이학석사학위논문

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이 논문을 이학석사 학위논문으로 제출함

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위 원 장 김 상 정 (인)

부 위 원 장 강 봉 균 (인)

위 원 Graham L. Collingridge (인)

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Yeseul Lee

Advisor

Professor Bong-Kiun Kaang, Ph.D.

Professor Graham L. Collingridge, Ph.D.

A Thesis for M.S. Degree in Brain and Cognitive Sciences

Graduate school

Seoul National University

Contents

Abstract

Behavioral studies on the effect of a GSK3β inhibitor, CT99021, in C57BL/6 mice

Yeseul Lee

Department of Brain and Cognitive Sciences,

Graduate School

Seoul National University

Abstract>

Glycogen synthase kinase 3 (GSK-3β) has been implicated in the pathogenesis of Alzheimer's disease, bipolar disorder, schizophrenia and Down's syndrome. Consequently, inhibition of this molecule has been considered as a treatment against the harmful effects or deficit seen in these diseases. One inhibitor of GSK-3β, CT99021, known to be highly specific and potent has been assessed behaviorally in C57BL/6 mice in this study. We found that inhibition of GSK-3β by CT99021 treatment during training enhances acquisition of spatial memory in

particular by increasing the learning rate during the hidden platform session of the Morris water maze task. CT99021 treatment also enhanced precise memory of the location of the platform. However, the treatment of CT99021 did not enhance contextual fear memory, and did not alter behavioral flexibility in the Morris water maze or the delayed match to place task in the T-maze. Taken together, we hypothesis that activation of GSK-3β may act as an impediment during spatial memory acquisition and that when the activity of GSK3β is inhibited by CT99021, learning rate and precise spatial memory can be enhanced. This finding gives an insight into how GSK-3β, which is involved in synaptic plasticity, can affect learning processes and behavior.

Key words: Glycogen Synthase Kinase 3β, CT99021 (CHIR99021), Morris water maze, Synaptic plasticity, NMDA receptor dependent LTD, Behavioral flexibility

Student number: 2011-24034

Introduction>

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine protein kinase that is involved in various central intracellular pathways. Mammalian GSK-3 encodes two isoforms, GSK-3α and GSK-3β, which are 95 % identical. GSK-3β is highly abundant in the central nerve system (CNS) (Woodgett 1990; Lau, Miller et al. 1999) and its expression in the brain increases with age (Lee, Chung et al. 2006). Because of the expression of active GSK-3β increases with in Alzheimer's disease patients (Pei, Tanaka et al. 1997; Pei, Braak et al. 1999), it has been extensively studied as a prominent target to treat neurodegenerative diseases such as Alzheimer's disease and other neurobiological conditions such as bipolar disorder, schizophrenia and Down's syndrome. GSK-3β has been found to be highly involved in cognitive deficits and mental retardation processes in these diseases. Two major forms of long lasting synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) which are thought to be crucial for learning and memory process (Andersen 2007) are often altered or changed in animal model of these diseases, thus deficits in these processes may be of relevance to the symptoms experienced by patients.

Recently, GSK-3β has been reported to have a role in synaptic plasticity (Peineau, Taghibiglou et al. 2007; Peineau, Bradley et al. 2008; Peineau, Nicolas et al. 2009; Collingridge, Peineau et al. 2010). Consequently, inhibition or activation of GSK-3β activity has been proposed to regulate synaptic plasticity and learning and memory related mechanisms in the brain. Since genetic disruption of GSK-3β

cause embryonic lethality (Hoeflich, Luo et al. 2000), inhibitors of GSK-3β or conditional knock out of GSK-3β have been studied instead. In the case of LTP, it has been found that the inhibitory phosphorylation at serine 9 (ser9) is important for the induction of LTP and robust serine-phosphorylation persists after LTP induction (Hooper, Markevich et al. 2007). Also inhibition of GSK-3β potentiated LTP (Son, Yu et al. 2003), and enhanced spatial learning in rat (Nocjar, Hammonds et al. 2007). In addition, decreased activity of GSK-3β in the amygdala was found to enhance fear memory (Maguschak and Ressler 2008). On the other hand, overexpression of GKS-3β or pharmacologically increased activity of GSK-3β led to an LTP deficit (Zhu, Wang et al. 2007) and a spatial learning deficit (Herna´ndez, Borrell et al. 2002; Liu, Zhang et al. 2003).

Lithium, which is a well-known inhibitor of GSK-3 (Klein and Melton 1996; Stambolic, Ruel et al. 1996; O'Brien and Klein 2009), has been used to correct LTP or learning deficits. For example, chronic treatment of lithium potentiated LTP (Son, Yu et al. 2003) or reversed deficits of LTP in animal models of Alzheimer's disease (Ma, Hoeffer et al. 2010), schizophrenia (Nadri, Dean et al. 2004; Lovestone, Killick et al. 2007; Mao, Ge et al. 2009), Down's syndrome (Contestabile, Greco et al. 2013), chronic stress (Silva, Mesquita et al. 2008) and early alcohol exposure (Sadrian, Subbanna et al. 2012).

Not only LTP, but LTD can be affected by GSK-3β activity. By using several inhibitors previous studies found that the induction of NMDA receptor dependent LTD is blocked (Peineau, Taghibiglou et al. 2007) and GSK-3β is solely involved

in NMDAR-LTD among various serine/threonine kinase inhibitors (Peineau, Nicolas et al. 2009).

Since LTP and LTD are important for learning and memory processes in the brain, we investigated the effect of GSK-3β inhibition in learning and memory related behavior in naïve mice with one of the GSK-3β inhibitors, CT99021 (also called CHIR99021). CT99021, which is brain blood barrier permeable, has been shown to be the most potent and specific inhibitor of GSK-3β so far identified (Bain, Plater et al. 2007). CT99021 has been reported to have no effect on LTP (Jo, Whitcomb et al. 2011) but to block the induction of NMDAR-LTD in whole-cell recording system (Peineau, Nicolas et al. 2009). Because CT99021 is known to block the induction of NMDAR-LTD, we began to seek the relation between the blockade of NMDAR-LTD by CT99021 and learning and memory. Therefore, we performed various learning and memory related behavioral tests with C57BL/6 mice to investigate its potential role on learning and memory related mechanism and behavioral outcomes.

< Methods >

Animal>

All of behavioral experiments were done in male C57BL/6 mice (9 - 11weeks for adult, 4 - 5 weeks for juvenile animal; Jackson Laboratories) in this study. 3 ~ 4 mice were housed per cage with 12:12-h light-dark cycle. Water and food were provided all the time except for during the T-maze task. All of experiments were done according to the policy and regulation for the care and use of laboratory animals approved by Animal Care and Use Committee of Seoul National University.

Drug treatment>

The specific GSK3β inhibitor CT99021 was purchased from Cayman (Cayman-13122). The drug was dissolved in DMSO first and then mixed with a solution consisting of 50 % Polyethylene glycol 400 (Fisher), 50 % saline (0.9 % NaCl). The final solution consisted of 10 % DMSO, 45 % PEG400, 45 % saline. The solution was administered by intraperitoneal injection (i.p). Drug treatment was conducted 1 h before behavioral experiments to allow full absorption of the drug. In the delayed match to place in the T-maze, however, the drug was injected 30 min before the test since the T-maze takes 1h per mouse at least. The concentration of CT99021 used in this study was 25 mg/kg. This dose was selected based on a previous behavioral study (Pan, Lewis et al. 2011). In addition, this concentration

was found to block the induction of LTD *in vivo* when administered i.p. to anaesthetized mice (Bortolotto & Collingridge, personal communication).

Behavior test>

All mice used for behavior tests were housed in a holding rack for 30min before tests to avoid unnecessary anxiety induction. For Morris water maze, contextual fear conditioning and delayed match to place in the T-maze, mice were handled for 3 min for 4 days by an experimenter before the test. Mice that underwent open field test were used for elevated plus maze. Except for that, all mice in this study underwent just one behavior test since continuous treatment of CT99021 could alter behavioral outcomes due to chronic inhibition of GSK-3β.

Open Field Test >

Mice were exposed to an open box for 10min and allowed to freely move and investigate the area. Exploratory behavior was assessed by time spent in three zones (10, 20 for center and 40 for peripheral) and locomotor activity was also assessed by total distance moved. Total distance moved and time spent in the box was recorded from the video mounted on the ceiling. The room for OFT (Open Field Test) was kept with dim lighting. The open box was cleaned with 75 % alcohol and distilled water between each trial. The box was comprised of five 40 cm x 40 cm boards (a cubic box without the ceiling).

Elevated plus maze>

A maze for this test was elevated 58 cm from the ground and consisted of two open arms and two closed arms. Mice were placed in the center of the maze facing one open arm and allowed to move freely. The EPM (Elevated plus maze) test was conducted under florescent light and each mice were recorded for 5 min. Time spent in two open arms or two closed arms were summed and averaged. Because the maze is elevated high from the ground, mice with normal level of anxiety tend to spend less time in the open arms compared to the closed arms. All of the recording was analyzed by an animal tracking program (Ethovision 3.1, Nodulus).

Contextual Fear Conditioning>

A Coulbourn chamber (Freeze Frame, Coulbourn, H10-24T) was used in this task and analyzed by Freeze Frame. Mice were handled for 4 consecutive days before the conditioning day. On the conditioning day, mice were placed in a chamber for 3 min. During the first 2 min 28 s, mice were exposed to a novel environment and allowed to explore the chamber without any harmful stimulation and then various intensities (0.2 mA, 0.6 mA, 1.8 mA) of foot shock were given for the following 2 s. Mice were left another 30 s to allow them to remember the context around them and they were immediately replaced back to their home cage (i.e., after conditioning process). 24 h later, on the retrieval day, mice were re-exposed to the environment for 3 min and their freezing behavior was recorded by the camera from the ceiling and analyzed using Freeze Frame.Threshold for freezing behavior

was set at 25 and bout was set at 0.5.

Morris water maze>

All mice that trained in the MWM (Morris water maze) were handled for 4 consecutive days to avoid additional anxiety caused by handling. After 4 days-handling, mice were trained in a gray circular water tank (Diameter: 140 cm, 100 cm in height) for 5days allowing them to remember spatial components around the water tank. Water was kept at 21±1℃ and dim light was used. Nontoxic fragrant-free white colored paint was diluted in the water so as to make it opaque. The submerged platform was painted white (Diameter: 10 cm) to keep it invisible and kept 1 cm below water level. The starting point for each training session was chosen in pseudo-random order. Animals were removed 5 s after they reached the platform during the first two days of training and removed 20 s after during the last three days of training and the reversal learning. On the sixth day (Probe day1) the platform was removed from the tank and mice were placed at the center of the tank and allowed to swim for 1 min. The mice tracking system recorded the time they spent in each quadrant and total distance moved. Four quadrants were designated as target quadrant, opposite quadrant, left quadrant and right quadrant with an imaginary line dividing the tank into four equal areas. On the seventh and eighth days, training for reversal learning was conducted. The platform was replaced to the opposite quadrant from the target quadrant and mice were exposed to the tank. In probe2 day, mice were placed in the center of the tank and allowed to swim for 1 min. All of swimming activity was recorded with a ceiling mounted camera and

analyzed with the program (Ethovision 3.1, Nodulus). The latency (s) to the hidden platform during training and reversal learning were calculated. Time spent in each quadrant and in a zone near to the platform were also recorded and analyzed by the program. The diameter of the water tank was reduced from 140 cm to 80 cm for young animal. All of the visual cues were kept the same as possible. Procedure for the young animal water maze was exactly the same as the procedure for the adult animal.

Delayed match to place in the T-maze task>

The body weight of mice was measured before the test was conducted and the food adjusted (1.5g pellet / 24 ~ 5 g body weight) to reduce the body weight to 85 % of their original body weight. A pellet was given to each mouse so every mouse had their own pellet. Feeding was conducted right after training finished. This feeding scheme was maintained until the last day of the task to maximize the motivation for reward and encourage the movement in the T-maze. The room for the T-maze test was kept under fluorescent light. The maze is a T-shaped maze that consisted of three arms; the starting arm, the left arm and the right arm. At the end of left and the right arms there was a drop of condensed milk (70ul, 1:1 diluted with D.W). Manually operated doors were located at the starting point of each arm. Prior to the test, mice were handled for 4 days for 3 min and habituated for 2 days. During the habituation days, they were exposed to the maze for 10 min to investigate the maze and find edible condensed milk drops. The reward, condensed milk drops, was refilled several times whenever it was consumed and thereby mice were allowed to

go into the same arm as many times as they wanted during habituation days. After 2 days of habituation, five days of training followed. One trial is composed of two sessions, a forced choice and a free choice session. In the forced choice session, mice are place in the starting arm. When the door located in the starting arm is open, they were allowed to forage for a condensed milk drop. Only one of two arms is open (right or left) so they had to go into the open arm to get the condensed milk drop. The following session, free choice session, they were replaced in the starting arm and two arms (right and left) were both open. They had 90s to decide which arm to go. When an animal passed the gate of one of the choice arms, the door closed. They had at least 30 s to consume the condensed milk drop and are then replaced back to the starting arm or its home cage. If they went into the new arm, it was marked as 'Correct' if not, marked as 'Fail'. Moreover, if they did not go into any arm, they are removed from the maze and the result was marked as 'Fail'. Four trials were conducted in a day and the interval between trials was 15 min to rule out the possibility of working memory involvement. After five days of training, reversal learning was performed. Because the open arm is fixed for 5 consecutive days (5 days of training), it was switched to the other side on day 6 and the test was carried on for further 3 days (3 days of reversal learning). If an animal learns and memorizes the location of the reward properly, the number of correct choice will be high and if it does not learn well, the error rate will be high. Also, flexible behavior of an animal can be assessed by counting correct choices they make over trials on reversal learning days. If an animal is able to learn a new rule and form a slightly different memory based on previous information, it may have a

higher chance to go into the new correct arm (previously wrong arm).

Statistics>

All of graphs in this study were created and statistics were calculated in GraphPad Prism 5.01. Mice that fell from the elevated maze were excluded from the analysis.

Results>

Basal anxiety is not altered by the GSK-3β inhibitor CT99021>

To test the basal level of anxiety, the EPM were used in this study. Mice that were treated with the vehicle or CT99021 were placed in the center of the EPM and allowed to investigate the maze. Mice in the control group and the CT99021 group both showed similar time in the closed arms and the open arms (Figure 1) (Treatment, n.s, $p = 0.9997$, 2Way-ANOVA). On average, both group had a tendency to stay in the closed arms more than in the open arms (closed arm: 59.7 ± 8.1 s for control, 69.4 ± 5.9 s for CT99021, open arm: 27.0 ± 8.0 s for control, 16.8 ± 5.3 s for CT99021) (Treatment, n.s, $p = 0.9987$, 2Way-ANOVA,).

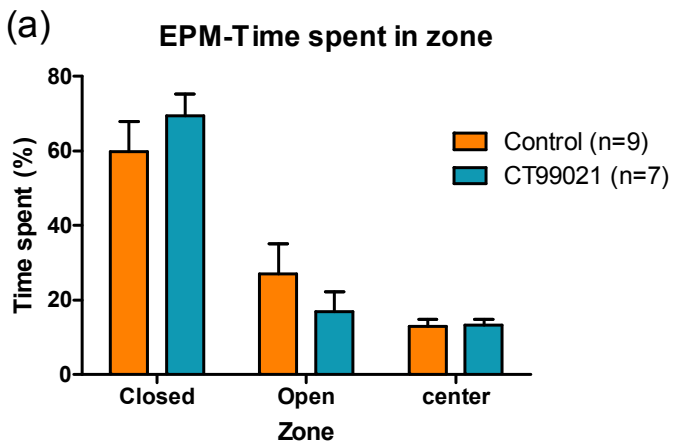


Figure1. CT99021 does not change basal anxiety in the EPM.

(a) Basal anxiety level is not changed in the EPM due to treatment with CT99021. Time spent in different zones (closed arm, open arm, center) (Treatment, n.s, p = 0.9987, 2Way-ANOVA, n = 9 for control, n = 7 for CT99021).

Specific GSK-3β inhibition does not change locomotor activity>

The OFT is widely used test for measuring locomotor activity and basal anxiety. The two groups of mice were tested in the OFT and the total distance moved (cm) and the time spent in zones were measured. Total distance moved by the CT99021-treated group was similar (Figure 2a) (4255.0 ± 225.4 cm for control, 3893.0 ± 236.6 cm for CT99021) compared to the control group (n.s, $p = 0.2784$, unpaired T-test). In addition, both groups had similar time spent in the center zone (10 cm) (10 cm, 2.4 ± 0.4 s for the control, 1.9 ±0.2 s for the CT99021, $n = 14$ for the control, $n = 15$ for the CT99021) and it was significantly less than the time spent in peripheral zone (40 cm) (Figure 2b) (40 cm, 91 ± 1.2 s for the control, 92.5 ± 1.0 for the CT99021). Overall, the treatment of CT99021 did not affect locomotor activity and basal anxiety.

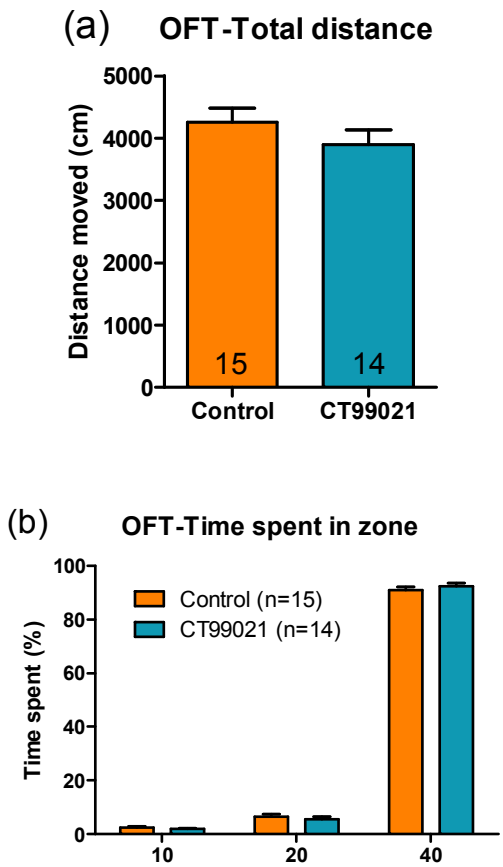


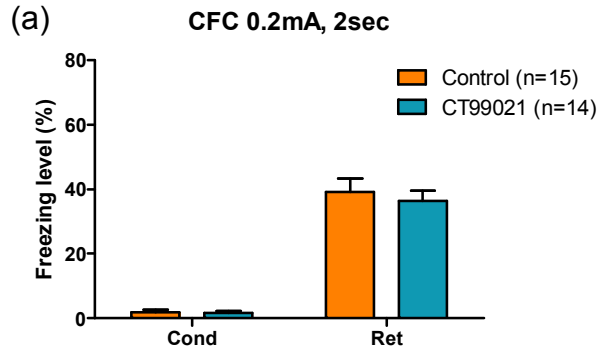
Figure2. CT99021 does not change locomotor activity and explorative behavior

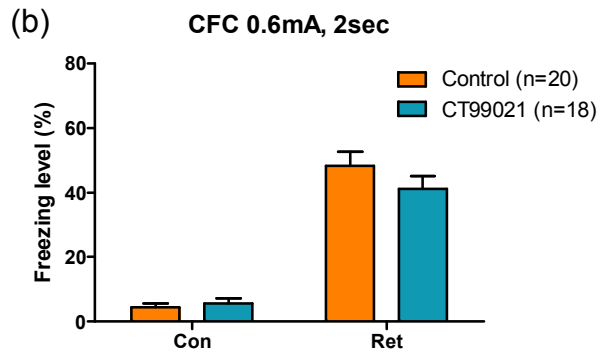
(a) Total distance moved (cm) in an open box. (Treatment, n.s, p = 0.2784, unpaired T-test, n = 15 for control, n = 14 for CT99021)

(b) Percentage time spent in 10, 20 and 40 cm zone. (Treatment, n.s, p = 0.9997, 2Way-ANOVA, n = 15 for control, n = 14 for CT99021)

Hippocampus dependent contextual fear memory>

We next looked into the role of inhibition of GSK-3β during hippocampus dependent memory tests. The contextual fear conditioning paradigm was chosen to assess the effect of GSK-3β inhibition in fear memory. CT99021 or vehicle was injected on the conditioning and retrieval day. Both groups showed low freezing level before foot shock delivery on the conditioning day (under 10 %) (Figure 3a, b, c). On the retrieval day when they were re-exposed to the context, CT99021-treated mice and vehicle treated mice froze about 40 ~ 60 % with various intensities of foot shock (0.2 mA shock, 39.2 ± 4.2 % for control 36.4 ± 3.2 % for CT99021, 0.6 mA shock, 48.2 ± 4.5 % for control, 41.0 ± 4.0 % for CT99021, 1.8 mA shock, 53.5 ± 4.8 % for control 52.5 ± 3.2 % for CT99021). There was no significant difference between vehicle treated group and CT99021-treated group on freezing levels. Therefore, we came to the conclusion that the GSK-3β specific inhibitor CT99021 does not affect acquisition and expression of hippocampus dependent fear memory in contextual fear conditioning paradigm regardless of foot shock intensity.


(a)
CFC 0.2mA, 2sec
Control (n=15)
CT99021 (n=14)
Freezing level (%)
80
60
40
20
0
Cond
Ret


(b)
CFC 0.6mA, 2sec
Control (n=20)
CT99021 (n=18)
Freezing level (%)
80
60
40
20
0
Con
Ret

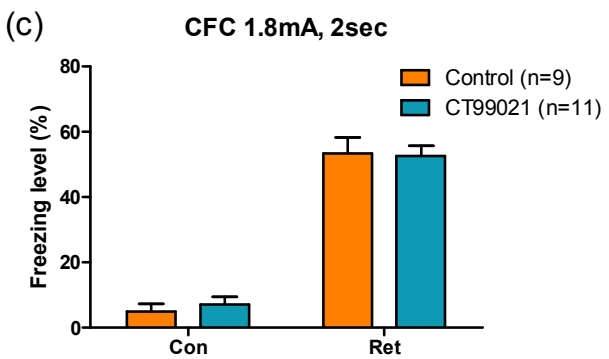

(c)
CFC 1.8mA, 2sec
Control (n=9)
CT99021 (n=11)
Freezing level (%)
80
60
40
20
0
Con
Ret

Figure3. CT99021 does not affect contextual fear memory associated with various intensities foot shocks

(a) Weak intensity (0.2mA) of single foot shock for 2 s (Treatment, n.s, $p = 0.5979$, Repeated measure 2Way-ANOVA)

(b) Single 0.6mA shock for 2 s (Treatment, n.s, $p = 0.3922$, Repeated measure 2Way-ANOVA)

(c) Strong intensity (1.8mA) of single foot shock for 2 s (Treatment, n.s, $p = 0.8607$, Repeated measure 2Way-ANOVA).

Delayed match to place in the T-maze test>

Behavioral flexibility is the ability to change behavior or strategies that have been learned as a result of information from a previous environment, when the animals are placed in a changed new environment. In rodents, many factors that may underlie behavioral flexibility have been investigated. One of the strongest possibilities for altered behavioral flexibility is an LTD deficit. Especially NMDAR-dependent LTD is known to be related with altered behavioral flexibility in transgenic mice that showed no NMDAR-LTD (Kim et al., 2011, Nicholls et al., 2008). Since a GSK-3β inhibitor can block NMDAR-LTD specifically (Peineau et al., 2007) leaving mGluR-LTD intact, we sought to determine whether behavioral flexibility involves GSK-3β signaling. One way to measure behavioral flexibility in mice is to use the 'Delayed match to place T-maze'. Delayed match to place T-maze is a well-established test that can assess one's spatial memory and behavioral flexibility. A calorie restricted diet is used to provide motivation in this test and the average weight of CT99021-treated group and vehicle treated group went down to approximately 75 ~ 80 % of their initial weight over time, but average weight from two groups did not significantly differ from each other (Figure 4a) (Treatment, n.s, $p = 0.1604$, 2Way ANOVA, Interaction, *, $p = 0.0338$, 2Way ANOVA). We speculated that CT99021-treated mice may make fewer correct choices during reversal learning days since there is a lack of NMDAR-LTD due to CT99021 treatment. However, when the CT99021-treated mice were tested in this form of memory test, they made correct choices more than chance level

(50 %) from the first day of training and were comparable to vehicle treated mice (Figure 4b) (Treatment, n.s, $p = 0.4815$, 2Way-ANOVA). Both groups reached a peak of correct choice on training day 4. On reversal learning days, CT99021-treated group mice and the control group leaned the new rule to a comparable extent with more correct choices made by both groups as training continued. We therefore concluded that CT99021 treatment does not impair the ability to learn a new rule, which can be interpreted as behavioral flexibility in this kind of test.

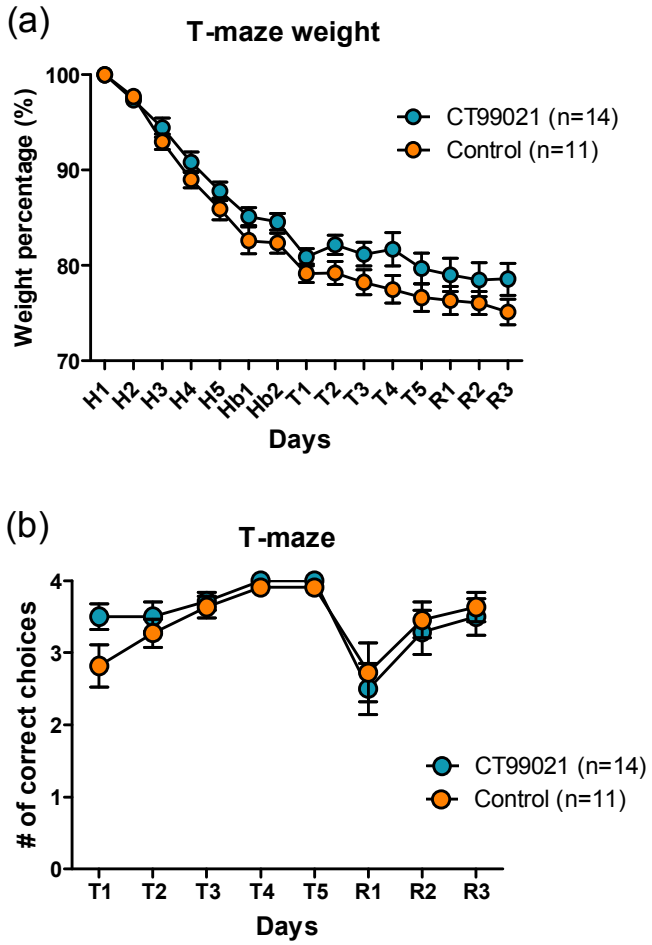


Figure 4. The effect of CT99021 in delayed match to place in the T-maze task.

(a) Averaged percentage of body weight change (Interaction, p = 0.0338, Treatment, p = 0.1605, n = 11 for control, n = 14 for CT99021).

(b) Number of correct choices in delayed match to place in the T-maze task (Treatment, n.s, p = 0.4815, n = 11 for control, n = 14 for CT99021)

Spatial learning rate in is enhanced with the CT99021 treatment >

The Morris water maze is a classic test that is widely used to assess an animal's ability in spatial learning and is dependent on the hippocampus (Morris et al., 1982). We systemically injected CT99021 (25 mg/kg, i.p) daily to mice 1 h before the first trial. Firstly, the escape latency to the hidden platform was measured. The control group and the CT99021-treated group did not show a significant difference in escape latency (Figure 5a, left panel) (Treatment, $p = 0.4761$, Repeated measure 2Way-ANOVA, $n = 15$ for control, $n = 14$ for CT99021). However, there was a strong trend that the CT99021-treated mice found the platform quicker on training day 5 (Figure 5a, right panel) (31.9 ± 2.9 s for control, 23.9 ± 3.1 s for CT99021, Treatment, $p = 0.0692$, unpaired T-test). However, when their path length to the platform was measured, two groups showed significant differences (Figure 5b, left panel). The path length to the hidden platform was significantly shorter in CT99021-treated mice compared to vehicle treated group (Figure 5b, right panel) (Treatment, $p = 0.0220$, Repeated measure 2Way-ANOVA, $n = 15$ for control, $n = 14$ for CT99021). On training day 5, the gap between the two groups was substantially different (Figure 4b) (561 ± 60 cm for Control, 372.8 ± 41.3 cm for CT99021, Treatment, $p = 0.0169$, Unpaired T-test) without velocity changes (Figure 4c) ($p = 0.107$, Unpaired T-test).

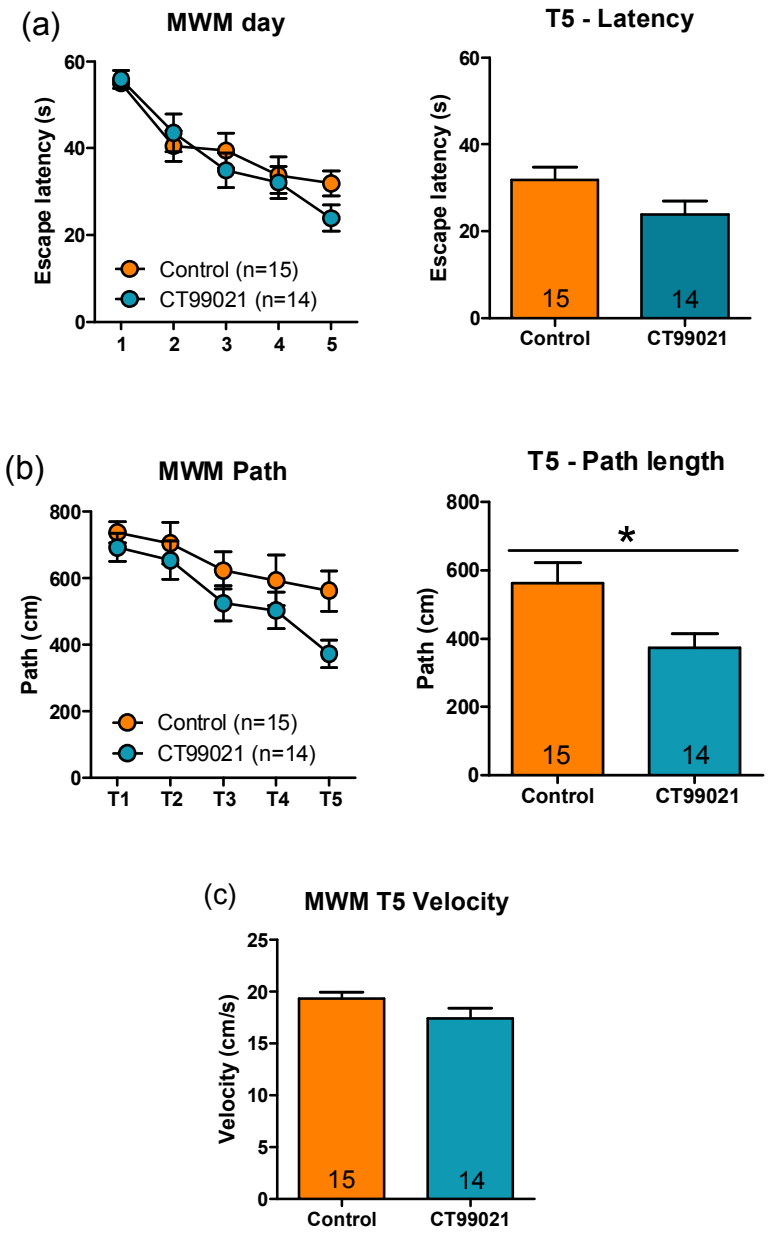

(a)
MWM day
Escape latency (s)
60
40
20
0
1
2
3
4
5
Control (n=15)
CT99021 (n=14)
T5 - Latency
Escape latency (s)
60
40
20
0
15
14
Control
CT99021
(b)
MWM Path
Path (cm)
800
600
400
200
0
T1
T2
T3
T4
T5
Control (n=15)
CT99021 (n=14)
T5 - Path length
Path (cm)
800
600
400
200
0
*
15
14
Control
CT99021
(c)
MWM T5 Velocity
Velocity (cm/s)
25
20
15
10
5
0
15
14
Control
CT99021

Figure5. CT99021 had no effect on latency but reduced path length to the platform during hidden platform session in Morris water maze task.

(a) The escape latency curve during the hidden platform session (Treatment, $p = 0.4761$, Repeated measure 2Way-ANOVA, $n = 15$ for control, $n = 14$ for CT99021) and the latency difference between the control group and the CT99021-treated group on training day 5 (Treatment, $p = 0.0692$, unpaired T-test)

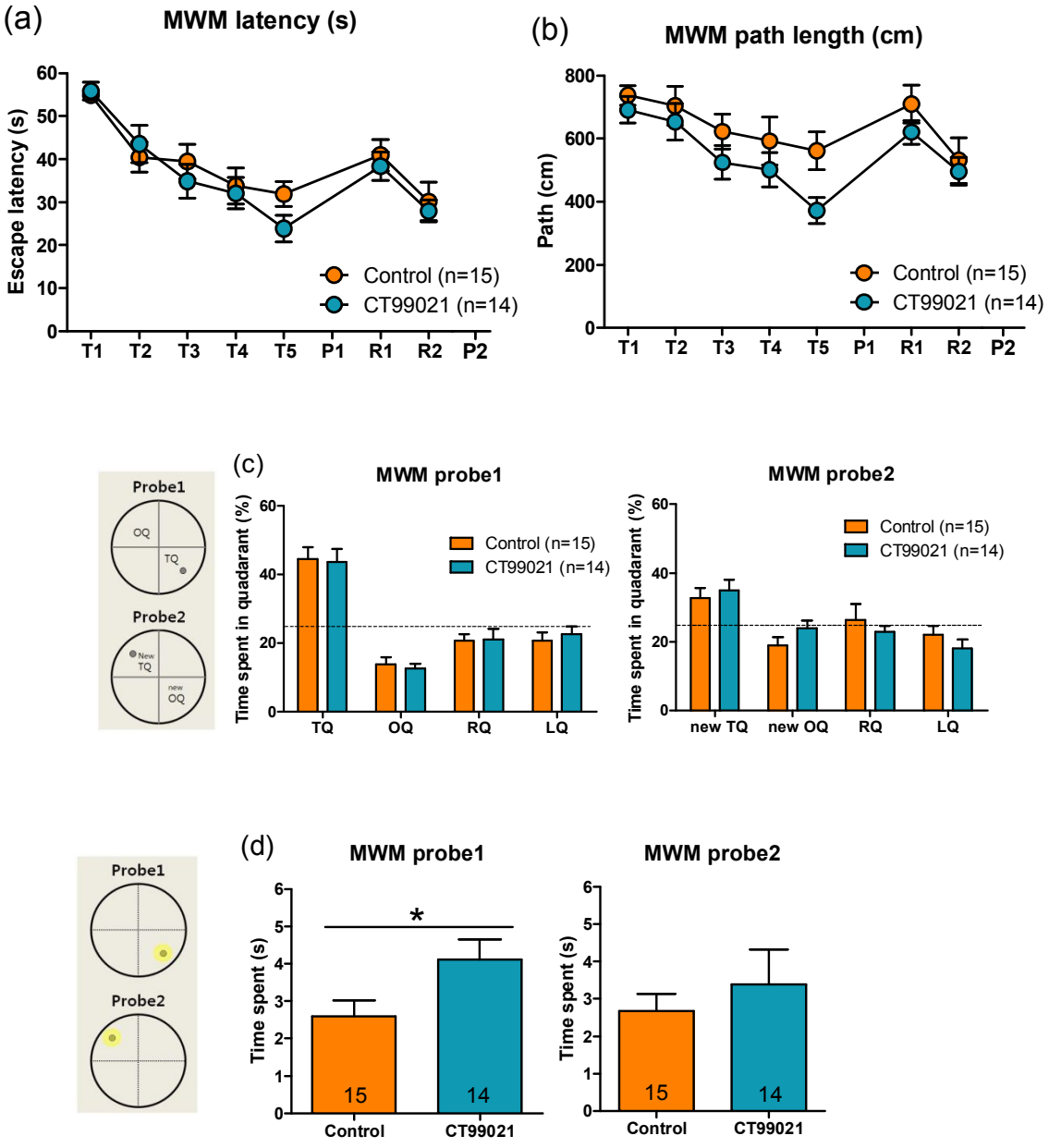
(b) The path length to the hidden platform. The CT99021-treated mice showed shorter path (Treatment, $p = 0.0220$, Repeated measure 2Way-ANOVA, $n = 15$ for control, $n = 14$ for CT99021). The CT99021 treated group showed significantly reduced path length (Treatment, $p = 0.0169$, unpaired T-test)

(c) The averaged velocity of two groups. The treatment of CT99021 did not affect general swim speed on the training day5 ($p = 0.107$, Unpaired T-test).

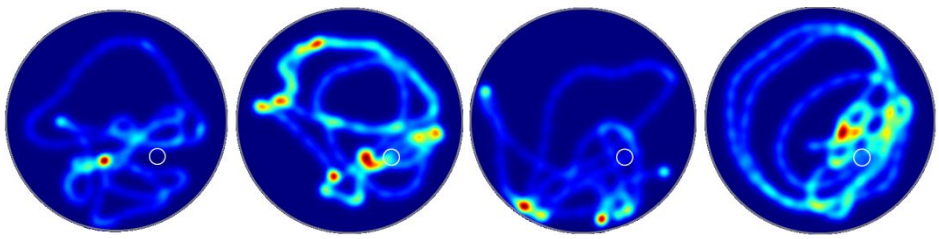
CT99021-treated mice have precise memory but behavioral flexibility is not altered>

In the Morris water maze task, spatial memory of an animal can be seen in the probe test and behavioral flexibility can be seen during the reversal learning. To test the spatial memory, a probe test was conducted on day 6 (probe1) and the time spent in the target quadrant was measured. Time spent in the target quadrant was not significantly different between two groups (Figure 6c, left panel) (Treatment, $p = 0.9996$, 2Way ANOVA). However, in the zone around the platform (defined as a 20 cm diameter area around a 10 cm diameter platform), CT99021-treated mice spent more time in the zone (Figure 6d, left panel) (2.6 ± 0.4 s for control, 4.1 ± 0.5 s for CT99021, Treatment, $p = 0.0341$, unpaired t-test, $n = 15$ for Control, $n = 14$ for CT99021), which implies CT99021-treated group of mice had a more precise memory of the location of the platform. To test behavioral flexibility, we moved the platform to the opposite quadrant and trained the mice for two days (reversal learning) and tested again without the platform (probe test 2). On probe day 2, the CT99021-treated group showed normal preference for the new target quadrant and spent less time in other quadrants including the previous target quadrant (new opposite quadrant) (Figure 6c, right panel). Moreover, the CT99021-treated mice did not spend more time in the zone near the platform (Figure 6d, right panel) (2.7 ± 0.4 s for control, 3.4 ± 0.9 s for CT99021, Treatment, $p = 0.0341$, unpaired t-test, $n = 15$ for Control, $n = 14$ for CT99021). We did not see any

differences in the CT99021-treated group mice during reversal learning indicating that CT99021 does not affect behavioral flexibility in our conditions (Treatment, $p = 0.9996$, 2Way ANOVA). Therefore, we conclude that daily injection of the GSK-3β inhibitor CT99021 has an effect spatial learning and memory, which can be shown by path length and probe test analyze. However, behavioral flexibility analyzed by reversal learning, remains unaltered in CT99021-treated mice.


(a)
MWM latency (s)
Escape latency (s)
Control (n=15)
CT99021 (n=14)
T1 T2 T3 T4 T5 P1 R1 R2 P2
(b)
MWM path length (cm)
Path (cm)
Control (n=15)
CT99021 (n=14)
T1 T2 T3 T4 T5 P1 R1 R2 P2
Probe1
OQ
TQ
Probe2
New TQ
new OQ
(c)
MWM probe1
Time spent in quadarant (%)
Control (n=15)
CT99021 (n=14)
TQ OQ RQ LQ
MWM probe2
Time spent in quadarant (%)
Control (n=15)
CT99021 (n=14)
new TQ new OQ RQ LQ
Probe1
Probe2
(d)
MWM probe1
Time spent (s)
*
15
14
Control
CT99021
MWM probe2
Time spent (s)
15
14
Control
CT99021

(e)



(f)

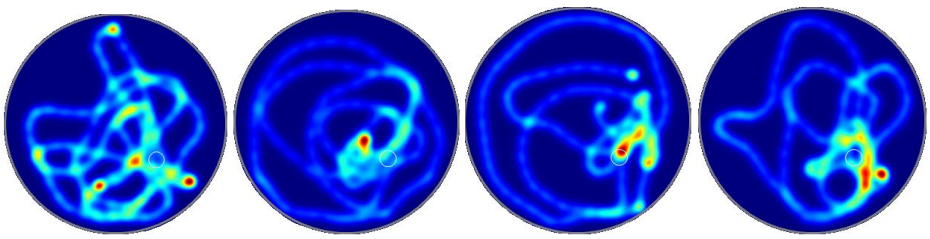


Figure6. GSK-3β inhibition enhances spatial memory but does not affect reversal learning in the Morris water maze task.

(a) The escape latency to the hidden platform during the Morris water maze task. (Treatment, p = 0.4015, Repeated measure 2Way-ANOVA, n = 15 for the control, n = 14 for CT99021) (Trials 1-5 are the same data as shown in Fig 5a).

(b) CT99021 reduced average path length to the platform during hidden platform session of the Morris water maze (Treatment, p = 0.0167, Repeated measure 2Way-ANOVA, n = 15 for control, n = 14 for CT99021), but does not affect the reversal learning (Trials 1-5 are the same data as shown in Fig 5b).

(c) Average time spent in quadrants on probe day 1 (Treatment, n.s, p = 0.9996, n = 15 for control, n = 14 for CT99021) and probe day 2 (Treatment, n.s, p = 0.9996, n = 15 for control, n = 14 for CT99021). (TQ = target quadrant, OQ = opposite quadrant, RQ = right quadrant, LQ = left quadrant)

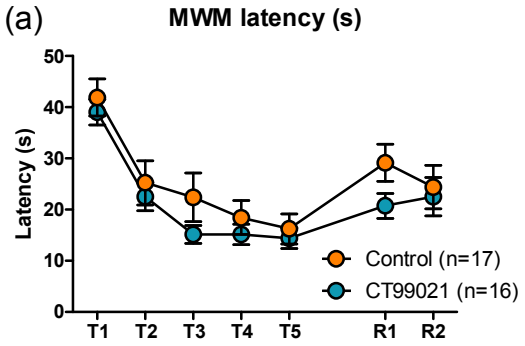
(d) Time spent in the zone around the platform location on probe day1 and probe day2. CT99021-treated mice spent significantly more time near the platform on probe day1 (Treatment, p = 0.0341, unpaired t-test, n = 15 for Control, n = 14 for CT99021) but not on probe day 2 (Treatment, n.s, p = 0.4924).

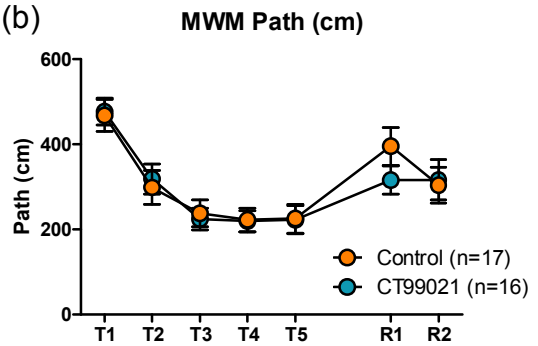
(e) Heat map analysis on the probe day 1 shows traces of control group animals in the water tank. Four representative animals were picked.

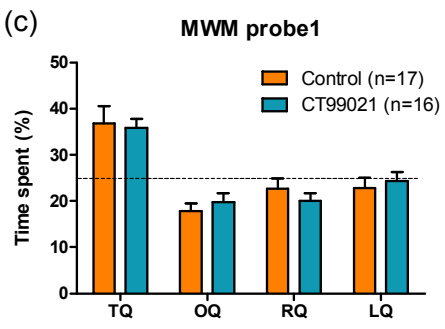
(f) CT99021 treated animals spent considerable time in the zone around the platform. (White circle = the platform)

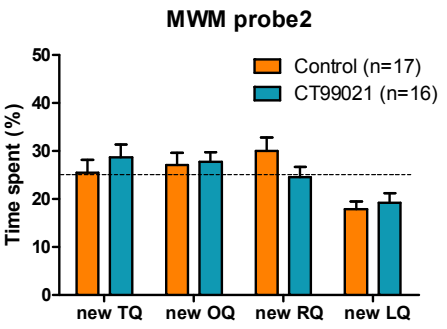
Hippocampus dependent spatial memory in juvenile animal>

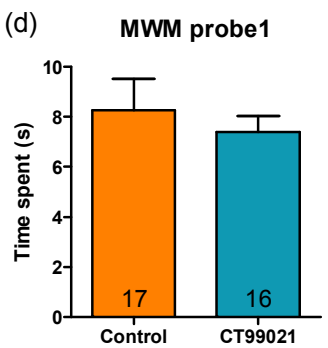
One possible reason why we did not see a reversal learning deficit even though blockade of NMDAR-LTD is expected in CT99021-treated mice is that NMDAR-LTD has been found to decrease developmentally, such that it is harder to obtain in slices from adult animals (Kemp, McQueen et al. 2000) To investigate this possibility, we used juvenile animal (4 - 5weeks old) in which LTD is readily induced in acute hippocampal slices. We shortened the diameter of the tank from 140 cm to 80 cm but all the visual cues and surrounding environment were kept as similar as possible. Due to the smaller tank, mice spent less time in the tank to find the hidden platform. Overall, the two groups of mice learned comparably, and were able to escape from the tank by finding a platform under the water over 5 days of training (Figure 7a, b). When they were tested on probe day 1, the control group mice and CT99021-treated group mice spent comparably more time in the target quadrant spending a lot less time in other quadrants (Figure 7c). When reversal learning was tested (ie. the platform was moved to another quadrant and they were retested) the CT99021-treated group had a shorter latency to find a platform than vehicle treated group on reversal day 1, but the difference was not statistically significant. On reversal day 2, both control group and CT99021-treated group spent similar time in all of quadrant regardless of the location of the hidden platform (Figure 7d). Therefore, control group and CT99021-treated group mice both did not learn the location of the moved platform suggesting juvenile mice are not as flexible in their spatial memory.


(a)
MWM latency (s)
Latency (s)
50
40
30
20
10
0
T1
T2
T3
T4
T5
R1
R2
Control (n=17)
CT99021 (n=16)


(b)
MWM Path (cm)
Path (cm)
600
400
200
0
T1
T2
T3
T4
T5
R1
R2
Control (n=17)
CT99021 (n=16)


(c)
MWM probe1
Time spent (%)
50
40
30
20
10
0
TQ
OQ
RQ
LQ
Control (n=17)
CT99021 (n=16)


MWM probe2
Time spent (%)
50
40
30
20
10
0
new TQ
new OQ
new RQ
new LQ
Control (n=17)
CT99021 (n=16)


(d)
MWM probe1
Time spent (s)
10
8
6
4
2
0
17
16
Control
CT99021

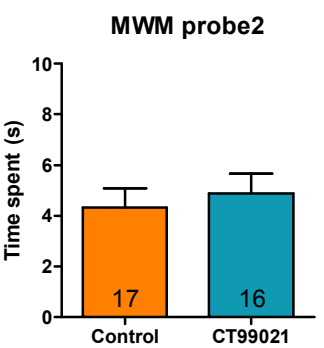

MWM probe2
Time spent (s)
10
8
6
4
2
0
17
16
Control
CT99021

Figure7. CT99021 does not alter path length or latency in Morris water maze in young animal.

(a) Averaged latency (s) to the platform during training and the reversal learning (Treatment, n.s, p = 0.2279, Repeated measure 2Way-ANOVA, n = 17 for control, n = 16 for CT99021).

(b) Averaged path length (cm) to the hidden platform in the Morris water maze (Treatment, n.s, p = 0.7747, Repeated measure 2Way-ANOVA, n = 17 for control, n = 16 for CT99021).

(c) Time spent in quadrants during probe1 and probe day2 (Treatment, n.s, p = 0.9992, Repeated measure 2Way-ANOVA, n = 17 for control, n = 16 for CT99021) and probe day2 (Treatment, n.s, p = 0.9995, Repeated measure 2Way-ANOVA, n = 17 for control, n = 16 for CT99021).

(d) Averaged time spent in the zone around the platform on the probe1 (Treatment, n.s. p=0.5602, Unpaired T-test, n=17 for control, n=16 for CT99021) and probe2 (Treatment, n.s. p=0.6011, Unpaired T-test, n=17 for control, n=16 for CT99021)

Discussion>

We sought to find a correlation between inhibition of glycogen synthase kinase-3β and hippocampus dependent learning and memory by using the most specific known GSK-3β inhibitor, CT99021. We have found that CT99021 reduces the path length to the platform in the Morris water maze task, during the hidden platform session. This suggests that a GSK-3β-dependent process, presumably LTD, is slowing the rate of acquisition of spatial information. In addition, since more time spent in the zone around the platform on probe days can be interpreted as better memory, CT99021 also enhances spatial memory. This is presumably a result of an increase of learning rate during the spatial learning test. In contrast, CT99021-treated mice showed comparable reversal learning rate and spent similar time in the zone around the platform on probe day 2 compared to the control group, suggesting that inhibition of GSK-3β is not involved in reversal learning rate and the behavioral flexibility.

Inhibition of GSK-3β enhances hippocampal spatial learning.

CT99021-treated mice showed a significantly reduced path length to the platform during the hidden platform session and spent significantly more time around the platform area on probe day1. However, we did not see any locomotor activity changes or motor malfunction in the OFT. This indicates that treatment of GSK-3β is specifically affecting the ability to remember the location of the platform.

Altered synaptic plasticity, including lack of LTD or enhanced LTP, could be one possible mechanism by which GSK-3β inhibition can enhance learning. Lithium, which has been extensively studied over many years, has been assessed, tested and used as a therapeutic drug in various diseases models with other GSK-3β inhibitors. It has been reported to enhance LTP in rats (Son, Yu et al. 2003) and switch synaptic plasticity from LTD to LTP-like plasticity in human cortex (Voytovych, Krivanekova et al. 2012). Not only this, but various GSK-3β inhibitors block the induction of LTD (Peineau, Taghibiglou et al. 2007).

Unlike lithium, previous studies of CT99021 have shown that CT99021 does not have an effect on LTP in electrophysiological condition (Jo, Whitcomb et al. 2011). However, we have found CT99021 can block NMDAR-LTD *in vivo* (data not shown). Therefore a possible explanation is that inhibition of GSK-3β can block the induction of LTD *in vivo* and that when spatial learning processes are occurring NMDAR-LTD may act as an impediment. If LTD is prevented by inhibiting GSK-3β, spatial learning processes are enhanced and the learning rate can be increased. This is consistent with previous findings that lithium enhanced learning in rats (Nocjar, Hammonds et al. 2007).

The advantage of using CT99021 to understand the relation between synaptic plasticity and learning & memory.

There have been many studies showing LTP is important for hippocampal spatial memory but fewer studies about the role of LTD on spatial memory. One study showed that a GluN2A-selective NMDA receptor subunit antagonists, which blocks specifically LTP does not affect long term spatial learning, whereas a GluN2B-selective antagonist, which blocks LTD impaired long term spatial learning in rats (Ge, Dong et al. 2010). However, GluN2B receptor is also involved in LTP and synaptic transmission. In addition, Tat-GluA2$_{3Y}$ peptide they used to block LTD in that study blocks not only NMDAR-LTD but also the AMPA receptor endocytosis that is involved in mGluR-LTD. Therefore, neither treatment is specific for NMDAR-LTD.

Another study on the role of NMDAR-LTD in behavior (Nicholls, Alarcon et al. 2008) showed that transgenic mice expressing the SV40 small t antigen exhibit an NMDAR-LTD deficit and also showed deficits in behavioral flexibility, presumably by inhibition of PP2A. . However, effects on other pathways cannot be excluded.

CT99021 does not affect LTP and mGluR-LTD. Even though inhibition of GSK-3β could affect many intracellular mechanism (glucose regulation, insulin secretion, and apoptosis), it is unlikely to impact on the time scale of pharmacological blockade. Based on this, treatment of CT99021 is a new approach that blocks only the induction of LTD. Taken together, this is the first study that shows the specific blockade the induction of NMDAR-LTD, sparing LTP and mGluR-LTD, enhances hippocampal spatial learning.

Juvenile animals showed altered behavioral flexibility.

Another finding we have made in this study is the difference in behavioral flexibility in juvenile animal compared to adult animals. Juvenile animals are often considered to be different from adult animals and vulnerable (Boitard, Etchamendy et al. 2012) since they are not fully developed. Therefore mice in their developmental stage are not often used for behavioral assay. We tested 4-5 weeks old mice in Morris water maze task since LTD is more easily induced in juvenile mice compared to fully developed adult mice. We hypothesized that inhibition of GSK-3β would block the induction of LTD in juvenile and that this alteration in plasticity would lead to behavioral changes during the learning tests. The results differ from our expectation. The vehicle treated juvenile animals showed similar learning rate in path length and latency plot compared to the CT99021-treated juvenile mice which implies that the blockade of LTD has no effects on learning and memory process in juvenile mice. In addition, the vehicle treated mice spent no more time in the new target quadrant than other quadrants on probe day2. This implies that juvenile mice are not flexible in their behavior. Previous studies with multiple reversal learning paradigms have reported that behavioral flexibility is greater in juvenile than adult mice (Johnson and Wilbrecht 2011). Therefore, our finding that behavioral flexibility in Morris water maze cannot be seen in juvenile mice regardless of GSK-3β inhibition requires further investigation.

GSK-3β as a therapeutic target of disease.

As mentioned earlier, GSK-3β has been implicated in the pathogenesis of Alzheimer's disease (Takashima 2006), schizophrenia, Aβ induced neuronal toxicity (Noh, Koh et al. 2009), and bipolar disorder (Gould, Zarate et al. 2004). Therefore, inhibition of GSK-3β activity through various inhibitors, or genetic manipulation using a viral approach, could correct memory deficits in those disease models. We have found that hippocampal spatial learning and memory can be enhanced by a specific inhibitor of this enzyme in naïve animals in this study. Conversely, activation or overexpression of GSK-3β causes spatial memory deficits and tau protein hyperphosphorylation (Herna´ndez, Borrell et al. 2002; Liu, Zhang et al. 2003) which leads to the early development of Alzheimer's disease. Consequently, appropriate activation or inactivation of GSK-3β seems to be crucial for learning and memory process. In conclusion, regulation of this molecule with inhibitors may be a future target for drug development to treat various neurological disorders.

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국문초록

GSK-3β 억제제인 CT99021의 영향에 대한 C57BL/6 생쥐에서의 행동학적 연구

이 예 슬

글리코겐생성효소인산화효소3 (GSK-3β)는 알츠하이머와 조울증, 정신분열, 다운증후군의 병리학기전과 연관이 되어 있음은 잘 알려져 있다. 그 결과, GSK-3β 의 억제가 질병들이 갖는 온갖 해로운 영향과 결함을 막을 수 있을 것인지에 대한 연구가 집중조명을 받아왔다. 이 연구에서는 지금까지 알려진 억제제들 중 가장 특이적이고 강력하다고 알려져 있는 GSK-3β 억제제인 CT99021를 C57BL/6 생쥐에 투여해 그 영향을 행동실험학적으로 평가하였다. 우리는 CT99021의 처리로 인한 학습과정 중 특히 모리스 수중미로 실험의 숨겨진 플랫폼 학습과정에서 학습능률을 높이는 것으로 GSK-3β의 억제가 공간기억의

습득을 향상시키는 것을 확인하였다. 하지만, CT99021의 투여가 조건화된 공포기억은 향상시키지 않았으며, 새로운 규칙을 얼마나 빨리 배우는지 알 수 있는 행동 유연성에도 영향을 미치지 않는 것을 확인하였다. 종합해보았을 때, GSK-3β의 활성은 공간기억의 학습을 늦추는 장애물 역할을 할 수 있으며, GSK-3β의 활성이 CT99021로 저해되었을 때, 학습능률이 오르는 것으로 공간기억을 향상시키는 것을 확인하였다. 이 연구결과는 GSK-3β와 같은 작은 분자의 활성이 시냅스가소성에 어떻게 영향을 주고 그것이 학습, 기억과정, 행동에 어떻게 영향을 줄 수 있는지에 대한 통찰을 돕는다.