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Hippocampal Damage Disrupts Eyeblink Conditioning in Mice Lacking Glutamate Receptor Subunit $\delta 2$

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Abstract. Cerebellar long-term depression (LTD) at the parallel fiber-Purkinje cell synapses has been proposed to be a neural substrate for classical eyeblink conditioning. Mutant mice lacking the glutamate receptor subunit $\delta 2$ (GluR$\delta 2$), in which the cerebellar LTD is disrupted, exhibited a severe impairment in the delay eyeblink conditioning with a temporal overlap of CS and US. However, they learned normally trace and delay conditioning without CS-US overlap, suggesting a learning mechanism which does not require the cerebellar LTD.

In the present study, we tested possible involvement of the hippocampus in this cerebellar LTD-independent learning. We examined effects of scopolamine and hippocampal lesion on the delay conditioning without CS-US overlap. The GluR$\delta 2$ mutant mice that received scopolamine or aspiration of the dorsal hippocampus together with its overlying cortex exhibited a severe impairment in learning, while the control mutant mice that received saline or aspiration of the overlying cortex learned normally. In contrast, wild-type mice that received either treatment learned as normally as the control wild-type mice. These results suggest that the hippocampus is essential in the cerebellar LTD-independent learning in the GluR$\delta 2$ mutant mice, indicating a new role of hippocampus in the paradigm with a short trace interval.

Key words: cerebellar LTD, eyeblink conditioning, gene-knockout mice, glutamate receptor subunit $\delta 2$, hippocampus, synaptic plasticity

1. Introduction

Classical eyeblink conditioning is one of the most extensively studied forms of associative motor learning [1–3]. These learning experiments can be divided into two distinct types, delay and trace paradigms, depending on the temporal relationship between the conditioned stimulus (CS) and the unconditioned stimulus (US) [2]. In the trace paradigm, a stimulus-free interval (denoted TI for 'trace interval') intervenes between the preceding CS offset and the US onset, forcing a subject to form a short-term memory ('trace') of the CS in order to successfully predict the

US onset. On the other hand, there is no interval between the two stimuli in the delay paradigm. In a standard delay paradigm, tone CS precedes and coterminates US (air-puff to the eye or periorbital electric shock). In a variant form of delay paradigm, however, US starts just at the offset of the preceding CS, without overlap or interval between the two stimuli.

The delay paradigm has been far more extensively used than the trace paradigm in studies on the neural mechanisms underlying this type of learning [3, 4]. The basic neural circuitry has been identified through lesion studies, and electrophysiological and anatomical characterizations of the projection pathways. The evidence accumulated thus far indicates that the memory trace is formed and stored in the anterior interpositus nucleus and overlying cerebellar cortex [3–5]. Several investigators have proposed that cerebellar long-term depression (LTD) at parallel fiber (PF)-Purkinje cell (PC) synapses is a key mechanism underlying eyeblink conditioning [6–8]. To test this hypothesis, gene-targeting technology in mice was introduced into this field in 1994 [9]. Although results of studies using cerebellar LTD-deficient mutant mice lacking the metabotropic glutamate receptor type 1 [9] and mice lacking glial fibrillary acidic protein [10] suggested that cerebellar LTD is a neural substrate for delay conditioning, these findings are inconclusive [11], as these molecules are also expressed in structures other than the cerebellar cortex in the wild-type mouse, including the hippocampus. In contrast, the glutamate receptor subunit $\delta 2$ (GluR$\delta 2$) is selectively expressed at the dendritic spines of PCs [12] and essential to the induction of cerebellar LTD [13]. Therefore, GluR$\delta 2$-null mice, in which cerebellar LTD is specifically impaired, provide a useful means for investigating the role of cerebellar LTD and other regions of the brain separately in eyeblink conditioning.

In previous papers [14, 15], we reported that mutant mice lacking GluR$\delta 2$ (GluR$\delta 2^{-/-}$ mice) exhibited impairment with standard delay eyeblink conditioning (Interstimulus interval of 252 ms). This gives a strong support for the cerebellar LTD hypothesis in the delay eyeblink conditioning. However, trace eyeblink conditioning with 500-ms and shorter TIs was normal. This rather surprising result has been confirmed in an experiment using mutant mice lacking phospholipase C$\beta 4$ which is expressed specifically in Purkinje cells in a rostral half of the cerebellum, a region known to be responsible for eyeblink conditioning [16]. Furthermore, we found that delay conditioning without temporal overlap of the CS and US is normal in GluR$\delta 2^{-/-}$ mutant mice, indicating that there is a distinct difference in the cerebellar mechanism involved in delay conditioning with CS-US overlap and delay as well as trace conditioning without CS-US overlap. Thus it may be more appropriate to redefine the delay conditioning without CS-US overlap as a limiting case of trace conditioning with zero TI, since its cerebellar mechanism is most likely the same as that of trace conditioning. Our results demonstrate that cerebellar LTD-independent learning is possible in paradigms without temporal overlap between the CS and US. On the other hand, GluR$\delta 2$ and cerebellar LTD are essential for learning when there is CS-US temporal overlap, suggesting that cerebellar neural substrates underlying

eyeblink conditioning may change, depending on the temporal overlap of the CS and US.

In the trace paradigm, in which a long stimulus-free trace interval (>500 ms) intervenes between the CS and the US, the hippocampus is required in addition to the cerebellum [17, 18]. Taking into account of this critical role of the hippcampus in trace conditioning, we hypothesized that the hippocampus may also play a critical role in the cerebellar LTD-independent learning in GluR$\delta 2^{-/-}$ mice. The purpose of the present study is to examine this hypothesis. In the first study (Experiment 1), we investigated effects of scopolamine in the eyeblink conditioning in GluR$\delta 2^{-/-}$ mice. Scopolamine is a muscarinic acetylcholine receptor antagonist and is known to impair trace eyeblink conditioning with a long trace interval but not delay conditioning [19, 20]. In the second experment, we investigated effects of hippocampal lesion on the delay conditioning without CS-US overlap.

2. Experiment 1: Effects of Systemically Administered Scopolamine

2.1. METHOD

GluR$\delta 2^{-/-}$ mice with 99.99% C57BL/6 genetic background [14] and wild-type C57BL/6 mice were kept on a 12-h light/12-h dark cycle with *ad libitum* access to food and water. Male and female mice, weighing 18–24 g at the time of surgery, were used.

Surgical procedure was the same as described previously [14]. Under anesthesia with ketamine (80 mg/kg, *i.p.*) and xylazine (20 mg/kg, *i.p.*), four Teflon-coated stainless-steel wires were implanted subcutaneously in the left upper eyelid. The rostral two wires were used to record eyelid EMG activity and the caudal two wires were to deliver the US.

Five days after the surgery, spontaneous eyeblink frequency was measured and on the following day conditioning was started. A daily conditioning consisted of 90 CS-US paired trials and 10 CS-alone trials on every 10th trial, with a pseudorandomized intertrial interval of 20–40 s. In the paired trials, a 352-ms tone CS (1 kHz, 90 dB) was followed by a 100-ms periorbital shock US (100 Hz square pulses), eliciting an eyeblink/head-turn response. In the present study, we used the delay paradigm without CS-US overlap, in which US started immediately after the termination of CS so that the interstimulus interval was 352 ms. Scopolamine hydrobromide (0.5 mg/kg) or saline (5 ml/kg) was administered intraperitoneally to the mice 15 min before the start of daily training.

The conditioned response (CR) was monitored by the eyelid EMG activities. Average + SD of the amplitude of EMG activities for 300 ms before CS onset in 100 trials was defined as the threshold, which was used in the further analysis below. In each trial, average values of EMG amplitudes above the threshold were calculated for a 300-ms period before the CS onset (pre-value), for a 30-ms period after the CS onset (startle-value), and for a 202-ms period before the US onset (CR-value). If the pre-value and startle-value were less than 10 and 30% of the

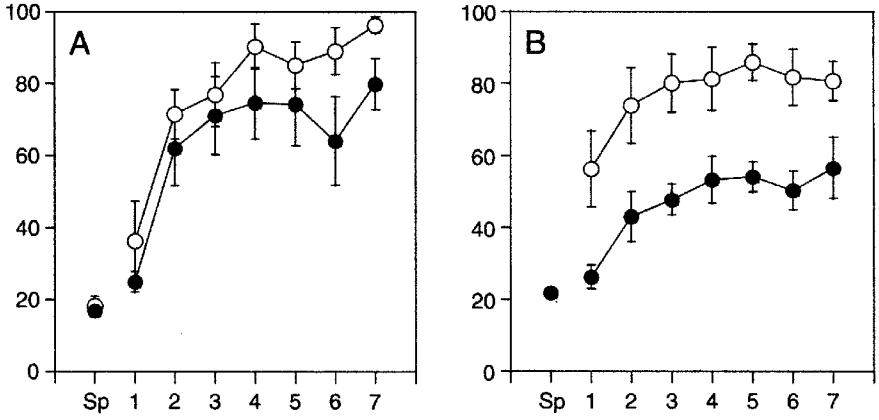

Figure 1. Effect of systemically administered scopolamine. Relative frequency of CR in a daily session (CR%) in the wild-type (A) and GluR$\delta 2^{-/-}$ mice (B). These mice were injected intraperitoneally with saline (open circle, n = 6 for wild-type mice and n = 9 for mutant mice) or scopolamine (filled circle, n = 6 for wild-type mice and n = 9 for mutant mice) 15 min before the start of daily conditioning. Sp, spontaneous eyeblink frequency.

threshold, respectively, the trial was regarded as the 'valid' trial. Among the valid trials, a trial was regarded to contain the CR if the CR value was larger than 1% of the threshold and exceeded 2-fold of the pre-value. In CS-alone trials, the period for CR-value calculation was extended to the CS offset. The frequency of CRs in the valid trials (CR%) was expressed as mean ± s.e.m. Statistical significance was determined by two-way and three-way repeated measures ANOVA using a statistical software, SPSS. The factors of the three-way repeated measures ANOVA were the drug (saline, scopolamine), the mouse genotype (wild-type mice, GluR$\delta 2^{-/-}$ mice), and the session. $p < 0.05$ was considered significant in this study.

2.2. RESULTS

As reported previously [15], GluR$\delta 2^{-/-}$ mice that had received intraperitoneal injection of saline learned normally delay eyeblink conditioning without CS-US overlap (Figure 1B, ○). The rate of learning was almost the same as that of the wild-type mice (Figure 1A, ○). Analysis with two-way repeated measures ANOVA revealed that there was no significant difference between the saline-treated wild-type mice and the saline-treated GluR$\delta 2^{-/-}$ mice ($F_{1,13} = 0.009$, $p > 0.9$). In contrast, intraperitoneal injection of scopolamine differentiated the wild-type mice and GluR$\delta 2^{-/-}$ mice. Scopolamine severely impaired the learning in GluR$\delta 2-/-$ mice (Figure 1B, ●) whereas a moderate impairment was observed in the wild-type mice (Figure 1A, ●). Three-way repeated measures ANOVA revealed that the value of CR% was significantly influenced by the drug ($F_{1,189} = 61.779$, $p < 0.001$) and the mouse genotype ($F_{1,189} = 8.813$, $p < 0.01$). This clearly indicates

that the effect of scopolamine on learning is different between the wild-type and GluR$\delta 2^{-/-}$ mice. There was no significant interaction between sessions and drugs, and between sessions and mice.

To check the basic sensory and motor performance concerning eyeblink conditioning, we examined spontaneous eyeblink frequency and startle response to the tone. The spontaneous eyeblink frequency of the scopolamine-treated GluR$\delta 2^{-/-}$ mice was quite similar to that of the saline-treated mice, as in the wild-type mice. Two-way ANOVA indicated no significant effects of interaction between mice and drugs, and no significant difference in mice [F (1, 26) = 0.502, $p > 0.1$] and in drugs [F (1, 26) = 0.165, $p > 0.5$]. With respect to startle response, the frequency of trials whose startle-value exceeded 20% of the threshold was calculated. The frequency of the startle response to the tone during conditioning was 4.2 $\pm$ 1.1% (n = 6) and 3.7 $\pm$ 1.1% (n = 6) for the saline- and scopolamine-treated wild-type mice, respectively, and 5.7 $\pm$ 1.0% (n = 9) and 3.7 $\pm$ 0.8% (n = 9) for the saline- and scopolamine-treated GluR$\delta 2-/-$ mice, respectively. Although the frequency of startle response of the scopolamine-treated mice tended to be lower than that of the saline-treated mice, there was no significant difference in mouse genotypic groups ($F_{1,26} = 4.325$, $p > 0.2$) and in drugs ($F_{1,26} = 3.187$, $p > 0.05$).

3. Experiment 2: Effects of Hippocampal Lesion

3.1. METHOD

The animal strains were the same as in Experiment 1. Prior to surgery, mice were anesthetized with ketamine (80 mg/kg, *i.p.*) and xylazine (20 mg/kg, *i.p.*). Under anesthesia, the head was shaved and then the mouse was mounted in a stereotaxic instrument with its head resting on the earbars. The skull was exposed by a mid-sagittal incision and bilateral holes were made. A hole was made by connecting four points in the following: a point 1 mm lateral from bregma, a point 4 mm from bregma, a point 5 mm lateral and 5 mm posterior to bregma and a point 1 mm lateral and 5 mm posterior to bregma. After removal of the overlying dura, the mouse group of hippocampal lesion received a bilateral aspiration of the dorsal hippocampus and the overlying neocortex. The control group of mice received only a bilateral aspiration of the neocortex and corpus callosum. After the surgery, all subjects were given an injection of Gentamicin sulfate (5 mg/kg, *i.p.*). All the animals were allowed to recover for 10 days and then implanted with stainless-steel wires as described in Experiment 1. The apparatus and behavioral training including conditioning paradigm were also the same as that used in Experiment 1.

After completing all behavioral experiments, the mice were deeply anesthetized and perfused transcardially with 0.9% saline followed by a 10% formalin. Brains were removed and placed in a 10% formalin solution. Prior to microtome sectioning, brains were placed in a 30% sucrose solution overnight. Coronal sections (40 μm thick) were made through the length of the hippocampus and stained with cresyl violet.

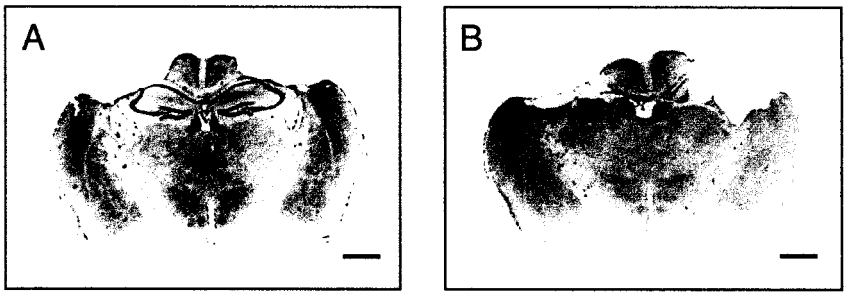

Figure 2. Photographs of the coronal brain section. After completing all behavioral experiments, coronal brain sections were made and stained with cresyl violet. The photographs show a typical brain section from a mouse in the control group (A) and hippocampal-lesion group (B). The bar indicates 1 mm.

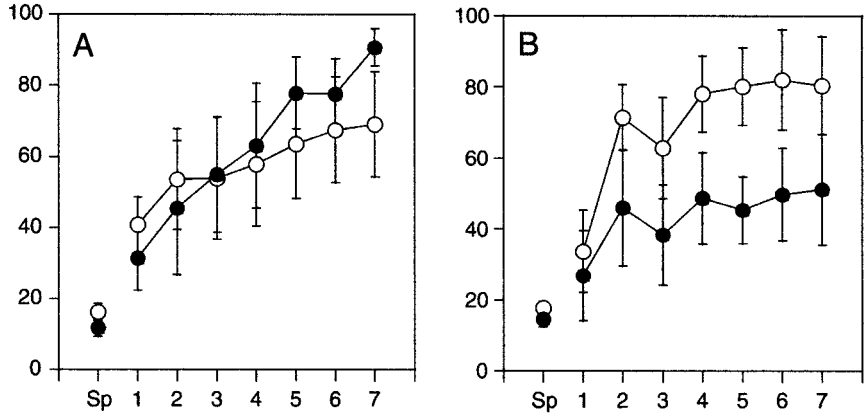

Figure 3. Effect of hippocampal lesion. Relative frequency of CR in a daily session (CR%) in the wild-type (A) and GluR$\delta 2^{-/-}$ mice (B). These mice were received a bilateral aspiration of neocortex (open circle, n = 5 for wild-type mice and n = 4 for mutant mice) or a bilateral aspiration of the dorsal hippocampus and the overlying neocortex (filled circle, n = 4 for wild-type mice and n = 4 for mutant mice).

3.2. RESULTS

Examination of brain sections revealed that hippocampal-lesion group had complete removal of the dorsal hippocampus. Although some of them spared the posteroventral portion of the hippocampus, the fimbria was bilaterally transected and ventral hippocampus isolated from the fornix. No damage to the thalamus was observed, and the cingulate/retrosplenial cortex was generally intact. Figure 2 shows the examples of a typical control (Figure 2A) and hippocampal-lesioned (Figure 2B) preparation.

As shown in Figure 3B, the GluR$\delta 2^{-/-}$ mice that had received aspiration of the dorsal hippocampus together with its overlying neocortex exhibited a severe

impairment in learning. In contrast, wild-type mice that received aspiration of the dorsal hippocampus learned as normally as the control wild-type mice (Figure 3A).

Effects on the spontaneous eyeblink frequency and startle response to the tone were also examined. The spontaneous eyeblink frequency was not influenced by hippocampal destruction in both wild-type mice and GluR$\delta 2^{-/-}$ mice. No effects were observed about the startle response to the tone. The startle response of the hippocampal-lesion group was little different from that of the control group.

4. Discussion

In the present study, we demonstrated that systemic administration of scopolamine and the ablation of the dorsal hippocampus prevent the acquisition of CR in eyeblink conditioning without temporal overlap of CS and US in GluR$\delta 2^{-/-}$ mice. Since scopolamine at the dose used in the present study and hippocampal lesion did not affect the frequency of spontaneous eyeblink and startle response to tone during conditioning, the disruption of CR acquisition was not due to defects in basic sensory and motor performance. Therefore, the results obtained in the present study indicate that the hippocampus plays a critical role in the learning mechanism which does not require the cerebellar LTD.

A little impairment of the learning observed in the wild-type mice by injection of scopolamine (Figure 1A) may be due to the effects to the brain region outside the hippocampus, because the hippocampal-lesion group in the wild-type mice learned normally as the control group (Figure 3A). This effect of scopolamine in the wild-type mice is consistent with that observed in the standard delay conditioning in rabbits [19–21].

The hippocampus has been suggested to be the most plausible candidate for the target of scopolamine [19, 20] in the standard delay eyeblink conditioning. Solomon et al. [20] reported that the learning rate in the standard delay conditioning was slowed by systemic administration of scopolamine, and that this impairment was not observed in hippocampectomized rabbit, suggesting that scopolamine affects the eyeblink conditioning via abnormal hippocampal activities. In the present study, scopolamine impaired severely the delay eyeblink conditioning without CS-US overlap in GluR$\delta 2^{-/-}$ mice. From the reason as mentioned above, the impairment in GluR$\delta 2^{-/-}$ mice is also likely due to an alteration of hippocampal activity.

It is well documented in rabbits that the trace conditioning with long TIs ($>$ 500 ms) requires the hippocampus but the trace conditioning with a short trace interval ($<$300 ms) or delay conditioning does not [17, 18]. In addition to this notion, Solomon and colleagues reported that lesion of the cingulate/retrosplenial cortex has effects similar to those caused by hippocampal lesion in the trace conditioning in rabbits [17]. We also investigated the effects of lesion of the neocortex including cingulate/retrosplenial cortex in GluR$\delta 2^{-/-}$ mice: GluR$\delta 2^{-/-}$ mice that received aspiration of the cingulate/retrosplenial cortex exhibited a severe impairment in learning as well as those that received hippocampal lesion (data not shown).

This result suggests that the participation of the hippocampus to cerebellar LTD-independent mechanism may be similar to that to the mechanism underlying the long-TI trace eyeblink conditioning. Since it remains an open question as to exactly how the hippocampus come into play in the trace conditioning, it is possible that investigating the mechanism of LTD-independent learning lead to help explain the role of hippocampus in the trace eyeblink conditioning.

In conclusion, the present study has clearly shown that the hippocampus is essential in the delay eyeblink conditioning without temporal overlap of CS and US (i.e., the trace conditioning with zero trace interval) in the GluRδ2-null mutant mice lacking the cerebellar LTD but not in wild-type animals. This indicates that the hippocampus is essential in the cerebellar LTD-independent learning but not in the learning where the cerebellar LTD is possible. Thus the trace eyeblink conditioning with zero or a short trace interval requires either of the cerebellar LTD or the hippocampus, indicating a new role of the hippocampus in the paradigm with a short trace interval.

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