CHAPTER 3

INCREASING PROTEIN ACETYLATION
FACILITATES LONG-TERM MEMORY FORMATION
WITH MASSED TRAINING
INTRODUCTION

Spacing Effect in Memory Formation

It is well established that memory formation is sensitive to the pattern in which the trials are applied during training. Wide temporal spacing of trials during training (spaced training) results in better long-term memory (LTM) formation compared to the same number of trials applied with little or no temporal spacing (massed training). The superiority of spaced training over massed training, in making better long-term memory, is commonly referred to as the spacing effect. This phenomenon was first observed by a psychologist, Herman Ebbinghaus, in 1882. Since then, this property of memory formation has been studied using several model systems and learning tasks. The spaced trained animal remembers a task for a longer time compared to the massed trained counterpart. A few examples are illustrated here. In an object recognition task, five trials spaced 15 min apart lead to better LTM than equal number of trials given with 5 min interval or no rest interval (Genoux et al., 2002). In a spatial memory task, rats given 16 trials across 4 days show better LTM than the animals that receive all the 16 trials on the same day (Commins et al., 2003). Similarly, in a fear conditioning task, animals given four trials with an interval of 8 min form better LTM than those, which receive the training trials with 10 sec interval (Josselyn et al., 2001). Collectively, research has shown that indeed the spacing effect is a conserved phenomenon from invertebrates to humans spanning diverse range of learning tasks (Naqib et al., 2012; Philips et al., 2013).
Spacing Effect and Synaptic Plasticity

Long-term potentiation (LTP) is considered to be the cellular mechanism of memory formation. LTP shares many molecular mechanisms with memory (Lynch, 2004). Depending on its requirement of protein synthesis and gene transcription, LTP could be either early phase LTP or late phase LTP. Similar to memory, LTP also displays pattern sensitivity. Spaced trains of electrical stimulations induce LTP that is higher in magnitude than the LTP induced with massed stimulation (Abraham et al., 2002; Scharf et al., 2002; Ajay and Bhalla, 2004; Kramar et al., 2012).

Even in invertebrates, synaptic plasticity induction is pattern sensitive. For example, in *Aplysia*, 5 pulses of serotonin applied in a spaced pattern are more effective for inducing long-term facilitation of sensory-motor synapses than the same number of serotonin pulses applied in a massed pattern (Mauelshagen et al., 1998). Thus, not only for memory, the spacing effect phenomenon appears to be conserved for synaptic plasticity also across different species.

Spacing Effect and Signaling Molecules

The spacing effect has been extensively studied with regards to memory tasks, but the underlying molecular mechanisms are not clearly understood. The late phase of LTP and long-term memory require changes in protein and RNA synthesis. Spaced tetanic stimulation-induced LTP is more sensitive to protein synthesis inhibition than the massed tetanic stimulation-induced LTP, suggesting that spaced stimulation recruits protein synthesis-dependent processes more than the massed stimulation (Scharf et al., 2002). The transcription factor, cAMP response element-binding protein (CREB), regulates
transcriptional events (Foulkes et al., 1991; Sassone-Corsi, 1995; De Cesare et al., 1999). Extensive evidence exists to show that CREB plays crucial roles in synaptic plasticity and memory (Frank and Greenberg, 1994; Silva et al., 1998; Lynch, 2004). The CREB activator isoform to CREB inhibitor isoform ratio determines LTM formation with spaced or massed training in *Drosophila* (Yin et al., 1995). Inhibition of CREB transcriptional activity produces profound deficits in LTP and memory (Impey et al., 1996; Jancic et al., 2009; Guzowski and McGaugh, 1997). Importantly, LTM deficits in CREB mutant mice are rescued by spaced training (Kogan et al., 1997). Activity of CREB is regulated by its phosphorylation, which is catalyzed by kinases such as cAMP-dependent protein kinase (PKA) and mitogen-activated protein kinases (MAPK) (Impey et al., 1998; Roberson et al., 1999). Both PKA and MAPK play important roles in LTP and memory formation (Lynch, 2004). Prolonged activation of MAPK is induced by spaced, but not by massed, stimulation of hippocampal neurons with KCl (Wu et al., 2001).

Overexpression of CREB improves LTM formation in massed-trained rats but it does not affect LTM formation after spaced training (Yin et al., 1995; Josselyn et al., 2001). Similarly, three trains of theta burst stimulations given at 1 h interval induces LTP, which does not improve any further upon increasing the number of stimulations (Kramar et al., 2012). Existence of this ‘ceiling effect’ or maximal LTP/LTM suggests a quantitative change in the molecular processes, perhaps during the rest interval, which accumulates over multiple training sessions and contributes to LTP and memory.
**Histone Acetylation in LTM Formation**

Protein acetylation plays crucial roles in synaptic plasticity and memory formation (Sharma, 2010). Histones are the most thoroughly studied acetylated proteins and the role of this histone modification in synaptic plasticity and memory is extensively studied. The late phases of LTP and memory require transcription of genes. Acetylated histones facilitate LTP and memory formation by enhancing transcriptional processes. Relative activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) regulate the acetylated histone level. Thus, whereas HAT activity facilitates transcription, HDAC activity inhibits this process. Indeed, HDACs constrain synaptic plasticity and memory (Guan et al., 2009). HDAC inhibition through genetic deletion or using pharmacological inhibitors enhances LTP and memory formation (Alarcon et al., 2004; Levenson et al., 2004; Yeh et al., 2004; Chwang et al., 2007; Fischer et al., 2007; Fontan-Lozano et al., 2008; Guan et al., 2009; Stefanko et al., 2009; Dagnas et al., 2013). Importantly, the HDAC inhibitors exhibit memory enhancing effects in a CREB-dependent manner (Vecsey et al., 2007). In addition, HDAC inhibitors reinstate memory in impaired conditions (Dash et al., 2009; Peleg et al., 2010; Graff et al., 2012). Although increasing acetylation level facilitates memory formation, the effects of increasing acetylation on memory induced after massed training is not understood. In this Chapter, we discuss the effects of enhancing acetylation by histone deacetylase inhibitors, on spatial memory induced by massed training.
MATERIALS AND METHODS

Animals

Two-three months old Sprague Dawley rats weighing 250-350 g were used for the task. Each rat was housed separately on a 12 h/12 h light/dark cycle. The rats were given free access to rodent chow and water. All experiments were conducted between 9 a.m. and 5 p.m.

Water Maze Apparatus

A circular water maze pool, 1.68 m diameter and 60 cm height, was used for the task. The tank was half-filled with water and the temperature was maintained at 24 ± 2°C. A white plexiglass, circular platform of 10 cm diameter was kept submerged, 2 cm below water level. Non-toxic white paint was used to make the water opaque, such that the platform becomes invisible. Four objects (cues) were placed at 40-60 cm distance around the pool. The objects were: a large black box (42 cm in height, 32 cm in length and 28 cm in width), a rectangular sheet of paper (50 cm in length and 40 cm in width) with black cross on white background, a large toy (60 cm in height, 40 cm in length and 13 cm in width) and a tall rack (200 cm in height, 92 cm in length and 46 cm in width). The room was illuminated from all the four corners using four 100 watt bulbs facing the ceiling. Across the trials, the position of the platform was kept fixed with respect to the cues. A camera was mounted on top of the pool to record animal activity during training and testing.
Handling

Rats were handled for 3 days before training (10 min each day). On the last day of handling, part of the head of the animal was painted black using hairdye so that they can be easily tracked during the experiment.

Training

Hidden platform version of the water maze task was used for the assessment of spatial memory. During the 5-day spaced training, each rat was given a total of 20 trials in 5 blocks. Each block consisted of 4 trials, which was given on a day with an inter-trial interval (ITI) of 1 min. Thus, the 5 blocks of training were delivered in 5 days. In the single-day spaced training protocol, rats were given 20 trials in 4 blocks, all in a single day with an inter-trial interval of 1 min. Each block comprised of 5 trials and a rest period of 2 h was allowed between the blocks. Rats in the massed group received all the 20 trials on the same day with an inter-trial interval of 1 min.

The release positions of the rats were pseudo-randomized such that none of the positions were repeated in a given block. Rats were released in the pool and were allowed to find the hidden platform within 90 seconds, failing which they were manually guided to it. Once on the platform, rats remained there for 30 seconds. The rats were towel-dried between the trials. The animals which showed no decrease in latency over the training were removed from the analysis. In the single day spaced and massed training experiment, out of the 30 animals used, 2 animals in the massed training group started drowning during the course of training and hence, were removed from the experiment. In addition, 2 animals from the massed-trained group were removed from the analysis since they did not show any decrease
in latency across the acquisition trials. In the experiments examining the effect of sodium butyrate on massed training, out of the 32 animals used, one animal in saline + massed-trained group and 2 animals in sodium butyrate + massed-trained group started drowning during the course of training, and hence, were removed from the experiment.

Probe trials were conducted 1 h and 24 h after the last training trial to assess short-term and long-term memory, respectively. During the probe trials, rats were released in the pool without the platform. They were allowed to swim for 60 seconds and their spatial memory was scored by three parameters: (1) goal (former platform area) latency, (2) preferential exploration of the platform quadrant, and (3) number of annulus crossings. Shorter goal latency indicates better memory. The amount of time rats spend in the platform quadrant is considered a better measure of memory for the platform. More time spent in the platform quadrant compared to other quadrants indicates strong spatial memory for the platform. The former platform area is known as annulus. Annulus crossings indicate memory of the precise location of the platform.

All the activities were recorded using ANYMAZE tracking software and the videos were analyzed manually. The measured values during acquisition trials and the probe tests were averaged for each group. Data are expressed as mean ± SEM.

**Sodium Butyrate Injections**

For the analysis of histone acetylation, sodium butyrate (1.2 g/kg body weight) was injected in the rats, intraperitoneally. This dose of sodium butyrate has been used in previous studies (Levenson, 2004; Lattal, 2007; Reolon, 2011; Hawk, 2011). A stock solution of 120 mg/ml of butyrate was prepared in normal saline. On an average, rats received 2.5 – 3.5 ml of
sodium butyrate solution. Control rats uniformly received 3 ml of saline. One hour post injection, the rats were killed according to the procedure approved by the Institutional Animal Ethics Committee, and their hippocampi were harvested in the lysis buffer containing Triton-X100 and used for isolation of nuclear fraction as described below.

For the behavioral experiments, rats were intraperitoneally injected with saline or sodium butyrate as mentioned above, every day for 13 days. On the 14th day, injections were given 1 h before training. Prolonged sodium butyrate treatment has been used previously to examine modulation of long-term memory in fear conditioning and water maze tasks (Fischer, 2007). Both saline- and sodium butyrate-injected groups were then subjected to massed training as described in the earlier section. Probe trials were conducted 24 h after training to assess long-term memory.

**Cell Fractionation**

The hippocampi of both saline- and sodium butyrate-injected rats were harvested in Triton-X100 containing lysis buffer (Triton-LB: 50 mM Tris-Cl pH 7.5, 137.5 mM NaCl, 10% glycerol, 50 mM NaF, 1 mM Na3VO4, 1 mM sodium butyrate, 5 mM EDTA, protease inhibitor cocktail and 0.5% Triton-X100). The tissue was homogenized using a Dounce homogenizer (VWR) and kept on ice for 30 min with intermittent vortexing. The homogenate was centrifuged at 100g for 5 min to remove un-lysed cells. The supernatant was further centrifuged at 1500g for 15 min. The supernatant after this centrifugation was considered as non-nuclear (cytosolic) fraction. The pellet was re-suspended in Triton-LB and centrifuged again at 1500g for 15 min. The resulting pellet was collected as the nuclear fraction. This pellet was lysed in SDS-containing lysis buffer (SDS-LB: 50 mM Tris pH 7.5,
150 mM NaCl, 50 mM NaF, 1 mM Na₃VO₄, 10 mM sodium butyrate, 1 mM EDTA, 2% sodium dodecyl sulfate (SDS) and protease inhibitor cocktail) and centrifuged at 14,000g for 10 min. The supernatant was collected as hippocampal nuclear lysate.

Protein content in the nuclear lysate was estimated using bicinchoninic acid (BCA, Pierce Biotechnology) method using bovine serum albumin (BSA, Sigma) as standard. The samples were mixed with sample buffer (5X sample buffer: 250 mM Tris pH 6.8, 11.5% SDS, 40% glycerol, 25% β-mercaptoethanol, 0.5% bromophenol blue) and boiled for 5 min before loading onto the gel.

**Western Blotting**

Equal amount of nuclear extracts from different samples were resolved on a polyacrylamide gel having 15% resolving and 4% stacking gels. The gel was run at a constant current of 20 mA in BioRad apparatus. Using wet transfer method, proteins were transferred to the nitrocellulose membrane (MDI) at 200 mA for 90 min. After transfer, the membranes were washed with Tris-buffered saline containing Tween-20 (TBST: 25 mM Tris-Cl pH 7.5, 137 mM NaCl, 2.7 mM KCl and 0.1% Tween-20). Blocking was done in 3% BSA prepared in TBST at room temperature for 1 h. The membranes were then incubated with primary antibody at 4°C overnight. The membranes were washed with TBST before incubating them with the secondary antibody (diluted in the blocking solution) for 1 h at room temperature. After this incubation, the blots were washed again and the bound antibody was detected using enhanced chemiluminescence reagent (Pierce). The signals were captured by exposing the blots to Hyperfilm (Amersham). Band intensities were quantified using NIH Image (NIH, Bethesda).
The blots were first incubated with total histone H3 antibody, which was stripped off before probing the blots for antibody against acetyl-histone H3 or H4. The antibody stripping was performed by incubating the blots in stripping buffer (62.5 mM Tris-Cl pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) at 65°C for 1.5 h with a change of buffer after 45 min of incubation. Stripping of antibody was confirmed by probing the blot with secondary antibody alone.

**Antibodies**

Antibodies that were used for the study were: acetyl-(lysine 14)-histone H3 (Upstate Biotechnology), acetyl-(lysine 5, 8, 12, 16)-histone H4 (Upstate Biotechnology) and histone H3 (Upstate Biotechnology). For the estimation of histone acetylation, acetyl-histone signal was divided by the total histone H3 signal in each lane, and then normalized with the control sample.

**Statistical Analysis**

In the behavioral experiments, student’s paired or unpaired t-test, as appropriate, was used to assess significant differences. The differences were considered significant when p value was less than 0.05.
RESULTS

Comparison of 5-Day and 1-Day Spaced Training on Memory Formation

Morris water maze is commonly used to assess spatial memory in the rodents. A typical paradigm that has been used in several studies is to conduct training over the course of several days. While this training protocol has been very effective to study different aspects of spatial memory formation, it possesses some limitations in examining molecular changes associated with memory formation. Indeed some previous studies have used a single-day training to study spatial memory formation in this task (Packard and Teather, 1997; Frick et al., 2000). Since 1-day spaced training offers a better comparison to 1-day massed training, we first established that training in a single day can lead to LTM formation in this task. A schematic diagram of training and testing is presented in Figure 1. We found that 20 training trials in 4 blocks (each block consisting 5 trials) with 2 h interval in between the training blocks produced LTM that lasted for at least 24 h. Both 5-day spaced training and 1-day spaced training showed decrease in escape latency over the course of training (Fig. 2A). Similar escape latency for the last block of trials suggests that both the groups learnt the task equally well. Both the groups showed short-term memory (1 h after the last training trial) and long-term memory (24 h after the last training trial), as determined by escape latencies. Although during the long-term memory test, the 5-day spaced-trained rats took lesser time to reach the platform area compared to single-day spaced-trained animals (26.5 sec versus 38.38 sec, Fig. 2A), the difference was not statistically significant. Figure 2B1 shows that during the LTM test, both the groups showed preferential exploration of the platform quadrant than other quadrants, and spent similar amount of time in the platform quadrant. Annulus crossings indicate the accuracy with which the rats remember the platform’s
Figure 1. Schematic depiction of the 5-day and 1-day spaced training paradigms in Morris water maze task. The 5-day spaced-trained group received 20 trials in 5 blocks, spread across 5 days. The inter-block interval was 24 h, and each block consisted of 4 trials with 1 min inter-trial interval (ITI). The 1-day spaced-trained group received 20 trials in 4 blocks, with an inter-block interval of 2 h. Each block consisted of 5 trials, with 1 min ITI. Probe trials were given 1 h and 24 h after the last training trial to assess short-term memory (STM) and long-term memory (LTM), respectively.
Figure 2A. Performance of 1-day spaced-trained and 5-day spaced-trained animals in the spatial memory task. The mean escape latency of both 5-day spaced-trained (n = 12) and 1-day spaced-trained (n = 8) rats reduced over the course of training indicating that animals in both the group learnt the task. The average latency to reach the platform area during the probe trial for short-term memory (STM, PT1) and long-term memory (LTM, PT2) are also shown. Both the groups showed STM as well LTM.
Figure 2B. Both 5-day and 1-day spaced-trained animals show long-term memory (LTM) for the platform. During the probe trial for LTM (PT2 in Figure 2A), the time spent by animals in different quadrants and annulus crossings, were also analyzed. Both the groups spent more time in the platform quadrant [Q4] than other quadrants (B1), indicating that both groups had formed LTM for the platform. There was no significant difference between the time spent in the platform quadrant by the two groups of animals. The average number of annulus crossings made by both the groups of animals also did not show any significant difference (B2). The representative track plots during the long-term memory test, are presented in B3. Asterisks denote significant difference from other quadrants (p < 0.05).
location. We found that rats in both the group crossed the annulus similar number of times (Fig. 2B2). The representative swim paths (tracks) taken by an animal from both the groups, during the probe trial is also shown (Fig. 2B3). Collectively, the results suggest that 1-day spaced training forms LTM comparable to 5-day spaced training.

**Single-day Spaced Training Forms Better Long-Term Memory than Single-Day Massed Training**

We compared the effects of 1-day spaced and 1-day massed patterns of training on LTM formation. The schematic diagram of the training and testing schedule is presented in Figure 3. Both spaced and massed trained groups showed a progressive decrease in the average escape latency across the acquisition trials (Fig. 4A). Rats in both the groups showed similar average latency in the last training block, suggesting that they learnt the task equally well.

Three different parameters – goal latency, exploration time of the four quadrants and the annulus crossings, were analyzed during the probe trial to get a better profile of the memory formed with either training paradigm (Kraemer et al., 1996; Frick et al., 2000). Long-term memory was tested in both the groups 24 h after the last training trial. During the probe trial, the 1-day spaced trained rats showed significantly shorter goal latency compared to the massed trained group (Fig. 4A). Time spent in the platform quadrant is a good measure of memory. Figure 4B1 shows that rats given 1-day spaced training spent significantly more amount of time in the platform quadrant as compared to the other quadrants. On the contrary, the massed-trained rats explored all the quadrants equally. Comparing the performances of rats in both the groups, 1-day spaced trained rats spent significantly more time in the platform quadrant compared to the massed trained group. In addition, 1-day
Figure 3. Schematic depiction of single-day spaced and massed training paradigms. The spaced-trained group received 20 trials in 4 blocks with an interval of 2 h between the blocks. Each block consisted of 5 trials with 1 min inter-trial interval (ITI). Massed group received 20 trials in 4 blocks with an interval of 1 min between the blocks. Each block consisted of 5 trials with 1 min ITI. Probe trials were conducted 24 h after the last training trial to assess long-term memory (LTM).
Figure 4A. Performance of spaced and massed trained rats in the spatial memory task. The rats given spaced or massed training (as described in Figure 3, \(n = 11\), both groups) presented progressively decreasing mean escape latency during the acquisition phase. During the last block of training, both spaced- and massed-trained rats showed similar escape latency indicating similar acquisition of the task. The average goal (platform area) latency of rats during the probe trials for long-term memory (PT) shows that the spaced-trained rats presented significantly shorter goal latency compared to the massed-trained group. Asterisk denotes significant difference between the groups (\(p < 0.05\)).
Figure 4B. Spaced-trained, but not massed-trained, animals preferentially explore the platform quadrant during the long-term memory test. During the probe trial for long-term memory (LTM; PT in Figure 4A), the time spent by animals in different quadrants and annulus crossings, were also analyzed. The spaced-trained rats spent significantly more time in the platform quadrant [Q4] than other quadrants, indicating that they had formed LTM for the platform (B1). However, the massed-trained rats did not show any preference in exploring the platform quadrant, indicating lack of LTM. There was a significant difference between the time spent in the platform quadrant by the two groups of animal. The average number of annulus crossings made by the spaced-trained rats was significantly more than the massed-trained group (B2). The representative track plots during the long-term memory test are presented in B3. Asterisk denotes significant difference (p<0.05) between time spent in Q4 compared to time spent in other quadrants [Q1, Q2 and Q3]. # denotes significant difference (p<0.05) between the groups.
spaced trained rats crossed the annulus significantly more number of times compared to the rats given 1-day massed training (Fig. 4B2). The representative swim paths (tracks) taken by an animal from both spaced and massed groups, during the probe trial is also shown (Fig. 4B3). Collectively, these results suggest that LTM formation, and not learning, is affected by different patterns of training.

We analyzed the swim speed of animals during training and probe trials. Rats in both the groups presented overall similar swim speed across the training and testing phases (Fig. 5), although during acquisition trials 6, 7 and 19, the swim speed of rats in both the groups showed significant difference. The difference in swim speed in 3 out of 20 trials did not affect acquisition of the task as indicated by similar escape latency of both the groups during the last training block (Fig. 4A). Similar speed indicates similar motivation of rats to reach the platform, irrespective of spaced or massed training.

**Systemic Administration of Sodium Butyrate Increases Histone Acetylation in the Hippocampus**

Our main aim of this study was to examine the effects of increasing protein acetylation on memory formation by massed pattern of training. Sodium butyrate has often been used to study the effects of increasing acetylation on synaptic plasticity and memory. Before conducting memory experiments, we examined whether systemic administration of sodium butyrate, a histone deacetylase inhibitor, enhances acetylation level in the hippocampus, a region critical for spatial memory formation. Rats were intra-peritoneally injected with sodium butyrate and their hippocampi were analyzed for the changes in acetylation of
Figure 5. Similar swim speed of spaced- and massed-trained animals. The average swim speed of spaced- or massed-trained groups during training and long-term memory test (PT, Figure 4A) were analyzed. Both the groups showed similar swim speed during acquisition and long-term memory test.
histones. We found that the hippocampi of butyrate-injected rat showed increased acetylation of histones, H3 and H4, compared to the saline-injected control (Fig. 6).

**Sodium Butyrate Improves Long-Term Memory Formation with Massed Training**

Next we asked whether increasing acetylation could affect LTM formation after massed training. The animals in the butyrate group received daily intraperitoneal injection of sodium butyrate for 13 days. The animals in the control group received equal volume of saline for the same duration. On the 14th day, 1 h after injections, animals in both the groups were subjected to massed training. Both butyrate- and saline-injected rats learnt the task equally well as indicated by similar decrease in latency to reach the platform over the course of training (Fig. 7A). The result suggests that sodium butyrate does not interfere with the learning abilities of the animals. When tested for LTM, the sodium butyrate-injected rats took lesser time to reach the former platform area compared to the saline-injected rats (Fig. 7A). Figure 7B1 shows that the sodium butyrate-injected massed-trained rats preferentially explored the platform quadrant than other quadrants (Fig. 7B1). On the contrary, the saline-injected massed trained control rats showed no preference for the platform quadrant. Additionally, rats in the sodium butyrate-injected group made more number of annulus crossings than the saline controls (Fig. 7B2). The representative swim paths (tracks) presented in Figure 7B3 also show that the animals in the sodium butyrate-treated group spent more time in the concentric rings around the annulus.

We also examined the swim speed, body weight and thigmotaxis in both the groups of animals. The sodium butyrate- and saline-injected massed trained groups presented overall similar swim speed during acquisition trials and during probe trial (Fig. 8), although during
Figure 6. Intraperitoneally injected sodium butyrate increases acetylation of histones in the hippocampus. Rats were injected with saline or sodium butyrate. One hour after injection, the hippocampi were collected and processed for nuclear extraction. The nuclear extracts were probed with antibodies against acetyl-histone H3 and acetyl-histone H4. Total histone H3 was used as loading control. The representative Western blots show that sodium butyrate increased acetylation of histones, H3 and H4, in the hippocampus.
Figure 7A. Performance of sodium butyrate-injected animals in the spatial memory task. The saline-or sodium butyrate-injected animals given massed training (saline + massed, n = 15; butyrate + massed, n = 13) showed progressively reducing mean escape latencies during the acquisition phase. During the last block of training, rats in both the groups presented similar escape latencies suggesting that both the groups of animals learnt the task equally well. The average goal (platform area) latencies of rats during the probe trial for long-term memory (PT, 24 h after training) are also shown. Sodium butyrate-injected massed trained animals presented shorter goal latencies compared to the saline-injected massed trained group. Asterisk denotes significant difference between groups (p < 0.05).
Figure 7B. Sodium butyrate-injected animals given massed training preferentially explore the platform quadrant during the long-term memory test. During the probe trial for long-term memory (LTM, PT in Figure 7A), the time spent by animals in different quadrants and annulus crossings, were also analyzed. The sodium butyrate-injected massed-trained animals spent more time in the platform quadrant [Q4] than other quadrants, indicating that they had formed LTM for the platform (B1). The saline-injected massed-trained animals did not show any preference in exploring the platform quadrant, indicating lack of LTM. The average number of annulus crossings made by the sodium butyrate-injected massed-trained animals was significantly more than the saline-injected massed-trained group (B2). The representative track plots during the long-term memory test are presented in B3. Asterisks denote significant difference (p < 0.05) from Q1 and Q3 (B1) or between groups (B2).
Figure 8. Similar swim speed of sodium butyrate-injected massed-trained and saline-injected massed-trained animals. The average swim speed of sodium butyrate-injected massed trained and saline-injected massed trained groups during training and long-term memory test (PT in Figure 7A) were analyzed. Both the groups showed similar swim speed during acquisition and long-term memory test.
one of the acquisition trials (trial 9), the swim speed between the two groups was significantly different. The difference in swim speed in 1 out of 20 trials did not affect acquisition of the task as indicated by similar escape latency of both the groups during the last several trials (Fig. 7A). We also found that rats in both the groups showed similar weight gain across the experimental period (Fig. 9). In addition, during the probe trial, the number of thigmotaxic events (Fig. 10A) and the time spent indulging in thigmotaxic activities (Fig. 10B) remained unchanged between the saline and sodium butyrate-injected massed-trained groups. Collectively, our results suggest that increasing protein acetylation improves LTM formation after massed training.
Figure 9. Sodium butyrate does not affect body weight. The body weight of animals in saline- or sodium butyrate- injected groups were recorded before training (days 1-13), on the day of training (day 14) and on the day of testing long-term memory (day 15). Both the groups of animals show similar body weight.
Figure 10. Sodium butyrate does not affect thigmotaxic activity. During the probe trial for long-term memory (LTM, PT in Fig. 7A), the number of thigmotaxic events (A) and the total time spent indulging in thigmotaxic activities (B) were analyzed. The sodium-butyrate-injected massed-trained and saline-injected massed-trained animals do not show any difference in these parameters.
DISCUSSION

Long-term memory is sensitive to the pattern in which training trials are delivered. Spreng and colleagues (Spreng et al., 2002) have shown that training trials spread across 5 days (5-day spaced training) form better LTM compared to when all the trials are given on the same day (massed training). An important disadvantage of this spaced paradigm is that the procedure requires several days of training which makes the study of underlying molecular mechanisms difficult. Compressing the training schedule to a single day may be better for molecular studies, and for comparison with massed training which is typically carried out in a single day. Broadly consistent with previous studies, we found that 4 blocks (each with 5 trials) with 2 h interval in between the blocks, given on a single day was sufficient to induce LTM formation. We also found that the single day spaced training forms LTM that is comparable to LTM formed after 5 day training. During the LTM test, although the 5-day trained rats took lesser time to reach the platform area than the 1-day trained animals, both the groups spent similar amount of time in the platform quadrant. To compare memory formation between 1-day spaced and 1-day massed-trained groups, the number of training trials was kept constant with inter-trial interval in both the groups being 1 min. The inter-block interval in the spaced group was 2 h. During the probe trial, the single-day spaced-trained rats performed better than the massed-trained rats when examined for latency to reach the former platform area (goal). The spaced-trained rats explored the platform quadrant more than other quadrants, whereas the massed-trained animals did not show any preference in exploration of the platform quadrant. These results are consistent with earlier reports that spaced and massed training procedures differ in their abilities to form long-term memory (Spreng et al., 2002; Commins et al., 2003). The previous studies used spaced
training spread over a number of days. The single-day spaced training paradigm used in our study offers a better comparison with the single-day massed training.

As stated earlier, several previous studies have shown that rest interval during training plays a deterministic role in LTM formation. The duration of rest interval may act as the regulator of cellular processes that contributes to LTM formation. The longer rest intervals may facilitate these processes. Histone acetylation is facilitatory for transcription and new RNA synthesis is required for long-term memory formation (Hebbes et al., 1988; Lynch, 2004). Since massed training is less effective in LTM formation, we hypothesized that increasing acetylation level may enhance memory formation by massed training. Despite receiving same amount of training, the sodium butyrate-injected massed-trained rats formed better LTM than the saline-injected controls. These results are consistent with the earlier studies that emphasize the role of acetylated proteins, especially histone acetylation in LTM formation (Levenson, 2004; Lattal, 2007; Reolon, 2011; Hawk, 2011). The fact that the swim speed of the sodium butyrate-injected animals was not affected, rules out any effect of sodium butyrate on locomotor activity of the animals. The results suggest that one reason why massed training is less effective in LTM formation is that it may not appropriately recruit histone acetylation which facilitates transcriptional events. Other processes, including acetylation of non-histone proteins, may also contribute to the facilitating effects of sodium butyrate on memory formation by massed training.

In summary, this study shows that a single-day spaced training leads to LTM formation in the animals. Importantly, the study shows that it is possible to enhance long-term memory formation with massed training by manipulating the acetylation level of proteins. It would be interesting to explore the possible cross-talks of acetylation with other signaling events that
may contribute to the enhancing effects of histone deacetylase inhibitors on synaptic plasticity and memory.
REFERENCES:


