이학박사학위논문

# 기억의 변형 및 만성 통증에서의 단백질 합성과 분해의 역할 연구

# Studies on the role of protein synthesis and degradation in memory modification and chronic pain

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최 준 혁

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# Studies on the role of protein synthesis and degradation in memory modification and chronic pain

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**ABSTRACT**

Protein synthesis and degradation is an essential process in cells underlying regulation of various cell functions. Neural functions are also heavily regulated by protein synthesis and degradation. In addition to basic functions of protein synthesis and degradation, there are critical periods in neuronal synaptic plasticity where protein synthesis and degradation of certain proteins are essential. Although decades of studies on brain there may still be many critical points we do not know. It is important to understand where and when protein synthesis and degradation of specific proteins are essential for specific function of brain.

Although some reports indicate that protein synthesis-dependent process may be induced by updating information, the role of protein synthesis and degradation in changing the content of pre-existing memory is still not clear. In this study, I utilized an object rearrangement task, in which partial information related to a pre-existing memory is changed, promoting memory modification. Inhibitors of both protein synthesis and protein degradation impaired adequate incorporation of the altered information, in a distinctive way. These results indicate that protein synthesis and degradation may play key roles in memory modification.

Change in synaptic strength is believed to be the fundamental mechanism of pain as well as memory. In this study, I also examined whether synthesis and degradation of synaptic proteins in the anterior cingulated cortex (ACC) is involved in chronic pain. I performed behavioral tests, immunoblot analysis, spine structure analysis and electrophysiological measurements in the ACC suffering from chronic pain. First, I found that blocking of protein synthesis with anisomycin

in the ACC decreased mechanical allodynia response in mice experiencing neuropathic or inflammatory pain. However, administration of protein degradation inhibitor prevented anisomycin from ameliorating the allodynia response. Second, I revealed that neuropathic pain leads to active degradation and synthesis of postsynaptic scaffolding proteins in the ACC. Finally, protein synthesis inhibition in ACC reversed the structural and functional changes in synapses induced by chronic pain. These results suggest that ongoing synthesis of synaptic scaffolding proteins in the ACC contributes to the chronic pain.

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Key words: Protein synthesis, Protein degradation, Learning, Memory, Memory reorganization, Neuropathic pain, Chronic pain, Anterior cingulate cortex, Scaffolding protein

## LIST OF FIGURES and TABLES

# CHAPTER 1. INTRODUCTION

## INTRODUCTION

The function of brain is orchestrated by the serial activation of neurons. The brain's billions of neurons connect with one another, composing complex neural networks. All physical and mental functioning depends on activation of neural networks, which can be newly established, modified, or maintained for years. Neurons communicate each other through a specialized structure called synapse. The actual neural network is defined not only by the physical connection of neurons composing the neural network but also by the strength of each synapses. The strength of synapses can be modulated by synaptic plasticity, which in turn changes the output of the neural network activation. This modulation is important for adapting the brain function to changing circumstances and accumulating experiences.

Depending on the maintained duration of the resulting change, synaptic plasticity is classified as short-term or long-term plasticity. In short-term plasticity, alteration of channel properties or distribution can increase or decrease the synaptic strength. However, long-term plasticity requires *de novo* mRNA and protein synthesis for long-term maintenance of the changed synaptic strength (Kandel, 2001; Nguyen et al., 1994; Sossin and Lacaille, 2010). Memory is also considered to be a result of synaptic plasticity, which requires mRNA and protein synthesis to last more than several hours (Bailey et al., 1996; Davis and Squire, 1984; Dudai, 1996; Flexner et al., 1965; Goelet et al., 1986; Hernandez and Abel, 2008; Kandel, 2001). Drugs such as protein synthesis inhibitors block stabilization of long-term memory and show an amnesic effect when they are given within a certain time

after learning, while short-term memories are not disturbed.

In contrast to the generally accepted role of protein synthesis in synaptic plasticity and memory, the role of protein degradation is not well established in diverse plasticity-related processes, although there are some evidences indicating involvement of protein degradation in synaptic plasticity and specific memory processes.

In this introduction chapter, I first introduce the known role of protein degradation in brain and neuronal functions. Next, I introduce the phenomenon called reconsolidation and the specific role of protein degradation during this process, which is the starting point for the researches described in chapters 2 and 3. Finally, how pain information is processed and modulated in the brain is reviewed. Studies on the role of protein synthesis and degradation in memory modification are the content of chapter 2, and studies on the role of protein synthesis and degradation in anterior cingulated cortex for chronic pain are the content of chapter 3.

***1) Protein degradation in neuron***

The ubiquitin proteasome system (UPS) is a ubiquitous, major pathway of protein degradation that governs the turnover of proteins, thereby inevitably affecting every process in which proteins are involved. In the UPS, the small protein ubiquitin is covalently conjugated to a substrate protein by the serial action of the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme and the E3 ubiquitin ligase. After a serial reaction to produce a polyubiquitin chain on the substrate, the polyubiquitinated substrate is directed to a large proteasome complex

that manages the degradation. E3 ubiquitin ligase seems to be the major component that determines substrate specificity (Figure 1). Emerging evidence indicates the critical involvement of protein degradation in specialized functions of the neurons. Ubiquitin proteasome-dependent degradation is known to play important roles in the regulation of synaptogenesis, the elimination of synapses in the development (DiAntonio et al., 2001; Ding et al., 2007; Liao et al., 2004; van Roessel et al., 2004; Schaefer et al., 2000; Wan et al., 2000), maintenance and modulation of neurotransmission functions (Arancibia-Cárcamo et al., 2009; Bedford et al., 2001; Burbea et al., 2002; Colledge et al., 2003; Dreier et al., 2005; Haas et al., 2007; Juo and Kaplan, 2004; Kato et al., 2005; Patrick et al., 2003; van Roessel et al., 2004; Speese et al., 2003; Tada et al., 2010; Willeumier et al., 2006; Yao et al., 2007) and the structural remodeling of the synapse (Cartier et al., 2009; Colledge et al., 2003; Hoogenraad et al., 2007; Hung et al., 2010; Pak and Sheng, 2003). Also, recent findings indicate that the UPS can be regulated by neuronal activity, suggesting a specific role for the UPS in plastic changes of synaptic strength (Bingol and Schuman, 2006; Bingol et al., 2010; Colledge et al., 2003; Deng and Lei, 2007; Djakovic et al., 2009; Ehlers, 2003; Fonseca et al., 2006; Hou et al., 2006; Karpova et al., 2006; Kato et al., 2005; Pak and Sheng, 2003; Patrick et al., 2003; Shen et al., 2007).

In accordance with the findings on the role of the UPS in synaptic plasticity in vitro, recent in vivo studies show an involvement of the UPS in memory (Artinian et al., 2008; Lee, 2008; Lee et al., 2008; Merlo and Romano, 2007; Wood et al., 2005). Some of these findings suggest a distinct role of protein degradation in a specific step of reconsolidation (Lee, 2008; Lee et al., 2008).

***2) Protein degradation in reconsolidation***

Memory can be stored for either a relatively short or a long period of time. For a memory to be stored long-term, it has to be 'consolidated'. Through the protein synthesis-dependent consolidation process, the information is stabilized as a long-term memory that is relatively insensitive to disruption. Maintaining a memory, without retrieval, does not require transient protein synthesis. After it is retrieved, however, it requires protein synthesis within a specific period, in order to recover the memory from a labile state. Without proper transient protein synthesis, the memory seems to be impaired, suggesting that protein synthesis is required to recover the memory from a certain state where the consolidated memory is destabilized. This post-retrieval, protein synthesis dependent process is termed reconsolidation (Misanin et al., 1968; Nader et al., 2000). The destabilization process is now demonstrated to rely on ubiquitin proteasome-dependent degradation (Lee et al., 2008).

Polyubiquitination of synaptic proteins in the hippocampus was specifically increased after the retrieval of consolidated contextual fear memory, which induces protein synthesis-dependent reconsolidation. As polyubiquitination is a key step of the ubiquitin proteasome-dependent protein degradation pathway, this result suggests that total ubiquitin proteasome-dependent protein degradation of synaptic proteins is increased under this condition.

The retrieval-induced degradation of synaptic proteins seems to be target specific (Lee et al., 2008). For example, the polyubiquitination of specific synaptic proteins, including Shank and GKAP, was increased, whereas that of PSD-95 was

not. This pattern resembles the results acquired in culture systems. Notably, the endogenous level of Shank in the synaptosomal fraction of the hippocampus decreased after retrieval, reaching the lowest level two hours after retrieval and recovering to basal levels at six hours after retrieval. This retrieval-induced decrease in the endogenous Shank level was blocked by clasto-lactacystin-β-lactone (β-lactone), a specific proteasome inhibitor, strongly suggesting that specific synaptic proteins are destabilized after retrieval through the ubiquitin proteasome-dependent degradation pathway.

The inhibition of proteasome activity in the hippocampus after retrieval seems to prevent the destabilization of memory (Lee et al., 2008). Post-retrieval, protein synthesis inhibitor anisomycin treatment leads to impairment of the previously formed memory. However, local treatment of proteasome inhibitor β-lactone along with anisomycin in the hippocampus after the retrieval of contextual fear memory prevented the amnesic effect of anisomycin. β-lactone treatment alone did not affect memory. These results suggest that ubiquitin proteasome-dependent protein degradation underlies the destabilization of a previously formed memory after it is retrieved. On the other hand, β-lactone treatment immediately after conditioning did not prevent the amnesic effect of anisomycin on consolidation. This result demonstrates that β-lactone does not have a critical role in the consolidation process of this fear memory and that the effect of β-lactone cannot be attributed to a direct compensation of the effects of anisomycin. This supports the hypothesis that protein degradation plays a critical role in the destabilization of previously formed memories after retrieval, rather than in the consolidation-like restabilization process. However, another study demonstrated that both consolidation and

reconsolidation of spatial memory in a water maze task were impaired by the inhibition of proteasome activity (Artinian et al., 2008), and the consolidation of learning in the crab *Chasmagnathus* was also interfered with by UPS inhibition (Merlo and Romano, 2007). These indicate that the involvement of proteasome-dependent degradation may differ between species and memory types.

Using proteasome inhibitor to block reconsolidation, a recent research has demonstrated that retrieval-induced destabilization of the previously formed memory is required to further strengthen the memory (Lee, 2008). After the first contextual fear conditioning, additional conditioning at the same context further strengthen the fear response the next day. The author showed that the strengthening mechanism resembles reconsolidation rather than consolidation, by inhibiting distinct molecular requirements for consolidation and reconsolidation. Then, the author showed that proteasome inhibitor locally treated in hippocampus after the second conditioning impaired further strengthening of the memory, leaving memory strength unchanged from the initial memory. Active weakening of the memory, or extinction, has also been demonstrated to be impaired by proteasome inhibitor (Lee et al., 2008). Local treatment of proteasome inhibitor in hippocampus after contextual fear memory extinction trials impaired the decrement of fear response, leaving the fear response level similar to the initial level.

The fact that strengthening reactivated memories requires protein degradation-dependent destabilization, together with the evidence that reconsolidation and extinction partly share a common mechanism, indicates that the maintenance, weakening, and strengthening of a reactivated original memory may be interpreted under a unified model of reorganization (Figure 2). After a memory is consolidated,

it can be retrieved by certain situations that include one or more components related to the original memory. These situations may be quite diverse and can determine the fate of the retrieved memory. In some cases, the memory seems to be maintained without being reactivated. When the memory retrieval is very brief, or when the memory is saturated by overtraining, it is not susceptible to the amnesic effect of protein synthesis inhibitors, even though the memory is well retrieved (García-DeLaTorre et al., 2009; Rodriguez-Ortiz et al., 2005, 2008; Suzuki et al., 2004; Wang et al., 2009). On the other hand, when a memory, usually unsaturated, is retrieved for more than a very brief period, it can be reactivated and reorganized. The reactivated memory first becomes destabilized by a mechanism that is likely initiated by the NMDA receptor, L-type voltage-gated calcium channel (LVGCC), or cannabinoid receptor type 1 (CB1) and involves protein degradation (Lee, 2008; Lee et al., 2008; Ben Mamou et al., 2006; Suzuki et al., 2008). The fate of the destabilized memory depends on the incoming information specific to the situation. In cases where the original memory is no longer valid, the destabilized memory will either passively remain in a destabilized state or the extinction information will be actively encoded, weakening the memory. In cases where the original memory is strengthened by additional training, the destabilized memory is restabilized into a stronger memory. Finally, in cases where there is no additional training, but there is not sufficient information to conclude that the original memory is no longer valid, the destabilized memory is restabilized to a similar level as the original memory.

The underlying molecular pathway of this reorganization mechanism is still under investigation. The destabilization process seems to be initiated by activation of NMDA receptors, LVGCC and CB1 receptors (Ben Mamou et al., 2006; Suzuki

et al., 2008). The UPS seems to have a critical role in this process, though the direct links of the upstream molecules have not been demonstrated in vivo. In vitro studies show the possibility that NMDA receptors and LVGCC can activate $Ca^{2+}$/calmodulin-dependent protein kinases II (CaMKII), which in turn activates and translocates the proteasome to the synaptic spines along with the autophosphorylated CaMKII (Bingol and Schuman, 2006; Bingol et al., 2010). The increase of degradation in the synaptosomal fraction can be well explained by this pathway (Lee et al., 2008). However, the pathway that links synaptic activity to the specificity of the substrate for degradation is unknown. Two substrates demonstrated to be actively degraded during the destabilization step are Shank and GKAP, both of which have been proven to be regulated by synaptic activity in vitro (Ehlers, 2003), where GKAP is especially ubiquitinated by TRIM3 ubiquitin ligase (Hung et al., 2010). Given the role of these proteins as scaffolding proteins of the synaptic spine, in which Shank specifically acts as a 'master' scaffolding protein that holds together intermediate scaffolding proteins such as GKAP and PSD-95, and also considering the fact that the UPS is involved in activity-dependent synaptic remodeling (Cartier et al., 2009; Pak and Sheng, 2003), it seems that during reconsolidation, reactivated synapses undergo synaptic remodeling, first being disassembled during the destabilization step and then being recovered to a state similar to the initial one or becoming stabilized as a modified state. This process might accompany morphological changes as well. Restabilization is basically protein synthesis dependent and shares many molecular mechanisms with the original consolidation, although some differences exist. The process of restabilization may be the key step that governs the fate of the memory (Figure 3).

The reorganization process investigated so far is focused on the postsynaptic site. The role of protein degradation on the presynaptic site is largely unknown.

***2) Pain processing***

Albeit some exception where pain occurs without function of nociceptors, pain is initiated by the stimulation of peripheral nociceptors in general. Nociceptors activate whenever there has been an injury, or even a potential injury, and these information are then delivered to the dorsal horn neurons in the spinal cord. Activation of nociceptor is transformed to electrical signals, which are delivered mainly through Aδ and C-fibers (Kandel et al., 2000). After these fibers innervate dorsal horn neurons, an electrical pain signal is transmitted to the brain via five ascending pathways (Kandel et al., 2000).

Several brain regions has role in the pain processing (Figure 4) (Jaggi and Singh, 2011; Kandel et al., 2000; Xie et al., 2009). In the lateral pain system, lateral thalamic nuclei send the nociceptive signal mainly to the somatosensory cortices and other brain regions. This system then processes the physical, sensory information such as location, intensity, and the duration of pain (Xie et al., 2009). On the other hand, in the medial pain system, nociceptive information from the peripheries is transmitted to the anterior cingulate cortex and insular cortex through the medial thalamic nuclei. These brain regions are known for processing the affective and emotional dimensions of pain and modulates the pain perception (Price, 2000). In addition, brain regions other than the thalamus, anterior cingulate cortex (ACC), insular cortex and somatosensory cortices are also known to be involved in processing pain. For example, the amygdala, hypothalamus, and

periaqueductal gray are activated by nerve injury and participate in the regulation of pain. Limbic system is also known to regulate the emontional response of the pain (Jaggi and Singh, 2011).

When the cause of injury ceases and the peripheries heal from injury, pain sensations would stop. This is because the nociceptors no longer detect any tissue damage or potential injury. This acute pain does not persist after the initial injury has been healed. Sometimes, however, pain is perceived for longer time. This can be caused by a disease or condition that continuously causes damage. In some cases, even in the absence of tissue damage the pain response is still present. In this chronic pain, neuropathic pain is caused by abnormalities or damages in the nerve system without direct injury of the peripheral organs. There are various known cause that can induce neuropathic pain such as external injuries, diabetes, vitamin deficiency, alcoholism, and cancer (Baron et al., 2010). These causes may lead to abnormal firing of pain processing neurons or reduce the firing threshold of these neurons. Symptoms such as allodynia, hyperalgesia, and the sensation of spontaneous burning and aching are known to be induced by neuropathic pain (Baron et al., 2010). Although there are some methods to alleviate these symptoms of neuropathic pain by drugs, neurectomy, or spinal cord stimulation, these treatments have limitations including side effects and short duration (Baron et al., 2010; Dworkin et al., 2007; Melnikova, 2010).

As mentioned earlier, the ACC is one of the brain regions involved in pain processing. Many studies have revealed the signaling pathway in the ACC that mediates pain, especially chronic pain (Zhuo, 2006). LTP contributes to the increased synaptic strength in the ACC resulting in sensitization of the pain

response (Descalzi et al., 2009; Zhuo, 2008). Digit amputation induces LTP-like changes and loss of LTD in the ACC (Wei and Zhuo, 2001; Wei et al., 1999). The induction mechanism of LTP in the ACC is similar to that of other brain regions. It has been demonstrated that NMDA receptor, AMPA receptor, kainite receptor, and the LVGCC initiate the induction of signaling pathway (Zhuo, 2007). Adenylyl cyclases, $Ca^{2+}$/calmodulin-dependent protein kinases (CaMKs), protein kinase A (PKA), and cAMP response element-binding (CREB) which are generally involved in LTP are also known to be involved in LTP signaling in the ACC (Zhuo, 2007). Recently, it has been shown that by inhibiting PKMzeta activity, increased synaptic strength and pain response can be reversed to basal level.

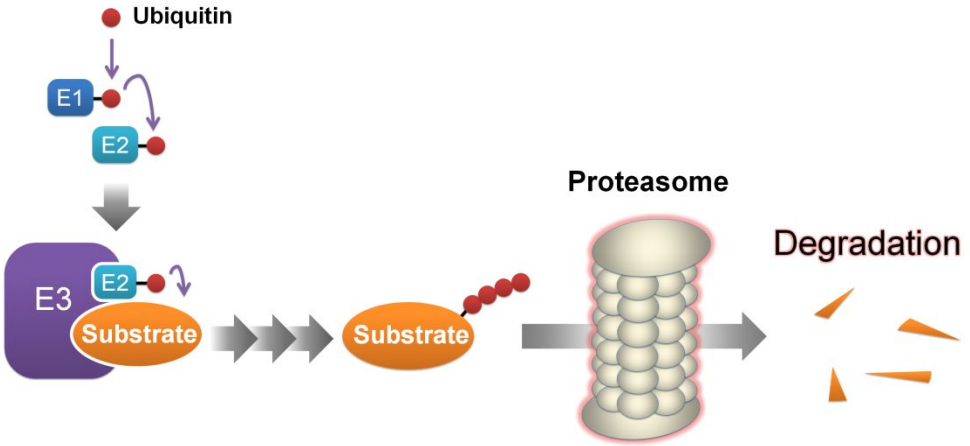


**Figure 1. Mechanism of the ubiquitin proteasome system**

Ubiquitin is first conjugated to E1 ubiquitin-activating enzyme (E1) in an ATP-dependent manner. The conjugated ubiquitin is then transferred to E2 ubiquitin-conjugating enzyme (E2). E3 ubiquitin ligase (E3) recognizes specific target proteins (substrates) and transfers and conjugates the ubiquitin from E2 to the substrate. E2 and E3 may also transfer the ubiquitin to a previously conjugated ubiquitin. After a serial reaction to produce a polyubiquitin chain on the substrate, the polyubiquitinated substrate is directed to a large proteasome complex that manages the degradation (Kaang and Choi, 2012).

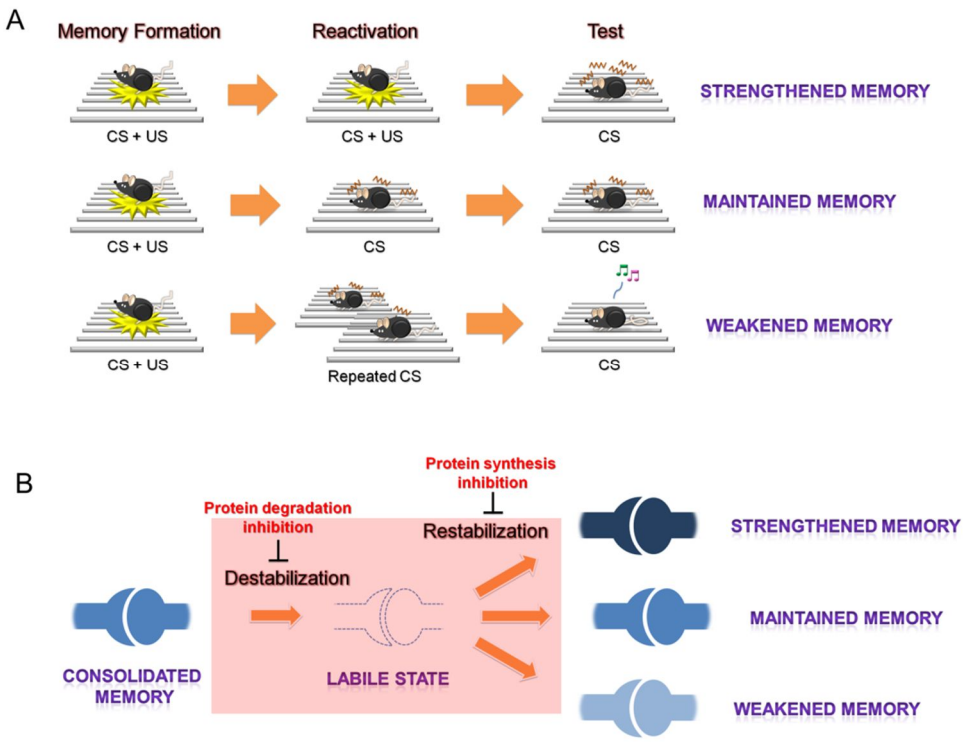


**Figure 2. A model for memory reorganization – strengthening, maintaining and weakening**

A. Cartoons of the behavioral scheme used to reveal the mechanism underlying memory strengthening, maintaining and weakening. After the original contextual fear conditioning, the memory is reactivated in various situations. In the scheme for memory strengthening, the animal receives an additional US shock. In the scheme for memory maintaining, it is exposed to the training context (CS) for a few minutes. In the scheme for memory weakening, it is repeatedly exposed to the training context (CS). Drugs are applied after memory reactivation, and the memory level is tested on the next day. B. The diagram represents the state of the memory during the strengthening, maintaining and weakening of the consolidated memory. Although the diagrams are shown with a single synapse, note that this is a

simple symbolic representation (Kaang and Choi, 2012).

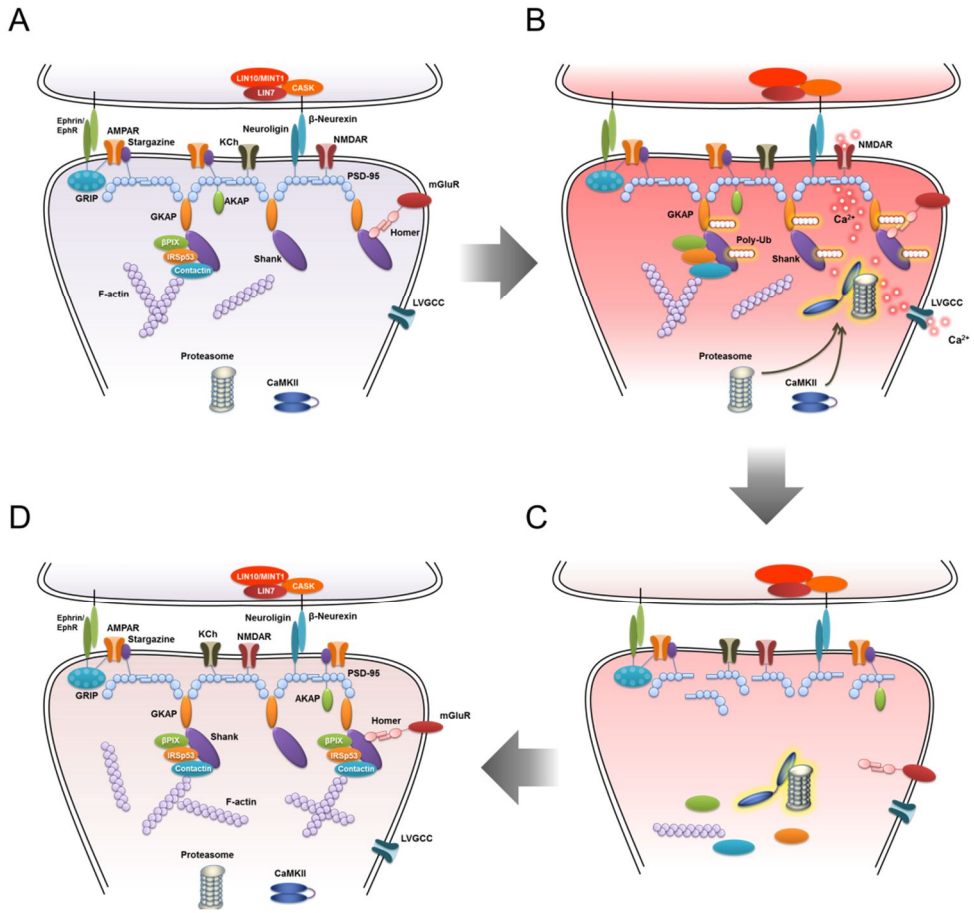


**Figure 3. A model for memory reorganization – synaptic remodeling**

A. Structure of a synapse encoding memory. B. When the memory is reactivated, NMDA receptor and LVGCC are opened, allowing calcium influx to the spine. These calcium ions activate CaMKII, which then phosphorylates the proteasome to increase the activity. The activated CaMKII may undergo autophosphorylation and can associate with and translocate the proteasome from the dendritic shaft to the spine. Meanwhile, target proteins are polyubiquitinated by the specific action of E3 ligases and other proteins. The known proteins that undergo polyubiquitination after memory reactivation are Shank and GKAP, as indicated. C. The recruited active proteasomes degrade these specifically polyubiquitinated targets. Since the

targets here are scaffolding proteins, it is a likely consideration that this spine undergoes structural remodeling. D. A protein synthesis-dependent process restabilizes the synapse either to a state similar to the initial state or to a modified state (Kaang and Choi, 2012).

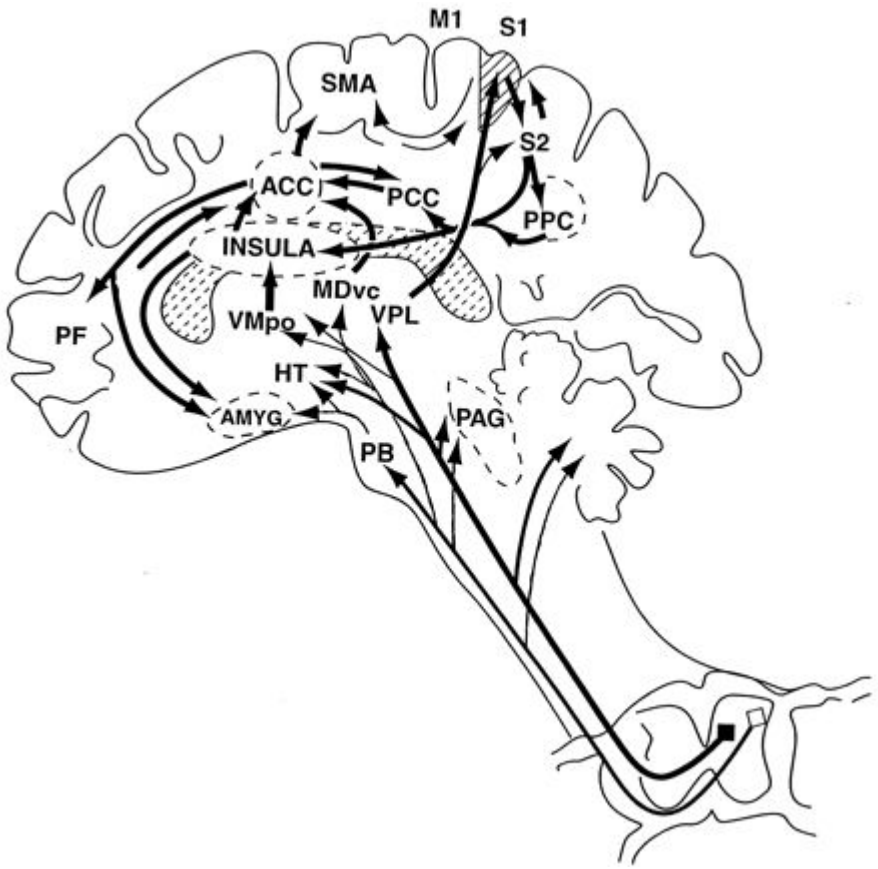


**Figure 4. Ascending pathway and the related brain regions involved in pain processing**

Schematic of ascending pathways, subcortical structures, and cerebral cortical structures involved in processing pain. PAG, periaqueductal gray; PB, parabrachial nucleus of the dorsolateral pons; VMpo, ventromedial part of the posterior nuclear complex; MDvc, ventrocaudal part of the medial dorsal nucleus; VPL, ventroposterior lateral nucleus; ACC, anterior cingulate cortex; PCC, posterior cingulate cortex; HT, hypothalamus; S-1 and S-2, first and second somatosensory cortical areas; PPC, posterior parietal complex; SMA, supplementary motor area; AMYG, amygdala; PF, prefrontal cortex (Price, 2000).

## PURPOSE OF THIS STUDY

In chapter 2, the role of protein synthesis and degradation during memory modification was investigated. Previous studies only focused on the strength of the memory for demonstrating the requirement of protein synthesis and degradation in the incorporation of changed information. Another major memory updating process in real-life is partial modification of the initial memory rather than simple strengthening or weakening of the memory. In the present study, I aimed to reveal the role of protein synthesis and degradation in the incorporation of partially modified information into the pre-existing memory, by using an object rearrangement task.

In chapter 3, the role of protein synthesis and degradation in chronic pain was investigated. Combining the fact that chronic pain mechanism in ACC resembles the memory mechanism, and the fact that the pain circuit underlying chronic pain may be constitutively 'reactivated' when suffering chronic pain, I hypothesized that protein synthesis and degradation may be increased for the scaffolding proteins in ACC, contributing to chronic pain and its resulting synaptic changes

# CHAPTER 2.

# Role of protein synthesis and degradation in memory modification

## INTRODUCTION

Memory formation in real life usually involves partial change of pre-existing memory. Though numerous studies have highlighted the molecular mechanism of memory formation, it is largely unknown how previously formed memory is altered or updated in the process.

A candidate mechanism of memory updating could involve dynamic regulation of memory stability after memory reactivation. Conventionally, it was thought that memory is consolidated by a protein synthesis-dependent process only once per item and persists thereafter in a stable state (Davis and Squire, 1984; Dudai, 1996; Flexner et al., 1965; Goelet et al., 1986). However, accumulating evidence suggests that memory reactivation induces a reconsolidating process that depends on the protein synthesis (Dudai, 2006; Milekic and Alberini, 2002; Nader et al., 2000; Suzuki et al., 2004). While protein synthesis has been shown to be required for the memory restabilization after retrieval, the destabilizing phase seems to require protein degradation. These studies showed that, using the contextual fear memory task, protein degradation in the hippocampus after memory reactivation is required for weakening or strengthening of the fear memory (Helton et al., 2008; Lee et al., 2008). This dynamic protein turnover after memory reactivation is hypothesized to be a molecular mechanism through which memory is updated or modified. Indeed, several reports have suggested that this protein-synthesis-inhibitor-sensitive reconsolidation process follows after reactivation only when there is an additional external stimulus that promotes updating of the original information (Morris et al., 2006; Rodriguez-Ortiz et al., 2005, 2008; Rossato et al., 2007).

However, the previous studies only focused on the strength of the memory for demonstrating the requirement of protein synthesis and degradation in the incorporation of changed information. Another major memory updating process in real-life is partially modifying the content of the initial memory rather than simply strengthening or weakening the memory. In the present study, I aimed to reveal the role of protein synthesis and degradation in the incorporation of partially modified information into the pre-existing memory, by using an object rearrangement task.

## EXPERIMENTAL PROCEDURE

### *1. Subjects*

Male C57BL/6NCrljOri mice purchased from Orient Bio at age of 10-11 weeks were used. Animals were housed in standard laboratory cages on a 12-hour light-dark cycle and provided with access to food and water *ad libitum*. All works were conducted according to the policy and regulation for the care and use of laboratory animals approved by Institutional Animal Care and Use Committee in Seoul National University.

### *2. Stereotaxic surgery*

Mice were implanted under deep ketamine and xylazine anesthesia with 24-gauge guide cannulae in the dorsal hippocampal CA1 region at coordinates AP -2.0 mm, L ± 1.5 mm, V 1.7 mm. Mice were given one week to recover after cannula implantation.

### *3. Behavioral procedures*

Mice were habituated to a context which is a 50 cm square plastic chamber with a white bottom and black walls, for five days, 15 minutes each. On day 6, mice were exposed to the same chamber with four distinct objects placed in the chamber for 15 minutes. On day 7, the mouse was re-exposed to the chamber for 15 minutes with the location of two adjacent objects inter-switched while the other two left unchanged. On day 8, the mouse was re-exposed for 15 minutes to the same object configuration as day 7. The behavioral scheme and the actual picture of the context

and four objects are shown in figure 5. The behavior of the mice was recorded by a camera installed above the chamber. Exploration time for each object was measured manually from the recorded video file as a time the mice placed its nose in proximity of each object. The duration that the mice climbed on top of the object and explored their surrounding were excluded from the exploration time.

***4. Drug injection***

The 30-gauge injection cannula was 0.1 mm lower than the guide. For intra-hippocampal infusion, 0.3 μl anisomycin in artificial cerebrospinal fluid (aCSF) (200 μg/μl), 0.3 μl β-lactone (32 ng/μl) in aCSF, or 0.3 μl vehicle (aCSF) was delivered bilaterally for 1 min each using a pump, and the cannula remained for an additional 1 min after drug infusion. Mice were slightly anesthetized by isofluorane during the injection process. After all experiments were completed, the mice brains were processed to assess the injection site (Fig. 11). Mice that were cannulated outside of the hippocampal CA1 were excluded from the analysis.

***5. Statistical analysis***

The preference of each object was calculated as exploration time for each objects divided by the total exploration time. Statistical significance were analyzed by unpaired or paired t-test, or one-way ANOVA. All data are presented as mean ± SEM.

## RESULTS

### *1. Setting up the behavioral task*

To assess the incorporation of changed information into the preexisting memory, I chose an object-location memory task (Dix and Aggleton, 1999). This task measures object-location associative memory utilizing the innate tendency of rodents to explore the novel aspects of the environment. The scheme of the task is depicted in Figure 5A.

After five days of 15 minutes habituation to a context, the mouse was exposed for 15 minutes to four objects located in one of the four positions in the context. Twenty-four hours later, the mouse was re-exposed to the context for 15 minutes with the location of two adjacent objects inter-switched while the other two left unchanged. Higher explorative preference to the switched objects was expected due to the novelty of the situation. On the next day, the mouse was re-exposed for 15 minutes to the same object configuration as the second day. If it had successfully incorporated the changed information on the second day, there would be no novel aspects in each object anymore, resulting in similar preference for each of them. The behavioral scheme and the actual picture of the context and four objects are show in Figure 5.

### *2. Preference of switched/unswitched objects*

To evaluate the preference for switched/unswitched objects, I measured the duration of exploration time for each object and calculated the percentage of the two switched/unswitched objects exploration time from the total exploration time.

The preference to the switched objects was higher on Day 2, indicating that the subjects have well recognized the original position or configuration of each object (Figure 6A). This returned to chance level at Day 3 when the same configuration as Day 2 was given. This implies that the subjects have well memorized the new, changed configuration to recognize it as familiar.

Although there was a slight difference in the preference for each of the four objects at the first day, it was statistically non-significant (Figure 6B). The two objects with slightly higher preference were always paired with the objects with lower preference, and the pair itself was counterbalanced for the next experiment in order to match the chance preference of switched and unswitched objects to be about 50 %.

***3. Behavioral scheme of the experiment***

As my aim was to focus on the associative memory between the objects and their location, I targeted hippocampus which seems to be more specifically involved in object-location memory (Bachevalier and Nemanic, 2008; Gilbert and Kesner, 2002, 2004; Jenkins et al., 2004; Lee and Solivan, 2008); although other regions such as mPFC and perirhinal cortex are also implicated in such tasks, they are probably more involved in novelty processing. Especially, it was recently reported that PKMzeta inhibitor destructed object location memory, but not object identity memory, when it was locally applied in hippocampus (Hardt et al., 2010). I implanted guide cannulae one week before the behavioral procedure by stereotaxic surgery and injected the protein synthesis inhibitor anisomycin (Ani; 200 μg/μl in aCSF, 0.3 μl) and/or the proteasome inhibitor clasto-lactacystin-β-lactone (βlac; 32

ng/μl in aCSF, 0.3 μl) or vehicle (Veh; aCSF, 0.3 μl) in the CA1 region of dorsal hippocampus, bilaterally, right after the exposure to the changed object location on Day 2 (Figure 7). Mice were slightly anesthetized by isofluorane during the injection process. Position of the cannulae tips are shown in Figure 8.

***4. Protein synthesis and degradation is required to appropriately incorporate partially modified information***

On Day 3, the anisomycin-injected group showed higher preference to the switched object compared to the vehicle-injected control group (Figure 9A), suggesting that subjects could not incorporate the information from the modified object configuration on Day 2. On the other hand, the βlac-injected group showed higher preference to the unswitched object compared to vehicle control group. This suggests that the preexisting information (unchanged configuration) was relatively weakened or somewhat impaired compared to the new information (changed configuration). This is consistent with the findings that βlac interrupted the reconsolidation process (Lee et al., 2008). It is still possible that the new information was more effectively stored in the presence of βlac, thus increasing the relative familiarity with the switched objects over with the unswitched objects. It is noteworthy that the effects of anisomycin and βlac on the memory change were opposite [preference to the switched objects (anisomycin) vs. to the unswitched (βlac)] as are the opposite effects of these drugs on protein level]. When anisomycin and βlac were injected together, the preference was similar to the control group. The treatment of anisomycin and βlac at the same time offset their effects on the behavioral change. The preference for switched object, on Day 2 was

similar among the four groups (Figure 9B). Taken together, my data suggest that protein synthesis and degradation have important roles in regulating the process of modifying the previously formed memory.

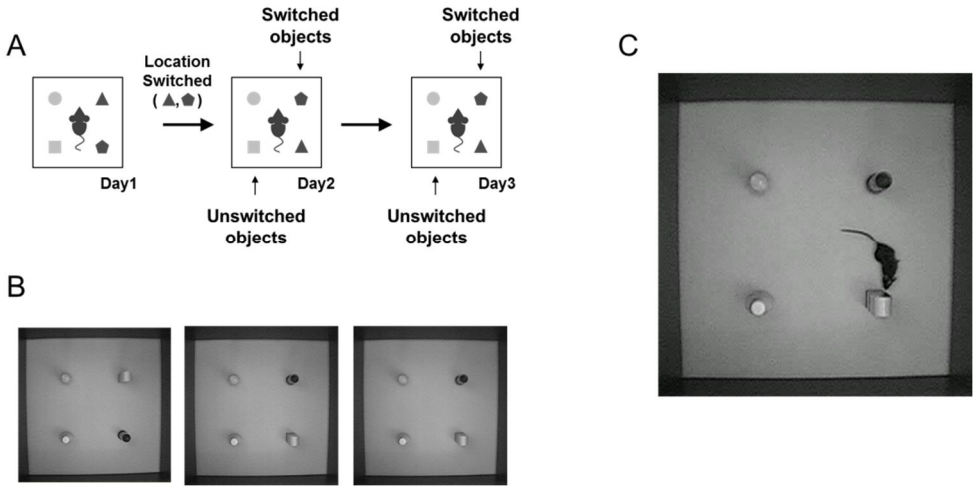


**Figure 5. Scheme of the object rearrangement task**

A. Schematic view of the task. After five days of habituation to the context, the mice were exposed to four distinctive objects each placed in one of the four positions for 15 minutes (Day 1). Two adjacent objects' positions were switched for the next two days (Day 2, Day 3), exposed for 15 minutes each. The object pair that is switched was counterbalanced between experiments. B. Picture of the context and objects for Day 1, 2, and 3. C, Example picture of exploration behavior.

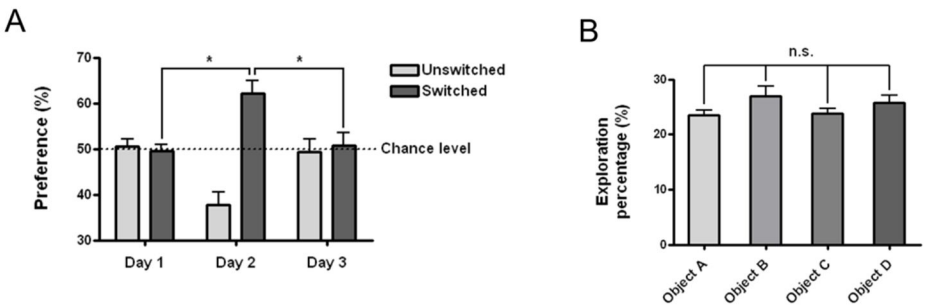


**Figure 6. Preference for objects in the object rearrangement task**

A. Preference to unswitched and switched objects over 3 days (n = 6, *p < 0.05; paired t test). Preference is the percentage of the two switched/unswitched objects exploration time from the total exploration time. B. Preference to each objects in the first day (n.s., non significant).

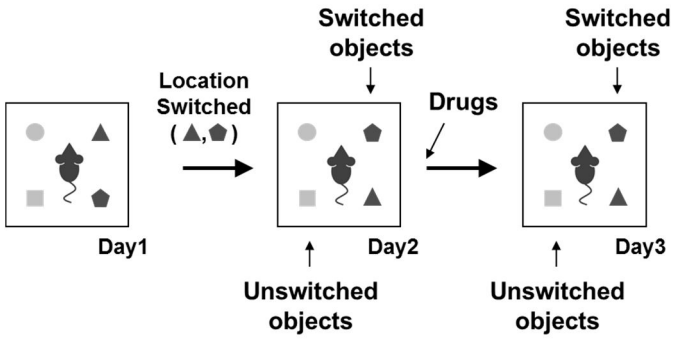


**Figure 7. Scheme of the object rearrangement task with drug injection**

Schematic view of the task. The process is similar as in Figure 5A, except that the mice have received intrahippocampal injection right after Day 2 exposure

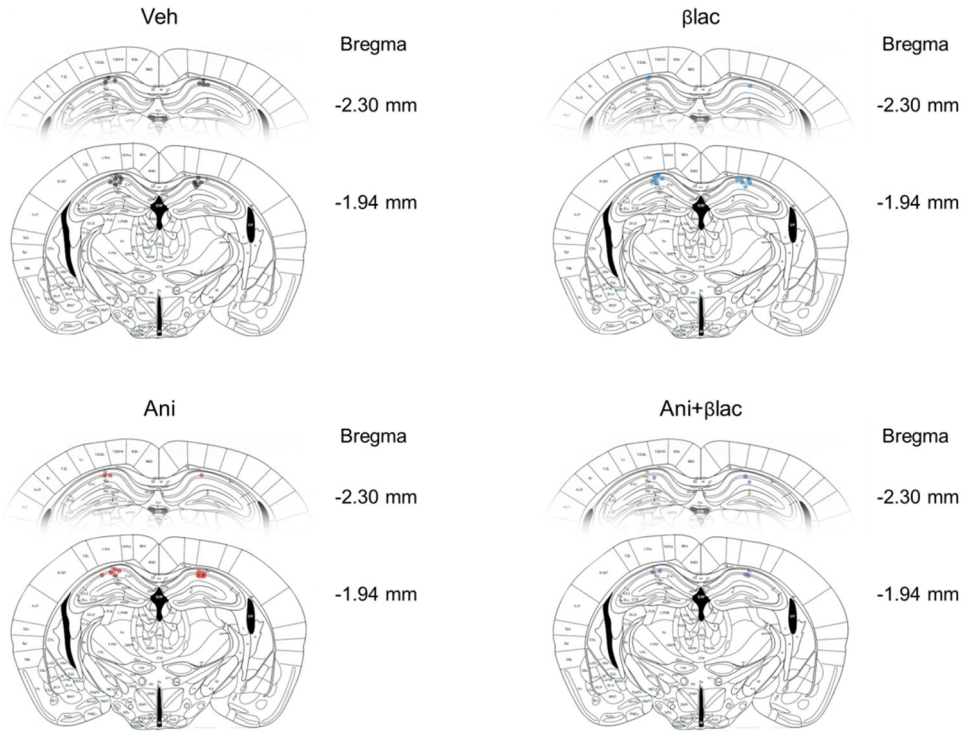


**Figure 8. Cannula location for each drug treatment group**

Cannula location in the hippocampus, at two different rostro-caudal planes. Numbers indicate the posterior direction from the bregma. Grey, blue, red, and purple circles indicate the infusion site of vehicle, βlac, anisomycin, and βlac plus anisomycin double infusion group, respectively.

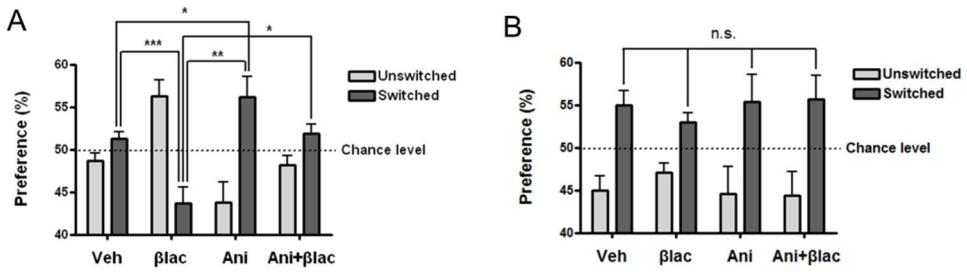


**Figure 9. Object rearrangement task requires protein synthesis and degradation**

A. Preference to unswitched and switched objects at the third day (Veh, vehicle, n = 10; Ani, anisomycin, n = 7; βlac, clasto-lactacystin-β-lactone, n = 7; Ani+ βlac, n = 4. $F = 9.869$, $p = 0.0002$; one-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; unpaired t test). B. Preference to unswitched and switched objects at the second day.

## DISCUSSION

In this study, I have provided a novel scheme of object-in-place memory task to assess whether the mice have correctly changed the contents of pre-existing object-location associative memory, making it possible to investigate the process of modifying memory. In previous studies focusing on the modification of pre-formed memory, the memory task commonly involved strengthening the memory by repeated training over days or weakening it by an experimental extinction protocol. The modified object rearrangement task has an advantage that the memory is not simply altered in its strength, but that the partial contents of the pre-formed memory are rapidly reorganized to incorporate changed information. Based on this advantage, this task is likely to be valuable for studies on dynamic memory modification process. Using this task, I have found that protein synthesis and degradation are required for incorporating partially modified information into the pre-existing memory.

Considering the effect of anisomycin on the memory modification, the original memory might be expected to be destabilized by anisomycin after reactivation on Day 2 as it would undergo a protein synthesis-dependent reconsolidation process, possibly resulting in the chance level preference for each object on Day 3. However, anisomycin-injected mice acted on Day 3 as if the second object configuration is novel, resulting in higher preference for the switched objects. One possible explanation for this is that not every component of the related memory becomes labile in this stage to be sensitive to protein synthesis inhibitor. For example, the memories for unswitched objects might have been less affected by anisomycin

being maintained relatively intact, while the memories for switched objects may have been more affected, being reactivated and destabilized. This may result in higher explorative preference for the switched objects, as the location for switched objects becomes more novel than the location for the unswitched objects. It is also possible that the consolidation-like process for incorporating additional information (i.e. switched location) is more sensitive to protein synthesis inhibitor than the reconsolidation process. In any case, my result indicates that incorporation of changed information into the pre-existing memory requires *de novo* protein synthesis.

The interpretation of the effect of proteasome inhibition is more complicated. If the process to incorporate the changed information is a simple additional learning of the novel information, the effect of a certain drug might be expected to be either impairment of modification leaving the memory unchanged or no effect leading to successful incorporation of the new information. In this study, however, the inhibition of protein degradation appeared to affect even the memory for unchanged information. Therefore, memory reactivation on Day 2 is likely to have induced complex processes involving modification to the previous memory, in which the protein degradation plays a major role.

Previous reports suggest that destabilization of the reactivated memory precedes the protein synthesis-dependent process. However, the retrieval process of four-object rearrangement task differs from that of contextual fear conditioning as it incorporates additional information rather than simply recovers the memory state. Although the memory reactivation-induced protein degradation seems to be critical to form successfully modified memory, the full process may also, in parts, include

consolidation-like process to encode the novel memory components. This process may be induced independently of destabilization of the previous memory. In this case, protein degradation inhibition would impair destabilization of the reactivated memory and possibly the following restabilization process, without affecting the independent consolidation-like process. The imbalance between these two essential components induced by protein degradation inhibition might have lead to higher preference for the unswitched objects on Day 3. In addition, the protein degradation might have a unique role in either restabilization of the previous memory or in a consolidation-like process to incorporate additional information. I cannot exclude the possibility that protein degradation is involved in the consolidation process in the four-object task used in the present study, although it does not seems to be the case, at least acutely, in contextual fear memory (Lee et al., 2008).

I have locally treated the inhibitors in hippocampus based on references that indicate hippocampus as a region specifically involved in object location memory rather than the object identity memory. The results using PKMzeta inhibitor demonstrates that hippocampus is at least one of the memory storage sites for object location memory while it is not a critical storage site for object identity memory (Hardt et al., 2010). Therefore, protein synthesis and degradation inhibition treatment in hippocampus is unlikely to have affected the object identity memory. However, there is a possibility that other brain regions are also critically involved in this memory task.

Although more studies are required to fully understand the mechanism, the present study suggests that protein synthesis and degradation play important roles

in modifying the memory in object-location memory paradigm. This evidence supports the hypothesis that memory dynamically changes after retrieval through protein turnover. In addition, I expect that the scheme used here or other modes of the object rearrangement task can be utilized for the investigation into the memory modification mechanism in the future.

# CHAPTER 3.

# Role of protein synthesis and degradation in chronic pain

## INTRODUCTION

There are some similarities between pain, especially chronic pain and memory mechanism (Ji et al., 2003). It has been demonstrated that adenylyl cyclase 1/8 (AC1/8), extracellular-signal-regulated kinase (ERK), and NMDA receptor subunit 2B are important for chronic pain as well as learning and memory in the ACC (Wei and Zhuo, 2008; Wei et al., 2001, 2002, 2006). Moreover, stimulation that induce pain causes electrophysiological changes in the ACC. Nerve injury, digit amputation, and inflammation, which all result in chronic pain, are known to induce plastic changes in the ACC (Descalzi et al., 2009; Kang et al., 2012; Li et al., 2010; Wei and Zhuo, 2001; Wei et al., 1999; Xu et al., 2008; Zhuo, 2008). Nerve injury by ligation of the peripheral nerve induces increase of synaptic transmission in the ACC (Li et al., 2010; Xu et al., 2008). It has also been demonstrated that PKC/Mzeta inhibiting peptide ZIP can relieve the enhance pain response induced by chronic pain when infused in the ACC (Li et al., 2010). This ZIP is also known to reverse the maintenance of LTP and memory in various brain regions and task (Bliss et al., 2006; Migues et al., 2010; Pastalkova et al., 2006; Sacktor, 2008; Shema et al., 2009).

During synaptic plasticity, some proteins are newly synthesized and some proteins are degraded. This turnover of synaptic proteins may contribute to the change in synaptic strength. Scaffolding proteins such as postsynaptic density protein 95 (PSD95), Shank, and guanylate-kinase-associated protein (GKAP) are especially responsible for these kinds of synaptic change (Kim and Sheng, 2004; Lee et al., 2008). It has been demonstrated that when memory is reactivated the

increased turnover of these proteins underlie the destabilizing and restabilizing process induced by memory reactivation (Lee et al., 2008).

Combining the fact that chronic pain mechanism in the ACC resembles the memory mechanism, and the fact that the pain circuit underlying chronic pain may be constitutively 'reactivated' when suffering chronic pain, I hypothesized that protein synthesis and degradation may be increased for the scaffolding proteins in the ACC, contributing to chronic pain and its resulting synaptic changes.

## EXPERIMENTAL PROCEDURE

### *1. Subjects*

Male C57BL/6NCrljBgi mice aged between 6 and 8 weeks were purchased from Charles River or Orient Bio. Animals were housed in standard laboratory cages on a 12-hour light-dark cycle with access to food and water *ad libitum*. Mice were used for experiments 1-2 weeks after being housed in the laboratory cage.

### *2. Surgery and drug infusion*

Guide cannulas (24 gauge) were implanted bilaterally into the ACC of the mice (AP + 0.7 mm, L ± 0.4 mm, V - 1.7 mm) under anesthesia with a ketamine/xylazine mixture. The mice were given at least 1 week to recover after cannula implantation. The 30-gauge injection cannula was 0.2 mm lower than the guide. For intra-ACC infusion, 0.5 μl anisomycin (100 μg/μl in aCSF), 0.5 μl β-lactone (100 μM or 1mM in aCSF), 0.5 μl anisomycin + β-lactone, or vehicle (aCSF) was delivered bilaterally within 2 min using a pump and cannula remained for an additional 2 min after drug infusion.

The surgical procedure used to induce neuropathic pain was substantially based on the previous report (Figure 10) (Vadakkan et al., 2005). Briefly, the mice were anesthetized with the ketamine/xylazine mixture in saline. Their eyes were protected by artificial tear jelly. The left leg of each mouse was shaven using scissors and sterilized with 70% alcohol and povidone iodine liquid. About 1cm of the left thigh skin was cut, exposing the muscles. Then, an incision was made in the muscle using scissors and sterile saline was applied to the exposed region. The

common peroneal nerve (CPN) was ligated with a wax coated braided suture 4-0 without disturbing or including the blood vessel. Next, the ligature was slowly tightened until twitching of the dorsiflexors of the foot became visible at the digit. After making a knot, the skin was sutured using a 5-0 silk suture and cleaned with povidone iodine liquid. To induce inflammatory pain, 10 μl complete Freund's adjuvant (CFA, 50% in saline; Sigma, St. Louis, Missouri) was injected subcutaneously into the dorsum of the left foot using a Hamilton syringe. Care was taken to avoid leakage of the injected solution. In a few case, the mice did not show an allodynia response within 4 days after CPN ligation or complete Freund's adjuvant (CFA) injection. These mice were excluded from further experiments.

***3. Behavioral experiments***

Mechanical allodynia response was measured 2 h after drug infusion into ACC on Day 4 or on Day 4 and Day 7 depending on experiments. Mice acclimatized to round bucket around 1 h prior to testing. Mechanical allodynia was assessed based on the responsiveness of the hind paw to the application of von Frey filaments (Stoelting, Wood Dale, Ill). The filament was applied over the dorsum of the foot while the animal was resting. Based on previous experiments, we used 1.65 filament to detect mechanical allodynia (Vadakkan et al., 2005). Positive responses included licking, biting, and sudden withdrawal of the hind paw. Nine trials were carried out every five minutes and the results were expressed as a percentage of positive responses. For each time point, the percentage response frequency of hindpaw withdrawal was expressed as follows: (number of positive responses)/18 × 100 per hindpaw. After all experiments were finished, brains were used for check

the tip of cannula (Figure 11). To test acute pain responses, the latency of response to heating of the tail (tail-flick test) or to placement on a hot plate (55°C) was measured as described (Wu et al., 2005).

***4. Antibodies***

PSD-95 (1:50,000; Thermo Scientific), Synaptophysin (1:1,000; Cell Signaling), SNAP-25 (1:5,000; Sigma), Shank (1: 100,000; the courtesy of Dr. Eunjoon Kim), GKAP (1:50,000; NeuroMab), GluA1 (1:500; Santacruz), GluA2 (1:1,000; Abcam), GluN1 (1:40,000; BD Bioscience), pan-Cadherin (1:5,000; Santacruz) and actin (1:5,000, Sigma).

***5. Subcellular fractionation***

The fractionation of postsynaptic fraction was performed as described previously (Li et al., 2010). 3 slices (400 μm) of ACC from sham or CPN-ligated mice near infusion site were collected per one mouse. Six ACC slices were used for fractionation. Briefly, slices were homogenated in the Frac buffer (30 mM pH 7.4 Tris-Cl, 4 mM EDTA, 1 mM EGTA) containing protease inhibitor cocktail. The homogenate were centrifuged at 500 g, 4 °C for 5 min, twice to remove nucleus fraction. The supernatant were centrifuged at 100,000 g, 4 °C for 1h and then, the pellet was lysed using Frac buffer containing 0.5 % Triton X-100 and protease inhibitor cocktail. After incubation for 20 min on ice, lysate were carefully loaded onto the surface of 1 M sucrose and then centrifuged again at 100,000 g, 4 °C for 1 hr. The pellet (postsynaptic) was used for blotting after lysed with Frac buffer containing 0.5 % Triton X-100 and protease inhibitor cocktail.

***6. Western blot analysis***

Western blot was performed essentially as described previously (Wang et al., 2007). Mice were lightly anesthetized with isofluorane and then decapitated. A region of ACC was dissected and then homogenized into RIPA buffer (50 mM pH 7.6 Tris-Cl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, 1mM DTT, 0.5% sodium dioxycholate) that contains protease inhibitor cocktail and protein phospatase inhibitor cocktail 1 and 2. After centrifuge, the supernatant was used for protein quantification by Bradford assay. Electrophoresis of equal amounts of total protein was performed on 4-12% SDS-polyacrylamide gels (Invitrogen). Separated proteins were transferred to nitrocellulose membrane at 4°C overnight. After blocking with 5% skim milk in TBST for 2 h at room temperature, membranes were incubated with primary antibody at 4°C overnight. After the wash, membranes were treated with HRP conjugated secondary antibody, and this was followed by enhanced chemiluminescence detection of proteins with Western lightning chemiluminescence reagent plus (PerkinElmer) according to the manufacturer's instruction. The density of immunoblots was measured using ImageJ program.

***7. mEPSC recording***

Two hours after drug infusion, the mouse was anesthetized by isoflurane and decapitated. Quickly extracted brain was coronally sectioned (300 μm thickness) by vibrating blade microtome (VT1200S; Leica). ACC slices were recovered for at least 1hr in submerged recovery chamber at room temperature with oxygenated

aCSF containing (in mM) 124 NaCl, 2.5 KCl, 1 $NaH_2PO_4$, 25 $NaHCO_3$, 10 Glucose, 2 $CaCl_2$ and 2 $MgSO_4$. After recovery, slices were transferred to recording chamber perfused with oxygenated ACSF and maintained at 30~32℃ for *ex vivo* recording. The recording pipettes (3~5 MΩ) were filled with an internal solution composed of the following (in mM): 145 K-gluconate, 5 NaCl, 10 HEPES, 1 $MgCl_2$, 0.2 EGTA, 2 MgATP and 0.1 $Na_3GTP$ (280~300 mOsm, adjust to pH 7.2 with KOH). Layer II/III neurons were discriminated between GFP positive and negative neurons using a cooled CCD camera (ProgRes MF cool; Jenoptik) and a fluorescence microscope (BX51WI; Olympus). Neurons were voltage-clamped at -70 mV in the presence of Picrotoxin (50μM) and tetrodotoxin (1μM) and measured mESPC for 3 min. MiniAnalysis program (Synaptosoft) was used for mEPSC analysis.

***8. Biocytin labeling***

For biocytin labeling, the slice were prepared identically as mEPSC recording with some modifications. The recording pipettes (3~5 MΩ) were filled with internal solution containing 145 K-Gluconate, 5mM NaCl, 0.2mM EGTA, 10mM HEPES, 2mM MgATP, 0.1mM $Na_3GTP$, 1mM $MgCl_2$, 2mg/ml Biocytin (pH 7.2 with KOH, 280~290 mOsm). The bath solution contained 124 mM NaCl, 2.5 mM KCl, 1 mM $NaH_2PO_4$, 25 mM $NaHCO_3$, 10 mM Glucose, 2 mM $CaCl_2$, 2 mM $MgSO_4$ saturated with 95% $CO_2$, 5% $O_2$. Coronal Slices (300 μm) were prepared with VT1000S (Leica microsystems). After incubation in room temperature (25~26°C) for 1 hr, slices were transferred to recording chamber (32~34°C) perfused with oxygenated aCSF at a flow rate of 2 ml/min. GFP positive pyramidal neurons in

Layer II/III were voltage clamped at -70 mV. After 15 min, recording pipette was detached from membrane and fixed with 4% paraformaldehyde (PFA), overnight at 4°C. 1~2 neurons per slice were labeled.

Fixed slices were washed three times with PBS shaking in 150 rpm, and then blocked and permeabilized with 5 % goat serum, 0.2 % Triton-X 100 in PBS for 1 hr, room temperature, while shaking in 80 rpm. Next, Streptavidin, Alexa Fluor® 488 conjugate (Life Technologies) was diluted (1:2000) in the same solution and the slices were agitated overnight at 4°C. Next day, slices were washed three times with PBS and mounted with VECTASHIELD® Mounting Media (Vector Laboratories) on slide glasses.

***9. Spine analysis***

Labeled neurons were imaged by Zeiss LSM700 with 100x oil immersion lens. Secondary/tertiary apical dendrites running horizontally to the coronal plane were imaged in Z-stack. One airy unit images were taken as stacks with 0.2 μm intervals. For each neurons, 100-300 μm sum segment of dendrites were imaged and analyzed, with several exceptional neurons with less than 100 μm segment analyzed due to lack of clearly labeled, horizontal dendrites. Z-stack images were reconstructed as 3D models and each parameters of the spines were measured using Imaris FilamentTracer (Bitplane) (Figure 12). Spines were classified to four types, stubby spine, mushroom spine, long thin spine, and short thin spine. Stubby spines were first defined as spines without neck. Next, among the remaining, mushroom spines were defined as spines with head width larger than 0.5 μm and spine neck less than 0.5 μm. Spines that are not classified as stubby or mushroom spines were

classified as long thin spine or short thin spine with a cutoff of 2 μm in total spine length.

### *10. Statistical analysis*

The alteration of allodynia response by drugs was analyzed using repeated measure two-way ANOVA with Bonferroni post-hoc test. The withdrawal latency in the tail flick and hot plate test were analyzed by one-way ANOVA with Bonferroni post-hoc test. Protein levels compared among more than two groups were analyzed by one-way ANOVA with Bonferroni post-hoc test, unpaired t-test for two groups. For analysis of the effect of anisomycin infusion on protein levels, anisomycin infused mice level was normalized with vehicle infused mice level for each sham surgery and nerve ligation groups. Spine structural analysis and mEPSC data were analyzed by two-way ANOVA with Bonferroni post-hoc test. All data are presented as mean ± SEM.

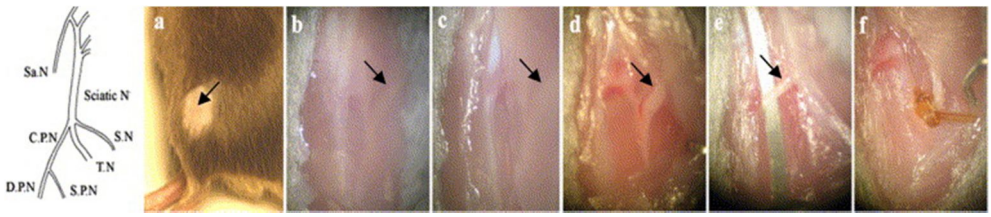


**Figure 10. Common peroneal nerve ligation as a model of neuropathic pain**

Diagram illustrating the ligation of CPN in mouse. Left panel: Schematic diagram of the left lumbar plexus, sciatic nerve and its branches. Abbreviations: Sa.N, saphenous nerve; C.P.N, common peroneal nerve; D.P.N, deep peroneal nerve; S.N, sural nerve; T.N, tibial nerve; S.P.N, superficial peroneal nerve. (a) Shows the area below and lateral to the knee joint before surgery. The head of the fibula can be palpated at the top of the shaved area. Note the groove between the anterior and posterior compartment of muscles. (b) Shows the view after dissecting the skin. Removal of the thin subcutaneous fascia will make it clearer. Note the white-colored fascia separating the anterior and posterior compartment of muscles. Note that the CPN runs 30 to 45° to the horizontal line and is accompanied by a blood vessel most of the time. The white fascia is lifted up with forceps and a vertical incision is made along the white fascia. (c) Incision along the white fascia separating the anterior and posterior compartment of muscles. Other white structures are tendons or connective tissue fascia. (d) Posterior group of muscles is pulled laterally to view the common peroneal nerve. (e) Suture needle is passed under the nerve. (f) The catgut ligature in place. Only one knot was necessary to maintain the knot in position. Arrows show the common peroneal nerve. (Vadakkan et al., 2005)

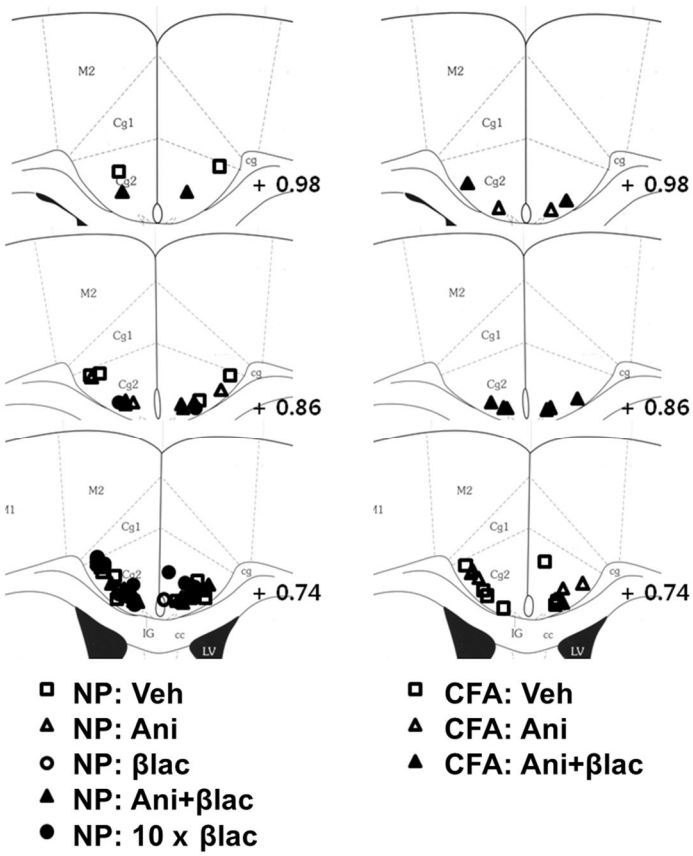


**Figure 11. Location of the infusion sites**

Empty rectangle, Vehicle (Veh); Empty triangle, anisomycin (Ani); Empty circle, β-lactone (βlac); Filled triangle, anisomycin + β-lactone (Ani+ βlac); Filled circle, 10 x β-lactone (1 mM) (10 x βlac). If the cannula tip was located outside of the ACC region, these mice were excluded from the data.

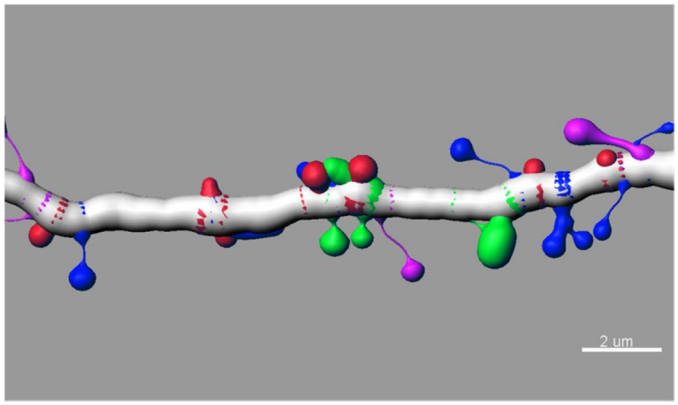


**Figure 12. 3D reconstruction of spine structure**

3D Reconstructed model of a dendrite segment with spines. Stubby spines (Red) were first defined as spines without neck. Next, among the remaining, mushroom spines (Green) were defined as spines with head width larger than 0.5 μm and spine neck less than 0.5 μm. Spines that are not classified as stubby or mushroom spines were classified as long thin spine (Violet) or short thin spine (Blue) with a cutoff of 2 μm in total spine length.

## RESULTS

### *1. Persistent protein synthesis in the ACC is required for neuropathic pain*

First, I examined whether protein degradation and synthesis in the ACC are required for neuropathic pain. Figure 13A shows the experimental procedure. Mechanical allodynia responses were measured 4 days after CPN ligation. Immediately after first allodynia test, anisomycin and/or β-lactone were infused into the ACC, and then a second allodynia response was measured 2 h after the drug was infused. CPN-ligated mice showed allodynia response 4 days after surgery (Figure 13B). When they were tested immediately before drug infusion, there were no differences among groups. However, allodynia response significantly decreased 2 h after anisomycin infusion into the ACC (repeated measure two-way ANOVA Bonferroni post-hoc test; $p < 0.001$, Figure 13B). This effect of anisomycin was blocked by co-infusion with β-lactone. Interestingly, β-lactone alone had no effect on the allodynia response in CPN-ligated mice even when its concentration was raised to 1 mM (paired t-test, $p = 0.4680$, Figure 13C).

In this neuropathic pain model, the pain signal is continuously transported from the peripheral nervous system to ACC. I therefore expected that allodynia response would be reinstated after anisomycin diffuses away. To examine this possibility, allodynia responses were re-tested 3 days after drug infusion (i.e. 7 days after surgery). On Day 7, decreased allodynia response in anisomycin-infused mice reached a level similar to what was measured at the pretest on Day 4 (repeated measure two-way ANOVA Bonferroni post-hoc test; $p < 0.001$ for response % between Day 4 pre and Day 4 post, Day 4 post and Day 7 pre, Figure 14 Ani→Veh

group). On the other hand, when Veh-infused mice (on Day 4) were microinjected with anisomycin into the ACC on Day 7, they showed reduced allodynia response 2 h after drug infusion (repeated measure two-way ANOVA Bonferroni post-hoc test; $p < 0.001$ for response % between Day 7 pre and Day 7 post, Figure 14 Veh→Ani group). Taken together, these results show that continuous protein synthesis is necessary to sustain neuropathic pain.

***2. Persistent protein synthesis in the ACC is required for inflammatory pain***

Next, I aimed to confirm these results using another chronic pain model. To induce inflammatory pain, the mice were injected with Complete Freund's adjuvant (CFA) into the left paw. Four days after CFA injection, allodynia responses were measured before and 2 h after drug infusion into the ACC. Consistent with the neuropathic pain model, anisomycin significantly reduced allodynia response to the level similar to saline-injected mice (repeated measure two-way ANOVA Bonferroni post-hoc test; $p < 0.001$, Figure 15). When anisomycin was infused simultaneously with β-lactone, there were no changes in allodynia responses. These results indicate that protein synthesis inhibition in ACC abolish chronic pain. Moreover, although protein synthesis is inhibited, pain is sustained when protein degradation is simultaneously inhibited by β-lactone.

***3. The effect of anisomycin and β-lactone on basal withdrawal threshold and acute pain***

Next, I asked whether protein synthesis inhibition by anisomycin could disturb the basal withdrawal threshold or response of acute pain. Anisomycin infusion did not

alter the threshold of paw-withdrawal response (repeated measure two-way ANOVA Bonferroni post-hoc test, Figure 16A). Also, microinfusion of anisomycin or β-lactone in the ACC did not induce any change of response latency in tail flick and hot plate test. (one-way ANOVA, Figure 16B and C). This indicates that protein synthesis and degradation is not required for acute pain processing.

***4. Level of synaptic proteins are maintained after CPN ligation***

To reveal whether the level of synaptic proteins in the ACC is altered by chronic pain, I examined level of several synaptic proteins in the ACC of CPN-ligated mice. The postsynaptic fraction of ACC was examined to detect the change of synaptic proteins after CPN ligation. However, I did not find any significant difference in the level of these synaptic proteins in sham and CPN ligation group (one-way ANOVA for each proteins, Figure 17). These results indicate that in the postsynaptic fraction, the level of synaptic proteins in the ACC are not altered by chronic pain.

***5. Turnover of scaffolding proteins in PSD is increased after CPN ligation***

Although the levels of synaptic proteins in the postsynaptic fraction are not changed by CPN ligation, this doesn't directly indicate that protein synthesis and degradation is not altered by chronic pain in the ACC. I examined turnover of synaptic proteins in the ACC after CPN ligation by inhibiting protein synthesis. Anisomycin was infused into ACC 4 days after CPN ligation and 2 h later, the ACC was sampled for western blot assay. I confirmed here again that CPN ligation did not directly alter the synaptic protein level (one-way ANOVA, Figure 18). However, scaffolding proteins Shank, GKAP and PSD95 were decreased by

anisomycin infusion in CPN ligation group compared to the sham group (unpaired t-test, $p < 0.05$, Figure 19). The level of GluA1, GluA2 and GluN1 did not significantly change by anisomycin infusion in CPN-ligated mice. These results indicate that although the level of synaptic proteins remain constant, synaptic proteins such as Shank, GKAP and PSD95 seems to be actively degraded and re-synthesized while chronic pain is induced.

***6. Increased protein synthesis supports structural changes induced by neuropathic pain***

Since I have found that synaptic scaffolding proteins Shank, GKAP, and PSD95 turnover is increased, I hypothesized that this increased turnover might affect the structural properties of spine and thus the synaptic strength. Previous works have shown that the glutamatergic synaptic transmission in the ACC is enhanced by CPN ligation or inflammation only in the activated neurons (Li et al., 2010). I used the same FosGFP transgenic mice to select and investigate the structural properties of spine in the activated neurons. Anisomycin or vehicle was infused 4 days after CPN ligation. 2 hrs later, mice were sacrificed and GFP positive, ACC layer II/III pyramidal neurons were filled with biocytin through a patch pipette. Secondary and tertiary apical dendrites with stained biocytin were imaged and analyzed for spine properties. First I have found that the spine density of these neurons was similar among four groups (two-way ANOVA, Figure 20). However, the length of the spine was decreased by neuropathic pain, which was recovered by anisomycin infusion (two-way ANOVA Bonferroni post-hoc test; $p < 0.001$, Figure 21). Anisomycin didn't affect the basal spine length confirmed by anisomycin infusion in mice with sham surgery.

I next classified spines into four types, stubby spine, mushroom spine, long thin spine, and short thin spine. I have found that neuropathic pain increase the proportion of stubby spine and mushroom spine (Figure 22A, B), while the proportion of long thin spine was decreased (Figure 22D), which is likely the reason I have observed decrease of spine length. The proportion of short thin spine was unaffected (Figure 22C). All of these changes were reversed by anisomycin infusion, while this infusion didn't affect the basal spine type proportion, supporting that the changed proportion was maintained by the increased protein synthesis, likely, of the scaffolding proteins (two-way ANOVA Bonferroni post-hoc test; $p < 0.05$ for stubby spine percentage, $p < 0.001$ for mushroom and long thin spine percentage, Figure 22).

***7. Increased protein synthesis supports heighten synaptic transmission induced by neuropathic pain***

Spine morphology is known to affect synaptic strength. I examined whether the structural changes of spines induced by chronic pain and reversed by protein synthesis inhibition is also reflected on synaptic strength measured in electrophysiological experiments. As mentioned above, previous works have shown that the glutamatergic synaptic transmission in the ACC is enhanced by CPN ligation or inflammation only in the activated neurons (Li et al., 2010). I used the FosGFP transgenic mice to compare the mEPSC frequency and amplitude of sham group injected with vehicle, CPN ligation group injected with vehicle or anisomycin (Figure 23). mEPSC frequency were unchanged by CPN ligation or protein synthesis inhibition in both GFP positive and negative neurons. However, the amplitude of the mEPSC was increased by CPN ligation which were reversed

to the basal level by anisomycin injection, only in the GFP positive neurons (two-way ANOVA Bonferroni post-hoc test; $p < 0.005$ between Sham-Veh and NL-Veh, $p < 0.05$ between NL-Veh and NL-Ani, Figure 23C). This finding is consistent with the altered spine type proportion as the mushroom spine is considered a more matured form with stronger synaptic strength compared to thin spines.

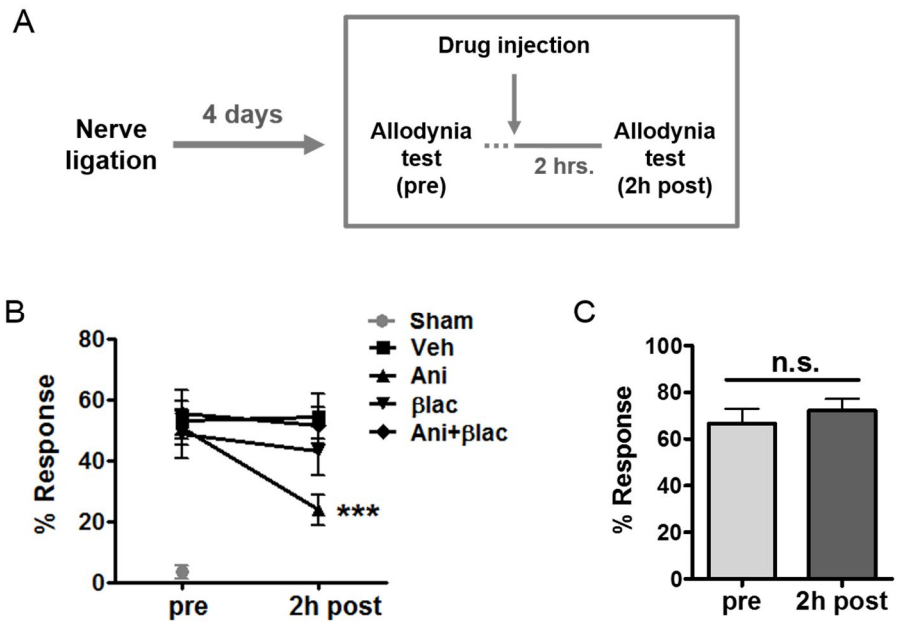


**Figure 13. Protein synthesis in the ACC is required for the maintenance of neuropathic pain**

A. Overall schedule for testing the effects of anisomycin and β-lactone on the mechanical allodynia. B. The mechanical allodynia response 2 h after drug (anisomycin or β-lactone or anisomycin + β-lactone) infusion into the ACC in the presence of neuropathic pain. Data were plotted as the percent positive response in both hind paws. In the CPN-ligated mice, only the anisomycin group showed different allodynia response before and after infusion. Moreover, it should be noted that infusion of anisomycin had no effect on the allodynia if the β-lactone was applied together with anisomycin (n = 7 per group, repeated measure two-way ANOVA Bonferroni post-hoc test; ***$p < 0.001$ between pre and post anisomycin infusion). C. Null effect of 10 x β-lactone (1 mM) in the ACC on allodynia in CPN-ligated mice. Protein degradation inhibition in ACC had no effect on the allodynia response in CPN-ligated mice (n = 7 per group, unpaired t-test). (in collaboration

with Hyoung-Gon Ko)

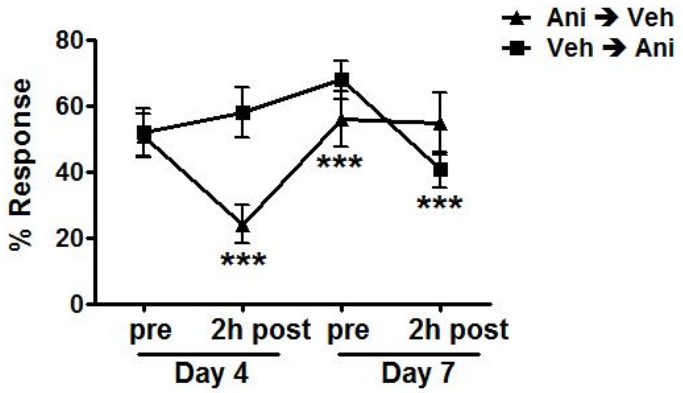


**Figure 14. The reinstatement of allodynia 3 days after anisomycin injection**

The allodynia response was reassessed before and after anisomycin or vehicle infusion into the ACC on Day 7. Mice with anisomycin injection on Day 4 were microinjected with vehicle on Day 7 and *vice versa*. The reduced response after anisomycin on Day 4 was reinstated on Day 7 ($n = 6$, repeated measure two-way ANOVA Bonferroni post-hoc test; ***$p < 0.001$ for response % between Day 4 pre and Day 4 post, Day 4 post and Day 7 pre). Consistent with the observation on Day 4, anisomycin reduced allodynia in CPN-ligated mice on Day 7 ($n = 6$, repeated measure two-way ANOVA Bonferroni post-hoc test; ***$p < 0.001$ for response % between Day 7 pre and Day 7 post). (in collaboration with Hyoung-Gon Ko)

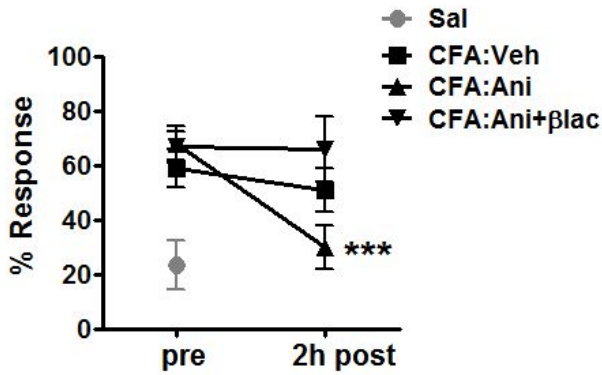


**Figure 15. Protein synthesis in the ACC is required for the maintenance of inflammatory pain**

To test the effect of protein synthesis and degradation on inflammatory pain, mice were injected with CFA on the top of the foot. Allodynia responses were assessed 4 days after CFA injection (pre). Two hours after drug infusion, the allodynia responses were retested (2h post). Consistent with the results in the neuropathic pain model, β-lactone inhibits the effect of anisomycin on the allodynia response (n = 3 - 5 per group, repeated measure two-way ANOVA Bonferroni post-hoc test; ***$p < 0.001$). (in collaboration with Hyoung-Gon Ko)

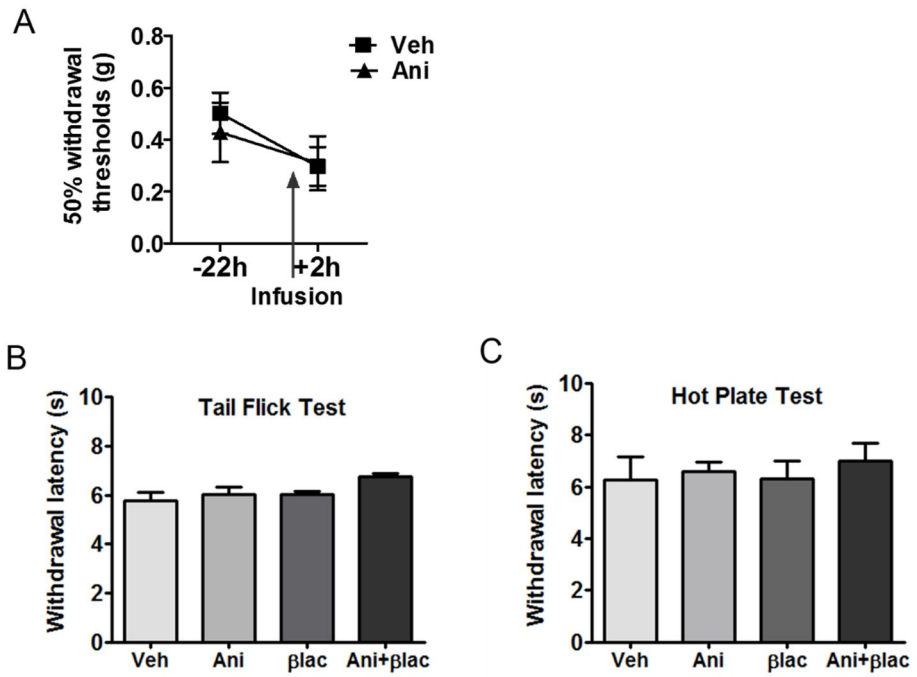


**Figure 16. Effects of inhibiting protein degradation and synthesis on basal withdrawal threshold and acute pain**

A. Effect of ACC anisomycin injection on basal withdrawal threshold was investigated by gradually increasing the von Frey filament size (n = 11 per group, repeated measure two-way ANOVA). B-C. Anisomycin alone, β-lactone alone, or coadministration of these two drugs into the ACC did not affect the withdrawal latency in the hot plate test (A) and tail flick test (B) (n = 5 - 6 per group, one-way ANOVA). (in collaboration with Hyoung-Gon Ko)

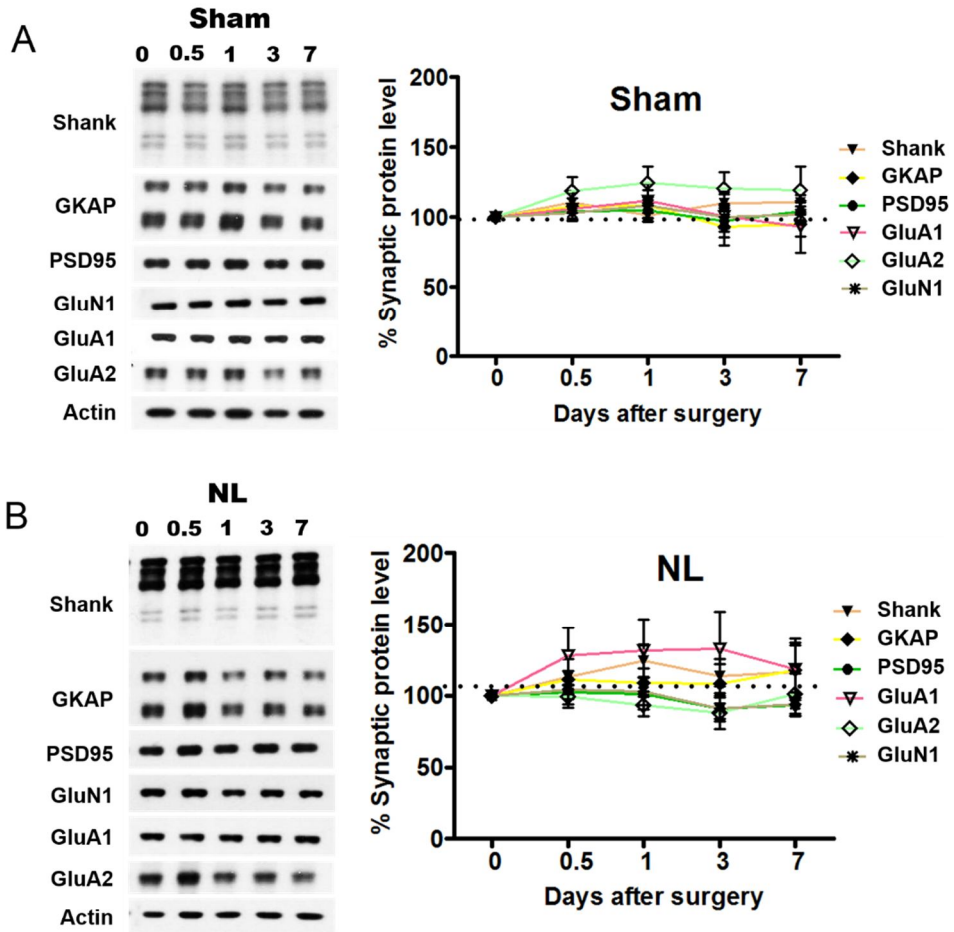


**Figure 17. Level of synaptic proteins in PSD is maintained after CPN ligation in various time point**

Western blot assay of synaptic proteins in postsynaptic fraction of ACC. A. Four days after sham surgery (Sham; n = 6 per group, one way ANOVA for each proteins). B. Four days after CPN ligation (NL; n = 6 per group, one way ANOVA for each proteins). (in collaboration with Hyoung-Gon Ko)

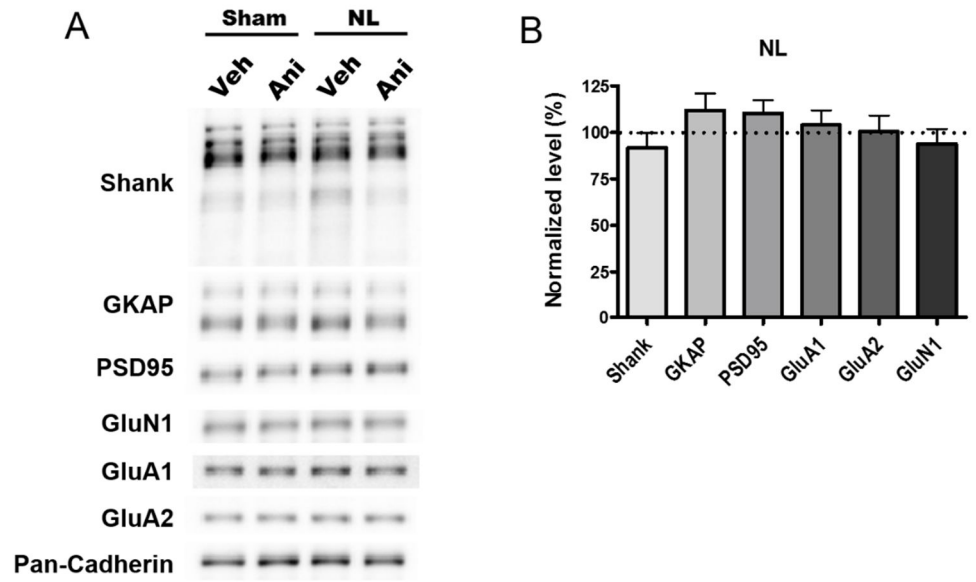


**Figure 18. Level of synaptic proteins in PSD is maintained four days after CPN ligation**

A. Western blot assay of synaptic proteins in postsynaptic fraction of ACC from sham or CPN-ligated mice after vehicle or anismoycin injection. Level of each proteins in CPN-ligated, vehicle injected mice, normalized by level of proteins in sham, vehicle injected mice (n = 9 per group, one-way ANOVA). (in collaboration with Hyoung-Gon Ko)

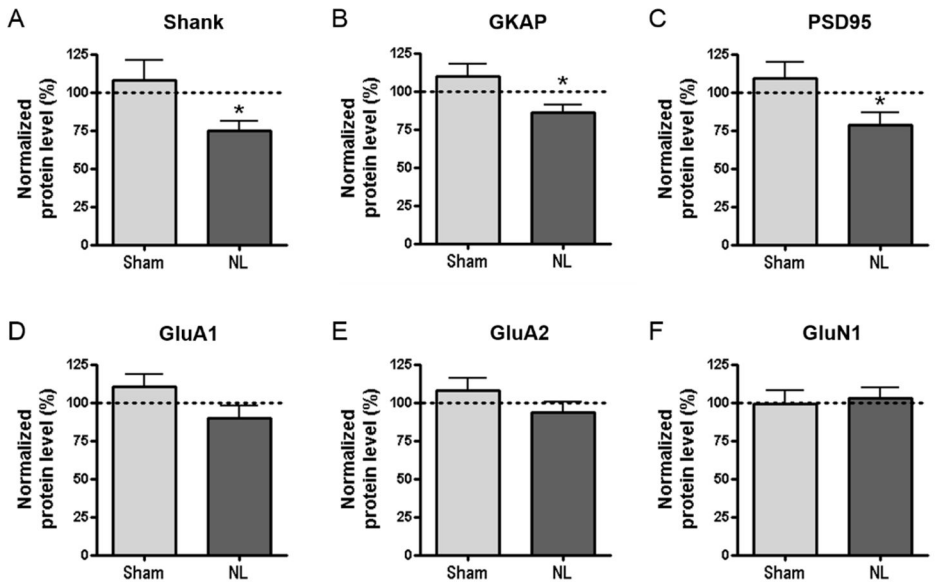


**Figure 19. Turnover of scaffolding proteins in PSD is increased after nerve injury**

A-F. For analysis of the effect of anisomycin infusion on protein levels, anisomycin injected mice level was normalized with vehicle injected mice level for each sham surgery (Sham) and CPN ligation (NL) groups. Level of Shank (A), GKAP (B), PSD95 (C), GluA1 (D), GluA2 (E), and GluN1 (F) was examined (n = 9 per group, unpaired t-test, *$p < 0.05$). (in collaboration with Hyoung-Gon Ko)

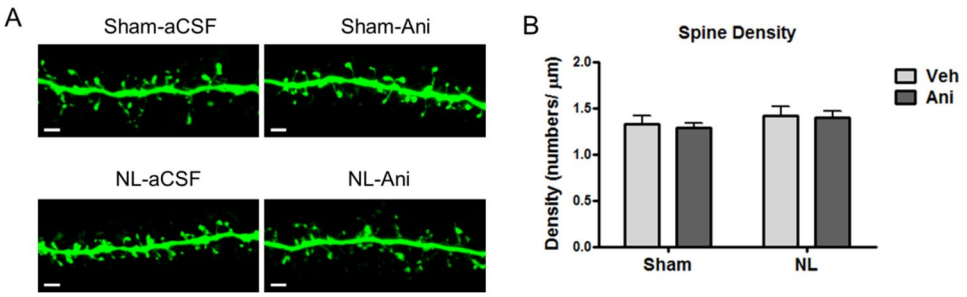


**Figure 20. Spine density of ACC neurons is stable after CPN ligation**

A.Biocytin staining sample images. Scale bar represents 2 μm. B. Spine density show no significant difference among groups (Sham-Veh, n = 11 neurons from 2 mice; Sham-Ani, n = 13 from 4 mice; NL-Veh, n = 17 from 3 mice; NL-Ani, n = 17 from 3 mice; two-way ANOVA).

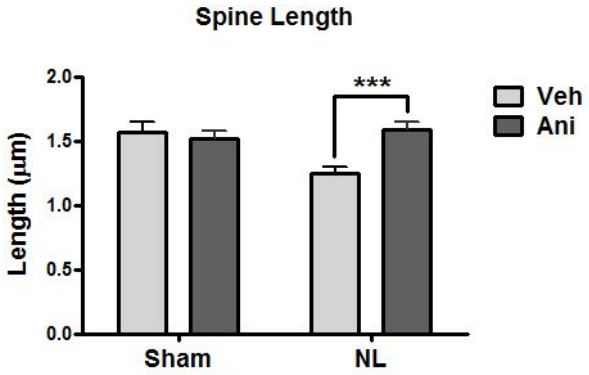


**Figure 21. Decreased spine length by CPN ligation was reversed by protein synthesis inhibition.**

Spine length for each group (Sham-Veh, n = 11 neurons from 2 mice; Sham-Ani, n = 13 from 4 mice; NL-Veh, n = 17 from 3 mice; NL-Ani, n = 17 from 3 mice; two-way ANOVA Bonferroni post-hoc test; ***$p < 0.001$). Surgery and drug treatment show significant interaction for spine length ($P < 0.005$). There was significant difference between vehicle and anisomycin treatment in CPN ligation groups (Bonferroni post-hoc test, $p < 0.001$)

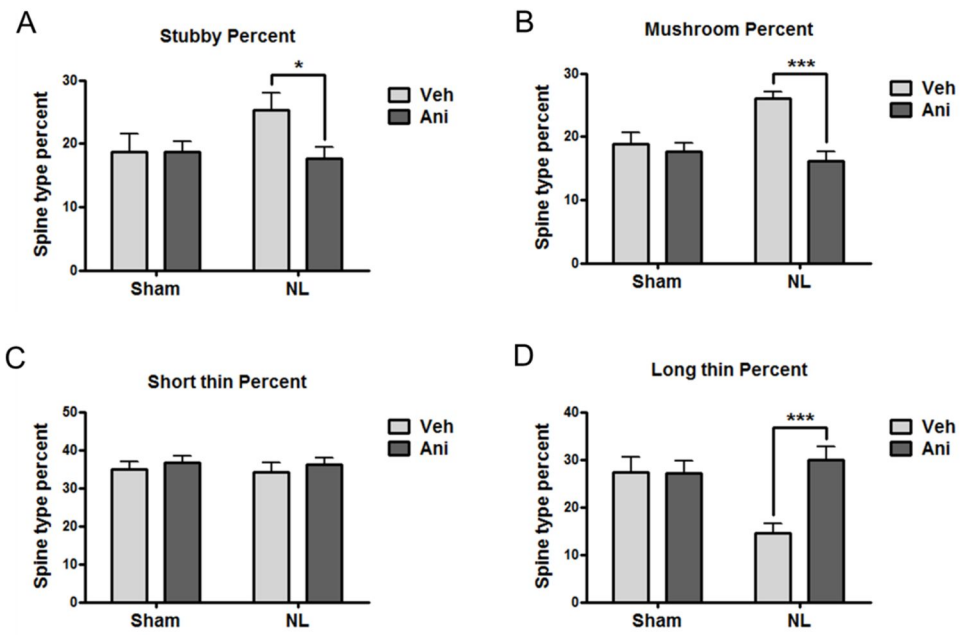


**Figure 22. Increased protein synthesis supports altered spine type composition induced by CPN ligation**

Percentage of four spine types for each group (Sham-Veh, n = 11 neurons from 2 mice; Sham-Ani, n = 13 from 4 mice; NL-Veh, n = 17 from 3 mice; NL-Ani, n = 17 from 3 mice, two-way ANOVA Bonferroni post-hoc test; *p < 0.05, ***p < 0.001). A. Stubby spine percentage show significant difference between vehicle and anisomycin treatment in CPN ligation groups (Bonferroni post-hoc test, P < 0.05) B, D. Surgery and drug treatment show significant interaction for Mushroom and Long thin spine percentage (P < 0.005). There was significant difference between vehicle and anisomycin treatment in CPN ligation groups (Bonferroni post-hoc test, P < 0.001).

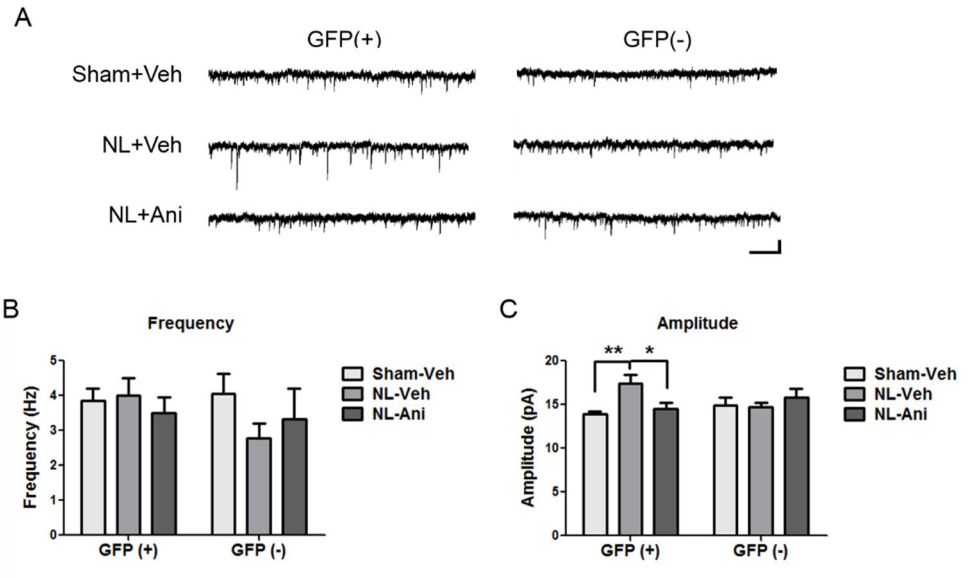


**Figure 23. Increased protein synthesis supports heightened synaptic transmission induced by CPN ligation**

A. mEPSC recording sample traces. Scale bar represents 20 pA, 0.5 s. B-C. Frequency and amplitude of mEPSCs recorded from GFP(+) and GFP(-) neurons in FosGFP transgenic mice (Sham-Veh, 12 GFP(+) and 13 GFP(-) neurons from 8 mice; NL-Veh, 12 GFP(+) and 11 GFP(-) neurons form 7 mice; NL-Ani, 16 GFP(+) and 11 GFP(-) neurons from 7 mice, two-way ANOVA, Bonferroni post-hoc test; *$p < 0.05$, **$p < 0.005$). B. Frequency shows no significant difference among groups. C. CPN ligation significantly increases the amplitude of mEPSC of GFP(+) neurons, reversed by anisomycin treatment. There is no significant difference in GFP(-) neurons. (in collaboration with Su-Eon Sim)

## DISCUSSION

Here, I examined whether chronic pain could induce any changes in synaptic proteins in ACC which is involved in the process of pain signal. I tested whether the synthesis and degradation of synaptic proteins are required for chronic pain using behavioral experiment. The synaptic protein changes induced by chronic pain and the effect of protein synthesis inhibition was also examined for its effect on structural changes of spines and synaptic strength in the ACC.

The reactivation of the memory circuit by retrieval is known to induce destabilization and restabilization of the memory. Especially in the hippocampus, reactivation of the contextual fear memory is demonstrated to induce destabilization of memory through protein degradation by the UPS, which seems to be restabilized by protein synthesis afterwards (Lee et al., 2008). Also, increased synaptic activity in cultured neurons has been demonstrated to increase the protein turnover of synaptic proteins (Ehlers, 2003).

Chronic pain is induced and maintained by persistent pain signal which is processed in many brain areas including ACC. The repeated activation of pain related circuits by chronic pain might lead to increased synaptic protein degradation and synthesis. My results demonstrate that the amount of synaptic proteins are stable after CPN ligation, but this is an outcome of increased protein turnover rate. I have also demonstrated that increased protein synthesis is required for maintenance of chronic pain. The effect of anisomycin injection strikingly resembles the result of intra-hippocampal anisomycin injection after contextual fear memory reactivation (Lee et al., 2008). When the protein degradation was

inhibited together with protein synthesis, the effect of protein synthesis inhibition was abolished in both in this experiments and the previous memory reactivation experiments. However, in case of chronic pain, pain was reinstated 3 days after anisomycin injection. It is not clear whether the effect of protein synthesis inhibition is transient compared to the case of fear memory, where the effect is permenant, and the circuit itself can be reinstated, or whether chronic pain is developed again since the source that induced this pain is remained as a nerve injury.

Although I have demonstrated that turnover rate of scaffolding proteins Shank, GKAP, and PSD95 are increased, there is no direct evidence that inhibition of these proteins synthesis itself could cause the reduction of pain response, because every *de novo* protein synthesis in ACC is affected by anisomycin. I am currently preparing shRNA tools to acutely and robustly inhibit further synthesis of these proteins. If acute protein synthesis inhibition of one of these proteins show similar effect as with anisomycin, the increased turnover of that protein would be critical for chronic pain.

The increased protein degradation and synthesis was shown indirectly by observing decrease of protein level after protein synthesis inhibition. It would be better to directly compare the turnover rate of each proteins in sham and CPN ligation group. I am currently examining this using a diet including $N^{15}$ isotope. After consumption of this diet, mice were sacrificed in various time points, and the isotope composition of each proteins will be examined to measure the turnover rate of each proteins.

Here I have found that CPN ligation induces increase of mushroom and stubby

spine number and decreases long thin spines. These findings are well reflected to the previous study demonstrating increased synaptic transmission in activated neurons in ACC after CPN ligation. Protein synthesis inhibition reversed these structural and functional changes of synapses, suggesting that increased protein turnover may be important to maintain the changed state. I am currently adding groups receiving both anisomycin and β-lactone injection to confirm whether β-lactone can counter the effect of anisomycin as in pain response experiments.

In summary, chronic pain induces increased turnover rate of synaptic proteins in ACC. Protein synthesis inhibition ameliorates allodynia response in mice with chronic pain. This is also consistent with the findings that structural and functional synaptic changes induced by chronic pain are reversed by protein synthesis inhibition.

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# CHAPTER 4. CONCLUSION

## CONCLUSION

In chapter 2, I have shown the critical involvement of protein synthesis and degradation in memory modification where the qualitative content of the memory is modified after it is initially formed using four-object rearrangement task. When protein synthesis inhibitor was injected into the hippocampus during the memory modification, the mice failed to incorporate the updated information and it still showed higher preference for the switched objects as in day 2. When protein degradation inhibitor was injected during the memory modification, the mice became to show higher preference to the unswitched objects. These two inhibitors counterbalanced the effect of each other. These findings demonstrate that protein synthesis and degradation have critical roles in memory modification process and that the balanced action of protein synthesis and degradation during this process may be essential for appropriate memory modification.

In chapter 3, I have demonstrated that upregulated protein synthesis is essential for chronic pain. The effect of protein synthesis inhibition on pain response was abolished by protein degradation inhibition. The protein synthesis and degradation of synaptic scaffolding proteins seems to be increased after CPN ligation. In accordance with the changes in turnover rate of scaffolding proteins which are essential components for synaptic structure, change in spine morphology and synaptic transmission were observed after CPN ligation, which was reversed by protein synthesis inhibition. These findings indicate that increased protein turnover of scaffolding proteins maintains the changes in synapses and increased pain response induced by chronic pain.

Although protein synthesis and degradation are fundamental, housekeeping

mechanism for regulating cellular functions, there are specific periods when synthesis and/or degradation of proteins are critically involved in plastic changes in the brain. Here I have revealed two behavioral changes that likely involves synaptic plasticity in the brain which was previously unknown for the requirement of protein synthesis and degradation. The present study not only gives insight for the mechanism of memory modification and chronic pain, but also gives possibility of novel therapeutic treatments, especially for chronic pain.

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**REFERENCES**

Arancibia-Cárcamo, I.L., Yuen, E.Y., Muir, J., Lumb, M.J., Michels, G., Saliba, R.S., Smart, T.G., Yan, Z., Kittler, J.T., and Moss, S.J. (2009). Ubiquitin-dependent lysosomal targeting of GABA(A) receptors regulates neuronal inhibition. Proc. Natl. Acad. Sci. U. S. A. *106*, 17552–17557.

Artinian, J., McGauran, A.-M.T., De Jaeger, X., Mouledous, L., Frances, B., and Roullet, P. (2008). Protein degradation, as with protein synthesis, is required during not only long-term spatial memory consolidation but also reconsolidation. Eur. J. Neurosci. *27*, 3009–3019.

Bachevalier, J., and Nemanic, S. (2008). Memory for spatial location and object-place associations are differently processed by the hippocampal formation, parahippocampal areas TH/TF and perirhinal cortex. Hippocampus *18*, 64–80.

Bailey, C.H., Bartsch, D., and Kandel, E.R. (1996). Toward a molecular definition of long-term memory storage. Proc. Natl. Acad. Sci. U. S. A. *93*, 13445–13452.

Baron, R., Binder, A., and Wasner, G. (2010). Neuropathic pain: diagnosis, pathophysiological mechanisms, and treatment. Lancet Neurol. *9*, 807–819.

Bedford, F.K., Kittler, J.T., Muller, E., Thomas, P., Uren, J.M., Merlo, D., Wisden, W., Triller, A., Smart, T.G., and Moss, S.J. (2001). GABA(A) receptor cell surface number and subunit stability are regulated by the ubiquitin-like protein Plic-1. Nat.

Neurosci. *4*, 908–916.

Bingol, B., and Schuman, E.M. (2006). Activity-dependent dynamics and sequestration of proteasomes in dendritic spines. Nature *441*, 1144–1148.

Bingol, B., Wang, C.-F., Arnott, D., Cheng, D., Peng, J., and Sheng, M. (2010). Autophosphorylated CaMKIIalpha acts as a scaffold to recruit proteasomes to dendritic spines. Cell *140*, 567–578.

Bliss, T.V.P., Collingridge, G.L., and Laroche, S. (2006). Neuroscience. ZAP and ZIP, a story to forget. Science *313*, 1058–1059.

Burbea, M., Dreier, L., Dittman, J.S., Grunwald, M.E., and Kaplan, J.M. (2002). Ubiquitin and AP180 regulate the abundance of GLR-1 glutamate receptors at postsynaptic elements in C. elegans. Neuron *35*, 107–120.

Cartier, A.E., Djakovic, S.N., Salehi, A., Wilson, S.M., Masliah, E., and Patrick, G.N. (2009). Regulation of synaptic structure by ubiquitin C-terminal hydrolase L1. J. Neurosci. Off. J. Soc. Neurosci. *29*, 7857–7868.

Colledge, M., Snyder, E.M., Crozier, R.A., Soderling, J.A., Jin, Y., Langeberg, L.K., Lu, H., Bear, M.F., and Scott, J.D. (2003). Ubiquitination regulates PSD-95 degradation and AMPA receptor surface expression. Neuron *40*, 595–607.

Davis, H.P., and Squire, L.R. (1984). Protein synthesis and memory: a review.

Psychol. Bull. *96*, 518–559.

Deng, P.-Y., and Lei, S. (2007). Long-term depression in identified stellate neurons of juvenile rat entorhinal cortex. J. Neurophysiol. *97*, 727–737.

Descalzi, G., Kim, S., and Zhuo, M. (2009). Presynaptic and postsynaptic cortical mechanisms of chronic pain. Mol. Neurobiol. *40*, 253–259.

DiAntonio, A., Haghighi, A.P., Portman, S.L., Lee, J.D., Amaranto, A.M., and Goodman, C.S. (2001). Ubiquitination-dependent mechanisms regulate synaptic growth and function. Nature *412*, 449–452.

Ding, M., Chao, D., Wang, G., and Shen, K. (2007). Spatial regulation of an E3 ubiquitin ligase directs selective synapse elimination. Science *317*, 947–951.

Dix, S.L., and Aggleton, J.P. (1999). Extending the spontaneous preference test of recognition: evidence of object-location and object-context recognition. Behav. Brain Res. *99*, 191–200.

Djakovic, S.N., Schwarz, L.A., Barylko, B., DeMartino, G.N., and Patrick, G.N. (2009). Regulation of the proteasome by neuronal activity and calcium/calmodulin-dependent protein kinase II. J. Biol. Chem. *284*, 26655–26665.

Dreier, L., Burbea, M., and Kaplan, J.M. (2005). LIN-23-mediated degradation of beta-catenin regulates the abundance of GLR-1 glutamate receptors in the ventral

nerve cord of C. elegans. Neuron *46*, 51–64.

Dudai, Y. (1996). Consolidation: fragility on the road to the engram. Neuron *17*, 367–370.

Dudai, Y. (2006). Reconsolidation: the advantage of being refocused. Curr. Opin. Neurobiol. *16*, 174–178.

Dworkin, R.H., O'Connor, A.B., Backonja, M., Farrar, J.T., Finnerup, N.B., Jensen, T.S., Kalso, E.A., Loeser, J.D., Miaskowski, C., Nurmikko, T.J., et al. (2007). Pharmacologic management of neuropathic pain: evidence-based recommendations. Pain *132*, 237–251.

Ehlers, M.D. (2003). Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. Nat. Neurosci. *6*, 231–242.

Flexner, L.B., Flexner, J.B., and Stellar, E. (1965). Memory and cerebral protein synthesis in mice as affected by graded amounts of puromycin. Exp. Neurol. *13*, 264–272.

Fonseca, R., Vabulas, R.M., Hartl, F.U., Bonhoeffer, T., and Nägerl, U.V. (2006). A balance of protein synthesis and proteasome-dependent degradation determines the maintenance of LTP. Neuron *52*, 239–245.

García-DeLaTorre, P., Rodriguez-Ortiz, C.J., Arreguin-Martinez, J.L., Cruz-

Castañeda, P., and Bermúdez-Rattoni, F. (2009). Simultaneous but not independent anisomycin infusions in insular cortex and amygdala hinder stabilization of taste memory when updated. Learn. Mem. Cold Spring Harb. N *16*, 514–519.

Gilbert, P.E., and Kesner, R.P. (2002). Role of the rodent hippocampus in paired-associate learning involving associations bet a stimulus and a spatial location. Behav. Neurosci. *116*, 63–71.

Gilbert, P.E., and Kesner, R.P. (2004). Memory for objects and their locations: the role of the hippocampus in retention of object-place associations. Neurobiol. Learn. Mem. *81*, 39–45.

Goelet, P., Castellucci, V.F., Schacher, S., and Kandel, E.R. (1986). The long and the short of long-term memory--a molecular framework. Nature *322*, 419–422.

Haas, K.F., Miller, S.L.H., Friedman, D.B., and Broadie, K. (2007). The ubiquitin-proteasome system postsynaptically regulates glutamatergic synaptic function. Mol. Cell. Neurosci. *35*, 64–75.

Hardt, O., Migues, P.V., Hastings, M., Wong, J., and Nader, K. (2010). PKMzeta maintains 1-day- and 6-day-old long-term object location but not object identity memory in dorsal hippocampus. Hippocampus *20*, 691–695.

Helton, T.D., Otsuka, T., Lee, M.-C., Mu, Y., and Ehlers, M.D. (2008). Pruning and

loss of excitatory synapses by the parkin ubiquitin ligase. Proc. Natl. Acad. Sci. U. S. A. *105*, 19492–19497.

Hernandez, P.J., and Abel, T. (2008). The role of protein synthesis in memory consolidation: Progress amid decades of debate. Neurobiol. Learn. Mem. *89*, 293–311.

Hoogenraad, C.C., Feliu-Mojer, M.I., Spangler, S.A., Milstein, A.D., Dunah, A.W., Hung, A.Y., and Sheng, M. (2007). Liprinalpha1 degradation by calcium/calmodulin-dependent protein kinase II regulates LAR receptor tyrosine phosphatase distribution and dendrite development. Dev. Cell *12*, 587–602.

Hou, L., Antion, M.D., Hu, D., Spencer, C.M., Paylor, R., and Klann, E. (2006). Dynamic translational and proteasomal regulation of fragile X mental retardation protein controls mGluR-dependent long-term depression. Neuron *51*, 441–454.

Hung, A.Y., Sung, C.C., Brito, I.L., and Sheng, M. (2010). Degradation of postsynaptic scaffold GKAP and regulation of dendritic spine morphology by the TRIM3 ubiquitin ligase in rat hippocampal neurons. PloS One *5*, e9842.

Jaggi, A.S., and Singh, N. (2011). Role of different brain areas in peripheral nerve injury-induced neuropathic pain. Brain Res. *1381*, 187–201.

Jenkins, T.A., Amin, E., Pearce, J.M., Brown, M.W., and Aggleton, J.P. (2004).

Novel spatial arrangements of familiar visual stimuli promote activity in the rat hippocampal formation but not the parahippocampal cortices: a c-fos expression study. Neuroscience *124*, 43–52.

Ji, R.-R., Kohno, T., Moore, K.A., and Woolf, C.J. (2003). Central sensitization and LTP: do pain and memory share similar mechanisms? Trends Neurosci. *26*, 696–705.

Juo, P., and Kaplan, J.M. (2004). The anaphase-promoting complex regulates the abundance of GLR-1 glutamate receptors in the ventral nerve cord of C. elegans. Curr. Biol. CB *14*, 2057–2062.

Kaang, B.-K., and Choi, J.-H. (2012). Synaptic protein degradation in memory reorganization. Adv. Exp. Med. Biol. *970*, 221–240.

Kandel, E.R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. Science *294*, 1030–1038.

Kandel, E.R., Schwartz, J.H., and Jessell, T.M. (2000). Principles of neural science (McGraw-Hill New York).

Kang, S.J., Liu, M.-G., Chen, T., Ko, H.-G., Baek, G.-C., Lee, H.-R., Lee, K., Collingridge, G.L., Kaang, B.-K., and Zhuo, M. (2012). Plasticity of metabotropic glutamate receptor-dependent long-term depression in the anterior cingulate cortex

after amputation. J. Neurosci. Off. J. Soc. Neurosci. *32*, 11318–11329.

Karpova, A., Mikhaylova, M., Thomas, U., Knöpfel, T., and Behnisch, T. (2006). Involvement of protein synthesis and degradation in long-term potentiation of Schaffer collateral CA1 synapses. J. Neurosci. Off. J. Soc. Neurosci. *26*, 4949–4955.

Kato, A., Rouach, N., Nicoll, R.A., and Bredt, D.S. (2005). Activity-dependent NMDA receptor degradation mediated by retrotranslocation and ubiquitination. Proc. Natl. Acad. Sci. U. S. A. *102*, 5600–5605.

Kim, E., and Sheng, M. (2004). PDZ domain proteins of synapses. Nat. Rev. Neurosci. *5*, 771–781.

Lee, J.L.C. (2008). Memory reconsolidation mediates the strengthening of memories by additional learning. Nat. Neurosci. *11*, 1264–1266.

Lee, I., and Solivan, F. (2008). The roles of the medial prefrontal cortex and hippocampus in a spatial paired-association task. Learn. Mem. Cold Spring Harb. N *15*, 357–367.

Lee, S.-H., Choi, J.-H., Lee, N., Lee, H.-R., Kim, J.-I., Yu, N.-K., Choi, S.-L., Lee, S.-H., Kim, H., and Kaang, B.-K. (2008). Synaptic protein degradation underlies destabilization of retrieved fear memory. Science *319*, 1253–1256.

Li, X.-Y., Ko, H.-G., Chen, T., Descalzi, G., Koga, K., Wang, H., Kim, S.S., Shang, Y., Kwak, C., Park, S.-W., et al. (2010). Alleviating neuropathic pain hypersensitivity by inhibiting PKMzeta in the anterior cingulate cortex. Science *330*, 1400–1404.

Liao, E.H., Hung, W., Abrams, B., and Zhen, M. (2004). An SCF-like ubiquitin ligase complex that controls presynaptic differentiation. Nature *430*, 345–350.

Ben Mamou, C., Gamache, K., and Nader, K. (2006). NMDA receptors are critical for unleashing consolidated auditory fear memories. Nat. Neurosci. *9*, 1237–1239.

Melnikova, I. (2010). Pain market. Nat. Rev. Drug Discov. *9*, 589–590.

Merlo, E., and Romano, A. (2007). Long-term memory consolidation depends on proteasome activity in the crab Chasmagnathus. Neuroscience *147*, 46–52.

Migues, P.V., Hardt, O., Wu, D.C., Gamache, K., Sacktor, T.C., Wang, Y.T., and Nader, K. (2010). PKMzeta maintains memories by regulating GluR2-dependent AMPA receptor trafficking. Nat. Neurosci. *13*, 630–634.

Milekic, M.H., and Alberini, C.M. (2002). Temporally graded requirement for protein synthesis following memory reactivation. Neuron *36*, 521–525.

Misanin, J.R., Miller, R.R., and Lewis, D.J. (1968). Retrograde amnesia produced by electroconvulsive shock after reactivation of a consolidated memory trace.

Science *160*, 554–555.

Morris, R.G.M., Inglis, J., Ainge, J.A., Olverman, H.J., Tulloch, J., Dudai, Y., and Kelly, P.A.T. (2006). Memory reconsolidation: sensitivity of spatial memory to inhibition of protein synthesis in dorsal hippocampus during encoding and retrieval. Neuron *50*, 479–489.

Nader, K., Schafe, G.E., and Le Doux, J.E. (2000). Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. Nature *406*, 722–726.

Nguyen, P.V., Abel, T., and Kandel, E.R. (1994). Requirement of a critical period of transcription for induction of a late phase of LTP. Science *265*, 1104–1107.

Pak, D.T.S., and Sheng, M. (2003). Targeted protein degradation and synapse remodeling by an inducible protein kinase. Science *302*, 1368–1373.

Pastalkova, E., Serrano, P., Pinkhasova, D., Wallace, E., Fenton, A.A., and Sacktor, T.C. (2006). Storage of spatial information by the maintenance mechanism of LTP. Science *313*, 1141–1144.

Patrick, G.N., Bingol, B., Weld, H.A., and Schuman, E.M. (2003). Ubiquitin-mediated proteasome activity is required for agonist-induced endocytosis of GluRs. Curr. Biol. CB *13*, 2073–2081.

Price, D.D. (2000). Psychological and neural mechanisms of the affective

dimension of pain. Science *288*, 1769–1772.

Rodriguez-Ortiz, C.J., De la Cruz, V., Gutiérrez, R., and Bermudez-Rattoni, F. (2005). Protein synthesis underlies post-retrieval memory consolidation to a restricted degree only when updated information is obtained. Learn. Mem. Cold Spring Harb. N *12*, 533–537.

Rodriguez-Ortiz, C.J., Garcia-DeLaTorre, P., Benavidez, E., Ballesteros, M.A., and Bermudez-Rattoni, F. (2008). Intrahippocampal anisomycin infusions disrupt previously consolidated spatial memory only when memory is updated. Neurobiol. Learn. Mem. *89*, 352–359.

Van Roessel, P., Elliott, D.A., Robinson, I.M., Prokop, A., and Brand, A.H. (2004). Independent regulation of synaptic size and activity by the anaphase-promoting complex. Cell *119*, 707–718.

Rossato, J.I., Bevilaqua, L.R.M., Myskiw, J.C., Medina, J.H., Izquierdo, I., and Cammarota, M. (2007). On the role of hippocampal protein synthesis in the consolidation and reconsolidation of object recognition memory. Learn. Mem. Cold Spring Harb. N *14*, 36–46.

Sacktor, T.C. (2008). PKMzeta, LTP maintenance, and the dynamic molecular biology of memory storage. Prog. Brain Res. *169*, 27–40.

Schaefer, A.M., Hadwiger, G.D., and Nonet, M.L. (2000). rpm-1, a conserved neuronal gene that regulates targeting and synaptogenesis in C. elegans. Neuron *26*, 345–356.

Shema, R., Hazvi, S., Sacktor, T.C., and Dudai, Y. (2009). Boundary conditions for the maintenance of memory by PKMzeta in neocortex. Learn. Mem. Cold Spring Harb. N *16*, 122–128.

Shen, H., Korutla, L., Champtiaux, N., Toda, S., LaLumiere, R., Vallone, J., Klugmann, M., Blendy, J.A., Mackler, S.A., and Kalivas, P.W. (2007). NAC1 regulates the recruitment of the proteasome complex into dendritic spines. J. Neurosci. Off. J. Soc. Neurosci. *27*, 8903–8913.

Sossin, W.S., and Lacaille, J.-C. (2010). Mechanisms of translational regulation in synaptic plasticity. Curr. Opin. Neurobiol. *20*, 450–456.

Speese, S.D., Trotta, N., Rodesch, C.K., Aravamudan, B., and Broadie, K. (2003). The ubiquitin proteasome system acutely regulates presynaptic protein turnover and synaptic efficacy. Curr. Biol. CB *13*, 899–910.

Suzuki, A., Josselyn, S.A., Frankland, P.W., Masushige, S., Silva, A.J., and Kida, S. (2004). Memory reconsolidation and extinction have distinct temporal and biochemical signatures. J. Neurosci. Off. J. Soc. Neurosci. *24*, 4787–4795.

Suzuki, A., Mukawa, T., Tsukagoshi, A., Frankland, P.W., and Kida, S. (2008). Activation of LVGCCs and CB1 receptors required for destabilization of reactivated contextual fear memories. Learn. Mem. Cold Spring Harb. N *15*, 426–433.

Tada, H., Okano, H.J., Takagi, H., Shibata, S., Yao, I., Matsumoto, M., Saiga, T., Nakayama, K.I., Kashima, H., Takahashi, T., et al. (2010). Fbxo45, a novel ubiquitin ligase, regulates synaptic activity. J. Biol. Chem. *285*, 3840–3849.

Vadakkan, K.I., Jia, Y.H., and Zhuo, M. (2005). A behavioral model of neuropathic pain induced by ligation of the common peroneal nerve in mice. J. Pain Off. J. Am. Pain Soc. *6*, 747–756.

Wan, H.I., DiAntonio, A., Fetter, R.D., Bergstrom, K., Strauss, R., and Goodman, C.S. (2000). Highwire regulates synaptic growth in Drosophila. Neuron *26*, 313–329.

Wang, H., Gong, B., Vadakkan, K.I., Toyoda, H., Kaang, B.-K., and Zhuo, M. (2007). Genetic evidence for adenylyl cyclase 1 as a target for preventing neuronal excitotoxicity mediated by N-methyl-D-aspartate receptors. J. Biol. Chem. *282*, 1507–1517.

Wang, S.-H., de Oliveira Alvares, L., and Nader, K. (2009). Cellular and systems mechanisms of memory strength as a constraint on auditory fear reconsolidation.

Nat. Neurosci. *12*, 905–912.

Wei, F., and Zhuo, M. (2001). Potentiation of sensory responses in the anterior cingulate cortex following digit amputation in the anaesthetised rat. J. Physiol. *532*, 823–833.

Wei, F., and Zhuo, M. (2008). Activation of Erk in the anterior cingulate cortex during the induction and expression of chronic pain. Mol. Pain *4*, 28.

Wei, F., Li, P., and Zhuo, M. (1999). Loss of synaptic depression in mammalian anterior cingulate cortex after amputation. J. Neurosci. Off. J. Soc. Neurosci. *19*, 9346–9354.

Wei, F., Wang, G.D., Kerchner, G.A., Kim, S.J., Xu, H.M., Chen, Z.F., and Zhuo, M. (2001). Genetic enhancement of inflammatory pain by forebrain NR2B overexpression. Nat. Neurosci. *4*, 164–169.

Wei, F., Qiu, C.S., Kim, S.J., Muglia, L., Maas, J.W., Pineda, V.V., Xu, H.M., Chen, Z.F., Storm, D.R., Muglia, L.J., et al. (2002). Genetic elimination of behavioral sensitization in mice lacking calmodulin-stimulated adenylyl cyclases. Neuron *36*, 713–726.

Wei, F., Vadakkan, K.I., Toyoda, H., Wu, L.-J., Zhao, M.-G., Xu, H., Shum, F.W.F., Jia, Y.H., and Zhuo, M. (2006). Calcium calmodulin-stimulated adenylyl cyclases

contribute to activation of extracellular signal-regulated kinase in spinal dorsal horn neurons in adult rats and mice. J. Neurosci. Off. J. Soc. Neurosci. *26*, 851–861.

Willeumier, K., Pulst, S.M., and Schweizer, F.E. (2006). Proteasome inhibition triggers activity-dependent increase in the size of the recycling vesicle pool in cultured hippocampal neurons. J. Neurosci. Off. J. Soc. Neurosci. *26*, 11333–11341.

Wood, M.A., Kaplan, M.P., Brensinger, C.M., Guo, W., and Abel, T. (2005). Ubiquitin C-terminal hydrolase L3 (Uchl3) is involved in working memory. Hippocampus *15*, 610–621.

Wu, L.-J., Toyoda, H., Zhao, M.-G., Lee, Y.-S., Tang, J., Ko, S.W., Jia, Y.H., Shum, F.W.F., Zerbinatti, C.V., Bu, G., et al. (2005). Upregulation of forebrain NMDA NR2B receptors contributes to behavioral sensitization after inflammation. J. Neurosci. Off. J. Soc. Neurosci. *25*, 11107–11116.

Xie, Y., Huo, F., and Tang, J. (2009). Cerebral cortex modulation of pain. Acta Pharmacol. Sin. *30*, 31–41.

Xu, H., Wu, L.-J., Wang, H., Zhang, X., Vadakkan, K.I., Kim, S.S., Steenland, H.W., and Zhuo, M. (2008). Presynaptic and postsynaptic amplifications of neuropathic pain in the anterior cingulate cortex. J. Neurosci. Off. J. Soc. Neurosci. *28*, 7445–7453.

Yao, I., Takagi, H., Ageta, H., Kahyo, T., Sato, S., Hatanaka, K., Fukuda, Y., Chiba, T., Morone, N., Yuasa, S., et al. (2007). SCRAPPER-dependent ubiquitination of active zone protein RIM1 regulates synaptic vesicle release. Cell *130*, 943–957.

Zhuo, M. (2006). Molecular mechanisms of pain in the anterior cingulate cortex. J. Neurosci. Res. *84*, 927–933.

Zhuo, M. (2007). A synaptic model for pain: long-term potentiation in the anterior cingulate cortex. Mol. Cells *23*, 259–271.

Zhuo, M. (2008). Cortical excitation and chronic pain. Trends Neurosci. *31*, 199–207.

## 국문초록

단백질 합성과 분해는 다양한 세포의 기능 조절하는 필수적인 현상이다. 신경 기능 또한 단백질 합성과 분해에 의해 조절된다. 신경세포에서는 단백질 합성과 분해의 기초적인 기능 외에도 시냅스 가소성을 위한 특정 단백질들의 합성과 분해가 필수적인 순간들이 있다. 수십 년의 뇌 연구에도 불구하고 특정 단백질들의 합성과 분해가 특정 뇌 기능에 필수적인 순간들에 대해 아직 모르는 경우가 많다.

일부 연구들에서 기존의 기억에 추가되는 정보 습득에 의해 단백질 합성을 동반하는 과정이 유도된다는 것을 밝혔지만, 기존 기억의 내용이 변화되는 과정에서 단백질 합성과 분해가 어떠한 역할을 하는지는 밝혀지지 않았다. 본 연구에서는 기존 기억과 연관된 부분적인 정보가 변화되어 기억의 변형을 유도하는, 물체 재배치 과제를 활용하였다. 단백질 합성과 분해 억제제 모두 변화된 정보의 적절한 수용을 각각 특정한 방식으로 차단하였다. 이 결과들은 단백질 합성과 분해가 기억의 변형에 주요한 역할을 한다는 것을 보여준다.

시냅스 단백질의 합성과 분해는 시냅스 구조의 재구성과, 그에 따른 시냅스의 강도의 변화를 조절한다. 기억과 마찬가지로 통증의 기본적인 메커니즘도 이러한 시냅스 강도의 변화로 알려져 있다. 본 연구에서 우리는 전대상피질(ACC)에서의 시냅스 단백질들의 합성과 분해가 만성 통증에 관여하는지 연구하였다. 만성 통증이 유도된 생쥐에서 행동실험, immunoblot 실험, 시냅스 돌기 구조 분석, 그리고 전기생리학적 측정을

수행하였다. 이를 통해 첫째, ACC에서의 단백질 합성 억제가 신경병증 통증과 염증 통증에 의한 이질통 반응을 감소시키는 것을 확인하였다. 하지만 단백질 분해 억제제는 단백질 합성 억제제의 이러한 이질통 반응 감소 효과를 상쇄하였다. 둘째로 신경병증 통증이 ACC에서 시냅스의 구조 단백질들의 활발한 단백질 합성을 유도한다는 것을 확인하였다. 마지막으로 ACC에서의 단백질 합성 억제가 만성통증에 의해 유도된, 시냅스의 구조적이고 기능적인 변화를 되돌리는 것을 확인하였다. 이 결과들은 ACC에서 시냅스 구조 단백질들의 지속적으로 증가된 합성이 만성 통증에 기여한다는 사실을 보여준다.

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주요어: 단백질 합성, 단백질 분해, 학습, 기억, 기억 변형, 신경 병증성 통증, 만성 통증, 전대상피질, 구조 단백질