Involvement of Muscarinic Acetylcholine Receptors and Hippocampal Theta Oscillation in Eyeblink Serial Feature-Positive Discrimination Task in Mice

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF GRADUATE SCHOOL of

INNOVATIVE LIFE SCIENCE, UNIVERSITY OF TOYAMA IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

MD. ASHRAFUR RAHMAN

Graduate School of Innovative Life Science
University of Toyama
Acknowledgments

The work described in these pages represents the products of a scientific journey I have taken as a graduate student with the mentorship, collaboration and support of many people. I am grateful for the remarkable group of talented, passionate, creative, caring and supportive friends and colleagues with whom I had the privilege of working at University of Toyama. During the 3 years of my tenure in Prof. Kawahara’s lab, my colleagues constantly impressed and inspired me with their sheer enthusiasm for science, and their energy was an invaluable inspiration and example for me through the years. I thank them not only for our shared scientific experiences whose results are described here, but also for the many intangible lessons and skills I have learned along the way. I was lucky to find in Norifumi Tanaka and Koji Usui, excellent collaborator. I learned a great deal from them to learn the basic of experiment and keenly critical approach to conditioning. I’ve learned to rely on Prof. Kawahara’s diligence and scientific honesty, as well as his unfailing good nature, and he has set a high standard for my future collaborators.

In my time at Toyama University, I was fortunate to interact with several students, who satisfied my need to talk about experimental problems and gave me hope that perhaps something new can truly come out of a chat in front of a whiteboard. Mr. Tanaka and Nishida are brilliant neuroscience thinkers with whom I worked on theoretical problems in the prof. kawahara’s lab. Before starting my work on neuroscience, I learned a great deal from a rotation experience in the group of Prof. Hishashi Mori, Prof. Masayuki Kaoru Ikeda, Prof Inokuchi. Nishida Takuma, Sano Katsunari, Ota Misako were wonderful colleagues.
# Table of Contents

<table>
<thead>
<tr>
<th>Topics</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>4</td>
</tr>
<tr>
<td><strong>Introduction</strong></td>
<td>5-8</td>
</tr>
<tr>
<td>- Research Background</td>
<td>5-6</td>
</tr>
<tr>
<td>- Aim of the study</td>
<td>7-8</td>
</tr>
<tr>
<td><strong>Experiment1: Behavioral Part</strong></td>
<td></td>
</tr>
<tr>
<td>- Figures</td>
<td>9-13</td>
</tr>
<tr>
<td>- Results</td>
<td>14-17</td>
</tr>
<tr>
<td>- Discussion</td>
<td>18-19</td>
</tr>
<tr>
<td>- Conclusion</td>
<td>20</td>
</tr>
<tr>
<td><strong>Experiment2: Hippocampal Local Field Potential</strong></td>
<td></td>
</tr>
<tr>
<td>- Figures</td>
<td>21-24</td>
</tr>
<tr>
<td>- Results</td>
<td>25-28</td>
</tr>
<tr>
<td>- Discussion</td>
<td>28-31</td>
</tr>
<tr>
<td>- Conclusion</td>
<td>31</td>
</tr>
<tr>
<td>References</td>
<td>32-35</td>
</tr>
</tbody>
</table>
Abstract

I investigated the involvement of muscarinic acetylcholine receptors (mAChRs) and hippocampal theta oscillation in eyeblink serial feature-positive discrimination learning in mice using the mAChR antagonist. A 2-s light cue was delivered 5 or 6 s before the presentation of a 350-ms tone paired with a 100-ms periorbital electrical shock (cued trial) but not before the tone-alone presentation (non-cued trial). Mice received 30 cued and 30 non-cued trials each day in a random sequence. I found that saline-injected control mice were successfully discriminating between cued and non-cued trials. In contrast, scopolamine-injected mice developed an equal number of conditioned response (CR) irrespective of the presence of the cue during conditioning. In addition, post training administration of scopolamine to the control mice did not impair the conditional discrimination and expression of pre-acquired CR. These results suggest that mAChRs may play a pivotal role in memory formation in the conditional brain state but not in the development of discrimination after conditional memory had formed in the serial feature-positive discrimination task during eyeblink conditioning.

Analysis of the hippocampal local field potential in cued trials revealed an existence of differential sub-bands: the higher (7–9 Hz) but not the lower (4–6 Hz) sub-band increased after the conditional cue. Further analysis of the hippocampal theta oscillation in non-cued trials revealed that the lower sub-band and the higher sub-band were increased before the CS during conditioning. These findings were consistent with their rising tendency before the conditional cue in cued trials. These results suggest that the lower and the higher sub-band might reflect an increase in general attention to the conditioning situation. Administration of a muscarinic acetylcholine receptor antagonist scopolamine after sufficient learning revealed that both sub-bands depend on mAChRs. In the present work, I suggest the existence and differential role of sub-bands of type 2 theta oscillation: one for general attentional increase and the other for recognition of conditional cue.
Research Background

Classical eyeblink conditioning is one of the preferred models of learning for studying the interaction between higher and lower levels of the nervous system [1–3]. Many studies have examined the underlying neural substrates for standard delay eyeblink conditioning, which essentially needs cerebellum and brainstem [4–6], the role of higher brain regions, such as the hippocampus and medial prefrontal cortex have also been under intense experimental scrutiny [7–14]. Top-down modulation of the ongoing input-output relationships of the higher brain regions to the lower regions of brain was thought to be the proposed roles of higher brain regions in eyeblink conditioning. This view is supported by studies of conditional discrimination tasks in patients with amnesia with temporal lobe lesions [15, 16]. Similar kinds of conditional discrimination tasks, also known as serial feature-positive discrimination or occasion setting [2], have been used in studies of rabbit eyeblink conditioning [17–21]. Recently in rat eyeblink conditioning, another experiment investigated the modulation of hippocampal local field potentials during the serial feature-positive discrimination task and found a significant correlation between an increase in the relative power of hippocampal theta oscillations after the light cue and a subsequent expression of the CR on a trial-to-trial basis [22]. This findings strongly suggested a hippocampal involvement in the top-down modulation of the CR in this conditional discrimination
task. Although the effect of hippocampal lesions on serial feature-positive discrimination has not been studied, the involvement of hippocampal is consistent with the impairment of the conditional discrimination in amnesic patients [15, 16], as well as with the pivotal role of the hippocampus in the simultaneous feature-positive discrimination, during which the light feature and tone targets were presented simultaneously [23]. These findings suggested the involvement of hippocampus in the top-down modulation of conditional response in eyeblink serial feature-positive discrimination. Hippocampal theta rhythm is one of the entrants for relevant neural activities that reflect a brain state contributing to the top-down modulation of the discriminative response. Recently, the dynamics of hippocampal local field potential (LFP) during eyeblink serial feature-positive discrimination task in rat eyeblink conditioning were investigated and found the enhancement of the relative power of theta oscillation after the light cue, which showed a significant correlation with the expression of CR on a trial-to-trial basis [22]. Based on the spectral peak around 5–8 Hz in immobile and attentive rats, the associated hippocampal theta oscillation was suggested to be the atropine-sensitive type 2 theta oscillation.
Aim of the study

The cholinergic system is known to be important in cognition and it has been proposed that the cholinergic system may be involved in attentional processes as well as in learning and memory. Several studies showed that systemic administration of muscarinic acetyl choline receptors antagonist, scopolamine disrupted acquisition of conditioned eyeblink responses. Salvatierra and Berry demonstrated that systemic scopolamine injections eliminate hippocampal responses during behavioral expression of the CR which indicated the role of hippocampus and forebrain acetyl choline system in memory. Also the hippocampus and septohippocampal ACh systems have proved to be much involved in eyeblink classical conditioning. In addition, it is well recognized that the cholinergic inputs originating from the medial septum [24] is projected to the hippocampus which is enriched with muscarinic acetylcholine receptors (mAChRs) [25]. In various types of hippocampus-dependent learning tasks [26–28], including eyeblink conditioning in rabbits [29] and mice [30], the crucial roles of mAChRs in learning and memory have been studied by using the mAChR antagonist scopolamine In addition, in rats [31] and humans [32], the role of mAChRs in attentional process has also been reported. Therefore, it is assumed that mAChRs play an important role in the serial feature-positive discrimination in eyeblink conditioning, which required much higher cognitive demand for memory formation.
and attentional power than the simple delay eyeblink conditioning, which does not necessary involve higher brain regions [5, 6].

Also, in order to investigate the neural mechanisms involved in eyeblink serial feature-positive discrimination task in mice, I used a muscarinic acetylcholine receptor (mAChR) antagonist, scopolamine. The rationality of this consideration is that scopolamine disrupts the hippocampal type 2 theta, and impairs various types of hippocampus-dependent learning tasks. I found that a systemic administration of scopolamine impaired the acquisition of the conditional discrimination, but did not affect the performance of the pre-acquired conditional discrimination. Analysis of behavioral result showed that mAChRs play a role in the formation of memory for the conditional discrimination rather than attentional role to the conditional cue required for discriminative performance. Analysis of hippocampal theta oscillation revealed that the higher sub-band of type 2 theta (7–9 Hz) was enhanced after the conditional cue and correlated significantly with the earlier onset of the CR. These results suggest that the higher sub-band of type 2 theta reflects the cognition of the conditional cue, leading to acquisition of the discriminative responses in mice.
**EXPERIMENT 1: FIGURES**

---

**Fig. 1.** The stimulus sequence in the eyeblink serial feature-positive discrimination task. Two kinds of trials were used in the task: cued trials and non-cued trials. In a cued trial, a light cue (2 s) was delivered 5 or 6 s randomly before a tone conditional stimulus (CS), which co-terminated with a periorbital electrical shock unconditional stimulus (US). In a non-cued trial, the CS was presented alone, without the preceding light or US. Inter-trial intervals were randomized between 60 and 70 s.
Fig. 2. Effects of scopolamine on the acquisition and expression of the conditioned response (CR). After 2 days of adaptation sessions (sp1–2), both saline– and scopolamine–treated mice underwent acquisition sessions for 7 consecutive days (acq1–7) followed by 3 days of expression sessions (exp1–3). (A,B) The left and middle panels represent the averaged CR frequency in both cued and non-cued trials. (A) Averaged data from cued trials (filled circles; n = 6) and non-cued trials (empty circles; n = 6) are illustrated for saline-injected control mice. (B) Averaged data from cued trials (filled circles; n = 6) and non-cued trials (empty circles; n = 6) are shown for scopolamine-injected mice. (C) The right panel represents the discrimination (%) to the CSs between the groups. Differences in discrimination percentage between cued and non-cued trials are plotted for the saline-treated (filled circles) and scopolamine–treated mice (empty circles). In panel A,B, the vertical axis represents the CR(%) frequency while the horizontal axis illustrates corresponding sessions and the vertical bars indicate the standard error of the mean. In panel C, the vertical axis represents discrimination to the CSs(%), while the horizontal axis illustrates corresponding sessions and the vertical bars indicate the standard error of the mean.

sp, spontaneous session; acq, acquisition session; exp, expression session.
Fig. 3. Effects of scopolamine on EMG amplitudes. (A) The left and middle panels represent the averaged EMG amplitude overall valid trials. (A) Averaged data from cued trials (filled circles; n = 6) and non-cued trials (empty circles; n = 6) are illustrated for saline-injected control mice. (B) Averaged data from cued trials (filled circles; n = 6) and non-cued trials (empty circles; n = 6) are shown for scopolamine-injected mice. (C) The right panel represents ΔEMG amplitude values between cued and non-cued trials in both groups. Filled rectangles represent ΔEMG amplitude values for the saline-treated group and empty rectangles represent ΔEMG amplitude values for scopolamine–treated mice. In panel A,B, the vertical axis represents averaged EMG amplitudes, while the horizontal axis illustrates corresponding sessions, and the vertical bars indicate the standard error of the mean. In panel C, the vertical axis represents the ΔEMG amplitude values, while the horizontal axis illustrates corresponding sessions and the vertical bars indicate the standard error of the mean.
Fig. 4. Modulation of CR dynamics in saline-treated mice.

onset latency data for cued (empty squares) and non-cued (filled squares) trials, respectively. Panel (B) represents peak latency data for cued (empty squares) and non-cued (filled squares) trials, respectively. Panel (C) represents the CR peak amplitude data for cued (empty squares) and non-cued (filled squares) trials, respectively. In panels, A and B, the vertical axis represents the average time (ms), while the horizontal axis illustrates corresponding sessions. In panel C, the vertical axis represents the CR peak, while horizontal axis shows corresponding sessions. Vertical bars indicate the standard error of the mean.
Fig. 5. Modulation of CR dynamics in scopolamine–treated mice. Panel (A) represents the onset latency data for cued (empty squares) and non-cued (filled squares) trials, respectively. Panel (B) represents peak latency data for cued (empty squares) and non-cued (filled squares) trials, respectively. Panel (C) represents the CR peak amplitude data for cued (empty squares) and non-cued (filled squares) trials, respectively. In panels, A and B, the vertical axis represents the average time (ms), while the horizontal axis illustrates corresponding sessions. In panel C, the vertical axis represents the CR peak, while horizontal axis shows corresponding sessions. Vertical bars indicate the standard error of the mean.
EXPERIMENT 1: RESULTS

Effects of scopolamine on the acquisition of the serial feature-positive discrimination in mice

During the 7 days of acquisition sessions, the saline-injected control mice learned to show a much higher number of CRs in cued trials than in non-cued trials (Fig. 2A), despite the CS was identical in both types of trials. The CR% comprised around 50–60% (51.2 ± 8.6% on the last day of acquisition) in cued trials, whereas it remained around 20% (19.8 ± 4.2% on the last day of acquisition) in non-cued trials. Furthermore, I have analyzed average EMG amplitudes between the CS onset and US onset over all valid trials, which do not depend on the criterion for CR detection, and confirmed successful discrimination learning during acquisition sessions (Fig. 3A). In contrast to observations in control animals, in scopolamine-injected mice, the CR% reached around 42% (42.1 ± 12% on the last day of acquisition) in cued trials, whereas it remained around 25% (23.6 ± 9% on the last day of acquisition) in non-cued trials (Fig. 2B). In addition, I have investigated differences in discrimination (difference in CR% between cued and non-cued trials) to the CSs between saline-injected control mice and scopolamine-injected mice (Fig. 2C). A two-way ANOVA with repeated measures demonstrated statistically significant effects of
session (P<0.05) and groups (P>0.05), but no significant session-group interaction (P>0.05), suggesting that scopolamine impaired acquisition of CS discrimination between cued and non-cued trials irrespective of the cue presence. To further confirm these results, I analyzed average EMG amplitudes over all valid trials in scopolamine-injected mice and revealed significant effects of session and trial type (P<0.05), as well as a significant interaction between session and trial type (P<0.05), Fig. 3B).

**Dynamics of the CR temporal pattern**

The average CR onset latency remained around 100 and 110 ms in cued and non-cued trials, respectively, during the 7 days of conditioning in saline-injected control mice (P<0.05, paired t-test, Fig. 4A). There were significant differences in the mean onset latency between cued and non-cued trials over the last 3 days of acquisition sessions (acquisitions 5–7): (P<0.05, paired t-test, Fig. 4A). In contrast, the CR onset latency remained around 111 in both cued and non-cued trials, during the 7 days of conditioning in scopolamine-injected mice (P>0.05, paired t-test, Fig. 5A). Additionally, there were no significant differences in the average onset latency between cued and non-cued trials over the last 3 days of acquisition sessions (P>0.1, paired t-test, Fig. 5A).

The average CR peak latency remained around 155 ms in cued and non-cued
trials, respectively, during the 7 days of conditioning in saline-injected control mice (P<0.01, paired t-test, Fig. 4B). There were no significant differences in the averaged peak latency between the cued and non-cued trials over the last 3 days of acquisition sessions (acquisitions 5–7): (P>0.05, paired t-test). The CR peak latency remained around 161 ms and 151 ms in cued and non-cued trials, respectively, during the 7 days of conditioning in scopolamine-injected mice. Additionally, there were no significant differences in the average onset latency between cued and the non-cued trials over the last 3 days of acquisition sessions (acquisitions 5–7): (P>0.1, paired t-test, Fig. 5B). Also, there were no significant differences in the averaged CR peak between the cued and non-cued trials, over the last 3 days of acquisition sessions in saline-injected control mice (acquisitions 5–7): (P>0.05, paired t-test, Fig. 4C). A lack of differences in averaged CR peak values was also noted in scopolamine-injected mice (acquisitions 5–7): P>0.05, paired t-test, Fig. 5C).

**Modulatory dynamics of the pre-acquired CR**

Post-learning administration of scopolamine did not impair the pre-acquired CR in cued trials but led to nominally higher average CR% in non-cued trials (P>0.05, paired t-test, Fig. 2A). In contrast, a gradual impairment of discrimination learning was observed in expression sessions after sequential administrations of scopolamine
on 3 consecutive days (in expression-1: P<0.05, paired t-test; in expression-2: P<0.01, paired t-test; in expression-3: P>0.1, paired t-test, Fig. 2A). A two-way repeated measures ANOVA across the expression sessions revealed a significant effect of trial type (P<0.05), indicating that scopolamine did not impair the pre-acquired discrimination between cued and non-cued trials. On the other hand, a progressive development of discrimination learning between cued and non-cued trials was observed in scopolamine-injected mice after switching to saline injections during expression sessions (P>0.1, paired t-test; in expression-2: P>0.1, paired t-test; in expression-3: P<0.05, paired t-test, Fig. 2B), indicate that after a switch from scopolamine to saline, mice developed discrimination learning between cued and non-cued trials.
EXPERIMENT 1: DISCUSSIONS

Serial feature-positive discrimination in mouse eyeblink conditioning

In the present study, control mice successfully acquired the discriminative response to the identical tone CS based on the presence/absence of preceding light stimuli: the frequency of the CR occurrence in cued trials was much higher than that in non-cued trials (Fig. 2A). I used the serial feature-positive discrimination eyeblink conditioning task with a temporal gap of 3–4 s between the end of the conditional cue and the CS onset, which corresponded to an inter-stimulus interval of 5–6 s between their onsets. This temporal relationship was almost equal to 3–5-s gap (5–7-s interval) used in rat serial feature-positive discrimination [22] and 3.4-s gap (4.2-s interval) required for the emergence of occasion setting in the serial conditional discrimination in rabbit [18]. At the same time, a much shorter gap of 1 s (5-s interval) [15] or even an overlap of the feature and target (3.3-s interval) [16] could be sufficient to detect the impairment of amnesic patients in the serial conditional discrimination. It should be noted that in rabbit serial conditional discrimination with a temporal gap of less than 3.4 s (4.2-s feature-target interval and 4.6-s feature-US interval), the control over the response to the target CS by the feature was weak and instead, a direct response to the feature was noted [18]. Consistent with the importance of the temporal gap for the top-down modulation of the eyeblink CR in animals, the temporal gap between the
feature and target stimuli enhanced the occasion setting ability of the feature stimulus in the feature-positive discrimination in rat appetitive conditioning [33].

**Involvement of mAChRs in the serial feature-positive discrimination**

I found that scopolamine impaired the acquisition of the conditional discrimination in the serial feature-positive discrimination task, as drug-treated mice manifested CRs to an equal degree in both the cued and non-cued trials (Fig. 2B). Therefore, the acquisition of the CR itself was not appreciably impaired. These results suggested that scopolamine impairs the formation of the memory for the top-down modulation, but not the acquisition of the CR itself. The latter process largely depends on the activity of the cerebellum. In addition, scopolamine did not significantly impair pre-acquired conditional discrimination performance and expression of the CR itself when tested in control mice after 7 days of acquisition sessions. These results paralleled the weak effect of scopolamine on the pre-acquired CR in the hippocampus-dependent trace eyeblink conditioning in rabbits [29]. Therefore, mAChRs might play an important role in the formation, but not the expression, of the memory for top-down modulation associated with the feature cue in the serial feature-positive discrimination task in mouse eyeblink conditioning.
EXPERIMENT 1: CONCLUSIONS

I have shown that mAChRs play a pivotal role in the acquisition of discrimination between cued and non-cued trials as pharmacological inhibition of their activity resulted in an indistinguishable expression of CRs in both trials. On the other hand, the administration of scopolamine did not disturb pre-acquired discrimination performance or expression of the CR itself. Collectively, these results, suggest that mAChRs may play an important role in the formation, but not expression, of the memory for top-down modulation in the serial feature-positive discrimination task in mouse eyeblink conditioning.
EXPERIMENT 2 FIGURES: Hippocampal Local Field Potential

Figure 6. Electrode position in the hippocampus. The picture of a brain section taken from one of the mice. The arrow showed the tips of a pair of the electrodes.

Figure 7. Effects of scopolamine on the expression of the conditioned response (CR). After 2 days of adaptation sessions (sp1–2), mice underwent acquisition sessions for 7 consecutive days (acq1–7) followed by 3 days of expression sessions (exp1–3). The figure represents the averaged CR frequency in both cued (filled circles; n = 6) and non-cued trials (empty circles; n = 6). In this figure the vertical axis
represents the CR(%) frequency while the horizontal axis illustrates corresponding sessions. The vertical bars indicate the standard error of the mean. sp, spontaneous session; acq, acquisition session; exp, expression session.

Figure 8. Changes in spectral power of type 2 theta sub-bands around the cue. The upper panels (A and B), represent the mean absolute theta power changes in type 2 theta sub-bands across the sessions. Panel A, represents the absolute theta power changes in 4–6 Hz frequency range and panel B, represents the absolute theta power changes in 7–9 Hz frequency range. In panels A and B, circles (empty; n = 6) indicate the data of the 3-s pre-cue, circles (filled; n = 6) indicate the data of the 3-s post-cue.

The bottom panels (C and D) represent the mean relative theta power changes in type 2 theta sub-bands across the sessions. Panel C, represents the relative theta power changes in 4–6 Hz frequency range and panel D, represents the relative theta power changes in 7–9 Hz frequency range. In panels C and D, circles (empty; n = 6) indicate the data of the 3-s pre-cue, circles (filled; n = 6) indicate the data of the 3-s post-cue.
In panels A and B, vertical axis represents the mean absolute theta power and the horizontal axis illustrates the sessions. In panels C, D, vertical axis represents the mean relative theta power and the horizontal axis illustrates the sessions. Error bars indicate SEM.

**Figure 9.** Changes in spectral power of type 2 theta sub-bands around the CS in cued trials. Panels A and B represent the mean absolute theta power changes of type 2 theta sub-bands in cued trials. Panel A represents the absolute theta power changes in 4–6 Hz frequency range and panel B represents the absolute theta power changes in 7–9 Hz frequency range. In panels A and B, circles (empty; n = 6) indicate the data of the 3-s pre-CS, circles (filled; n = 6) indicate the data of the 3-s post-CS. The bottom panels C and D represent the mean absolute theta power changes of type 2 theta sub-bands in non-cued trials. Panel C represents the absolute theta power changes in 4–6 Hz frequency range and panel D represents the absolute theta power changes in 7–9 Hz frequency range. In panels A and B, circles (empty; n = 6) indicate the data of
the 3-s pre-CS, circles (filled; n = 6) indicate the data of the 3-s post-CS. In panels A, B, C, D vertical axis represents the mean absolute theta power and the horizontal axis illustrates the sessions. Error bars indicate SEM.

Figure 10. Correlation of CR onset latency with the post-cue theta power. The scattergram (A) represents the relationship between CR onset latency and post-cue relative theta power of higher sub-band (acq 4-7). The scattergram (B) represents the relationship between CR onset latency and pre-cue relative theta power of lower sub-band (acq 4-7). Filled circles correspond to onset latency with respect to post-cue relative theta power of higher sub-band and filled triangles correspond to onset latency with respect to pre-cue relative theta power of lower sub-band in cued trials. The vertical axis represents the onset latency while horizontal axis represents the relative theta power.
EXPERIMENT 2: RESULTS

Effects on acquisition in serial feature-positive discrimination task in mice

During the 7 days of acquisition session, mice learned to show a much higher number of CRs in cued trials than in non-cued trials (Figure 7), despite the CS was identical in both types of trials. The CR% comprised around 70–80% in the last day of cued trials, whereas it remained around 15–25% in the last day of non-cued trials. A statistical comparison across sessions using a two-way ANOVA with repeated measures revealed effects of session (P<0.001) and trial type (P<0.001), as well as significant interaction between session and trial type, (P<0.001), suggesting mice responded more frequently to the tone in the cued trials than in the non-cued trials.

Power spectrum around the cue

I have subdivided the type 2 theta frequency (4–9 Hz) into two sub-bands, lower sub-band (4–6 Hz) and higher sub-band (7–9 Hz) and compared the changes of absolute power of each sub-band between 3-s pre-cue and 3-s post-cue across the sessions (Figure 8). In case of lower sub-band of type 2 theta (Figure 8A), a comparison across the 9 sessions using a two-way analysis of variance (ANOVA) with repeated measures revealed no significant effect of session (P>0.05) and time course (P>0.05). In contrast, the lower sub-band of type 2 theta showed an increasing
tendency before the cue during 7 days of conditioning. The higher sub-band (7–9 Hz) of type 2 theta was enhanced across the sessions and further by the cue (Figure 8B). A two-way repeated measures ANOVA across the sessions demonstrated statistically significant effects of session (P>0.05) and time course, (P>0.05).

Post-training administration of scopolamine suppressed the pre-cue power (Figure 8A) but not the post-cue power (Figure 8A) of lower sub-band (4–6 Hz) of type 2 theta. Scopolamine suppressed the pre- and post-cue (P<0.05, t-test, Figure 8B) power of higher sub-band (7–9 Hz) of type 2 theta. Together these results implied that scopolamine suppressed both the sub-bands of type 2 theta. To further substantiate these findings, I analyzed the effects of scopolamine on dominance alteration (Figure 8C,D). I found that scopolamine diminished the dominance alteration, because the theta oscillation was almost suppressed (P>0.05, t-test, Figures 8C,D).

**Power spectrum around the CS**

To assess the changes in spectral power of type2 theta sub-bands around the CS, I have considered two different time course, 2-s duration before the CS onset (pre-CS) and 2-s duration after 2-s of CS offset (post-CS). I have compared the absolute power in two sub-bands of type 2 theta between pre- and post-CS in cued trials (Figures 9A,B). A comparison across the 9 sessions using a two-way analysis of
variance (ANOVA) with repeated measures revealed significant effect of time course (P<0.05) but no effect of sessions (P>0.05). As CS was identical in both the trials, I have analyzed the changes in spectral power of type 2 theta sub-bands around the CS in non-cued trials also (Figure 9C). In case of non-cued trial, a steeper rising tendency of lower sub-band was detected around the CS. A two-way analysis of variance (ANOVA) with repeated measures revealed significant effect of time course (P<0.05) but no effect of sessions (P>0.05).

In case of higher sub-band of type 2 theta (Figure 9B), a comparison across the 9 sessions around the CS in cued trials using a two-way analysis of variance (ANOVA) with repeated measures revealed significant effect of session (P<0.05) but no effect of time course (P>0.05), which implied that the higher sub-band of type 2 theta was enhanced before the CS, preceded by the conditional cue. In non-cued trials, a two-way analysis of variance (ANOVA) with repeated measures revealed significant effects of session (P<0.05), but no effect of time course (P>0.05)

Post-training administration of scopolamine suppressed the pre-CS power of both the sub-bands of type 2 theta (expression 1: P<0.05, t-test, Figures 9A,B) but not the post-CS power (expression 1: P>0.05, t-test, P>0.05) in cued trials. On the other hand, in non-cued trials scopolamine suppressed the pre- and post-CS power of both the sub-bands of type 2 theta (expression 1: P<0.05, t-test, Figures 9A,B).
**Correlation between CR onset latency with the pre- and post-cue theta power**

To quantitatively evaluate the relationship between the hippocampal state and behavioral dynamics, I have examined the correlation between the relative theta power of higher sub-band around the cue and the CR onset latency in cued trials. The scattergram illustrates that the CR onset latency distribution was negatively skewed with respect to the post-cue relative theta power \((r = -0.21, P = 0.0004)\) of higher sub-band, while there was no correlation with the pre-cue relative theta-power \((r = -0.16, P = 0.02)\) of lower sub-band.

**EXPERIMENT 2: DISCUSSIONS**

Many studies have suggested that hippocampal theta oscillations correlated with the cognition [34] as well as the learning speed in classical eyeblink conditioning [35-36]. The relative power of theta oscillation increased after the light cue in eyeblink serial feature-positive discrimination task in rats, which showed a significant correlation with the expression of CR on a trial-to-trial basis [22]. Two types of hippocampal theta oscillations have been distinguished, type 1 (atropine-resistant) and type 2 (atropine-sensitive) [37-38]. The type 1 theta is associated with motor movements and the type 2 theta is associated with an immobile, attentive state.

In the present study, the spectral peak of the observed hippocampal theta is
around 4–9 Hz which was suppressed after administration of scopolamine. Thus 4–9 Hz band frequency would be considered as “type 2 theta” [39-40] which is supported by the hypothesis that scopolamine disrupts the hippocampal type 2 theta.

**Elevation of general attention to the conditioning situation by the lower sub-band of type 2 theta**

In the present study, the lower sub-band (4–6 Hz) was not increased after the conditional cue in cued trials as well as prior to the CS in cued trials. Together these results suggest that the lower sub-band did not reflect the cue recognition as well as the prior enhancement by the conditional cue in cued trials. In contrast, I have observed that the lower sub-band was increased before the conditional cue in conditioned trials during conditioning. In addition, analysis of the hippocampal theta oscillation in non-cued trials revealed that the lower sub-band was increased before the CS during conditioning. Together these findings suggest that the lower sub-band of type 2 theta reflected an elevation of the general attention to the conditioning situation.

**Cognition of the conditional cue by the higher sub-band of type 2 theta**

In the present study, I observe that the higher sub-band of type 2 theta (7–9
Hz) was increased after the conditional cue. In addition, I observed that the spectral pattern from the lower- to the higher-band theta dominance was shifted and the higher sub-band was correlated with the earlier onset of the following conditioned response (Figure 10). In accordance with this finding, I observed a significant difference in the frequency of the CR occurrence between cued and non-cued trials, despite the presence of an identical tone in both trials. Therefore, together these results suggested that the higher sub-band of type 2 theta related to the cognition of the conditional cue.

In addition to the increase of higher sub-band before the CS in non-cued trials, I observed that the higher sub-band was increased significantly before the CS in cued trials, suggesting the prior enhancement of higher sub-band by the conditional cue in cued trials. This finding was consistent with the enhancement of higher sub-band of type 2 theta after the cue, reflecting an increased level of attention to the coming CS by the conditional cue.

**Dependency of the sub-bands of type 2 theta on mAChRs**

In the present study, I used a muscarinic acetylcholine receptor (mAChR) antagonist, scopolamine, because it disrupts the hippocampal type 2 theta, and impairs various types of hippocampus-dependent learning tasks, including those in eyeblink conditioning in rabbits, and mice. I observed that scopolamine suppressed the elevation of pre- and post-CS power of both the sub-bands of type 2 theta in non-cued (Figure 9) and pre-CS power in cued trials (Figure 9). Together these findings suggest
that both the sub-bands of type 2 theta depend on mAChRs which play an important role in the conditional discrimination learning through activation of sub-bands of type 2 theta oscillation.

In addition, I observed that scopolamine did not impair the pre-acquired discrimination despite suppressed the sub-bands of type 2 theta in the serial feature-positive discrimination task. In contrast, it could impair the acquisition of discrimination between cued and non-cued trials in eyeblink conditioning. As scopolamine disrupted the type 2 theta which was found to be strongly correlated with the expression of CR during eyeblink serial feature-positive discrimination task in rat eyeblink conditioning [22], therefore it could be clearly understood the involvement of hippocampal type 2 theta state in acquiring the discrimination between cued and the non-cued trials but not tightly in expression, where the association of other structures other than the hippocampus might be involved.

**EXPERIMENT 2: CONCLUSIONS**

My results suggested the existence of differential sub-bands of type 2 theta. The higher sub-band reflects the cognition of the conditional cue in addition to the general attention to the conditioning situation, leading to acquisition of the discriminative responses in mice whereas the lower sub-band reflects an increase in general attention to the conditioning situation or the behavioral context.
REFERENCES


hippocampus plays an important role in eyeblink conditioning with a short trace interval in glutamate receptor subunit δ2 mutant mice. J Neurosci. 2003; 23(1): 17–22.


18. Wagner AR, Terry WS. Backward conditioning to a CS following an expected versus a surprising UCS. Anim Learn Behav. 1975; 3: 370-374.


