Synopsis

Protein Modifications: Relevance to Synaptic Plasticity and Memory

Memory is the process of archiving experiences for using them at a later stage. Information is stored for short time (short-term memory) or for longer time (long-term memory). Long-term potentiation (LTP), an increase in synaptic strength, is considered a cellular mechanism of memory formation. Similar to memory, LTP is also divided into at least two phases, the early phase of LTP and the late phase of LTP (Sweatt, 1999; Lynch, 2004).

Posttranslational modifications of proteins such as phosphorylation play crucial roles in LTP and memory formation (Soderling and Derkach, 2000; Sweatt, 2004). Modification of proteins through acetylation also has emerged as a key event regulating various cellular processes including synaptic plasticity and memory (Sharma, 2010). Relative activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) determine the protein acetylation level in a cell. HDACs negatively regulate LTP and memory formation (Guan et al., 2010). Additionally, increase in protein acetylation through deacetylase inhibitors facilitates synaptic plasticity, improves long-term memory and rescues memory impairments associated with neurodegenerative conditions (Sharma, 2010; Fischer et al., 2010).

Histones are the prominent targets of HDAC inhibitors. Histone acetylation is correlated with the permissive state of chromatin that favors transcription. Considerable efforts have been made towards elucidating the role of histone acetylation in the processes related to synaptic plasticity and memory formation. However, the relevance of other acetylated proteins remains poorly understood. Moreover, unavailability of specific protein deacetylase

inhibitors has restricted our understanding about the role of protein acetylation in plasticity-related processes and their cross-talk with other signaling processes.

The present study elucidates the importance of protein acetylation in synaptic plasticity and memory formation. A combination of molecular techniques and behavioral tasks has been employed for the study. The study has been broadly divided under four chapters.

The first chapter describes the identification of a 55 kDa protein (p55), acetylation of which increases upon deacetylase inhibition. As stated earlier, HDAC inhibition facilitates LTP and memory. Since histones are not the only substrates of commonly referred histone deacetylase inhibitors, we wanted to identify HDAC targets in addition to histones. Hippocampal slices were treated with trichostatin A (TSA), a commonly used HDAC inhibitor. The increase in acetylation was examined using an antibody that recognizes proteins containing acetylated lysine residues. The results showed that TSA increased acetylation of histones as expected. In addition to histones, there was a prominent increase in the acetylation of a 55kDa protein. Taste learning as well as TSA increase acetylation of a 55kDa protein in the insular cortex (Swank and Sweatt, 2001). Since the identity of p55 was not known, we aimed to identify p55 in the hippocampus. Based on its molecular weight and the tendency to get acetylated, we considered the possibility of p55 being either p53 or alpha tubulin. We observed that p55 had different mobility than p53 and was present in non-nuclear fraction. On the other hand, both p55 and alpha tubulin resolved at the same position on SDS-PAGE. Copolymerization of p55 with microtubule fraction suggested it to be a microtubule-related protein. Sodium butyrate, a deacetylase inhibitor that does not target alpha tubulin, did not affect the acetylation of p55. Finally, in two-dimensional SDS-PAGE,

acetyl-p55 and alpha tubulin showed identical mobility. Based on these criteria, we conclude that p55 is most likely alpha tubulin.

We next examined whether acetylation of alpha tubulin is regulated in activity-dependent manner. Alpha tubulin is known to be post-translationally modified by covalent addition of an acetyl group to its lysine 40 residue. Acetylation of alpha tubulin was examined using an antibody that recognizes acetylated alpha tubulin and an antibody against unmodified alpha tubulin. We used KCl as activity-mimicking stimulus since it leads to calcium influx in neurons (Wu et al., 2001; Nashat et al., 2003), and it induces LTP in the hippocampal slices in NMDA receptor-dependent manner (Fleck et al., 1992). Results showed that KCl treatment increased acetylation of alpha tubulin in the hippocampus. NMDA receptor is known to play crucial roles in different forms of LTP and memory (Lynch, 2004). We found that hippocampal slices treated with NMDA also showed increased alpha tubulin acetylation which was blocked by APV, a specific NMDA receptor antagonist. To determine the involvement of synaptic NMDA receptors, slices were treated with a combination of bicuculline, nifedipine and glycine (BNG) (Ivanov et al., 2006). Results showed that synaptic glutamate release increased acetylation of alpha tubulin. Furthermore, APV blocked BNG-induced increase in alpha tubulin acetylation. Collectively, these results suggest that acetylation of alpha tubulin in the hippocampus is regulated in an activity-dependent manner.

We next examined the regulation of activity-dependent alpha tubulin acetylation. Sirtuin2, a tubulin deacetylase (North et al., 2003), is phosphorylated by cyclin-dependent kinase 5 (CDK5). This phosphorylation event inhibits sirtuin2 activity (Pandithage et al., 2008). Thus, we considered the involvement of CDK5 in activity-dependent regulation of alpha

tubulin acetylation. To determine the role of CDK5 in alpha tubulin acetylation, we used two CDK5 inhibitors - roscovitine and olomucine. We found that both inhibitors blocked KCl-induced increase in alpha tubulin acetylation. We next examined whether CDK5 activity is required for NMDA receptor-mediated increase in alpha tubulin acetylation. NMDA treatment failed to increase acetylation of alpha tubulin in the presence of roscovitine. Thus, CDK5 regulates KCl- and NMDA-induced alpha tubulin acetylation. We next asked whether KCl treatment indeed affects sirtuin2 enzyme activity. KCl treatment significantly reduced sirtuin2 activity in the hippocampal slices. Importantly, in the presence of roscovitine, KCl failed to reduce sirtuin2 activity. Thus, CDK5 plays important role in activity-dependent alpha tubulin acetylation.

We next examined whether memory formation leads to acetylation of alpha tubulin. Novel object recognition task utilizes the tendency of the rodents to explore a novel object more than a familiar object (Ennaceur and Delacour, 1988). Before training rats were habituated to the arena. During training, the rats were exposed to two copies of the same object. After 1 h or 24 h post training, the rats were tested for short-term and long-term memory, respectively. During the test phase, one copy of the familiar object was replaced with a new object. Similar to earlier reports, our results showed that the rats trained in this task formed long-term memory that lasted for at least 24 h. We next determined the effect of learning on acetylation of alpha tubulin in the hippocampus. One hour after training, rats were sacrificed and alpha tubulin acetylation was examined in the hippocampus and insular cortex. Results showed that learning event significantly increased acetylation of alpha tubulin in the hippocampus. However, acetyl-alpha tubulin level in the insular cortex was unchanged. Collectively, this chapter shows that alpha tubulin is acetylated in activity-dependent manner

and identifies the signaling pathway that regulates this event. Finally, we show that alpha tubulin acetylation is indeed regulated by memory training.

The second chapter elucidates the role of acetylation in processes important for synaptic plasticity and memory. Surface recruitment of GluR1-containing AMPA receptors is associated with LTP and memory formation (Hayashi et al., 2000; Yeh et al., 2006; Nedelescu, 2010). Therefore, we asked whether acetylation plays a role in surface recruitment of AMPA receptors. Results showed that treatment of hippocampal neurons with deacetylase inhibitor, TSA, increased surface abundance of GluR1. Since TSA targets proteins in addition to tubulin, we used a specific tubulin deacetylase inhibitor, tubacin, to assess the effect of acetylated tubulin on surface insertion of AMPA receptors. Tubacin also increased surface expression of GluR1. These results suggest a role for acetylated tubulin-based transport in surface recruitment of AMPA receptors.

Earlier studies have shown that the delivery of GluR1-containing AMPA receptors to the membrane surface is regulated in an activity-dependent manner (Rumpel et al., 2005; Appleby et al., 2011). Consistent with these results, we found that KCl significantly increased surface expression of GluR1 in cultured hippocampal neurons. We next examined whether surface expression of AMPA receptors is regulated in the same manner as alpha tubulin acetylation. Results showed that the CDK5 inhibitor, roscovitine blocked KCl-induced increase in surface GluR1 expression in the hippocampal neurons. Thus, this chapter shows that alpha tubulin acetylation facilitates surface expression of AMPA receptors.

With our interest in protein acetylation and differential memory formation by massed and spaced training, the third Chapter of the thesis describes the effects of increasing acetylation on memory induced by massed training. Spaced training is superior to massed training in memory formation in different model systems from invertebrates to vertebrates (Philips et al., 2013). This phenomenon is referred to as the "spacing effect". In our studies, we used Morris water maze task to examine the role of acetylation in spatial memory formation. After establishing memory assessment protocol in our laboratory, we examined memory formation by spaced and massed training paradigms. Using a protocol in which during the spaced training the training trials are distributed over 5 days and during massed training the trials are restricted to a single day, our results showed that spaced training was more effective in long-term memory formation than the massed training. This is consistent with previous studies examining the effects of training patterns on memory formation.

The molecular mechanisms underlying the spacing effect are poorly understood. To examine molecular changes it would be helpful if the training can be completed in a shorter time. Thus, we scaled down a 5-day training task to a single day. Single day spaced training also offers a better strategic comparison to the single day massed training. Results showed that the single day spaced trained rats performed similar to the 5-day spaced trained rats. We then compared memory formation between the single day spaced and massed trained group. Similar to the 5-day spaced versus massed training results, the single day spaced trained group formed better long-term memory as compared to the massed trained group.

We next asked whether increasing protein acetylation has any effect on long-term memory formation by massed training. Rats were intra-peritoneally injected with a histone deacetylase inhibitor, sodium butyrate (SB). The SB-injected rats showed enhanced level of

histone acetylation in the hippocampus. Rats were then given SB or saline before massed training. Although both groups showed similar extent of learning, during the long-term memory test SB-injected rats showed shorter escape latencies and more annulus crossings as compared to the saline controls. Thus, enhancing acetylation level enhances memory formation by massed training in spatial memory task.

Chapter four of the thesis examines the interaction of histone acetylation and poly(ADP)-ribosylation, another important posttranslational modification of histones. Like acetylation, poly(ADP)-ribosylation of histones facilitates transcription (Martinez-Zamudio and Ha, 2012). Membrane depolarization enhances poly(ADP)-ribosylation in neurons (Homburg et al., 2000). Importantly, inhibition of the enzyme, poly(ADP)-ribose polymerase (PARP) inhibits memory formation (Cohen-Armon et al, 2004; Goldberg et al., 2009). Thus, we examined the interaction between acetylation and poly(ADP)-ribosylation in memory formation. Increasing acetylation with sodium butyrate enables memory formation by a sub-threshold training (Stefanko et al., 2009). To determine the role of poly(ADP)-ribosylation in HDAC inhibitor-mediated effects on memory, rats were intra-peritoneally injected with SB or TiQ-A (a specific PARP inhibitor) or both (SB + TiQ-A). The rats were subjected to sub-threshold object recognition training and memory was tested 1 h or 24 h after training. Across the groups, rats spent almost equal amount of time in exploring the objects during the training session. Short-term memory test showed that rats in all the groups explored novel object more than the familiar object. However, long-term memory test showed clear differences in the exploratory preferences of different groups. The SB-injected rats explored novel object more than the familiar object. However, in presence of the PARP inhibitor, SB-

injected group failed to form long-term memory. Thus, HDAC inhibitor-facilitated memory formation requires PARP activity.

Thus, in this work, we have identified activity-dependent acetylation of alpha tubulin, examined its mechanism and its role in AMPA receptor surface expression. We show that alpha tubulin acetylation is enhanced by memory training. These experiments suggest a role for tubulin acetylation in synaptic plasticity and memory possibly through enhanced transport. We further show that enhancing acetylation levels facilitates spatial memory formation by massed training. Finally, we have examined the role of poly(ADP)-ribosylation in HDAC inhibitor-facilitated memory formation in object recognition task. Collectively, the study has provided novel insights into the role of protein acetylation in synaptic plasticity and memory formation.

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