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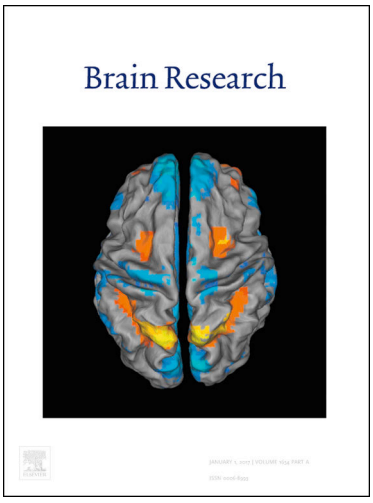

Research report

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The fluctuations of metabotropic glutamate receptor subtype 5 (mGluR5) in the Amygdala in fear conditioning model of male Wistar rats following sleep deprivation, reverse circadian and napping

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Abstract

Sleep is involved in metabolic system, mental health and cognitive functions. Evidence shows that sleep deprivation (SD) negatively affects mental health and impairs cognitive functions, including learning and memory. Furthermore, the metabotropic glutamate receptor subtype 5 (mGluR5) is a metabolic biomarker, which is affected by various conditions, including stress, sleep deprivation, and cognitive and psychiatric disorders. In this research, we investigated the effect of SD and reverse circadian (RC), and two models of napping (continuous and non-continuous) combined with SD or RC on fear-conditioning memory, anxiety-like behavior and mGluR5 fluctuations in the amygdala. 64 male Wistar rats were used in this study. The water box apparatus was used to induce SD/RC for 48 hours, and fear-conditioning memory apparatus was used to assess fear memory. The results showed, fear-conditioning memory was impaired following SD and RC, especially in contextual stage. However, anxiety-like behavior was increased. Furthermore, mGluR5 was increased in the left amygdala more than the right amygdala. Additionally, continuous napping significantly improved fear-conditioning memory, especially freezing behavior. In conclusion, following SD and RC, fear-conditioning memory in contextual stage is more vulnerable than in auditory stage. Furthermore, increase in anxiety-like behavior is related to increase in the activity of left amygdala and mGluR5 receptors.

***Keywords*:** Sleep deprivation; Napping; Amygdala; Fear-conditioning; mGluR5

1. Introduction

Sleep modulates neuronal activity, and synaptic renormalization and neurotransmission in the brain (Puentes-Mestril and Aton, 2017; Qureshi and Jha, 2017). Sleep is also involved in learning and memory processes (Li et al., 2017; Ognjanovski et al., 2018). It has been revealed that oscillations in sleep-related network activity are critically involved in memory storage (Aton, 2013; Inostroza and Born, 2013). Sleep deprivation (SD) impairs memory function. SD can disrupt the consolidation of different types of memory, including fear memory (Chowdhury et al., 2011; Qureshi and Jha, 2017; Rosier et al., 2018; Tripathi and Jha, 2016). On the other hand, the Circadian rhythm is a 24 hours' rhythm, which is generated by molecular clocks and involved in coordinating internal time (Jagannath et al., 2017). Different studies have shown that, reverse circadian (RC) impairs various types of memory, including emotional memory (Albrecht and Stork, 2017; Xia and Storm, 2017). For example, acute shifts in the light-dark cycle impair contextual fear memory in mice (Loh et al., 2010). Furthermore, impaired cued fear memory has been reported in jet-lag model of rodents (Harrison et al., 2017).

Different sub-regions in the amygdala are involved in mediating fear memory (Bergstrom and Johnson, 2014; Fanselow, 2010; Morena et al., 2018). In the amygdala, the metabotropic glutamate receptor subtype 5 (mGluR5) plays a significant role in conditioned fear memory and anxiety-like behavior. In the lateral amygdala (LA), mGluR5 has a significant role in synaptic plasticity and fear memory formation (Rodrigues et al., 2002). In addition, short-lasting form of the long-term potentiation (LTP) can transform into a persistent form via mGluR5 activation (Cohen et al., 1998; Raymond et al., 2000). Given these findings, mGluR5 inhibition may disrupt the function of the amygdala and induce memory deficit. It has been reported that administration of mGluR5 antagonist into the LA impairs the acquisition of auditory fear-conditioning memory (Rahman et al., 2017). Furthermore, NMDA-mediated LTP in the CA1 hippocampal region is completely abolished in transgenic mice lacking mGluR5 (Jia et al., 1998; Lu et al., 1997). Additionally, these transgenic mice are poor in the acquisition of spatial information in the Morris Water Maze (MWM) apparatus (Lu et al., 1997). Further, it has been reported

that injection of the selective mGluR5 antagonist, MPEP (2-methyl-6-(2-phenylethynyl)-pyridine), blocks the acquisition of cued fear-conditioning memory in rats (Schulz et al., 2001).

mGluR5 is related to sleep/wakefulness cycle, so that mGluR5 availability is increased following SD (Holst et al., 2017). However, it is still unknown that increase in mGluR5 availability is a compensatory mechanism to promote wakefulness during SD period, or is a signal which shows the need for sleep, or even both? (Holst et al., 2017). To answer this question, previous study has reported that the positive allosteric modulator of mGluR5, ADX47273, promotes wakefulness and reduces Non-REM sleep and total sleep time, but on the contrary, the selective mGluR5 antagonist, MPEP, increases the duration of total sleep and improves sleep efficiency (Ahnaou et al., 2015). Thus, it seems that changes in sleep/wakefulness cycle can affect the function of mGluR5, or vice versa.

Napping has therapeutic effects on SD-induced memory impairment. Napping also improves various cognitive functions, including memory, emotion regulation, procedural skills and attention (Cellini et al., 2015; Cellini et al., 2016; Gujar et al., 2011). Sometimes, napping is as effective as sleep to facilitate memory processes (Nishida and Walker, 2007). Furthermore, napping facilitates the encoding of new episodic memories, via reinvigorating the networks which are involved in memory (Antonenko et al., 2013). Previous study has shown more generalized fear response after a daytime nap (Davidson et al., 2018). In addition, napping enhances visual working memory performance and verbal memory (Lau et al., 2018; MacDonald et al., 2018).

According to the mentioned findings, the goal of the present study is to investigate mGluR5 signaling fluctuations in the right and the left amygdala following SD and RC, combined with two models of napping.

2. Results

2.1. Latency to first freezing

Latency to first freezing shows the speed of memory retrieval. In fact, it does not specify the amount of memory. One rat can have a poor memory, but show it so fast. On the other hand, another rat can have a strong memory, but show it too late. In this evaluation, low seconds of latency means a strong memory retrieval, not necessarily a strong memory performance; but high seconds of latency means a poor memory retrieval, not necessarily a poor memory performance.

Three-way ANOVA analysis showed that, the main effect of sleep deprivation ($F_{2,35} = 21.75$, $P<0.001$), continuous napping ($F_{1,35} = 8.13$, $P<0.01$) and non-continuous napping ($F_{1,35} = 4.13$, $P<0.05$) in contextual stage was significant. Furthermore, three-way ANOVA analysis showed that, the main effect of sleep deprivation ($F_{2,35} = 34.09$, $P<0.001$) and non-continuous napping ($F_{1,35} = 4.88$, $P<0.05$) in auditory stage was significant. On the other hand, three-way ANOVA analysis showed that, the main effect of reverse circadian ($F_{2,35} = 131.02$, $P<0.001$) and continuous napping ($F_{1,35} =741.83$, $P<0.001$) in contextual stage was significant. Furthermore, three-way ANOVA analysis showed that, the main effect of reverse circadian ($F_{2,35} = 35.67$, $P<0.001$) and non-continuous napping ($F_{1,35} =91.86$, $P<0.001$) in auditory stage was significant (Fig. 1). Post hoc analysis reported that, the sham group had higher latency in contextual stage compared with the intact group. However, in auditory stage, showed lower latency. 48-hour SD group (SD48) had higher latency in auditory stage and lower latency in contextual stage compared with the intact and the sham groups. Furthermore, 48-hour reverse circadian (RC) group (R48) had higher latency in auditory stage and lower latency in contextual stage compared with the intact and the sham groups. Moreover, continuous and non-continuous napping in SD48 group increased latency in contextual stage, but only non-continuous napping decreased latency in auditory stage. In R48 group, continuous napping increased latency in contextual stage and non-continuous napping decreased latency in auditory stage (Fig. 1).

[Fig. 1 somewhere here]

2.2. Freezing

Three-way ANOVA analysis showed that, the main effect of sleep deprivation ($F_{2,35} = 67.81$, $P<0.001$) and continuous napping ($F_{1,35} = 25.54$, $P<0.001$) in contextual stage was significant. Furthermore, three-way ANOVA analysis showed that, the main effect of sleep deprivation ($F_{2,35} = 5.86$, $P<0.01$) and continuous napping ($F_{1,35} = 4.62$, $P<0.05$) in auditory stage was significant. On the other hand, three-way ANOVA analysis showed that, only the main effect of reverse circadian ($F_{2,35} = 176.45$, $P<0.001$) in contextual stage was significant. Furthermore, three-way ANOVA analysis showed that, only the main effect of non-continuous napping ($F_{1,35} = 6.66$, $P<0.05$) in auditory stage was significant (Fig. 2). Post hoc analysis showed that, the sham group had lower freezing only in contextual stage compared with the intact group. Both SD48 and R48 groups had lower freezing in contextual and auditory stages compared with the intact group. Only SD48 group had lower freezing in contextual stage compared with the sham group. Moreover, continuous napping in SD48 group increased freezing in contextual and auditory stages, while non-continuous napping increased freezing only in contextual stage. Furthermore, in R48 group, non-continuous napping increased freezing in auditory stage (Fig. 2).

[Fig. 2 somewhere here]

2.3. Grooming

Three-way ANOVA analysis showed that, the main effect of sleep deprivation ($F_{2,35} = 124.89$, $P<0.001$), continuous napping ($F_{1,35} = 108.60$, $P<0.001$) and non-continuous napping ($F_{1,35} = 85.23$, $P<0.001$) in contextual stage was significant. Furthermore, three-way ANOVA analysis showed that, only the main effect of non-continuous napping ($F_{1,35} = 4.32$, $P<0.05$) in auditory stage was significant. On the other hand, three-way ANOVA analysis showed that, only the main effect of reverse circadian ($F_{2,35} = 87.60$, $P<0.001$) in contextual stage was significant. Furthermore, three-way ANOVA analysis showed that, only the main effect of reverse circadian ($F_{2,35} = 6.21$, $P<0.01$) in auditory stage was significant (Fig. 3). Post hoc analysis showed that, the sham group had higher grooming in contextual stage compared with the intact group. Both SD48 and R48 groups had higher grooming in contextual

stage compared with the intact group, but in auditory stage, only R48 group had higher grooming. Moreover, in SD48 group, non-continuous and continuous napping decreased grooming only in contextual stage (Fig. 3).

[Fig. 3 somewhere here]

2.4. Rearing

Three-way ANOVA analysis showed that, the main effect of sleep deprivation ($F_{2,35} = 4.41$, $P<0.05$) and non-continuous napping ($F_{1,35} = 5.36$, $P<0.05$) in contextual stage was significant. Furthermore, three-way ANOVA analysis showed that, only the main effect of sleep deprivation ($F_{2,35} = 6.60$, $P<0.01$) in auditory stage was significant. On the other hand, three-way ANOVA analysis showed that, the main effect of reverse circadian ($F_{2,35} = 18.43$, $P<0.001$) and non-continuous napping ($F_{1,35} = 5.60$ $P<0.05$) in contextual stage was significant. Furthermore, three-way ANOVA analysis showed that, the main effect of reverse circadian ($F_{2,35} = 13.91$, $P<0.001$) and non-continuous napping ($F_{1,35} = 12.67$, $P<0.001$) in auditory stage was significant (Fig. 4). Post hoc analysis showed that, in SD48 group, rearing was increased compared with the intact group in both contextual and auditory stages, while increased only in auditory stage compared with the sham group. In R48 group, rearing was increased compared with both intact and sham groups in both contextual and auditory stages. Moreover, in R48 group, non-continuous napping decreased rearing in auditory stage (Fig. 4).

[Fig. 4 somewhere here]

2.5. mGluR5 protein blotting

2.5.1. Results of the right hemisphere

Three-way ANOVA analysis showed that, the main effect of sleep deprivation ($F_{2,10}$ =60.94, $P<0.001$) and continuous napping ($F_{1,10}$ = 29.89, $P<0.001$) in dimer band was significant. Furthermore, three-way ANOVA analysis showed that, the main effect of sleep deprivation ($F_{2,10}$ =47.70, $P<0.001$) and continuous napping ($F_{1,10}$ = 6.49, $P<0.05$) in monomer band was significant. On the other hand, three-way ANOVA analysis showed that, the main effect of reverse circadian ($F_{2,10}$ = 46.83, $P<0.001$) and continuous napping ($F_{1,10}$ =20.80, $P<0.001$) in dimer band was significant. Furthermore, three-way ANOVA analysis showed that, the main effect of reverse circadian ($F_{2,10}$ = 36.28, $P<0.001$) and continuous napping ($F_{1,10}$ =14.58, $P<0.001$) in monomer band was significant (Fig. 5). The results of the independent t-test showed that, blotting of mGluR5 protein was increased in the sham group compared with the intact group only in the dimer band. In both SD48 and R48 groups, blotting of mGluR5 protein was increased compared with both intact and sham groups in both dimer and monomer bands. Furthermore, only continuous napping in the dimer band decreased blotting in the SD48 group, while in the R48 group, continuous napping decreased blotting in both dimer and monomer bands (Fig. 5).

2.5.2. Results of the left hemisphere

Three-way ANOVA analysis showed that, the main effect of sleep deprivation ($F_{2,10}$ =114.40, $P<0.001$), continuous napping ($F_{1,10}$ = 22.76, $P<0.001$) and non-continuous napping ($F_{1,10}$ = 7.75, $P<0.05$) in dimer band was significant. Furthermore, three-way ANOVA analysis showed that, the main effect of sleep deprivation ($F_{2,10}$ =76.37, $P<0.001$) and continuous napping ($F_{1,10}$ = 4.80, $P<0.05$) in monomer band was significant. On the other hand, three-way ANOVA analysis showed that, the main effect of reverse circadian ($F_{2,10}$ = 86.13, $P<0.001$) and continuous napping ($F_{1,10}$ =40.37, $P<0.001$) in dimer band was significant. Furthermore, three-way ANOVA analysis showed that, the main effect of reverse circadian ($F_{2,10}$ = 56.64, $P<0.001$) and continuous napping ($F_{1,10}$ =6.61, $P<0.05$) in monomer band was significant (Fig. 5). The results of the independent t-test showed that, blotting of

mGluR5 protein was increased in the sham group compared with the intact group in both dimer and monomer bands. In SD48 group, blotting of mGluR5 protein was increased compared with both intact and sham groups in both dimer and monomer bands, while in R48 group, blotting of mGluR5 protein was increased compared with both intact and sham groups only in the dimer band. In the monomer band, blotting of mGluR5 protein was increased only compared with the intact group. Furthermore, only continuous napping decreased blotting in both SD48 and R48 groups only in dimer band (Fig. 5).

2.5.3. Results of the comparison between the two hemispheres

To compare two hemispheres, the results of the independent t-test showed that, in the sham group, blotting of mGluR5 protein in the left hemisphere was more increased than the right hemisphere in both dimer and monomer bands. Furthermore, in the left hemisphere, mGluR5 blotting was more increased than the right hemisphere in both SD48 and R48 groups in the dimer band, while in the monomer band, only SD48 group showed this effect. Additionally, non-continuous and continuous napping increased blotting in the left hemisphere more than the right hemisphere in SD48 group, only in the dimer band. However, in the monomer band, only continuous napping showed this effect. On the other hand, in R48 group, continuous and non-continuous napping increased blotting only in the monomer band. In the dimer band, only non-continuous napping showed this effect (Fig. 5).

[Fig. 5 somewhere here]

Description of western blot images is also provided in details (Fig. 6).

[Fig. 6 somewhere here]

3. Discussion

3.1. 48-hour SD/RC decreased latency to first freezing and the rate of freezing

As the results showed, 48-hour SD/RC decreased latency to first freezing in contextual stage, but increased it in auditory stage. As mentioned, latency to first freezing shows the speed of memory retrieval. In fact, it does not specify the amount of memory. One rat can have a poor memory, but show it so fast. On the other hand, another rat can have a strong memory, but show it too late. In this evaluation, low seconds of latency means a strong memory retrieval, not necessarily a strong memory performance; but high seconds of latency means a poor memory retrieval, not necessarily a poor memory performance. It seems that 48-hour SD/RC increased the speed of memory retrieval in contextual stage, but not in auditory stage. This is a novel finding; however, previous reports have shown similar results. For example, a previous study has reported chronic fluoxetine treatment impairs contextual fear memory in adult mice, but spares auditory fear memory (Sanders and Mayford, 2016). It has been also revealed that Rac (Ras-related C3 botulinum toxin substrate) in the BLA (basolateral amygdala) is crucial for the reconsolidation of auditory Pavlovian fear memory, while Rac in the CA1 hippocampal region is crucial for the reconsolidation of contextual Pavlovian fear memory (Wu et al., 2014). It seems that contextual fear memory is mediated by the hippocampus, while auditory fear memory is mediated by the amygdala (Kim and Fanselow, 1992). The BLA is an important brain area that is required for memory reconsolidation of auditory fear conditioning (Duvarci et al., 2005), the central amygdala is involved in the acquisition, consolidation, and expression of fear memory (Pitts et al., 2009), and the CA1 region in the hippocampus is critically involved in the retrieval of contextual fear memory (Hoeffer et al., 2011). Thus, we can suggest that 48-hour SD/RC can potently disrupt the function of the amygdala, but not the hippocampus (at least, as much as the amygdala) in fear-conditioning memory task. Furthermore, the decrease in latency to first freezing in contextual stage following 48-hour SD/RC is maybe associated with anxiety (in the following, we will report 48-hour SD/RC increases anxiety-like behaviors). Further, many studies have revealed that SD and sleep disorders can increase irritability in humans and rodents (Marks and Wayner, 2005; Schwarz et al., 2019; Waxmonsky et al., 2017). It's possible that anxiety in contextual stage is more than auditory stage. Thus, we can also suggest this result may be related to 48-hour SD/RC-induced increment in anxiety, irritability and excitability. In general, more detailed reports

are needed to better understand the possible different mechanisms in contextual and auditory fear memory.

Furthermore, the results showed that 48-hour SD impaired fear-conditioning memory (decreased freezing). As we know, sleep has a main role in memory formation and neuroplasticity (Blissitt, 2001). It has been revealed that, after learning a task, activation of the hippocampus during sleep is increased (Gais et al., 2007). Furthermore, there is a significant correlation between the REM phase and memory consolidation (McCarley, 2011). Many studies have shown that SD impairs various types of memory (Nabaee et al., 2018; Ocalan et al., 2019; Patti et al., 2010). SD has also negative effects on attention and decision making (Chen et al., 2017; Goel et al., 2013; Whitney et al., 2015). SD impairs memory formation and decreases hippocampal activity, after learning a task (Kim et al., 2005; Yoo et al., 2007). In addition, REM SD impairs hippocampus-dependent memory processes (Alhaider et al., 2010; Zhang et al., 2013). REM SD also impairs fear-conditioning memory (Nasehi et al., 2018). Thus, a normal sleep per day is crucial for learning and memory formation. Additionally, the results showed that 48-hour RC impaired fear-conditioning memory (decreased freezing). As we know, Circadian rhythm is critically involved in physiology and health by coordinating cellular functions and behavior (Manoogian and Panda, 2017). As expected, disrupted circadian rhythm induces a wide-range of negative effects on cognitive functions. For example, disrupted circadian rhythm attenuates sleep quality (Dijk et al., 2001; Farajnia et al., 2012). Disrupted circadian rhythm also induces cognitive deficits in rodents, including impairment of hippocampal learning and memory (Antoniadis et al., 2000; Craig and McDonald, 2008). In mice, disrupted circadian rhythm decreases dendrite length and neuronal complexity in the prelimbic prefrontal cortex, which is involved in executive functions and emotion (Karatsoreos et al., 2011). Furthermore, disrupted circadian rhythm impairs different types of fear memory (Harrison et al., 2017; Loh et al., 2010). Additionally, rodent models of jet-lag have shown long-term cognitive impairments due to inhibition of hippocampal neurogenesis (Gibson et al., 2010). Thus, circadian rhythm plays an important role in learning, formation of memory and modulating cognitive functions.

3.2. First-night effect: negative effect of environmental changes on memory (the sham group)

Considering the significant difference between the sham group and the intact group, we can point to the role of the new environment or environmental stress in the quality of sleep. Impairment of fear memory in the sham group, due to the fact that no other intervention has been performed except changing the environment, can justify the "first-night effect" phenomenon. This phenomenon says if an animal (or person) is placed in unfamiliar environment for the first time, experiences insomnia at the first night (Lee et al., 2016). Although it (he/she) may experience sleep, but not a complete sleep, because the left hemisphere of the brain (in most animals) has high levels of stimulation for the survival. This phenomenon occurs in all animals which experience sleep, as a physiological response when facing a dangerous or new situation (Tamaki et al., 2016).

3.3. Contextual memory was more vulnerable than auditory memory following 48-hour SD and RC

As the results showed, impairment of fear-conditioning memory in contextual stage was more than auditory stage. The basolateral nucleus of the amygdala mediates environment-related fear memory, and has a significant role in contextual stage due to its' greater distribution of glutamatergic neurons compared with the other parts of the amygdala, and also, due to its' interaction with GABAergic neurons of the central nucleus of the amygdala. It has been shown that, decrease in the activity of GABAergic inhibitory system ultimately increases the activity of glutamatergic system, which leads to memory impairment and lack of fear-conditioning memory consolidation in contextual stage in the basolateral amygdala (Morrison and Ressler, 2014). In this research, we suggest that increase in the activity of glutamatergic system and decrease in the activity of GABAergic system following SD, induced more impairment in fear-conditioning memory in contextual stage.

3.4. Positive effects of continuous napping on SD-induced fear-conditioning memory impairment

We performed two models of napping to evaluate its effect on fear-conditioning memory impairment. Note that, duration of continuous napping was 27mins, which included both REM and Non-REM phases of the rat's sleep. Furthermore, duration of non-continuous napping was 3mins, equivalent only to the REM phase (Twyver, 1969). We did not use EEG (electroencephalogram) to exactly specify the duration of REM/Non-REM phases; however, we cite to the previous research. In future studies, we will definitely use EEG to exactly assess the duration of REM/Non-REM periods. Furthermore, there was a limitation to induce only REM sleep (3mins non-continuous napping). During non-continuous napping REM phase occurred, but Non-REM phase was also possible to induce. Note that, wakefulness is followed by Non-REM phase. Thus, although we induced a REM phase via 3mins non-continuous napping, induction of Non-REM phase (even a slight period) was also possible. The results showed that, continuous napping attenuated fear-conditioning memory impairment induced by 48-hour SD, while non-continuous napping had less positive effect.

At first, it should be noted that, the effect of napping on mGluR5 blotting or SD-induced fear memory impairment has not been investigated yet. Furthermore, there is no published paper about this topic. Napping has enhancement effect on memory, emotion regulation, alertness and other cognitive functions (Cellini et al., 2015; Cellini et al., 2016; Smith-Coggins et al., 2006). In previous studies, beneficial effects of napping have been reported on declarative and emotional memories (Alger et al., 2012; Lahl et al., 2008; Lemos et al., 2014). In a recent study, daytime napping prevents deterioration of procedural memories (van Schalkwijk et al., 2019). In addition, routine mid-day napping may enhance the reaction time on spatial memory task (Ji et al., 2018). In young adults, napping in the afternoon has enhancement effect on the retention of episodic memory (Scullin et al., 2017). Interestingly, previous study has reported that napping enhances knowledge acquisition in young children, with this condition

that, napping should be long-term and continuous to entry into the adequate REM phase to be full of profit (Spano et al., 2018). Furthermore, napping enhances working memory in college students; this enhancement effect is positively correlated with the duration of napping, that should be long-term and continuous to entry into the sufficient REM phase (Lau et al., 2015). Thus, many studies have shown that long-term and continuous napping (including both REM and Non-REM phases) induces more positive effects than non-continuous napping. In other words, although non-continuous napping has some positive effects, but in a wide-range of SD side effects cannot induce significant protective effects, because short-term and non-continuous napping (3mins in this study for the rats) only consists of the REM phase. Thus, we suggest both phases of REM and Non-REM are crucial for the consolidation of emotional memories.

3.5. The opposite relationship between fear-conditioning memory and anxiety-like behavior

As the results showed, in all groups following 48-hour SD and RC, in addition to decrease in freezing (fear-conditioning memory impairment), increase in stereotyped behaviors including grooming and rearing (anxiety-like behaviors) was also observed. Grooming and rearing are equivalent to anxiety index (Lee and Lee, 2018; Lee et al., 2018). Increase in grooming and rearing is related to higher level of GluR5 in the left amygdala via increase in the function of glutamatergic system in this region (Schulz et al., 2001). Anxiety-like behaviors in long-term may lead to serious cognitive and psychiatric disorders, including schizophrenia, obsessive compulsive, substance addiction and behavioral addiction (Fineberg et al., 2018; Ioannidis et al., 2016; Yadav et al., 2018). Furthermore, many studies have reported the relationship between sleep disorders and psychiatric disorders. For example, SD may lead to higher level of impulsivity and increase in unplanned suicidal behavior (Porras-Segovia et al., 2018). SD has an important role in the etiology of postpartum OCD (obsessive-compulsive disorder) (Sharma, 2019). Furthermore, sleep loss is significantly involved in depressive behavior, insomnia, different cognitive impairments and other mood disorders (Benca and Peterson, 2008; Euston and Steenland,

2014; Li et al., 2018). Thus, in this part of the study, it can be noted that despite the impairment of fear-conditioning memory following 48-hour SD and RC, increase in anxiety-like behaviors was also observed. According to this finding, it's recommended that SD should not be used as an adjunct therapy to suppress fear memory, because ignoring the increase in anxiety-like behaviors may lead to serious psychiatric disorders.

3.6. Molecular findings: Increase in mGluR5 blotting in the left amygdala

As the results showed, in most of groups, there was a significant difference between two hemispheres of the brain in mGluR5 blotting (in the left amygdala, mGluR5 blotting was higher than the right amygdala). As mentioned, mGluR5 is associated with the sleep/wakefulness cycle, so that mGluR5 availability is increased during SD (Holst et al., 2017). It has been revealed that the positive allosteric modulator of mGluR5, ADX47273, promotes wakefulness and reduces Non-REM sleep and total sleep time, but on the contrary, the selective mGluR5 antagonist, MPEP, increases the duration of total sleep and sleep (Ahnaou et al., 2015). Thus, it seems that any changes in the sleep/wakefulness cycle may affect the function of mGluR5. It's important to note that the exact correlation between mGluR5 in the left or the right amygdala and contextual or auditory fear memory is not clear and more detailed studies are needed. Furthermore, the exact effect of mGluR5 function on sleep and circadian rhythm is still unknown. On the other hand, we can point to the "laterality". Laterality is the development of specialized functioning in each hemisphere of the brain. In this research, we can point to the laterality as an important mechanism which affects functional anatomy, neurobiology and neurotransmitter systems in the brain. The process of negative emotions including fear is more performed in the right amygdala (Ocklenburg et al., 2016), and also, mGluR5 mediates contextual fear memory (Schulz et al., 2001; Xu et al., 2009). Thus, the more increase in mGluR5 blotting in the left amygdala (versus less increase in the right amygdala) may lead to this result. We suggest doing more detailed studies in future to better understand the interaction between mGluR5 function and fear memory.

Furthermore, the results showed that stereotypes behaviors (anxiety-like behaviors) were increased following 48-hour SD and RC. A large number of clinical and preclinical studies have shown that glutamate has a main role in the pathophysiology of anxiety (Barbosa Neto et al., 2012; Herlenius and Lagercrantz, 2004; McQuillen and Ferriero, 2004). In addition, many basic studies in animals have revealed a strong connection between anxiety and glutamatergic system (Busse et al., 2004; Cortese and Phan, 2005; Nordquist et al., 2008). Further, metabotropic and ionotropic glutamate receptors including mGluR5 can be neurobiological targets in anxiety and stress-related disorders (Harvey and Shahid, 2012; Riaza Bermudo-Soriano et al., 2012). Previous study has reported that anxiety-like behavior potentiates the expression of metabotropic and ionotropic glutamate receptors in the amygdala in both male and female offspring rats, but on the contrary, reduces the expression of these receptors in the hippocampus and the prefrontal cortex (Wang et al., 2015). Additionally, mGluR5 antagonists show efficacy in different animal models of anxiety (Brodkin et al., 2002; Varty et al., 2005). Thus, we can suggest that, higher level of mGluR5 blotting in the left amygdala via increase in the function of glutamatergic system in this region may induce emotional irritability, such as grooming and rearing (Schulz et al., 2001). It should be noted that, in the present study, it's difficult to understand the clear role of the right and the left amygdala in the contextual or the auditory fear memory, and anxiety-like behaviors. In future studies, we will need to assess selective inhibition or activation of mGluR5 in the left or the right amygdala to provide further information on the role of these receptors.

4. Conclusion

In conclusion, our data showed that fear-conditioning memory was impaired following 48-hour SD and RC, especially in contextual stage; while, anxiety-like behaviors were increased. mGluR5 was more increased in the left amygdala than the right amygdala. Furthermore, continuous napping significantly improved fear-conditioning memory, especially freezing behavior; and also, showed more positive effects than non-continuous napping. Thus, under SD and RC conditions, fear-conditioning

memory in contextual stage is more vulnerable than in auditory stage. Moreover, increase in anxiety-like behaviors is due to the increase in the activity of the left amygdala and mGluR5 receptors.

5. Material & Method

5.1. Animals

64 male Wistar rats weighing approximately 200-250g were used. Rats were kept in the standard temperature (22±3°C) and the cycle of light/darkness was 12/12h. Furthermore, this study consisted of eight groups and each group consisted of eight rats. All experiments were performed in two ways: In the SD group, rats were under sleep deprivation for 48 hours. But in the RC group, rats were under sleep deprivation only in the lighting cycle. All rats had ad libitum access to food and water except during the experiments. Our experimental protocol was approved by the Research and Ethics Committee of the School of Advanced Technologies in Medicine, Tehran University of Medical Sciences and done in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 80–23).

5.2. Sleep deprivation apparatus

The water box, an automatic total SD apparatus (BorjSanatAzma Co. Tehran, Iran) was used to induce total SD (Norozpour et al., 2016). The water box was a water tank made of clear Plexiglas (120×30×50 cm) with 4 equal-sized boxes (30×30×50 cm). The water temperature was 30 centigrade degrees. Two small platforms with a diameter of 15 cm and 3 mm edge depth were located next to each other in the middle of the tank. The platforms had some holes at their surface with a diameter of 2 mm to help rats avoid slipping by facilitating water discharge. At first, platforms were submerged a little into the water. Then, platforms regularly moved up and down with the speed of 1 m/s to force rats move constantly, to prevent getting wet. In fact, during using this technique, rats are forced to stay awake to

constantly change their position to avoid getting wet (because platforms are regularly moved up and down into the water). Completion of platforms rotation needed 30 seconds. The peak height of the platforms was 10 cm over the surface of the water to get food and water (holding time = 10 sec). After this time, the platforms were moved 60 mm down in 2.5 seconds and immediately raised to the first position. All rats were familiarized with the apparatus 24h before performing the experiments, for 30 minutes. Behavioral observations during SD have shown that rats are awake 100 percent of the time during using this technique (Norozpour et al., 2016).

5.3. Sleep deprivation models

48h sleep deprivation model: Rats were placed in the SD apparatus for 48 hours. Each rat had free access to water and food.

48h reverse circadian model: Rats were placed in the SD apparatus for 48 hours. In every 24 hours, each rat was deprived from sleep for 12 hours in the opposite of the circadian rhythm. In this model, rats were deprived from 7 am to 7 pm.

5.4. Napping models

Continuous napping: Rats were allowed to sleep for 27 minutes in every 3 hours by turning off the apparatus. This opportunity was given during each model of SD and RC. The duration of continuous napping (27 min) consisted of both REM/Non-REM phases.

Non-continuous napping: Rats were allowed to sleep for 3 minutes in every 20 minutes (totally 27 min in 3 hours) by turning off the apparatus. This opportunity was given during each model of SD and RC. The duration of non-continuous napping (3 min) only consisted of the REM phase.

5.5. Fear-conditioning memory

5.5.1. Fear-conditioning memory apparatus

In this study, fear-conditioning memory apparatus was used to evaluate fear memory. This apparatus included a box (67×53×55 cm). The inner walls of the box were covered with acoustic plates. On the ceiling, there was a camera for viewing the rat and two speakers for audio playback. The loudspeakers were connected to the computer. A light bulb with 24 watts was installed in the corner of the box. The conditioning box (25×25×25 cm), also called training chamber, was placed inside the acoustic box. At the bottom of the box, the parallel bars made of steel with a diameter of 0.3 cm and a parallel spacing of 1 cm were attached to the shock absorber. After each use, the floor was cleaned with water and alcohol. The protocol which was used in this study consisted of three sessions: one session for training and two for tests. At the first session, rats were trained. 2 hours after training, each rat was placed in the SD apparatus (under SD or RC condition) for 48 hours. 2 hours after the completion of 48h SD/RC period, contextual fear memory test was performed. 1 hour later, auditory fear memory test was performed.

5.5.2. Fear-conditioning training

In the first session (training), each rat was placed in the fear-conditioning apparatus and allowed to explore for 5 min for habituation purpose. Then, each rat was placed in the training chamber for 150 seconds and four consecutive shocks with sound (every 30 seconds, one shock) were broadcasted. 30 seconds after the last shock, the rat was removed from the chamber. Each shock was 1 mA with a frequency of 50 Hz for 5 seconds and each sound was 4 kHz, 35 dB and played for 5 seconds. Approximately, this step lasted 5 minutes.

5.5.3 Fear-conditioning memory evaluation

2h after the completion of SD/RC period, in the second session (contextual fear memory test), each rat was placed in the training chamber for 5 minutes without any stimulus. The rate of freezing of each rat was recorded per second. Other behaviors of the rat, grooming and rearing, were also recorded in time.

One hour after the contextual fear memory test, the third session (auditory fear memory test) was performed. The rats were individually transferred to a new chamber with different shape and color and allowed to habituate for 150 seconds in the absence of shocks. Then, 4 consecutive sounds without shock were broadcasted (every 30 seconds: one sound, 4 kHz, 35 dB). 30 seconds after the last broadcast, rats were removed from the new space. Each sound was played for 5 seconds, and this period approximately lasted 5 minutes. The latency to first freezing (speed of memory retrieval) and the freezing time were recorded.

5.6. Extracting the right and the left amygdala

After performing memory tests, rats were placed in a special chamber and killed with CO2 gas. Then, the rats' brain was removed. After a brief wash with normal saline, each brain was placed inside a coronal section matrix suitable for the brain of the rat weighing 200-250g, and a coronal cut was created under the hypothalamus. Then, the right and the left cores of the amygdala were separated and entered into the nitrogen tank (Note: all stages of tissue extraction should not last longer than 2 minutes). After 24 hours, all the specimens were transferred from the tank to an -80°C freezer.

5.7. Experimental procedure

The experimental procedure has been shown in Fig. 7. The rats of all groups were trained in fear-conditioning apparatus. 2h later, they were placed in SD apparatus for 48h to perform 48h SD/RC. 2h after the completion of SD/RC, each rat was placed in fear-conditioning apparatus to assess fear-

conditioning memory in contextual stage. 1h later, fear-conditioning memory in auditory stage was also evaluated. At the end, the amygdala of each rat was extracted to assess mGluR5 blotting.

[Fig. 7 somewhere here]

5.8. Molecular and enzymatic experiments (Western blotting)

As mentioned, after the completion of behavioral experiments, the amygdala was dissected and frozen in liquid nitrogen immediately, and then, stored at −80°C freezer until needed for later analysis.

Protein concentration was measured by spectrophotometry at 230 nm using Picodrop instrument (Picodrop, Hinxton, UK), and the results were acquired as microgram per milliliter. All the samples were loaded in SDS-8% polyacrylamide gel (PH=8.3) and electrophoresis was done at 120 V for 120 minutes, then transferred to polyvinylidene fluoride membrane (Chemicon Millipore Co., Temecula, CA, USA). To block non-specific protein binding sites, membranes were incubated in 5% skim milk (PH=8.6) for 90 minutes. Then, blots were incubated overnight at 4°C with a primary antibody (Abcam, 1:1000 diluted in skimmed milk). On the next day, Tris-buffered saline and Tween 20 (TBST) were used to wash membranes three times and then, blots were incubated for 60 minutes, with secondary antibody (Horseradish peroxidase-linked goat anti-rabbit IgG, Abcam, 1:5000). To detect bounds, blots were exposed by enhanced chemiluminescence (ECL; Amersham, UK) and visualized by exposure to autoradiographic film for adequate time (Ashabi et al., 2017; Mahboubi et al., 2019).

5.9. Statistical analysis

The results of this study are set in two parts: behavioral and molecular. Three-way ANOVA and Post hoc Tukey tests were used to compare the groups. Independent t-test was used to compare molecular changes in the two hemispheres of the brain. The statistically significance level was considered as $P<0.05$.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Legends

Fig. 1. Effect of 48-hour sleep deprivation (SD48), 48-hour reverse circadian (R48) and two models of continuous (+) and non-continuous (-) napping on latency to first freezing in contextual and auditory stages of fear-conditioning memory apparatus. The results are indicated as Mean±S.E.M considering normal distribution of data and homogeneity of variance. *P<0.5, **P < 0.01 and ***P < 0.001 different from the intact group. ^P<0.5, ^^P < 0.01 and ^^^P < 0.001 different from the sham group. $P<0.5, $$P < 0.01 and $$$P < 0.001 different from SD48/R48 groups.

Fig. 2. Effect of 48-hour sleep deprivation (SD48), 48-hour reverse circadian (R48) and two models of continuous (+) and non-continuous (-) napping on freezing in contextual and auditory stages of fear-conditioning memory apparatus. The results are indicated as Mean±S.E.M considering normal distribution of data and homogeneity of variance. *P<0.5, **P < 0.01 and ***P < 0.001 different from the intact group. ^P<0.5, ^^P < 0.01 and ^^^P < 0.001 different from the sham group. $P<0.5, $$P < 0.01 and $$$P < 0.001 different from SD48/R48 groups.

Fig. 3. Effect of 48-hour sleep deprivation (SD48), 48-hour reverse circadian (R48) and two models of continuous (+) and non-continuous (-) napping on grooming in contextual and auditory stages of fear-conditioning memory apparatus. The results are indicated as Mean±S.E.M considering normal distribution of data and homogeneity of variance. *P<0.5, **P < 0.01 and ***P < 0.001 different from the intact group. ^P<0.5, ^^P < 0.01 and ^^^P < 0.001 different from the sham group. $P<0.5, $$P < 0.01 and $$$P < 0.001 different from SD48/R48 groups.

Fig. 4. Effect of 48-hour sleep deprivation (SD48), 48-hour reverse circadian (R48) and two models of continuous (+) and non-continuous (-) napping on rearing in contextual and auditory stages of fear-

conditioning memory apparatus. The results are indicated as Mean±S.E.M considering normal distribution of data and homogeneity of variance. *P<0.5, **P < 0.01 and ***P < 0.001 different from the intact group. ^P<0.5, ^^P < 0.01 and ^^^P < 0.001 different from the sham group. $P<0.5, $$P < 0.01 and $$$P < 0.001 different from SD48/R48 groups.

Fig. 5. Effect of 48-hour sleep deprivation (SD48), 48-hour reverse circadian (R48) and two models of continuous (+) and non-continuous (-) napping on mGluR5 blotting in both dimer and monomer bands in two hemispheres of the brain. The results are indicated as Mean±S.E.M considering normal distribution of data and homogeneity of variance. *P<0.5, **P < 0.01 and ***P < 0.001 different from the intact group. ^P<0.5, ^^P < 0.01 and ^^^P < 0.001 different from the sham group. $P<0.5, $$P < 0.01 and $$$P < 0.001 different from SD48/R48 groups. #P<0.5, ##P < 0.01 and ###P < 0.001 different from the right hemisphere.

Fig. 6. Description of western blot images. In this figure, we have provided a description of western blot images in details. In all boxes (as mentioned in the figure), the blotting from left to right is correlated with the order of groups (from left to right), which is shown in the graph.

Fig. 7. The experimental procedure of study. The rats of all groups were trained in fear-conditioning apparatus. 2h later, they were placed in SD apparatus for 48h to perform 48h SD/RC. 2h after the completion of SD/RC, each rat was placed in fear-conditioning apparatus to assess fear-conditioning memory in contextual stage. 1h later, fear-conditioning memory in auditory stage was also evaluated. At the end, the amygdala of each rat was extracted to assess mGluR5 blotting. (SD = sleep deprivation, RC = reverse circadian, + = continuous napping, - = non-continuous napping).

Author Contributions

P. Kordestani-Moghadam and M. Khani collected animal data. F. Khodagholi analyzed data. S. Vaseghi wrote the manuscript and managed the literature searches. M. Nasehi and M.R. Zarrindast designed the study and the methodology. All authors have approved the final manuscript.

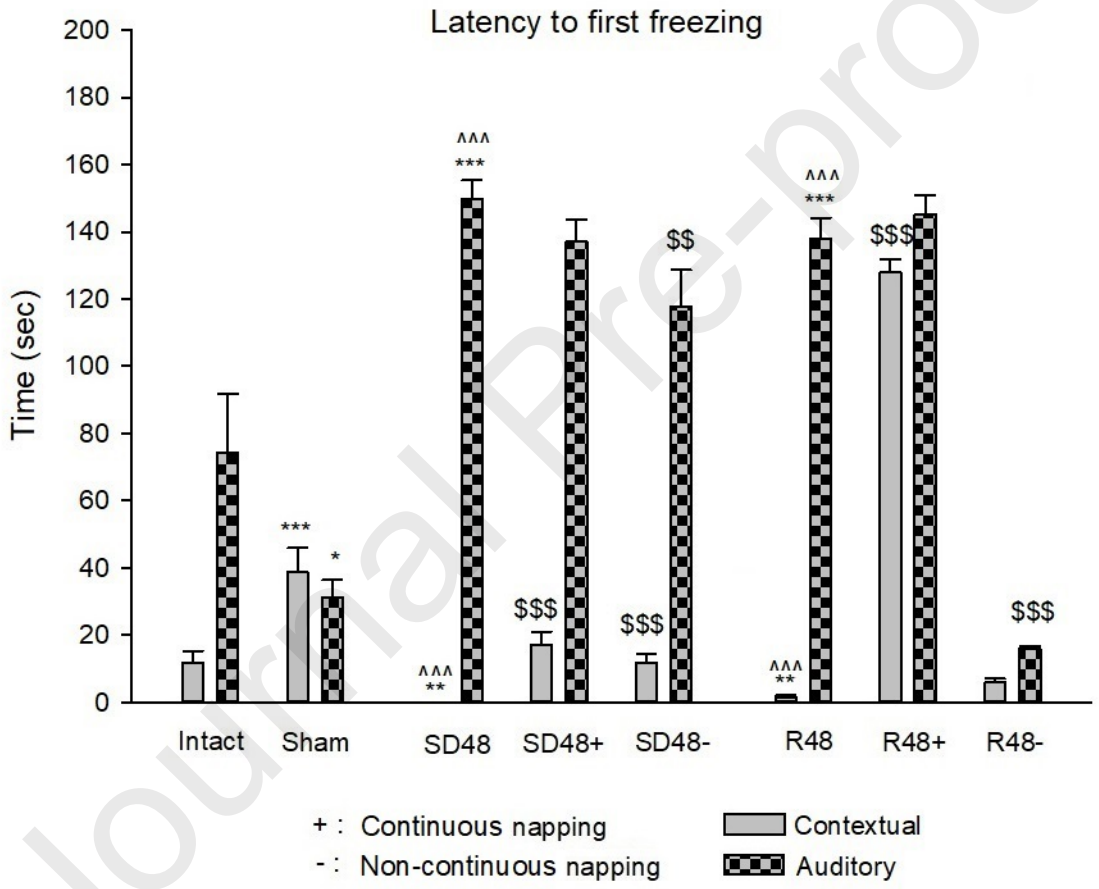


Fig. 1

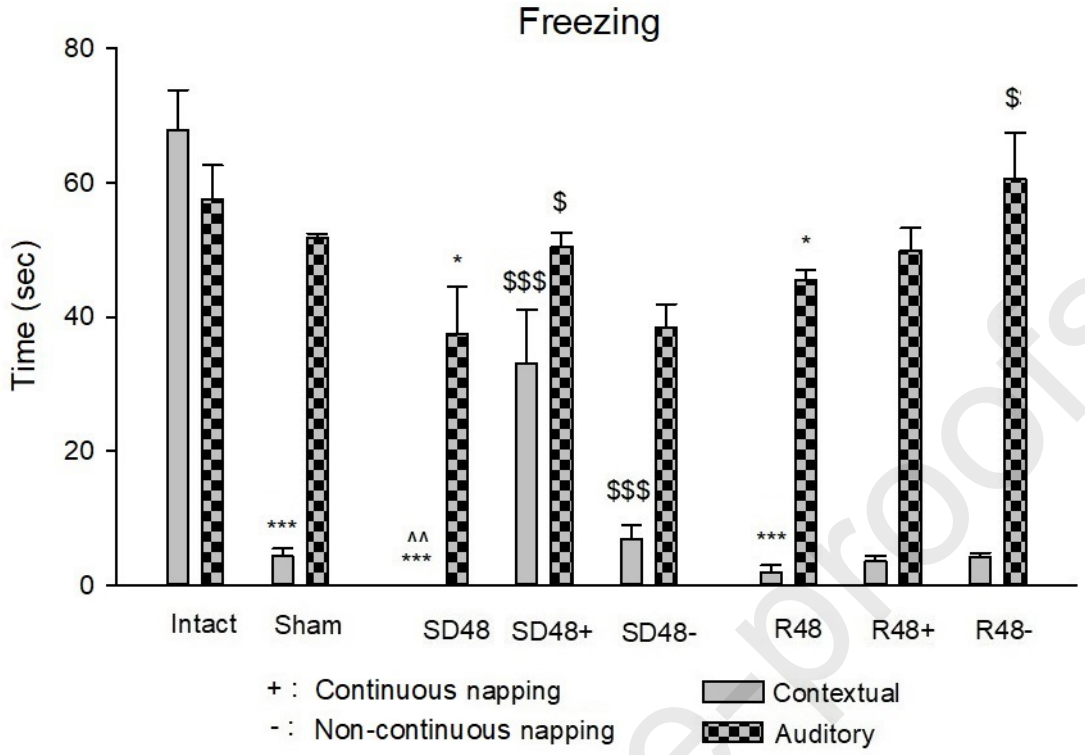


Fig. 2

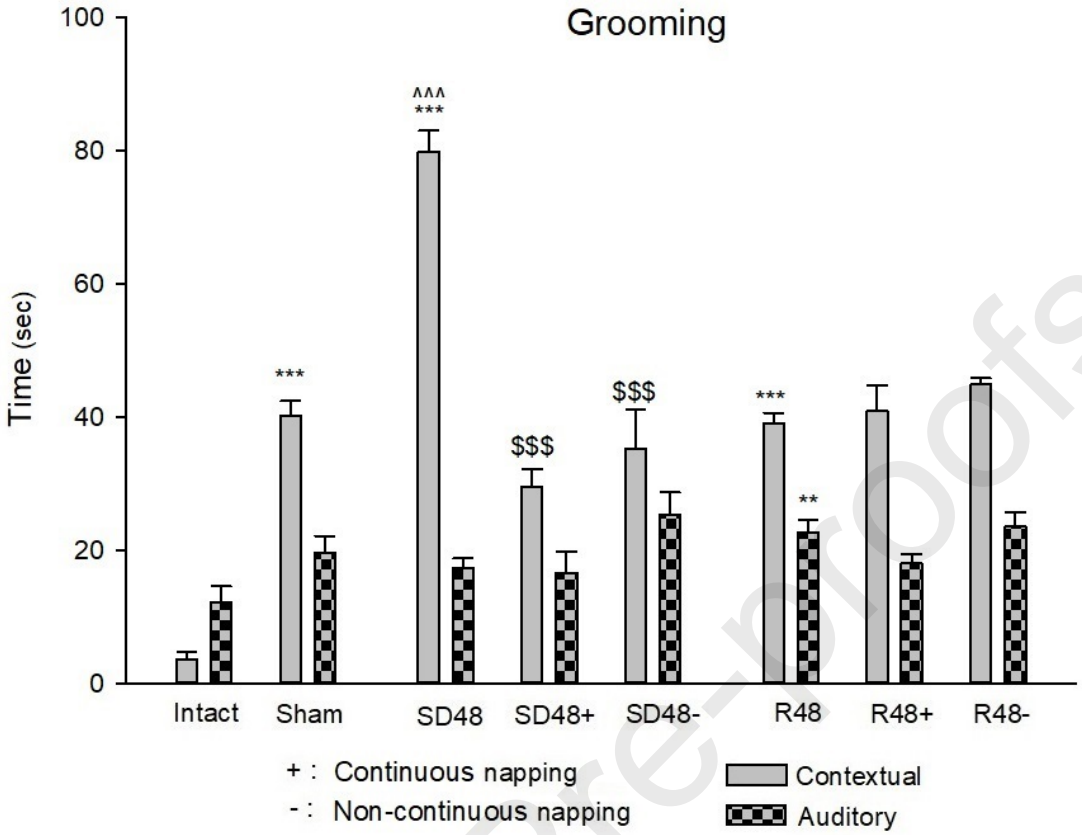


Fig. 3

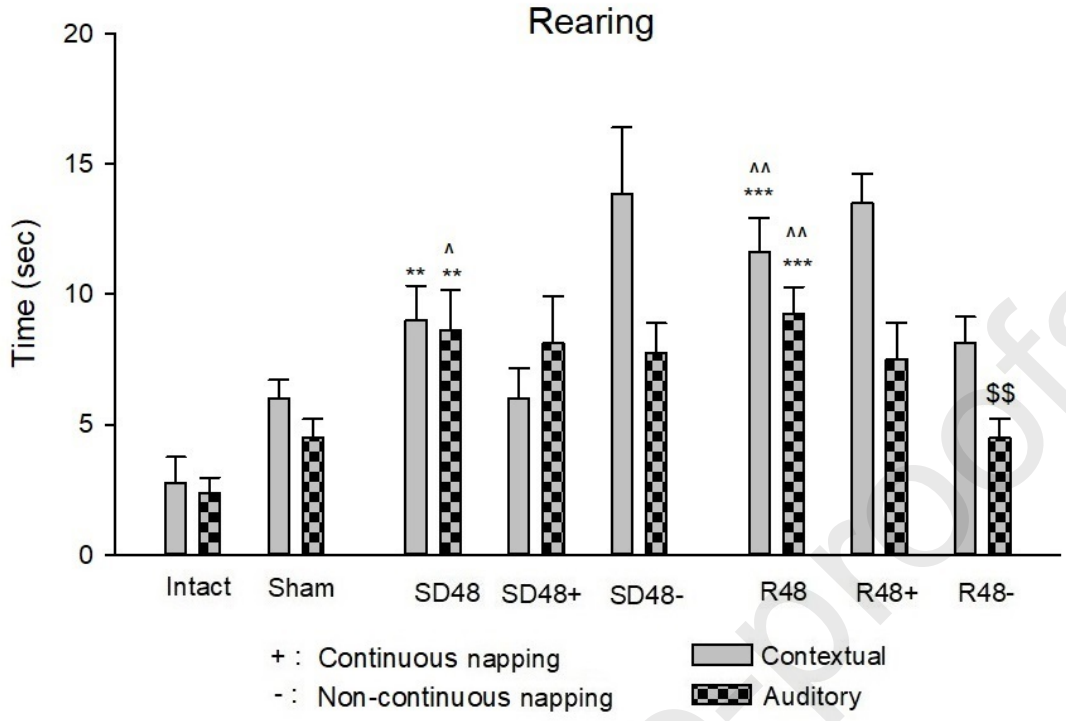
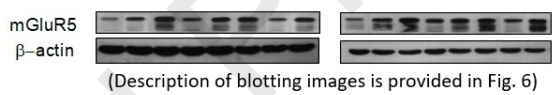


Fig. 4



(Description of blotting images is provided in Fig. 6)

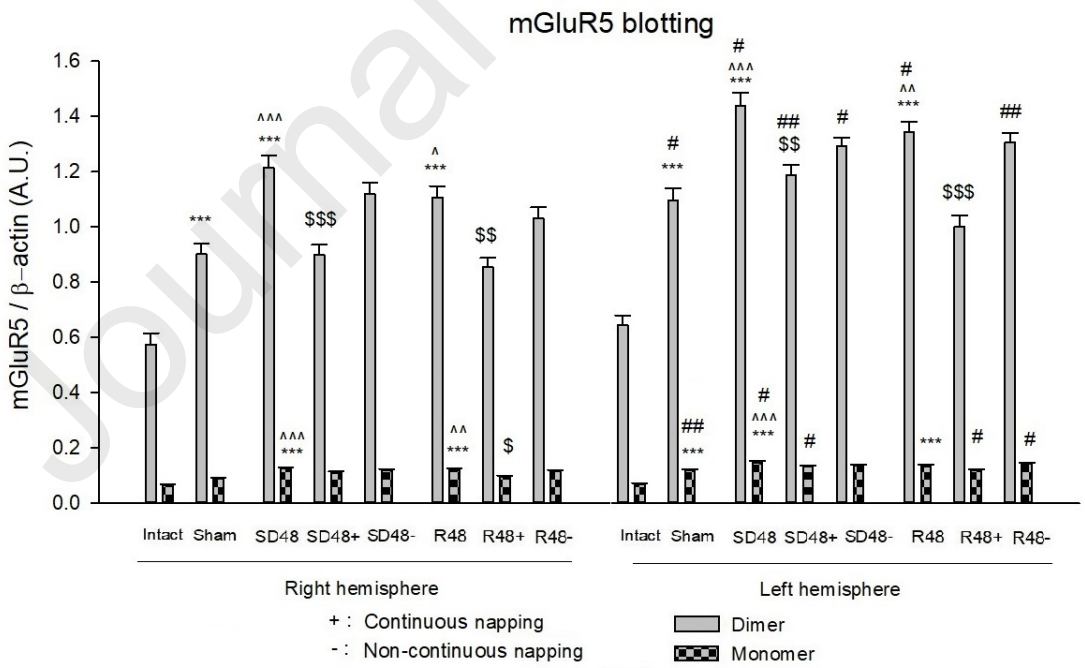


Fig. 5

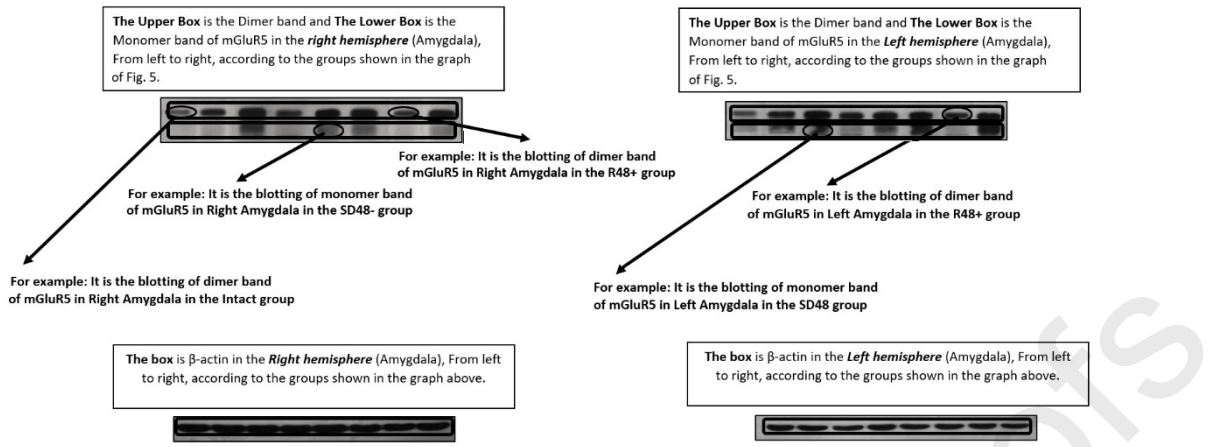


Fig. 6

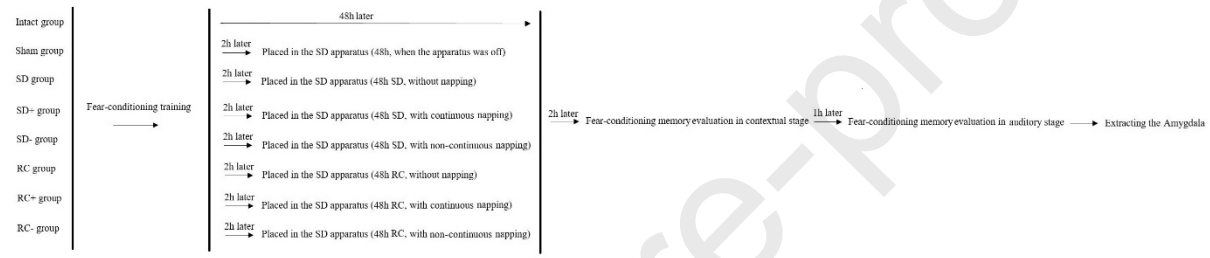


Fig. 7

Highlight Research

- Sleep deprivation (SD)/reverse circadian (RC) impaired fear-conditioning memory
- Anxiety-like behaviors were increased following SD and RC
- mGluR5 was increased in left amygdala more than the right amygdala following SD/RC
- Continuous nap improved fear-conditioning memory more than non-continuous nap
- Fear-conditioning memory in contextual stage was more vulnerable