Master's Thesis of Natural Science

Dependence of trace fear conditioning on Kv1.2 potassium channel subunit in mice

Kv1.2 칼륨 채널 소단위에 대한 생쥐의 흔적 공포 조건화 의존성

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ABSTRACT

Hippocampal CA3 region is believed to play an important role in learning and memory. It is supposed that plasticity contributes to the function of CA3, but it is not clear how plasticity, in particular heterosynaptic plasticity, contributes to the function of CA3. One example of heterosynaptic plasticity is heterosynaptic long-term potentiation (LTP) in CA3 pyramidal cells induced by Kv1.2 downregulation. Because this heterosynaptic LTP could induce long-term changes in CA3, it is likely that the heterosynaptic LTP associates discrete events separated by time interval, but it has not been confirmed in behavioral experiments. To test whether the heterosynaptic LTP induced by Kv1.2 downregulation mediates association of events separated by time interval, two behavioral experiments, trace fear conditioning experiment and transfer of fear experiment, were performed in C3H KCNA2 heterozygous knockout mice, which are deficient in Kv1.2. As a result, trace fear conditioning is impaired in mice lacking Kv1.2. This result indicates that Kv1.2 is required to associate discrete events separated by time interval.

Keywords: CA3 pyramidal cell, Kv1.2, heterosynaptic long-term potentiation, trace fear conditioning, time interval

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LIST OF ABBREVIATIONS

α	Bonferroni adjusted *p*-value
A/C	Associational/Commissural
ACC	Anterior cingulate cortex
CA	Cornu ammonis
CS	Conditional stimulus
DG	Dentate gyrus
EC	Entorhinal cortex
EPSP	Excitatory postsynaptic potential
GC	Granule cell
ITI	Inter-trial interval
LTP	Long-term potentiation
LTP-IE	Long-term potentiation of intrinsic excitability
MF	Mossy fiber
min	Minutes
mPFC	Medial prefrontal cortex
n	Number of samples
P	*p*-value
PC	Pyramidal cell
PP	Perforant pathway
PP-EPSP	Perforant pathway-evoked excitatory postsynaptic potential
PTK	Protein tyrosine kinase
SC	Schaffer collateral
sec	Seconds
STDP	Spike time-dependent plasticity
US	Unconditional stimulus
WT	Wild-type

INTRODUCTION

1. Hippocampus and CA3 subregion

The hippocampus plays an important role in learning and memory, and neural circuits of the hippocampus contribute to the function of the hippocampus (Fig. 1A). Major circuit of hippocampus is trisynaptic circuit, which is a relay of three glutamatergic synaptic transmission: Perforant pathway (PP), mossy fibers (MFs), and schaffer collaterals (SCs). PP mainly projects from pyramidal cells in the entorhinal cortex (EC) to granule cells in the dentate gyrus (DG). Specifically, axons of layer II pyramidal cells of EC project to DG and CA3, whereas axons of layer III pyramidal cells of EC, also called the temporoammonic pathway, project directly the subiculum, CA1, and CA3. MFs project from granule cells in DG to CA3 pyramidal cells, and SCs project from CA3 pyramidal cells to CA1 pyramidal cells. In addition, CA3 pyramidal cells also make synapses with associational/commissural (A/C) fibers other than MF and PP. A/C fibers project from CA3 pyramidal cells to CA3 pyramidal cells and contralateral hippocampus.

CA3 is the subregion of the hippocampus located between DG and CA1, relaying signals from MF and PP to CA1 and CA3 themselves. CA3 is believed to play a critical role in encoding spatial information (Kesner, 2013; O'keefe and Nadel, 1978) and episodic memory (Rolls, 2013; Scoville and Milner, 1957). The function of CA3 may be related to its inputs. CA3 pyramidal cells have three major inputs: MFs, PP, and A/C fibers. MFs have strong but sparse synapses with CA3 pyramidal cells, consisting of the giant bouton of MF and thorny excrescence of pyramidal cells (Henze et al., 2000; Rebola et al., 2017), therefore information can be stored in CA3

pyramidal cells with A/C fibers (Treves and Rolls, 1992). Also, sparse property of synapses between MFs and CA3 pyramidal cells could contribute to pattern separation, which makes discrete representation to similar inputs by amplifying small difference of input patterns (Rolls, 2013). PP is directly connected with EC and provide information about external stimuli from the cortex, therefore PP is important to learning and recall (Rolls, 2013). A/C fibers have the largest number of synapses with CA3 pyramidal cells, therefore autoassociative recurrent network become a key property of CA3 as an attractor. Recurrent property of synapses between A/C fibers and CA3 pyramidal cells could store autoassociative memory and contribute to pattern completion, which makes partial inputs elicit overall activation (Rolls, 2013). It is believed that such property contribute to rapid association like contextual fear conditioning (Lee and Kesner, 2004).

2. Heterosynaptic LTP induced by Kv1.2 downregulation

MFs play role as detonator synapse (Bischofberger et al., 2006), which evokes action potentials in CA3 pyramidal cells and is supported by various types of plasticity between MF and CA3 pyramidal cells (Rebola et al., 2017). MF synapses exhibit plasticity of presynaptic mechanisms such as short-term plasticity involving kainate receptors (Sachidhanandam et al., 2009), post-tetanic potentiation (PTP), depolarization-induced potentiation of excitation (DPE) (Carta et al., 2014), NMDAR-independent mossy fiber LTP (Nicoll and Schmitz, 2005). MFs also induce postsynaptic plasticity to CA3 pyramidal cells like AMPAR-LTP, NMDAR-LTP (Rebola et al., 2011; Rebola et al., 2017). In addition, other synaptic inputs incoming together with MF inputs can induce action potentials of CA3 pyramidal cells through

the mechanisms of associative and heterosynaptic plasticity.

One example of heterosynaptic plasticity is non-hebbian heterosynaptic LTP that is induced after burst stimulation for MF (Fig. 1B). In place fields, peak firing rate of CA3 pyramidal cells and granule cells is more than 10-20 Hz in vivo (Evstratova and Tóth, 2014; Leutgeb et al., 2007; Neunuebel and Knierim, 2012). To observe effects of the firing, soma of CA3 pyramidal cells was stimulated at theta frequency in vitro. As a result, long-term potentiation of intrinsic excitability (LTP-IE) which reduces the input conductance, occurred with a decrease in the D-type K+ current (Hyun et al., 2013). When stimulating presynaptic MF at 20 Hz, the LTP-IE occurred similarly in CA3 pyramidal cells. It is supposed that MF inputs from proximal apical dendrites induce back-propagating action potentials to distal apical dendrites, and then Ca2+ increases. Increased Ca2+ activates protein tyrosine kinase (PTK). PTK phosphorylates the D-type K+ channel subunit Kv1.2, then internalizes and eventually downregulates Kv1.2. LTP-IE induced by MF inputs in CA3 pyramidal cells results in heterosynaptic LTP of PP-evoked excitatory postsynaptic potential (PP-EPSP), because Kv1.2 subunits are concentrated in the distal apical dendrites, where PP synapses are located (Hyun et al., 2015). Unlike spike time-dependent plasticity (STDP) as hebbian LTP, which presynaptic neurons and postsynaptic neurons spike in sequence at short time interval, the LTP-IE and the heterosynaptic LTP are maintained longer. Therefore, even if there is a time gap between MF inputs and PP inputs or PP inputs themselves, they could be associated by the heterosynaptic mechanism in CA3 pyramidal cell.

3. Behavioral experiments with time interval

The heterosynaptic LTP induced by Kv1.2 downregulation is likely to associate discrete events separated by time intervals in animal behavior. If events evoke PP inputs to CA3 pyramidal cells, discrete events separated by time interval evoke temporally discontinuous PP inputs to CA3 pyramidal cells. Each set of PP inputs corresponding to each event separated by time interval can't be associated via hebbian mechanism alone, because PP inputs are weak and discontinuous to induce hebbian LTP between PP and CA3 pyramidal cells. With the support of Kv1.2 dependent heterosynaptic LTP, however, PP inputs could be associated with each other. Once MFs stimulate CA3 pyramidal cells at appropriate frequencies above 20 Hz, Kv1.2 dependent heterosynaptic LTP could occur and amplify PP-EPSPs. Increased PP-EPSP by the heterosynaptic LTP allows synapses between PP and CA3 pyramidal cells to be enhanced by hebbian LTP induced by PP inputs. Therefore, hebbian LTP could occur at multiple synapses between PP and CA3 pyramidal cell by multiple sets of PP input corresponding to events, as long as Kv1.2 dependent heterosynaptic LTP is maintained. In addition, it is likely that Kv1.2 dependent heterosynaptic LTP is maintained for a long time in vivo as in vitro. Therefore, it is possible that discontinuous PP inputs evoked from discrete events can be associated with specific neuronal assemblies of CA3 pyramidal cells where repetitive MF inputs are reached and then Kv1.2 dependent heterosynaptic LTP has occurred. This hypothesis can be tested as behavioral experiments that require association of events separated by time intervals. There are two behavioral tasks, in which animals should associate episodic memories with time interval: trace fear conditioning experiment and 'transfer of fear' experiment (Cai et al., 2016).

In trace fear conditioning, the unconditional stimulus (US) is delivered after time

interval in sec after the end of the conditional stimulus (CS). After successful trace fear conditioning, mice should have a freezing behavior for the unconditional stimulus, even if these stimuli are separated by time interval. Trace fear conditioning depends on several regions of brain (Raybuck and Lattal, 2014), like medial prefrontal cortex (mPFC) (Gilmartin and Helmstetter, 2010; Gilmartin and McEchron, 2005b), anterior cingulate cortex (ACC) (Han et al., 2003), entorhinal and perirhinal cortices (Esclassan et al., 2009; Kholodar-Smith et al., 2008), amygdala (Selden et al., 1991), and hippocampus (Chowdhury et al., 2005; Cox et al., 2013; Misane et al., 2005). Trace fear conditioning depends on DG and CA1 region among the hippocampus (Gilmartin and McEchron, 2005a; Huerta et al., 2000; Rogers et al., 2006; Weitemier and Ryabinin, 2004), but there was little experiment with CA3.

In transfer of fear experiments, mice are exposed to two discrete contexts separated by time interval in hours (5 hours), then they are shocked in one context. As a result, mice show contextual fear not only in context in which mice were shocked, but also in context in which mice were previously exposed, as if the fear had transferred. It is supposed that the two contextual memories are linked because the CA1 neuronal ensembles for each context are likely to overlap when mice experience each context in 5 hours.

4. Aim of this study

To observe whether the heterosynaptic LTP assists animals to associate discrete events separated by time interval, trace fear conditioning experiment and transfer of fear experiment were performed in heterozygous KCNA2 knockout (KCNA2 +/-)

mice in which only half of the Kv1.2 subunits exist compared to wild-type (WT) mice, resulting in MF-induced heterosynaptic LTP of PP-EPSPs being impaired. Experiment results indicate trace fear conditioning of KCNA2 +/- mice is impaired, which could mean that LTP-IE induced by Kv1.2 downregulation mediates the association of discrete events separated by short time interval.

MATERIALS AND METHODS

1. Subjects

Fifty one male C3H mice (10-20 weeks old) were used. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University. Animals were maintained on a 12-hour light/dark cycle, and experiments were performed during light phase.

Before the behavioral experiment, all animals were handled for 2 min per day for 3 days. On the day of the experiment, the animals were brought to the laboratory with a home cage, and then habituated for at least 20 min.

Then the animals were transferred to individual cages, and then transported to a room with a conditioning chamber and habituated for 5 min (Burman et al., 2014).

Heterozygous Kv1.1 knockout mice (KCNA1 +/-) and heterozygous Kv1.2 knockout mice (KCNA2 +/-) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA; Donating Investigator: Dr Bruce Tempel, Univ. of Washington School of Medicine), and maintained by intercrossing heterozygous knockout mice. KCNA1 +/+ mice or KCNA2 +/+ mice were used as wild-type mice. KCNA1 gene encodes Kv1.1 potassium channel subunit and KCNA2 gene encodes Kv1.2 potassium channel subunit.

Heterozygous knock-out mice were used instead of homozygous knock-out mice, because homozygous knockout mice have a lifespan of only 13-18 days after birth (Brew et al., 2007).

2. Behavioral apparatus

The conditioning chamber (45 cm wide × 45 cm long × 60 cm high) can be configured as three context. One context (Context A) is a rectangular test cage (18 cm wide × 18 cm long × 28 cm high; H10-11M-TC; Coulbourn Instruments, USA) which is rectangular and consists of a metal grid floor, aluminum side walls, and a transparent front door and back wall. The electrical stimulator (H13-15-220, Coulbourn Instruments) shocks the animal through metal grid floor in the context A. Another context (Context B) is rectangular (16 cm wide × 16 cm long × 20 cm high) and consists of a white acrylic floor, and a white acrylic wall with a black check pattern. The other context (Context C) is cylindrical (15 cm diameter × 18 cm high) and consists of a white acrylic floor, and a white acrylic wall.

The experimental protocol was performed by Ethovision XT 13 software.

3. Trace fear conditioning experiment

Four KCNA1 +/- mice, six KCNA2 +/- mice, and sixteen wild-type mice were used for trace fear conditioning experiment.

In the trace fear conditioning protocol, the intensity of the tone has been reduced to decrease the aversive nature of the tone itself, and longer inter-trial interval (ITI) were used to reduce the interference at training (Burman et al., 2014; Seo et al., 2015). Within wild-type mice, unpaired training were performed to eight mice on day 1 as control group.

Trace fear conditioning experiment were performed for three days (Fig. 2A).

On day 1, training was performed during 1400 sec session. Mice were placed in context A, then the tone (20 sec, 75 dB) was delivered at 300, 490, 740, 1020, 1180 sec. 20 sec after each tone ends, footshock (2 sec, 0.5 mA) was delivered. ITI were

ranged from 160 to 280 sec. Unpaired training was performed during 1400 sec session, which was consisted of random sequence of five tones and five foot shocks. Interval between stimuli were ranged from 60 to 160 sec. The tone was delivered at 300, 490, 740, 1020, 1180 sec. The footshock was delivered at 240, 420, 620, 940, 1280 sec. After each training, the chamber was cleaned with 70% ethanol.

On day 2, context fear test was performed during 5 min session. Mice were placed for 5 min in the same context A in the same chamber. After each test, the chamber was cleaned with 70% ethanol.

On day 3, tone fear test was performed during 840 sec session. Mice were placed in the novel context B in the other chamber. The tone (20 sec, 75 dB) was delivered at 300, 400, 510, 630, 740 sec. After each test, the chamber was cleaned with 1% ammonia.

4. Transfer of fear experiment

Seven KCNA1 +/- mice, six KCNA2 +/- mice, and seven wild-type mice were used for transfer of fear experiment (Cai et al., 2016).

Trace fear conditioning experiment were performed for five days (Fig. 4A).

On day 1, context exploration was performed. Mice were placed in pre-exposed context (Context B) for 5 min. After mice were removed, the chamber was cleaned with 70% ethanol. After 5 hours, mice were place in context A for 5 min. After mice were removed, the chamber was cleaned with 30% isopropanol.

On day 3, two day later, training was performed during 6 min session. Mice were placed in context A, and then the footshock (2 sec, 0.7 mA) was delivered at 3, 4, 5 min. After each training, the chamber was cleaned with 30% isopropanol.

On day 5, two day later again, context fear test was performed. Mice were placed in the pre-exposed context (Context B) for 5 min. After each test, the chamber was cleaned with 70% ethanol. After 2 hours, mice were placed in novel context (context C) for 5 min. After each test, the chamber was cleaned with 1% ammonia. After 2 hours again, mice were placed in context A for 5 min. After each test, the chamber was cleaned with 30% isopropanol.

5. Data analysis

Animal behavioral experiments were recorded in video and then analyzed. Freezing behavior in which the movement of the animal stops was calculated manually in time. Freezing ratio is defined as the percentage freezing behavior time to total time.

Statistical data were expressed as means ± standard error of means (SEM) and error bar indicates the standard error of means. *n* represents the number of samples. Nonparametric tests were used to determine the difference in distribution between groups. The two-tailed Wilcoxon rank-sum test (Mann–Whitney U test) was used for two independent groups and the two-tailed Wilcoxon signed-rank test was used for two paired groups. The Kruskal–Wallis H test was used to compare three or more independent groups, and Wilcoxon rank-sum test with the Bonferroni correction was used as *post hoc* test. The Friedman test was used to compare three or more paired groups, and Wilcoxon signed-rank test with the Bonferroni correction was used as *post hoc* test. P indicates *p*-value, and significance was determined to be less than P=0.05. α indicates Bonferroni adjusted p-value, and significance was determined to be less than α when applying the Bonferroni correction. Statistical tests were performed using IBM SPSS Statistics 25 software.

RESULTS

1. Deficiency of Kv1.2 impairs trace fear conditioning

Trace fear conditioning experiment was performed in three genotype mouse groups, KCNA1 +/-, KCNA2 +/-, and wild-type groups. Unlike KCNA2 +/- mice, the mossy fiber-induced LTP-IE still occurred in KCNA1 +/- mice (Hyun et al., 2013), but trace fear conditioning experiments were performed on KCNA1 +/- mice as well as KCNA2 +/- mice, because general excitability changes of neuron caused by modification of Kv1 channels can affect animal behavior (Robbins and Tempel, 2012). For trace fear conditioning, the tone and footshock separated by 20 sec time interval were delivered to mice. As a control group, some wild-type mice were subjected to unpaired conditioning instead of trace fear conditioning (Burman et al., 2014). For the unpaired training group, footshocks and tones were delivered like trace fear conditioning groups, but sequence of them and time intervals between them were randomized.

On day 1, mice were subjected to paired (Fig. 2C) or unpaired training (Fig. 2D) in context A. When paired training in trace fear conditioning groups, 20 sec tone was delivered first as a conditioned stimulus. 20 sec after the tone ended, a 2 sec foot shock (0.5 mA) was delivered as an unconditioned stimulus (Fig. 2B). This procedure was repeated five times. Unpaired training consists of five tones and five footshocks like the trace fear conditioning, but temporal order of them is irregular with variable time interval. When mice were pre-exposed to the conditioning chamber for 5 min, they showed active behavior ($\chi^2_{(3)}$=3.249, P=0.355; WT: 20.82±2.28%, n=8; KCNA1 +/-: 15.79±6.86%, n=4; KCNA2 +/-: 18.83±3.02%, n=6;

Unpaired WT (4 min): 17.44±2.79%, n=8), but after five footshocks were delivered to the mice, they showed an increase in freezing behavior.

On day 2, to test the mice for a contextual fear memory formation, the mice were put in the same context A for 5 min (Fig. 2E). All groups showed similar and high levels of freezing behavior ($\chi^2_{(3)}$=2.261, P=0.52; WT: 46.19±3.76%, n=8; KCNA1 +/-: 61.97±6.28%, n=4; KCNA2 +/-: 49.07±5.72%, n=6; Unpaired WT: 52.60±8.58%, n=8) compared to before shock (Before shock vs. After shock, WT: P=0.012, n=8; KCNA1: P=0.028, n=4; KCNA2: P=0.069, n=8; Unpaired WT: P=0.012, n=8), although KCNA1 group shows no significant difference.

On day 3, the fear response to a tone was tested for the mice in a novel context B (Fig. 3). In the tone fear test, five 20 sec tones were delivered to the mice at 100 to 120 sec intervals. During the baseline period for 5 min, all groups showed similar low levels of freezing behavior ($\chi^2_{(3)}$=1.817, P=0.611; WT: 23.51±3.47%, n=8; KCNA1 +/-: 24.60±7.58%, n=4; KCNA2 +/-: 17.43±8.61%, n=6; Unpaired WT: 20.35±3.99%, n=8). But when tones were delivered, each group shows different levels of freezing behavior. During the tone period, wild-type and KCNA1 +/- groups showed higher levels of freezing behavior, while freezing behavior of KCNA2 +/- group was lower than wild-type and KCNA1 +/- group ($\chi^2_{(3)}$=10.234, P=0.017; WT: 62.41±5.61%, n=8; KCNA1 +/-: 58.22±13.89%, n=4; KCNA2 +/-: 26.35±5.09%, n=6; Unpaired WT: 41.67±8.59%, n=8; WT vs. KCNA2 +/-: P=0.003; KCNA1 +/- vs. KCNA2 +/-: P=0.038; α=0.0083), although the difference between KCNA1 group and KCNA2 group was not significant compared to α. During the trace period, which are defined as 20 sec period after tone ends in which animals expect US timing (Raybuck and Lattal, 2014), wild-type group showed higher levels of freezing

behavior, while freezing behavior of KCNA2 +/- and unpaired training group was lower than wild-type group ($\chi^2_{(3)}$=10.61, P=0.014; WT: 72.01±5.77%, n=8;KCNA1 +/-: 62.42±14.67%, n=4; KCNA2 +/-: 38.57±7.29%, n=6; Unpaired WT: 38.84±7.36%, n=8; WT vs. KCNA2 +/-: P=0.008; WT vs. Unpaired WT: P=0.005; α=0.0083). During the inter-trial interval (ITI) period, defined as a period between the trace period and the next tone period, wild-type group showed higher levels of freezing behavior, while freezing behavior of KCNA2 +/- and unpaired training group was lower than wild-type group behavior ($\chi^2_{(3)}$ =7.979, P=0.046; WT: 59.05±6.68%, n=8; KCNA1 +/-: 47.09±12.30%, n=4; KCNA2 +/-: 29.84±4.27, n=6; Unpaired WT: 36.18±6.79%, n=8; WT vs. KCNA2 +/-: P=0.013; WT vs. Unpaired WT: P=0.028; α=0.0083), although differences were not significant compared to α. Altogether, the KCNA2 +/- group showed lower levels of freezing behavior similar to unpaired training control group within tone and trace periods, indicating that KCNA2 deletion impairs the trace fear conditioning.

2. Deficiency of Kv1.1, not Kv1.2, impairs transfer of fear

For the transfer of fear experiment, mice are placed in a neutral context and in a context where footshocks were delivered with a time interval of 5 hours. After successful training, mice are expected to show high levels of freezing behavior in the neutral context as well as in the footshock context. Transfer of fear experiment were performed in three genotype mouse groups, KCNA1 +/-, KCNA2 +/-, and wild-type groups, like trace fear conditioning experiment.

On day 1 (Fig. 4B), mice were exposed to context B and then context A for 5 min each, which were separated by 5 hours interval. In these contexts, all groups showed

active behavior in context B ($\chi^2_{(2)}$=0.274, P=0.872; WT: 9.35±3.36%, n=7; KCNA1 +/-: 8.24±1.56%, n=7; KCNA2 +/-: 7.68±1.00%, n=6). In context A, likewise, all groups showed active behavior ($\chi^2_{(2)}$=4.084, P=0.13; WT: 16.97±1.57%, n=7; KCNA1 +/-: 10.98±2.73%, n=7; KCNA2 +/-: 11.00±2.44, n=6).

On day 3, mice were placed in context A (Fig. 4C), in which footshocks were delivered after a 3 min baseline period. During the baseline period for 3 min, they also showed active behavior ($\chi^2_{(2)}$=1.144, P=0.564; WT: 19.36±5.15%, n=4; KCNA1 +/-: 12.32±6.21%, n=4; KCNA2 +/-: 16.72±3.70%, n=3). After three footshocks (2 sec, 0.7 mA) were delivered to the mice, they had high levels of freezing behavior ($\chi^2_{(2)}$=1.144, P=0.564; WT: 85.29±4.95%, n=4; KCNA1 +/-: 82.55±5.62%, n=4; KCNA2 +/-: 73.66±10.77%, n=3).

On day 5 (Fig. 4D), the mice were tested for freezing behavior in context B, a novel context C, and then context A with two hours interval each. Firstly, mice were placed in context B for 5 min. In context B, wild-type group showed higher levels of freezing behavior than the KCNA1 +/- group ($\chi^2_{(2)}$=6.208, P=0.013; WT: 46.88±4.50%, n=7; KCNA1 +/-: 27.96±4.83%, n=7; KCNA2 +/-: 44.14±9.52%, n=6; WT vs. KCNA1 +/-: P=0.011; α=0.017). Two hours later, mice were placed in a novel context C for 5 min. In a novel context, all groups showed low levels of freezing behavior ($\chi^2_{(2)}$=1.58, P=0.454; WT: 22.16±4.87%, n=7; KCNA1 +/-: 17.74±3.28%, n=7; KCNA2 +/-: 13.92±2.00%, n=6). Lastly, 2 hours later, the mice were tested for contextual fear memory formation in context A for 5 min. In context A, all groups showed high levels of freezing behavior ($\chi^2_{(2)}$=0.927, P=0.629; WT: 53.61±10.43%, n=7; KCNA1 +/-: 47.94±9.65%, n=7; KCNA2 +/-: 42.01±7.50%,

n=6). In the KCNA2 +/- group ($\chi^2_{(2)}$=9, P=0.011, n=6; context A vs. context B: P=0.753; context A vs. C: P=0.028; context B vs. C: P=0.028; α=0.017) as well as the wild-type group ($\chi^2_{(2)}$=10.571, P=0.005, n=7; context A vs. context B: P=0.612; context A vs. context C: P=0.018; context B vs. context C: P=0.018; α=0.017), the freezing ratio in context B and context A was higher than the freezing ratio in context C, although differences were not significant compared to α, and there was no difference between the freezing ratio in context B and context A. In the KCNA1 +/- group, the freezing ratio in context B and context A was also higher than the freezing ratio in context C, but the freezing ratio in context A was higher than that of context B ($\chi^2_{(2)}$=12.286, P=0.002, n=7; context A vs. context B: P=0.043; context A vs. context C: P=0.018; context B vs. context C: P=0.018; α=0.017), although differences were not significant compared to α.

Altogether, wild-type and KCNA2 +/- groups showed high levels of freezing behavior in context B as well as in context A that delivered footshocks. However, in the KCNA1 +/- group, mice showed lower levels of freezing behavior in context B than that in context A that delivered footshocks.

DISCUSSION

How downregulation of Kv1.2 subunits induces heterosynaptic LTP has been studied in vitro. However, it is not known whether the heterosynaptic LTP can affect animal behavior. In this study, it is revealed that Kv1.2 mediates trace fear conditioning in mice. Therefore, LTP-IE mediated by Kv1.2 could play an important role in association of two episodic memories with time interval of tens of sec.

Since general KCNA2 knockout mice were used in experiments instead of CA3 region-specific knockout mice, it is difficult to observe the relationship between behavioral experimental results and heterosynaptic LTP in CA3 pyramidal cells induced by Kv1.2 downregulation. Because KCNA2 deficiency is present in overall body, it is not allowed to rule out the possibility that trace fear conditioning is regulated by Kv1.2 subunits in brain area other than the hippocampal CA3 region, because Kv1.2 is expressed in other areas which are related with trace fear conditioning like mPFC, ACC, entorhinal and perirhinal cortices, and amygdala (Finnegan et al., 2006). Nevertheless, the hippocampal CA3 region exhibits the highest expression of Kv1.2 (Grosse et al., 2000) (Sheng et al., 1994), and it is established that Kv1.2 is essential for heterosynaptic LTP (Hyun et al., 2015), the discussion will mainly focus on the role of Kv1.2 in CA3 pyramidal cells. To observe the relationship between behavioral experimental results and heterosynaptic LTP in CA3 pyramidal cells induced by Kv1.2 downregulation, behavioral experiments should be performed in CA3 region-specific knockout mice.

1. Trace fear conditioning depends on Kv1.2

In the trace fear conditioning experiment, KCNA2 +/- mice group showed low levels of freezing behavior like unpaired control than that of wild-type and other voltage gated potassium channel subunit knockout (KCNA1 +/-) mice group. Dependence of trace fear conditioning on Kv1.2 might be explained by heterosynaptic LTP induced by MF stimulation.

In the trace fear conditioning experiment, it is likely that PP inputs reflect external stimuli in CA3 pyramidal cells, therefore PP inputs from footshocks and PP inputs from tones should be associated to learn relationship between US and CS. Such association is usually supported by LTP. In general, LTP occurs via hebbian mechanism such as spike-timing-dependent plasticity (STDP), which requires delivering inputs in close time in ms. In trace fear conditioning, however, PP inputs conveying from footshocks and tones may be delivered to CA3 with time interval in sec. Such temporally discontinuous synaptic inputs cannot be associated by STDP. In mPFC, neurons keep firing at trace interval, therefore it is likely that such sustained firing can help associate two discontinuous stimuli (Gilmartin et al., 2013). However, such firing pattern was not found in hippocampus during trace intervals (Gilmartin and McEchron, 2005b).

With the support of Kv1.2 dependent heterosynaptic LTP, however, PP inputs can be associated by hebbian LTP. It makes sense that MF could stimulate CA3 pyramidal cells during trace fear conditioning, resulting from novel context or stimuli like footshocks or tones. Once MFs stimulate CA3 pyramidal cells at adequate frequency more than 20 Hz, the heterosynaptic LTP could occur between PP and CA3 pyramidal cells. As long as the heterosynaptic LTP is maintained, sets of PP inputs evoked by external events can induce hebbian LTP at synapses between PP and CA3

pyramidal cells. The Kv1.2 dependent heterosynaptic LTP is maintained for at least 30 min in vitro. Although the heterosynaptic LTP may be maintained shorter in vivo than in vitro, it is likely to be maintained at least 20 sec, trace interval in trace fear conditioning experiment. Therefore, sets of PP inputs from events, like tones and footshocks, can induce hebbian LTP and then can be associated with specific neuronal assemblies of CA3 pyramidal cells during the training session of the trace fear conditioning experiments. Because PP inputs mainly induce hebbian LTP in CA3 pyramidal cells receiving repetitive MF inputs, neuronal assemblies evoked from tones could overlap large portion of neuronal assemblies evoked from footshocks. Therefore, only PP inputs from tones can induce activation of neuronal assemblies of CA3 pyramidal cells associated with both tones and footshocks. Similarly, freezing behavior can occur when animals receive only tones, as if they have received footshocks. If CA3 pyramidal cells do not have enough Kv1.2 subunits, the heterosynaptic LTP is not induced by MF inputs (Hyun et al. 2015). This may be the reason why KCNA2 +/- group show low levels of freezing behavior.

2. Transfer of fear is not affected by Kv1.2

In the transfer of fear experiment, freezing levels of KCNA2 +/- mice group were not different from freezing levels of wild-type mouse group. In other words, KCNA2 +/- mice responded differently in experiments with time interval, such as trace fear conditioning and transfer of fear experiment. Different behaviors in KCAN2 +/- group can be caused by difference in the length of the time intervals. After mice are exposed to external stimuli such as novel contexts, MF could stimulate CA3 pyramidal cells, then LTP-IE could occur in both transfer of fear experiment and

trace fear conditioning experiment. In the trace fear conditioning experiment, time interval between stimuli are 20 sec, which is enough to assume that LTP-IE was maintained. In the transfer of fear experiment, however, time interval between stimuli can be considered 5 hours, which is much longer than in the trace fear conditioning experiment. The longer the time interval, the less likely the LTP-IE is to be maintained. In particular, during 5 hours rest or sleep, sharp wave ripples could occur in the hippocampus, and then could eliminate LTP-IE (Norimoto et al., 2018). Thus, decreased LTP-IE cause decreased heterosynaptic LTP, resulting in no effect to transfer of fear. Consequently, transfer of fear does not require heterosynaptic LTP induced by Kv1.2 downregulation, therefore behavior of KCNA2 +/- group were not different from that of wild-type group.

Transfer of fear experiment could have different mechanisms than trace fear conditioning. Although mechanisms in transfer of fear experiment is not clear, it is obvious that contextual fear conditioning is the background of transfer of fear experiment. In general, results of contextual fear conditioning and trace fear conditioning performed in the same animal are dissociated (Burman et al., 2014; Curzon et al., 2009; Lugo et al., 2014). Difference between contextual fear conditioning and trace fear conditioning implicates that transfer of fear and fear control of traces are done through different processes.

3. Deficiency of Kv1.1 affects transfer of fear

The KCNA1 +/- group showed high levels of freezing behavior in the trace fear conditioning experiment, similar to wild-type group. Neither pharmacological block of Kv1.1 subunits nor hetero-insufficiency of KCNA1 affected the LTP-IE in vitro

(Hyun et al., 2013), supporting the notion that LTP-IE is induced by Kv1.2 downregulation (Hyun et al, 2015). Thus, the normal trace fear conditioning in KCNA1 +/- further supports the notion that Kv1.2 is involved in association of US and CS.

However, the transfer of fear decreased unexpectedly in the KCNA1 +/- group. Thus, Kv1.1 could help the transfer of fear occur. Deficiency will not be related to CA3 pyramidal cells, since it does not affect LTP-IE in CA3 pyramidal cells. When mice were exposed two contexts within a day, the hippocampal CA1 ensembles activated by each context were more overlapped than ensembles activated by each context when contexts are separated in a week. Therefore, the sharing of neural ensembles for each context is likely to cause memory linking, then the transfer of fear occurs. Thus, it is likely that Kv1.1 subunits could increase overlapping ensembles in CA1. However, it is also likely that Kv1.1 works in other regions (Finnegan et al., 2006). It is reported that Kv1.1 subunits mediate learning (Kourrich et al., 2001; Meiri et al., 1997) but it is not clear at this time how Kv1.1 assists in the transfer of fear.

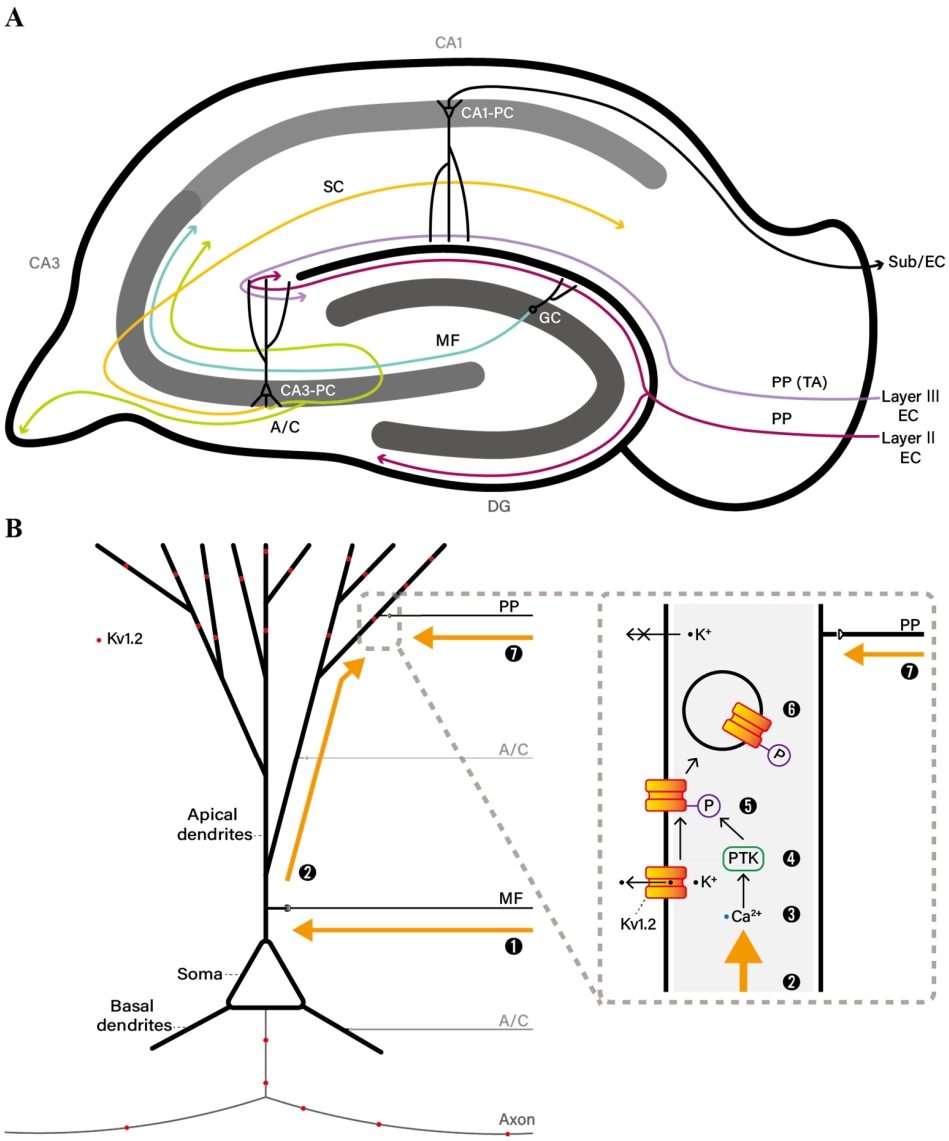

Figure 1. Heterosynaptic LTP induced by Kv1.2 downregulation in CA3 pyramidal cell. (A) Anatomy of the transverse section of the ventral hippocampus. PP (violet) projects from layer II pyramidal cells of EC to granule cells of DG and CA3 pyramidal cells. PP projecting from layer III pyramidal cells of EC to CA1 pyramidal cells and CA3 pyramidal cells is also called TA (purple). MFs (cyan) project from granule cells of DG to CA3 pyramidal cells. SCs (yellow) project from

CA3 pyramidal cells to CA1 pyramidal cells. A/C (green) project from CA3 pyramidal cells to CA3 pyramidal cells and contralateral hippocampus. **(B)** LTP-IE in CA3 pyramidal cell is induced by Kv1.2 downregulation after MF burst inputs, resuting in PP-specific heterosynaptic LTP. (1) MF inputs at the frequency higher than 20 Hz cause sufficient action potentials in CA3 pyramidal cell. (2) The action potentials propagate backward to reach distal apical dendrites. (3) Back-propagating action potentials open voltage-dependent calcium channels and increase calcium concentration. (4) Increased calcium concentration activates PTK. (5) Activated PTK phosphorylates the D-type K+ channel subunit Kv1.2 (red dot). (6) Endocytosis occurs on phosphorylated Kv1.2. (7) Without Kv1.2, the number of potassium ions that move out from the dendrites is reduced, resulting in LTP-IE in CA3 pyramidal cell. Because surface expression of Kv1.2 subunits is polarized to the distal apical dendrite where synapses between PP and CA3 pyramidal cell are located, this LTP-IE induces heterosynaptic LTP, which amplifies PP-EPSP; Associational /Commissural fibers, A/C. Dentate gyrus, DG. Entorhinal cortex, EC. Excitatory postsynaptic potential, EPSP. Granule cell, GC. Long-term potentiation of intrinsic excitability, LTP-IE. Long-term potentiation, LTP. Mossy fiber, MF. Perforant pathway, PP. Protein tyrosine kinase, PTK. Schaffer collateral, SC. Pyramidal cell, PC. Subiculum, Sub. Temporoammonic pathway, TA.

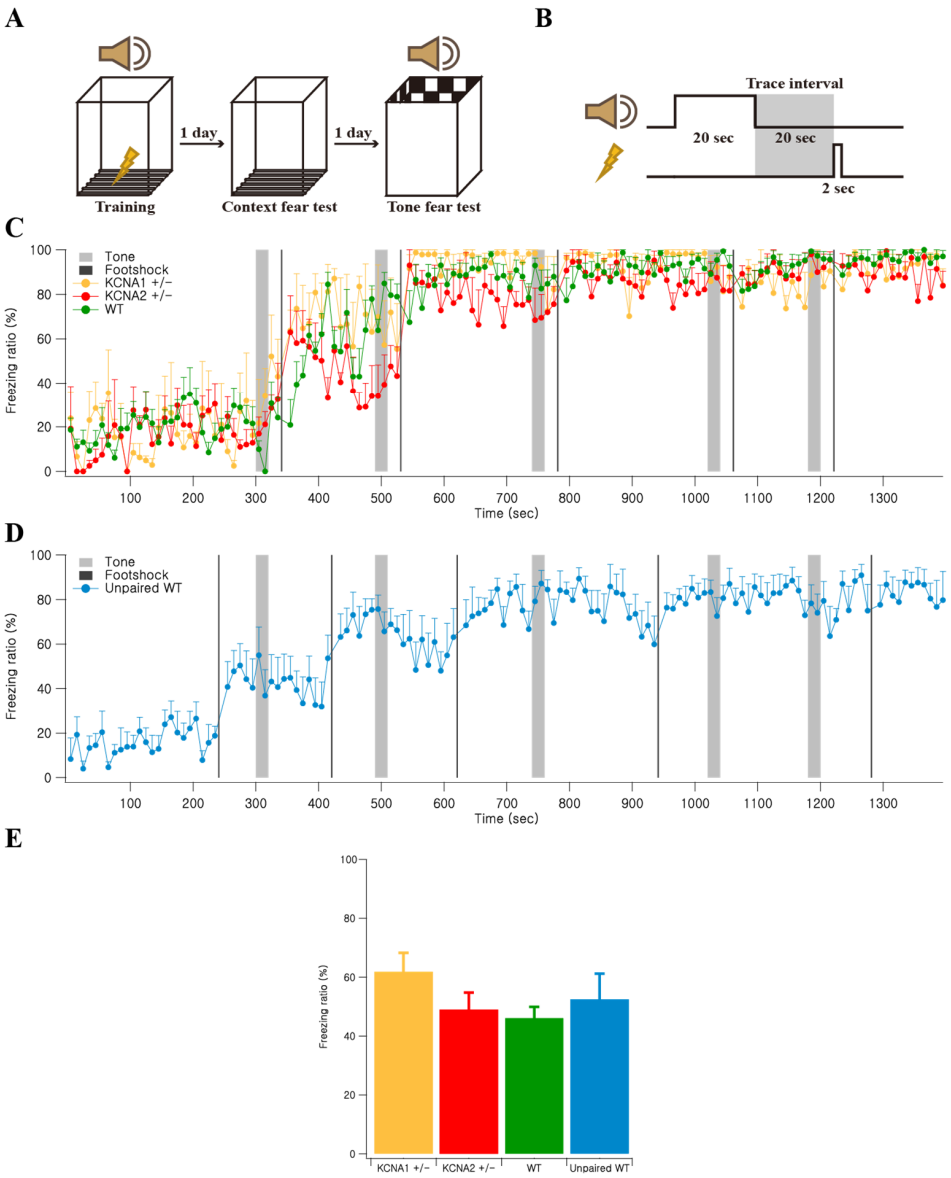

Figure 2. Behavior of mice during training and context fear test in the trace fear conditioning experiment. (A) Experimental design for the trace fear conditioning experiment. In the training session, mice were exposed to five pairs of tone and footshock, which are separated by 20 sec. In the context fear test session, mice were placed in the same context where they were shocked. In the tone fear test session,

mice were placed in a novel context and exposed to tones. **(B)** Experimental design for the training. After each 20 sec tone (speaker shape) ends, a 2 sec footshock (lightning shape) was delivered, but tone and footshock were separated by trace interval. Trace interval is time interval between tone and footshock where no stimulus was given. **(C)** Freezing ratio change during the training. After 5 min baseline period, 20 sec tone was delivered (gray). 20 sec after the tone ended, a 2 sec footshock was delivered (black). The tone was delivered at 300, 490, 740, 1020, 1180 sec. **(D)** Freezing ratio change during the unpaired training. After 4 min baseline period, tones and footshocks were delivered. The tone was delivered at 300, 490, 740, 1020, 1180 sec (gray). The footshock was delivered at 240, 420, 620, 940, 1280 sec (black). **(E)** Freezing ratio changes during context fear test. All groups show similar freezing ratios; Wild-type, WT.

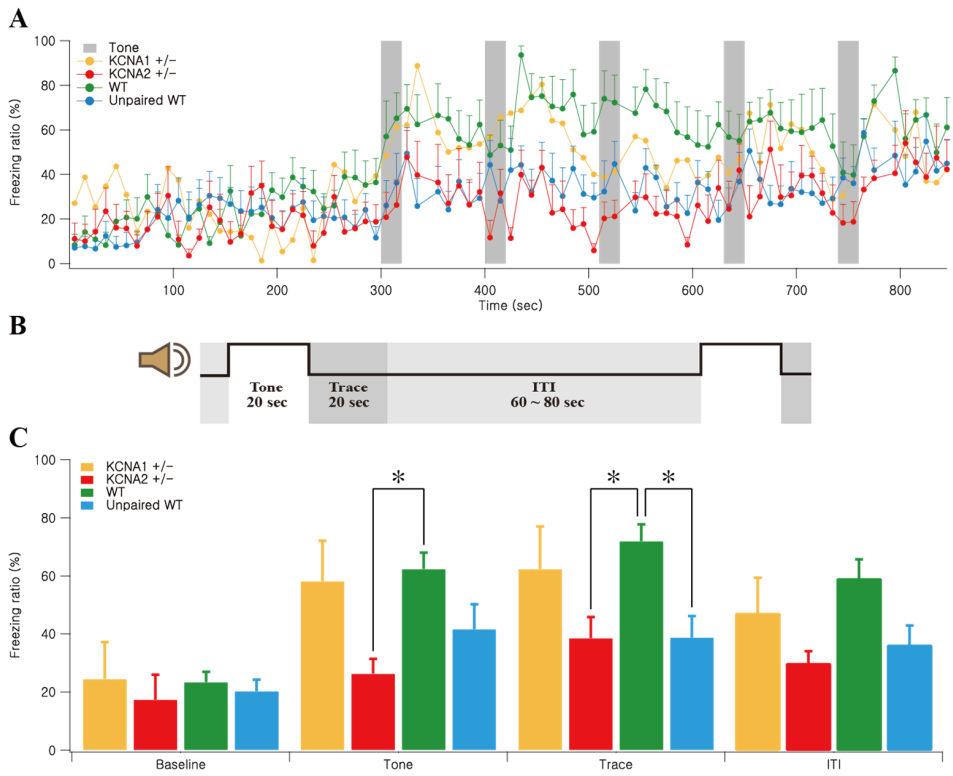

Figure 3. Behavior of mice during tone fear test in the trace fear conditioning experiment. (A) Freezing ratio change during the tone fear test. After 5 min baseline period, the tone was delivered at 300, 400, 510, 630, 740 sec (gray). **(B)** Period separation in the tone fear test. Tones began to be delivered after a 300 sec baseline period from the start of the test. The tone period was defined as the 20 sec period in which five tones were delivered. The trace period was defined as 20 sec after tones end. The ITI period was defined as 60 to 80 sec period between the trace period and the next tone period. **(C)** Average freezing ratios during the tone fear test. During the baseline period, all groups showed similar freezing ratios. During tone periods, KCNA2 +/- group have lower freezing ratio than wild-type group (WT vs. KCNA2 +/-: P=0.003). During trace periods, KCNA2 +/- and unpaired wild-type group have lower freezing ratio than wild-type group (WT vs. KCNA2 +/-: P=0.008; WT vs.

Unpaired WT: P=0.005). During ITI, all groups showed similar freezing ratios. * indicates P<α=0.0083; Wild-type, WT. Inter-trial interval, ITI.

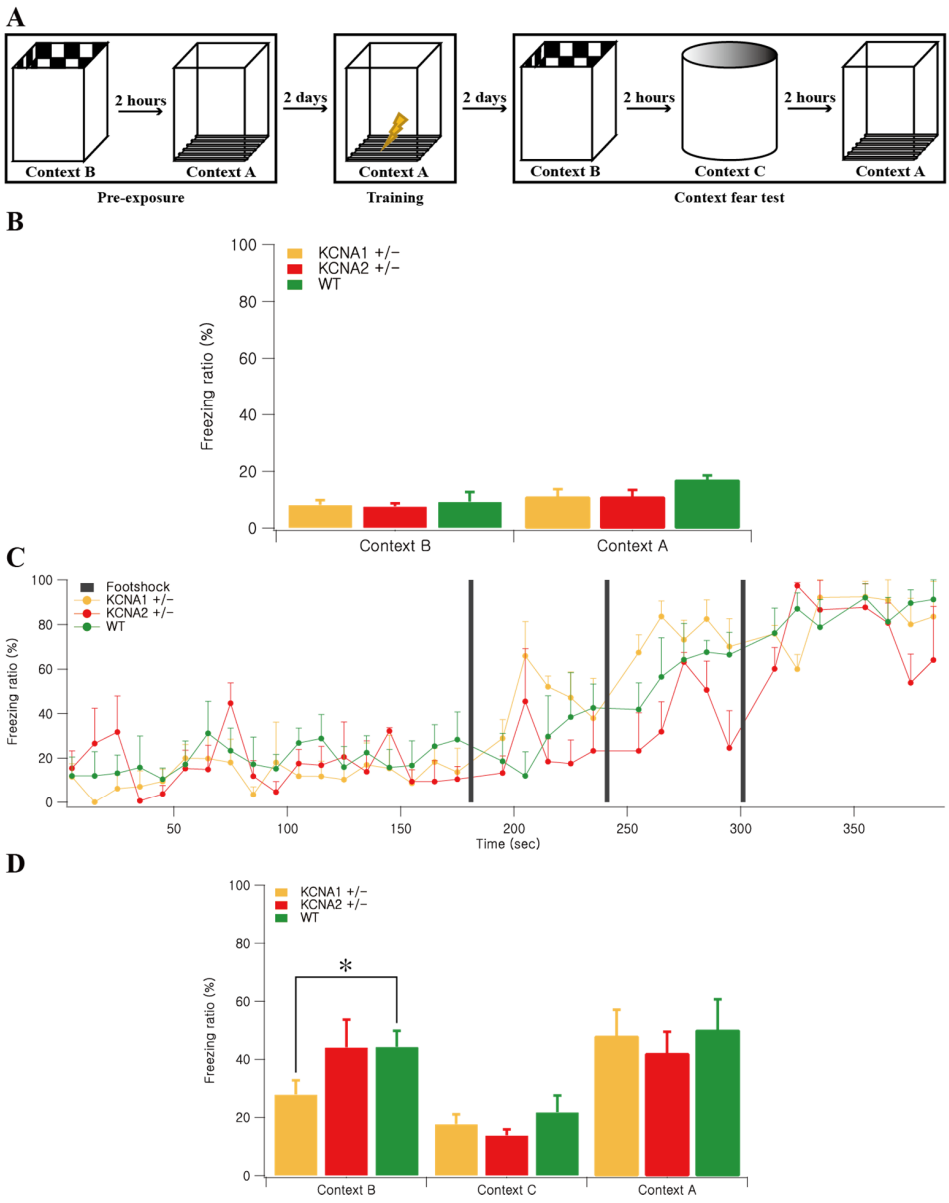

Figure 4. Behavior of mice during the transfer of fear experiment. (A) Experimental design for the transfer of fear experiment. In the pre-exposure session, mice were exposed to context B and context A. In the training session, mice were shocked in context A. In the context fear test session, mice were exposed to context B, context C, and context A. **(B)** Freezing ratios during the pre-exposure session. On

first day of the transfer of fear experiment, mice were exposed to context B and context A without footshock. Freezing ratios of all groups were similarly low in context B and in context A. **(C)** Freezing ratio change during the training session. On third day of the transfer of fear experiment, mice were shocked in context A. The footshock was delivered at 180, 240, 300 sec (black). **(D)** Average freezing ratios during the context fear test session. On fifth day of the transfer of fear experiment, the context fear test were performed in context B, context C, and context A. In context B, KCNA1 +/- group have lower freezing ratio than wild-type and KCNA2 +/- groups (WT vs. KCNA1 +/-: P=0.011). In context C and context A, all groups have similar levels of freezing ratio for each context. * indicates $P<\alpha=0.017$; Wild-type, WT.

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국문초록

해마의 CA3 영역은 학습 및 기억에 중요한 역할을 할 것으로 여겨진다. CA3의 기능에 신경가소성이 기여할 것으로 추정되나, 특히 이종시냅스 가소성이 어떻게 기여할지는 분명하지 않다. 이종시냅스 가소성의 한 예는 D형 칼륨 채널 Kv1.2 소단위의 하향 조절로 인해 유도되는 CA3 피라미드 세포에서의 이종시냅스간 장기강화이다. 이 이종시냅스간 장기강화는 CA3에서 장기간의 변화를 유도할 수 있기 때문에 시간 간격으로 분리된 사건들을 연합시키는 데 도움을 줄 가능성이 있지만, 아직 행동실험으로 확인해 본 바는 없다. Kv1.2의 하향 조절로 인해 유도되는 이종시냅스 가소성이 시간 간격으로 분리된 사건들을 연합시키는 데 영향을 끼치는지 보기 위해, 흔적 공포 조건화 실험과 공포 전이 실험을 KCNA2 유전자가 이형 접합으로 결손 되어 Kv1.2의 양이 줄어든 C3H 생쥐에게 시행하였다. 실험 결과 Kv1.2가 부족한 생쥐들의 경우 흔적 공포 조건화가 일어나지 않았다. 이 연구 결과는 Kv1.2가 시간 간격으로 분리된 사건들을 연합시키는 데 필요하다는 것을 보여준다.

키워드: CA3 피라미드 뉴런, Kv1.2, 이종시냅스간 장기강화, 흔적 공포 조건화, 시간 간격

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