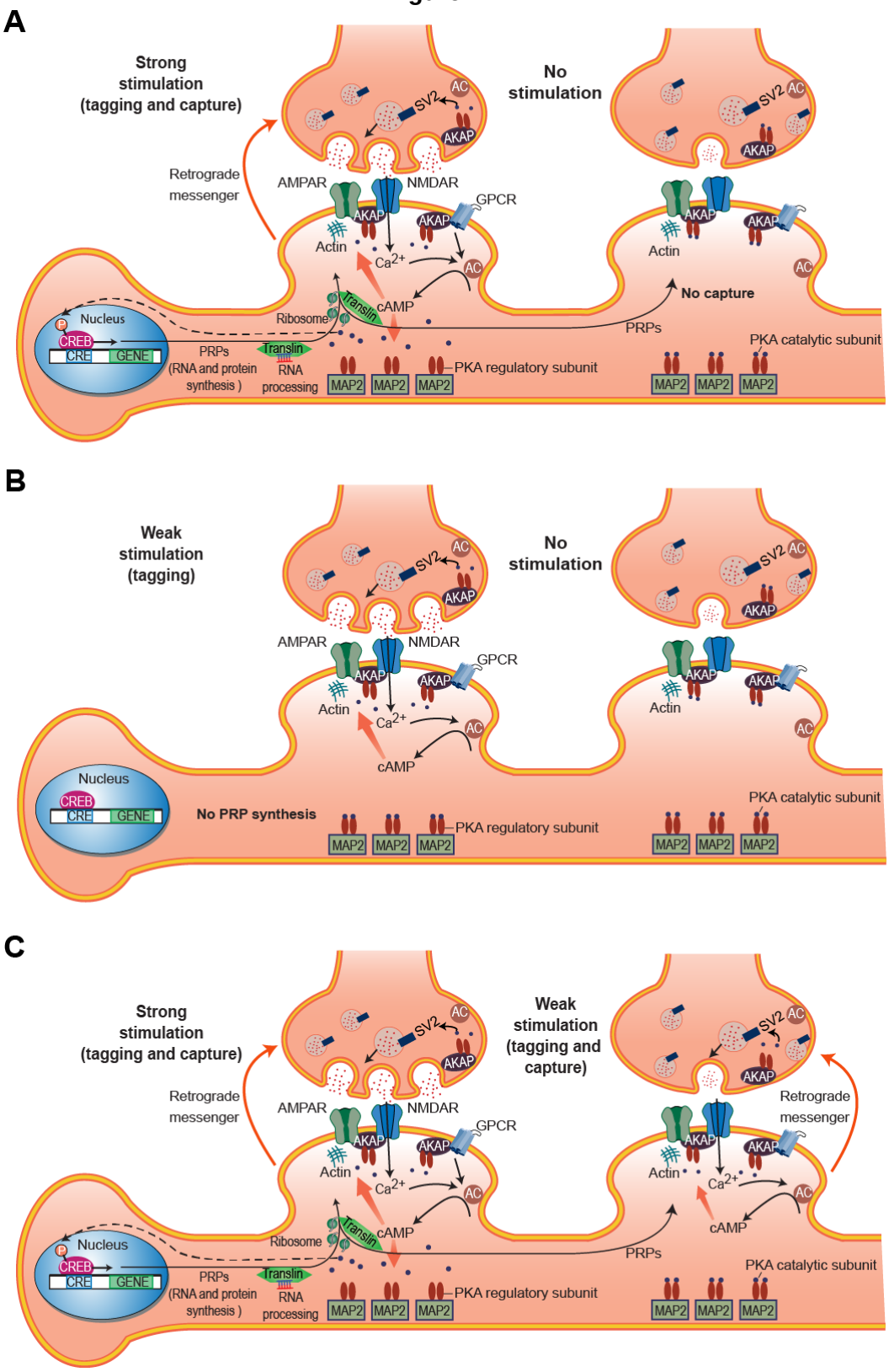
Figure 4.1. Mechanisms of synaptic tagging and capture.

A. In the strong pathway, cAMP is produced by adenylyl cyclases (ACs) in both presynaptic and postsynaptic neurons. In postsynaptic neurons, a large amount of cAMP activates PKA not only from the local AKAP pool in the spine (tagging), but also from the dendritic MAP2 pool. PKA induces CREB-mediated gene transcription, and translin transports new transcripts to activated dendrites. Translin then controls subsequent dendritic expression plasticity related proteins (PRPs) through miRNA-mediated RNA processing. PRPs then coordinate compartmentalized PKA signaling in the spine (capture). In presynaptic neurons, local pool of PKA maintains the readily releasable pool of synaptic vesicles through SV2 (tagging). As retrograde messengers, PRPs from postsynaptic neurons enhance synaptic vesicle release from the readily releasable pool (capture). These presynaptic and postsynaptic mechanisms strengthen the synaptic connection. ***B***. weak stimulation induces the sub-threshold level of cAMP that can only tag the synapses, but is unable to elicit PRP production. ***C***. The weak pathway can be strengthened if it captures PRPs from the strong pathway when the two independent pathways are paired.

Author Contributions

Jung Whan (Alan Jung) Park wrote this chapter with comments and suggestions by Robbert Havekes, Vincent Luczak, Kyle Krainock, and Ted Abel.

Figure 4.1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No stimulation
Retrograde messenger
AMPAR
NMDAR
GPCR
SV2
AC
AKAP
Actin
Ca2+
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Translin
Ribosome
No capture
PRPs
Nucleus
CREB
CRE
GENE
PRPs (RNA and protein synthesis)
Translin
RNA processing
PKA regulatory subunit
PKA catalytic subunit
MAP2
B
Weak stimulation (tagging)
No stimulation
No PRP synthesis
C
Strong stimulation (tagging and capture)
Weak stimulation (tagging and capture)
Retrograde messenger

Appendix

I had been involved in many other projects that were not directly related to the topic of my dissertation but were critical to understand the mechanism of synaptic plasticity and memory. In this section, I will briefly describe the background and show data I collected for projects that are already published or in a preparation for journal submission.

1. The role of PKA anchoring protein Gravin in hippocampal synaptic plasticity

As discussed in **Chapter 1**, A-kinase anchoring proteins (AKAPs) play important roles in synaptic plasticity and memory by compartmentalizing PKA signaling molecules. PKA anchoring protein Gravin mediates PKA phosphorylation of β2-adrenergic receptors (β2-ARs) that leads to desensitization of the receptor and activation of the MAPK pathway (**Chapter 1, section 1.7**, Baillie et al., 2003; Daaka, Luttrell, & Lefkowitz, 1997). Pairing β-AR agonist isoproterenol (ISO) with 5Hz stimulation establishes long-lasting LTP (L-LTP) via the activation of β2-ARs (Qian et al., 2012), PKA (Gelinas et al., 2008) and ERK1/2 (Winder et al., 1999). To determine whether PKA anchoring is required for this form of synaptic plasticity, we treated hippocampal slices with a PKA-anchoring disrupting peptide Ht31 (Carr, Stofko-Hahn, et al., 1992). stHt31 treatment impaired this form of plasticity (**Fig. 5.1*A***; both groups, $n = 4$; Kruskal–Wallis ANOVA, $p < 0.05$). The mean fEPSP slope over the last 20 min of the recording was significantly reduced due to treatment with stHt31 (vehicle treatment, 154.9 ± 13.5%; stHt31, 98.9 ± 4.2%; Mann–Whitney *U* test, $p = 0.029$). This reduction was not observed with bath application of stearated pseudo-stHt31 control peptide (data not shown). We found that Gravin mutant mice showed impaired L-LTP induced by pairing ISO with 5Hz stimulation or theta-burst stimulation (Havekes et al., 2012). To determine whether theta-burst LTP requires β2-ARs, we delivered the β2-AR antagonist ICI 118551 (100 nM). Theta-burst LTP was impaired in slices from wild-type slices

treated with the β2-AR antagonist (**Fig. 5.1*B***; WT, *n* = 5; GT, *n* = 6; Kruskal–Wallis ANOVA, *p* < 0.05). The mean fEPSP slope over the last 20 min of the recording was significantly reduced under conditions of the β2-AR blocker (vehicle, 156.3 ± 10.1%; ICI 118551, 119.3 ± 7.1%; Mann–Whitney *U* test, *p* = 0.007). Because Gravin compartmentalizes PKA signaling molecules, we anticipated that a PKA-independent form of LTP induced by four high-frequency trains of stimuli applied at 5 s intervals (massed four-train LTP, Woo et al., 2003) is not affected in Gravin mutant mice. As expected, massed four-train LTP was not altered in these mice (**Fig. 5.1*C***; WT, *n* = 5; GT, *n* = 5; Kruskal–Wallis ANOVA, *p* > 0.05). We next wanted to establish whether loss of Gravin impairs a form of synaptic potentiation that is induced by forskolin, a pharmacological activator of adenylyl cyclase. The synaptic potentiation following forskolin treatment was similar in wild-type littermates and Gravin mutant mice (**Fig. 5.1*D***; WT, *n* = 6; GT, *n* = 5; Kruskal–Wallis ANOVA, *p* > 0.05). These findings suggest that cell-wide elevation of cAMP to supraphysiological levels overcomes the mislocalization of PKA caused by the deletion of Gravin in the mutant mice.

Materials and Methods

Animals

The gravin mutant mice were a gift from Dr. Jacqueline Barra (Institut Pasteur, Paris, France) and backcrossed to a C57BL/6J background for >10 generations. Male and female 3- to 5-month-old Gravin mutant mice and wild-type littermates had *ad libitum* access to food and water and were maintained on a 12 h light/dark cycle with lights on at 7:00 (ZT0). All experiments were conducted according to National Institutes of Health guidelines for animal care and use and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Electrophysiology

Electrophysiological recordings were performed as described previously (Vecsey et al., 2009). LTP was induced using the following protocols: massed four-train (four 1 s 100 Hz trains delivered 5 s apart), theta-burst (bursts of four 100 Hz pulses delivered for a total of 3 s at 5 Hz). LTP was also induced by pairing a 5 Hz, 3 min stimulation with application of the 1 μM β-adrenergic agonist isoproterenol (ISO) for 25 min. The electrical stimulation was given 15 min into ISO treatment. ISO (Sigma-Aldrich) was prepared daily as a 10 mM stock solution in Milli-Q water. Synthesis of stearated Ht31 (stHt31) (St-N-DLIEEAASRIVDAVIEQVKAAGAY-C) and pseudo-Ht31 peptide (stHt31P) (St-N-DLIEEAASRPVDAVPEQVKAAGAY-C) (Carr, Stofko-Hahn, et al., 1992) was conducted by Quality Controlled Biochemicals. Lyophilized powder was resuspended at a stock concentration of 10 mM in 50 mM Tris-HCl, pH 7.0, and 0.05% DMSO and used at 10 μM final concentration in aCSF. Forskolin was dissolved in 100% ethanol to make 50 mM stock solution and used at 50 μM final concentration in aCSF. The β2-AR antagonist 3-(isopropylamino)-1-[(7-methyl-4-indanyl)oxy]butan-2-ol (ICI 118551) (Sigma-Aldrich) was dissolved in H_2O at a stock concentration of 100 μM and used at a 100 nM final concentration in aCSF.

Data analysis

Data analysis was performed using SPSS. Electrophysiological data were analyzed using nonparametric tests (Nie et al., 2007). The initial slope of the fEPSP at each time point was analyzed. For comparisons of the average slope over the last 20 min of the recording, the Mann–Whitney *U* test was used. Differences were considered statistically significant when $p < 0.05$. Data are plotted as mean ± SEM.

This study was published as:

Robbert Havekes, David A. Canton, Alan J. Park, Ted Huang, Ting Nie, Jonathan P. Day, Leonardo A. Guercio, Quinn Grimes, Vincent Luczak, Irwin H. Gelman, George S. Baillie, John D. Scott, and Ted Abel. 2012. Gravin Orchestrates Protein Kinase A and β2-Adrenergic Receptor Signaling Critical for Synaptic Plasticity and Memory. *The Journal of Neuroscience*.

2. Forskolin-induced synaptic potentiation is impaired in transgenic mice expressing Ht31

Our collaborator Dr. Blackwell at George Mason University found that PKA anchoring close to the source of cAMP is critical for synaptic plasticity using their computational modeling. Additionally, the model predicts that inhibition of cAMP degrading enzyme phosphodiesterases (PDEs) would rescue the LTP deficit caused by disruption of PKA anchoring. To test this model, we used forskolin, an adenylyl cyclase activator, to induce synaptic plasticity in mice expressing Ht31 peptide in the hippocampus and in wildtype controls. If Ht31 disrupts PKA anchoring close to the source of cAMP, forskolin-induced potentiation should be impaired in the mice expressing Ht31. Indeed, this form of potentiation was blocked in the Ht31-expressing transgenic mice (**Fig. 5.2*A***, $p = 0.012$). Furthermore, in the presence of IBMX (3-isobutyl-1-methylxanthine), a non-specific PDE inhibitor, Ht31 expression does not impair forskolin-induced synaptic plasticity (**Fig. 5.2*B***, $p = 0.65$). Therefore, we confirmed the computational model prediction.

Materials and Methods

Animals

Adult (3-5-month-old) Ht31(1) mice and their littermates (Nie et al., 2007) were used to verify the modeling predictions. All animal care and experiments were approved by the Institutional Animal

Care and Use Committee of the University of Pennsylvania and conducted in accordance with the National Institutes of Health guidelines.

Electrophysiology

Electrophysiological recordings were performed as described previously (Vecsey et al., 2009). Forskolin (Molecular grade FSK, Sigma) was dissolved in 100% ethanol to make 50 mM stock solution and used at 50 μM final concentration in aCSF. FSK was applied to hippocampal slices for 15 minutes to induce chemical LTP as previously described (Vecsey et al., 2009). 3-Isobutyl-1-methylxanthine (IBMX, Sigma) was dissolved in 50 mM Tris–HCl (pH 7.0) and 0.05% DMSO and used at 30 μM final concentration in aCSF. FSK and IBMX were used together to induce a form of chemical LTP (T. Huang et al., 2006)

Data analysis

All electrophysiology data were analyzed with SigmaStat (Systat Software, San Jose, CA). A repeated-measure ANOVA compared fEPSP slopes, normalized against baseline, during the first 20 min and during the last 20 min of the recordings. Differences were considered statistically significant when $p < 0.05$. Data are plotted as mean ± SEM.

This study was published as:

Kim M, Park AJ, Havekes R, Chay A, Guercio LA, Oliveira RF, Abel T, Blackwell KT. 2011. Colocalization of protein kinase A with adenylyl cyclase enhances protein kinase A activity during induction of long-lasting long-term-potentiation. *PLoS Computational Biology*

3. Daily acclimation handling does not affect hippocampal long-term potentiation

An important approach to study sleep function is to perform sleep deprivation in rodents. Gentle handling is a commonly used approach to sleep deprive animals as completely as possible over a relatively short period of time (≤ 6 h). In this method, animals are kept awake manually by tapping on the cage, shaking the cage gently, or disturbing the nesting material (Graves, Heller, Pack, & Abel, 2003; Vecsey et al., 2009). Because this method has the potential to be stressful to the animal, it is often preceded by a number of days of brief handling to acclimate the animal to what it will experience during the sleep deprivation period. We previously reported that the brief 5 hours of sleep deprivation impairs hippocampal synaptic plasticity and hippocampus-dependent memory (Vecsey et al., 2009). However, a recent study raises a concern that acclimation handling could itself result in behavioral and neurochemical alterations that would affect the subsequent response to sleep deprivation (Longordo, Fan, Steimer, Kopp, & Lüthi, 2011). To determine whether the method of acclimation handling used in our previous studies causes disturbances in hippocampal function, we examined hippocampal synaptic plasticity in handled or undisturbed animals.

We compared the effect of handling on spaced 4-train (four 100 Hz trains with 5 minutes interval)-induced L-LTP, a PKA-dependent form of plasticity that is impaired by brief sleep deprivation (Vecsey et al., 2009). We found that acclimation handling did not affect the induction (no overall effect of handling, $F = 0.13$, $p = 0.73$) or maintenance (no overall effect of handling, $F = 0.07$, $p = 0.8$) of LTP (**Fig. 5.3*A***). We also assessed the effects of handling on basal synaptic properties, including the maximum response amplitude (**Fig. 5.3*B***), paired-pulse facilitation (PPF) (**Fig. 5.3*C***), and input-output relationship (**Fig. 5.3*D***). No effects of handling were observed on any of these measures (Max response — no overall effect of handling, $F = 0.17$, $p = 0.69$; PPF — no overall effect of handling, $F = 5.16$, $p = 0.053$, and no interaction between handling and interstimulus interval, $F = 1.31$, $p = 0.29$; Input-output — no effect of handling, $F = 0.25$, $p = 0.63$). These findings demonstrate that brief acclimation handling alone for 6 days does not produce any

effects on synaptic plasticity or baseline synaptic function that could explain the deficits observed due to sleep deprivation.

Materials and Methods

Animals

Adult (2-3-month-old) male C57BL/6J mice were used for all experiments. Mice were individually housed with ad libitum food and water on a 12 h-12 h light-dark schedule. All animal care and experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and conducted in accordance with the National Institutes of Health guidelines.

Handling

Mice were handled daily beginning on the 2nd day of individual housing, whereas the other mice were left undisturbed in their home cages. Acclimation handling was performed at approximately zeitgeber time (ZT) 4.5, and lasted 2-3 min per mouse. Acclimation of all animals was completed within 30 min for all experiments. Handling consisted of all techniques used during our gentle handling sleep deprivation method (Graves et al., 2003; Vecsey et al., 2009). Cages were removed in pairs from their racks and placed on carts for the handling, which included gentle tapping of the exterior of the cage, removing the cage lid, light rattling of the wire cage top and rummaging through the food, removing the wire cage top and gently stroking the mouse as it moved freely around the cage, and disturbing the bedding.

E*lectrophysiology*

Mice were brought to the electrophysiology room on the day after the last handling session at ZT 5-6 and killed by cervical dislocation, and hippocampal slices were prepared. Electrophysiological recordings were performed as described previously (Vecsey et al., 2009). LTP was induced by

spaced four-train (four 1 s 100 Hz trains delivered 5 minutes apart). PPF was induced by paired stimuli, with interstimulus intervals of 300, 200, 100, 50, and 25 ms. The ratio of the slope of the second response relative to the first was recorded.

Data analysis

All electrophysiology data were analyzed with SigmaStat (Systat Software, San Jose, CA). For input-output and maximum response data, one-way analyses of variance (ANOVAs) were used, with handling as the main factor. For PPF data, a repeated-measure ANOVA was used, with interval as the within subject factor and handling as the between-subject factor. For LTP analysis, a repeated-measure ANOVA compared fEPSP slopes during the first 20 min after the last tetanus (induction), and during the last 20 min of the recordings (maintenance), normalized against baseline, with time as the within-subject factor and handling as the between-subject factor. Differences were considered statistically significant when $p < 0.05$. Data are plotted as mean ± SEM.

This study was published as:

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4. The negative impact of sleep deprivation on hippocampal synaptic plasticity is reversed by overexpression of an inactive form of cofilin

It is generally acknowledged that sleep is of critical importance for proper brain function. The general consensus is that sleep is crucial for neuronal recovery, maintenance, and synaptic plasticity thereby supporting memory processes. Indeed, loss of sleep attenuating neurocognitive,

psychological and behavioral processes (Bonnet & Arand, 2003). We previously reported that brief 5 hours of sleep deprivation impairs PKA-dependent forms of hippocampal L-LTP (Vecsey et al., 2009), and our subsequent study suggests that this phenomenon is due to the increased activity of an actin depolymerizing enzyme cofilin (in a manuscript preparation). To examine whether the suppression of cofilin activity prevents the negative effect of sleep deprivation on hippocampal LTP, we engineered a phosphomimetic inactive form of cofilin (cofilin^{S3D}) (Moriyama, Iida, & Yahara, 1996; Pontrello et al., 2012). We expressed the inactive cofilin^{S3D} or enhanced green fluorescent protein (eGFP), which served as a control, selectively in hippocampal excitatory neurons of adult male C57BL/6J mice using Adeno-Associated Viruses (AAVs). Five hours of sleep deprivation impaired LTP induced by 4 high-frequency trains of electrical stimuli applied at 5-minute intervals (spaced 4-train stimulation) in hippocampal slices from mice expressing eGFP (**Fig. 5.4*A***; n = 6 for each group, two-way ANOVA, effect of virus $F_{1,10} = 21.685$, $p < 0.001$). In contrast, spaced 4-train LTP was unaffected by sleep deprivation in hippocampal slices from mice expressing cofilin^{S3D} (**Fig. 5.4*B***; n = 5, two-way ANOVA, effect of virus $F_{1,8} = 0.016$, $p < 0.902$). The expression of cofilin^{S3D} did not alter basal synaptic properties (data not shown). Therefore, the suppression of cofilin activity in hippocampal excitatory neurons is sufficient to prevent the sleep deprivation-induced impairments in synaptic plasticity.

Materials and Methods

Animals

Two- to three-month-old C57BL/6J male mice had *ad libitum* access to food and water and were maintained on a 12 h light/dark cycle with lights on at 7:00 (ZT0). Mice were handled for 6 days and sleep-deprived in their home cages for 5 h by gentle handling beginning at ZT0 or left undisturbed (non-sleep-deprived mice). All experiments were conducted according to National Institutes of Health guidelines for animal care and use and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Electrophysiology

Electrophysiological recordings were performed as described above. LTP was induced by spaced four-train (four 1 s 100 Hz trains delivered 5 minutes apart) stimulation.

Data analysis

Data analysis was performed using SPSS. Electrophysiological data were analyzed using a two-way repeated-measures ANOVA. The initial slope of the fEPSP at each time point was analyzed. Differences were considered statistically significant when $p < 0.05$. Data are plotted as mean ± SEM.

This work is in a manuscript preparation as:

Robbert Havekes, Alan J. Park, Vibeke M. Bruinenberg, Jennifer H.K. Choi, Jonathan P. Day, Shane G. Poplawski, Kyle S. Krainock, Angela Zhu, Sara J. Aton, Kasia Radwańska, Peter Meerlo, Miles D. Houslay, George S. Baillie, Ted Abel. Sleep deprivation causes memory deficits by negatively impacting spine dynamics in the hippocampus.

5. The age-dependent effect of sleep deprivation on hippocampal and cortical gene expression

The consequences of sleep deprivation (SD) and aging receive great attention today because acute/chronic or partial sleep restriction and increase in average life span are characteristics of developed countries. Indeed, many studies have reported detrimental effects of SD and aging especially in cognitive functions. Rodent studies show that SD or aging impairs hippocampus-dependent memory consolidation and hippocampal LTP (Barnes & McNaughton, 1985; Burke & Barnes, 2006; Graves et al., 2003; Guan, Peng, & Fang, 2004; Ruskin, Liu, Dunn, Bazan, & Lahoste, 2004; Vecsey et al., 2009). Interestingly, basal performance of aged people is much lower than that of the young, and SD significantly affects the performance level of the young, not

the aged (Philip et al., 2004). Physiological data indicates that sleep deprivation disrupts carbohydrate metabolism and endocrine function in young adults, both of which are characteristics of aging (Spiegel, Leproult, & Cauter, 1999). Collectively, it is interesting that the consequences of SD and aging are similar to each other. Here, we hypothesize that if there are correlations between SD and aging, hippocampal or cortical gene expression changes in young SD mice will be similar to that of aged non-SD mice and there will be either no SD effects or enhanced SD effects in aged mice.

We previously found genes whose expression levels are specifically affected by 5 hours of SD and are restored after 2.5 hours of recovery sleep in the hippocampus. These genes are *Prkab2, Tsc22d3, Arc*, *Hspb1*, *and Hspa5* from upregulated gene lists and *Rbm3, Hnrpdl, and Usp2* from down regulated gene lists. *Prkab2* encodes AMP activated kinase (AMPK) β-2 subunit. As a metabolic sensor, AMPK inhibits lipid synthesis under low energy state (G. Gao et al., 1996). *Arc* (Activity regulated cytoskeleton associated protein) is a synaptic plasticity gene (Tzingounis & Nicoll, 2006). *Tsc22d3* is a leucine zipper containing transcription factor gene that is activated by glucocorticoid, thereby mediating immunosuppression (Macedo et al., 2008). Heat shock proteins (HSP) are important in the unfolded protein response (UPR). Hspa5 (Bip or Grp78) is a member of the HSP70 family undergoing refolding of damaged proteins by utilizing ATP (Gething, 1999). Hspb1 is a member of small heat shock protein assisting protein refolding by HSP70 in ATP independent manner (Gusev, Bogatcheva, & Marston, 2002). Rbm3 (RNA binding motif 3) and Hnrpdl (Heterogeneous nuclear ribonucleoprotein D like) have roles in mRNA metabolism and trafficking (Kawamura et al., 2002; Smart et al., 2007). Usp2 (ubiquitin specific peptidase 2) is involved in deubiquitination (Löw, 2011). These gene expression changes suggest that SD induces increases in synaptic activity, stress response and UPR and decreases in mRNA metabolism, trafficking and ubiquitin recycling in the hippocampus. Similarly, cortical gene expression studies on rats suggest that genes involved in synaptic plasticity, energy metabolism, stress response, and transcriptional activation are upregulated by SD while genes

involved in synaptic downscaling, macromolecule synthesis, and membrane maintenance are upregulated during sleep (Cirelli, Gutierrez, & Tononi, 2004; Cirelli & Tononi, 2004).

In both the hippocampus and the cortex, SD induced significant increase in the expression levels of *Prkab2* (energy metabolism), *Arc* (synaptic plasticity), *Hspa5, Hspb1,* and *Tsc22d3* (stress response) (**Fig. 5.5**; two-way ANOVA, $p < 0.05$). The genes displayed significantly decreased expression levels after SD are involved in mRNA metabolism and trafficking (*Rbm3, Hnrpdl*) and recovery (*Usp2*) (**Fig. 5.5**; two-way ANOVA, $p < 0.05$). However, the effect of aging was observed only for certain genes (**Fig. 5.5**; two-way ANOVA, $p < 0.05$; Hippocampus: *Rbm3, Hnrpdl*, and *Usp2*; Cortex: *Prkab2*, *Rbm3, Hnrpdl*, and *Usp2*). The correlation between aging and SD was found only in the hippocampal expression of *Hspb1* (**Fig. 5.5*A***; two-way ANOVA, $p = 0.007$). We expected an interaction between SD and aging, such that SD could have either no or enhanced effects in aged mice. But our data suggests that this is not generally the case. Our data suggests that SD and aging may influence neuronal function in different molecular pathways.

Materials and Methods

Animals

Two (young)- or twenty-three (old)-month-old C57BL/6J male mice had *ad libitum* access to food and water and were maintained on a 12 h light/dark cycle with lights on at 7:00 (ZT0). Mice were handled for 5 days and sleep-deprived in their home cages for 5 h by gentle handling beginning at ZT2 or left undisturbed (non-sleep-deprived mice). All experiments were conducted according to National Institutes of Health guidelines for animal care and use and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Quantitative real-time RT-PCR (qPCR)

RNA preparation, cDNA synthesis, and qPCR analysis was performed as previously described (Vecsey et al., 2007). Reactions were prepared in 96-well optical reaction plates (ABI, Foster City, CA) with optical adhesive covers (ABI). Each well contained 11.4 pi cDNA, 1 pi of 5 uM primer mix solution, and 12.4 µl Power SYBR Green PCR Master Mix (ABI). Three technical replicates were used. Reactions were carried out in the ABI Prism 7000 with an initial activation at 50°C for 2 minutes followed by incubation at 95°C for 15 minutes and 40 subsequent cycles of 95°C for 15 sec, 56°C for 30 sec, and 72°C for 30 sec. Primer sequences were as follows: Hnrpdl: forward - AAGAACCAGCAGGATGACGGT, reverse - TGCAGTCTACCACTTCCCCAA. Rbm3: forward - AGGACTTGCCTTCTGCCATGT, reverse - ATAGGCCCAAAGCTGCTGAA. Hspb1: forward - CACAGTGAAGACCAAGGAAGG, reverse -GGTGAAGCACCGAGAGATGT. Hspa5: forward - GGAGACTGCTGAGGCGTATT, reverse - TGGGCATCATTGAAGTAAGC. Arc: forward - AGCAGCAGACCTGACATCCT, reverse - GGTGTCATTCTCCTGGCTCT. Usp2: forward - AAAGAGTGCCCAGGGTCTG, reverse - TCTCTCAGCTCTCGGGTGTT. Prkab2: forward - GGGAAAGGAGCACAAGATC, reverse - CTGCTGCCAGGGTACAAAC. Tsc22d3: forward - TTCTCTGCTTGGAGGGGATT, reverse - GCTCACGAATCTGCTCCTTT. Data was normalized to Actgl, Hprt and Tuba4a prior to calculation of differences, using the same primers as described previously (Vecsey et al., 2007). These housekeeping genes were chosen because their expression was not affected by sleep deprivation in our microarray experiment. Relative quantification of gene expression was performed according to ABI's User Bulletin #2. Fold change was calculated from the delta Ct values with corrections for standard curve data from each gene and housekeeping gene expression levels for each sample based on the relative standard curve method described in the Applied Biosystems manual.

Data analysis

A two-way ANOVA was performed to compare fold change values for each gene in each comparison of interest using Statistica 7 (Statsoft, Inc., Tulsa, OK). Differences were considered statistically significant when $p < 0.05$. Data are plotted as mean ± SEM.

This work is in a manuscript preparation as:
Christopher Vecsey, Alan J. Park, Ted Abel.

Figure Legends

Figure 5.1. PKA anchoring is required for certain forms of hippocampal synaptic plasticity.

A. The disruption of PKA anchoring using membrane-permeable stearated Ht31 peptide impairs a form of hippocampal synaptic plasticity induced by pairing 1 μM ISO with a 5 Hz stimulation for 3 min (Kruskal–Wallis ANOVA, $p < 0.05$). ***B***. Bath application with the β2-AR antagonist ICI 118551 (100 nM) impairs a long-lasting form of LTP induced by theta-burst stimulation (15 bursts) (Kruskal–Wallis ANOVA, $p < 0.05$). ***C***. Massed four-train LTP, a PKA-independent form of long-lasting LTP was not impaired in slices from Gravin mutant mice (Kruskal–Wallis ANOVA, $p > 0.05$). ***D***. Forskolin-mediated synaptic potentiation was not impaired in slices from Gravin mutant mice (Kruskal–Wallis ANOVA, $p > 0.05$). In all sample sweeps, black traces indicate baseline, and red traces were acquired 2 h after stimulation. Calibration: 2 mV, 10 ms. Error bars indicate SEM.

Figure 5.2. The disruption of PKA anchoring impairs forskolin-induced long-lasting potentiation, but does not overcome the impact of phosphodiesterase inhibitors.

A. Forskolin (FSK)-induced synaptic potentiation is impaired 2 hours after FSK treatment in Ht31 mice (squares) compared to wildtype littermates (triangles) (n = 5 for each group, $p = 0.012$). ***B***. The impairment in FSK-induced potentiation was rescued in the presence of IBMX, which inhibits phosphodiesterases. There was no difference in fEPSP between Ht31 mice and wildtype littermates 2 hours after FSK treatment (n = 5 for each group, $p = 0.65$).

Figure 5.3. Handling does not disrupt hippocampal plasticity.

A. Handling did not affect induction or maintenance of spaced four-train long-term potentiation as measured by field excitatory postsynaptic potentials (fEPSPs) from hippocampal Schaffer collateral CA1 synapses (n = 5 for each group, F = 0.07, p = 0.8). Inset shows representative traces depicting fEPSP responses during baseline (dashed lines) and at 160 min posttetanization (solid lines). Handling also did not alter basal synaptic properties, such as the maximum response amplitude (***B***; n = 5 for each group, F = 0.17, p = 0.69), paired-pulse facilitation (PPF) (***C***; n = 5 for each group, F = 5.16, p = 0.053), or input-output relationship (***D***; n = 5 for each group, F = 0.25, p = 0.63). Insets in ***C*** and ***D*** show overlaid representative fEPSP responses from PPF and input-output experiments, respectively. Shown are means ± standard error of the mean.

Figure 5.4. The suppression of cofilin activity in the hippocampus prevents synaptic plasticity deficits that are associated with sleep deprivation.

A. Following 5 hours of sleep deprivation, long-lasting LTP was induced by spaced 4-train stimulation in the hippocampal Schaffer collateral pathway. Sleep deprivation impaired long-lasting LTP in slices from mice expressing eGFP (n = 6, two-way ANOVA, effect of virus $F_{1,10}$ = 21.685, p < 0.001). ***B***. In contrast, virally delivered cofilin^{S3D} prevents sleep deprivation-induced deficits in this form of LTP (n = 5, two-way ANOVA, effect of virus $F_{1,8}$ = 0.016, p < 0.902). NSD: non-sleep deprived, SD: sleep deprived. Values represent the mean ± SEM.

Figure 5.5. The effect of sleep deprivation and aging on hippocampal and cortical gene expression.

Sleep deprivation (SD) caused significant effects (t-test, $p < 0.05$) on both upregulated genes (***A*** and ***B***) and downregulated genes (***C*** and ***D***) in the hippocampus and the cortex. The significant effect of aging (t-test, $p < 0.05$) was found in the expression levels of *Rbm3, Hnrpdl*, and *Usp2* in the hippocampus (***C***) and *Prkab2*, *Rbm3, Hnrpdl*, and *Usp2* in the cortex (***B*** and ***D***). The correlation between aging and SD was found only in the hippocampal expression of *Hspb1* (***A***; t-test, $p = 0.007$). Fold changes are relative to the expression level of the young non-sleep deprived (NSD) group. Expression levels are normalized relative to the level of housekeeping genes (***E*** and ***F***; *Tubulin, Hprt* and *Actg*). n = 8 for all groups.

Figures

Figure 5.1

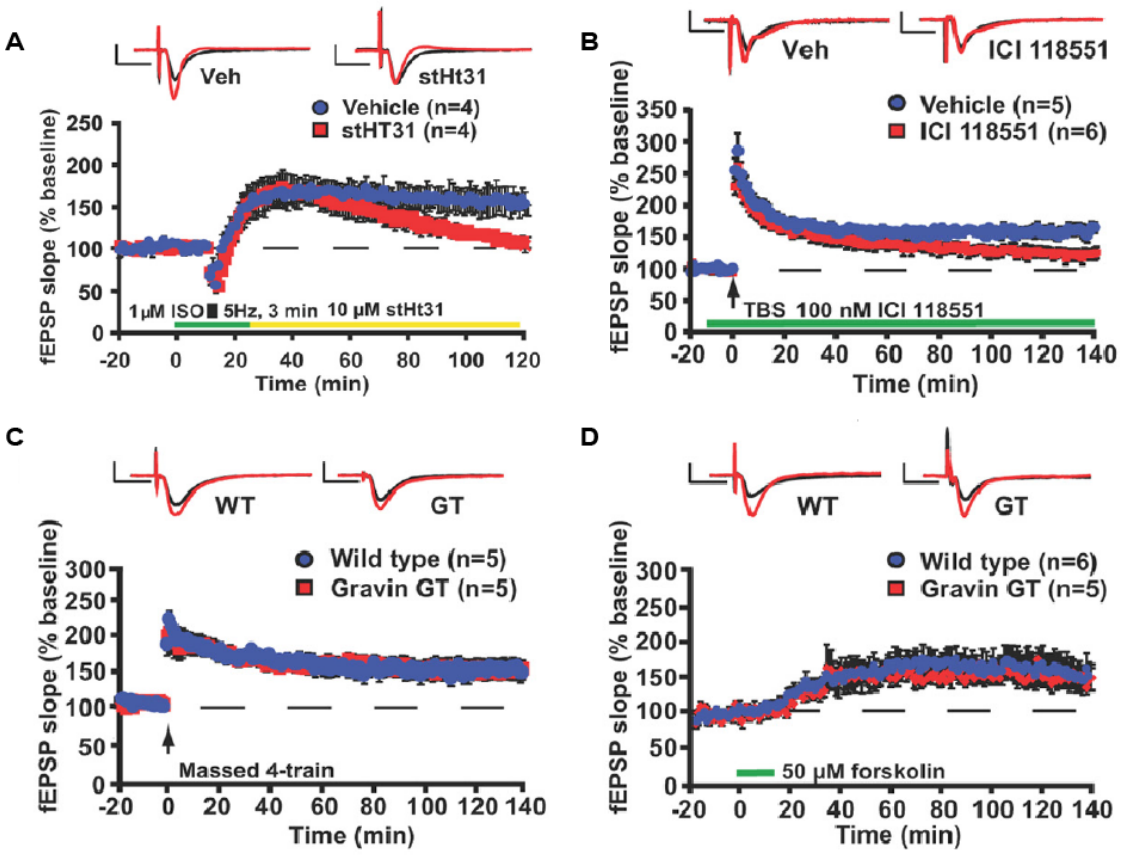


Figure 5.2

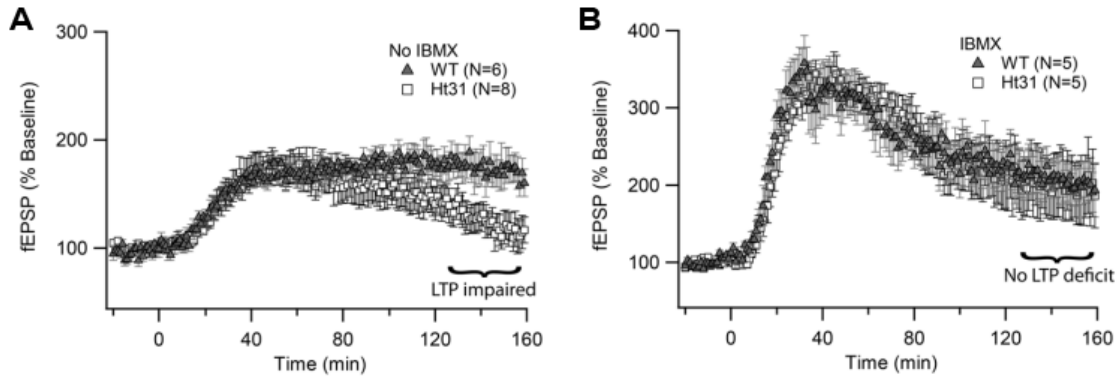


Figure 5.3

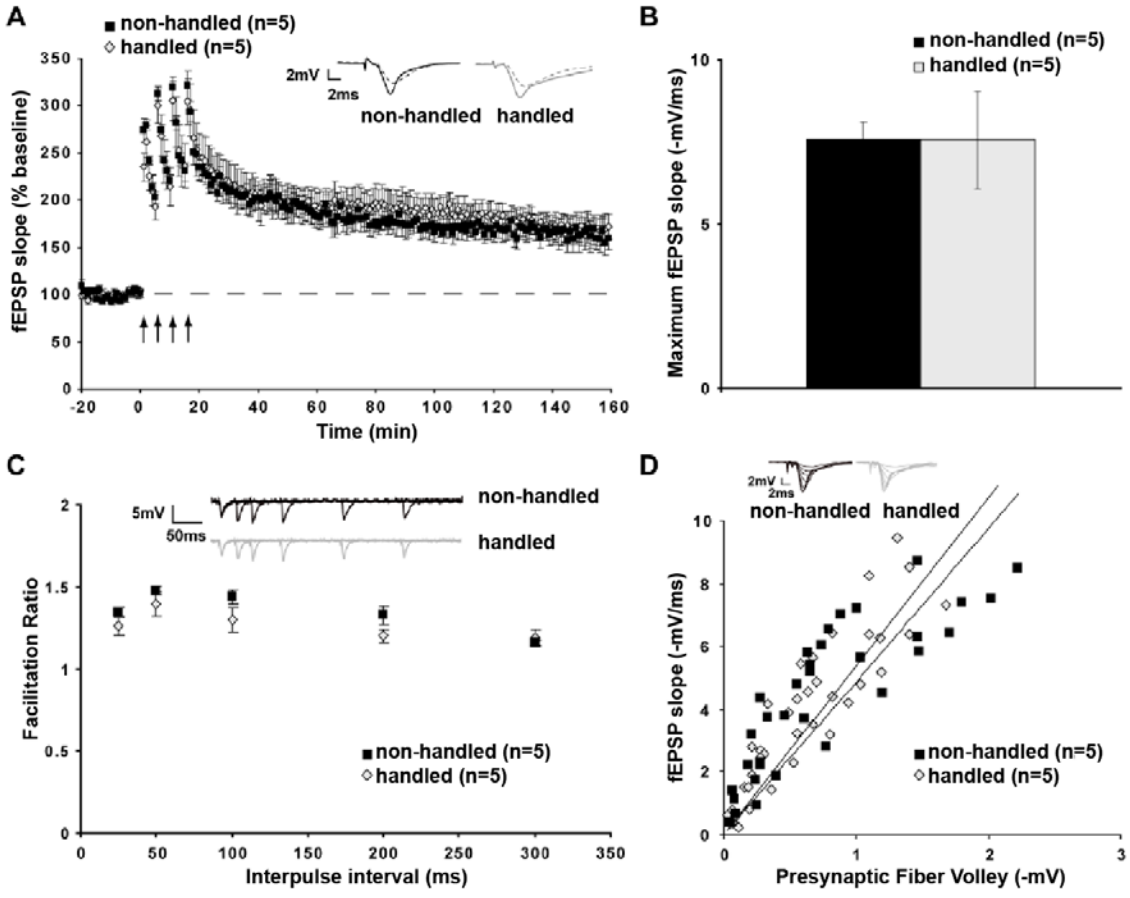
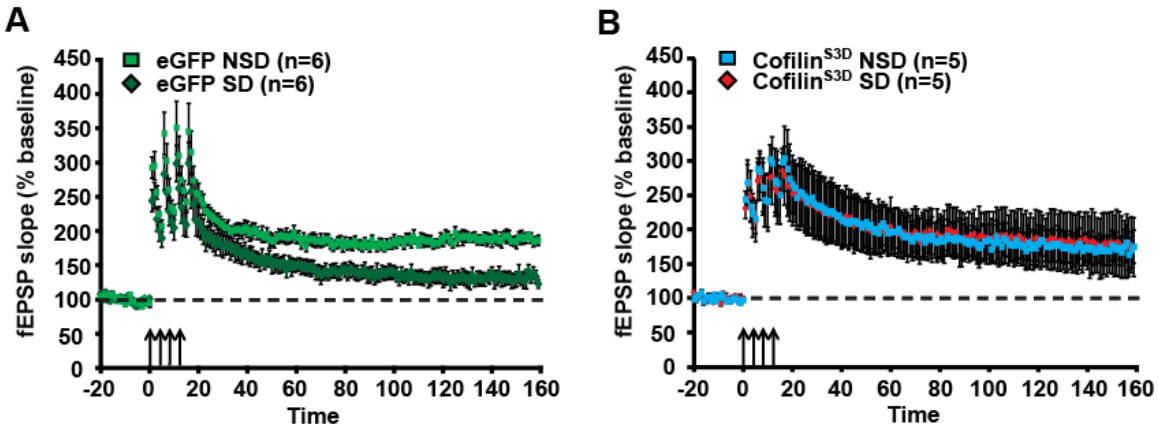


Figure 5.4


A
eGFP NSD (n=6)
eGFP SD (n=6)
fEPSP slope (% baseline)
450
400
350
300
250
200
150
100
50
0
-20
0
20
40
60
80
100
120
140
160
Time
B
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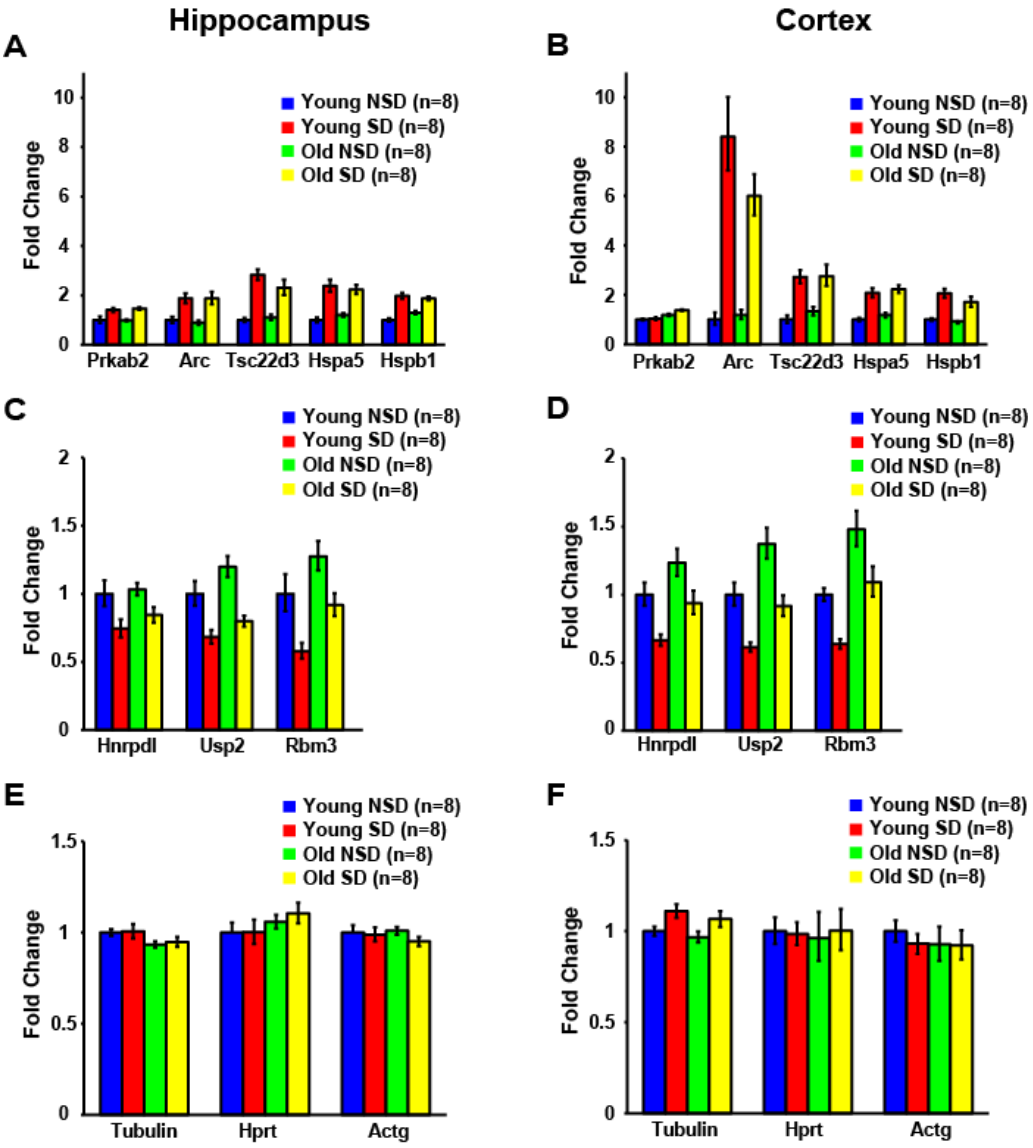
Cofilin^{S3D} SD (n=5)
fEPSP slope (% baseline)
Time

Figure 5.5


Hippocampus
Cortex
A
B
C
D
E
F
Young NSD (n=8)
Young SD (n=8)
Old NSD (n=8)
Old SD (n=8)
Fold Change
Prkab2
Arc
Tsc22d3
Hspa5
Hspb1
Hnrpdl
Usp2
Rbm3
Tubulin
Hprt
Actg

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