The NMDA receptor GluRε2 is important for delay and trace eyeblink conditioning in mice.

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ABSTRACT

It has been proposed that the *N*-methyl-D-aspartate (NMDA)-type glutamate receptor (GluR) plays an important role in synaptic plasticity, learning and memory. The four GluRε (NR2) subunits, which constitute NMDA receptors with a GluRζ (NR1) subunit, differ both in their expression patterns in the brain and in their functional properties. In order to specify the distinct participation of each of these subunits, we focused on the GluRε2 subunits, which are expressed mainly in the forebrain. We investigated delay and trace eyeblink conditioning in GluRε2 heterozygous mutant mice whose content of GluRε2 protein was decreased to about half of that in wild-type mice. GluRε2 mutant mice exhibited severe impairment of the attained level of conditioned response (CR) in the delay paradigm, for which the cerebellum is essential and modulation by the forebrain has been suggested. Moreover, GluRε2 mutant mice showed no trend toward CR acquisition in the trace paradigm with a trace interval of 500 ms, in which the forebrain is critically involved in successful learning. On the other hand, the reduction of GluRε2 proteins did not disturb any basic sensory and motor functions which might have explained the observed impairment. These results are different from those obtained with GluRε1 null mutant mice, which attain a normal level of the CR but at a slower rate in the delay paradigm, and showed a severe impairment in the trace paradigm. Therefore, the NMDA receptor GluRε2 plays a more critical role than the GluRε1 subunit in classical eyeblink conditioning.

INTRODUCTION

Classical eyeblink conditioning is one of the most extensively studied models of associative learning. In the delay paradigm, in which the unconditioned stimulus (US) is delayed and coterminates with the conditioned stimulus (CS), the cerebellum is essential for acquisition of the conditioned response (CR) in rabbits [11] and mice [3]. Although animals can acquire the CR normally without the forebrain [8], several lines of evidence indicate the involvement of the hippocampus [1, 14]. In the trace paradigm, in which the CS and the US are separated by a stimulus-free trace interval, conditioning with a long trace interval requires an intact hippocampus [13, 16, 17] and medial prefrontal cortex [17, 25] in addition to the cerebellum [17, 26] for successful acquisition and retention of the CR. Although this important basic framework of the learning mechanism has been established in rabbits, recent progress in molecular biological techniques promotes application of this learning to mice, in which we can elucidate more detailed mechanisms at the molecular level.

The *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor (GluR) channel plays key roles in synaptic plasticity, learning and memory [10]. Pharmacological systemic blockade of the NMDA receptors retards delay eyeblink conditioning and severely impairs trace eyeblink conditioning in both rabbits [20] and mice [15]. Moreover, microinfusion of an NMDA antagonist into the cerebellum severely impairs delay CR acquisition [4]. Of the four GluRε (NR2) subunits which constitute NMDA receptors with a GluRζ (NR1) subunit [12], the GluRε1 subunit is important for important for this learning, since mutant mice lacking a GluRε1 subunit (but not those lacking a GluRε3 subunit) exhibit impairment similar to that produced by pharmacological blockade [6, 7]. However, the role of the GluRε2 subunit in eyeblink conditioning has not yet been examined, although it is strongly expressed in the forebrain [22] and has been implicated in several kinds of learning and in long-term potentiation in the hippocampus [19]. Here we investigated classical eyeblink conditioning in heterozygous GluRε2 mutant mice, which we had generated previously [9].

MATERIALS AND METHODS

Animals: Since the homozygous GluRε2 mutation is lethal [9], we used littermates derived from the crossing of male heterozygous GluRε2 mutant mice and female heterozygous mutant mice, female wild-type mice, or female C57BL/6 mice. We used both male and female mice, which were 11-24 weeks of age and had greater than a 99.9% genetic background of the C57BL/6 strain [18]. Since the learning performances did not differ by gender, the data for males and females were combined. Genotypes of the mice were determined by polymerase chain reaction, as described previously [21]. The animals were housed individually in standard plastic cages in a colony room with a 12-h light/dark cycle. Water and food were available *ad libitum*. All experiments were performed in accordance with the guidelines established by the Institutional Animal Investigation Committee at the University of Tokyo and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to optimize comfort and to minimize the use of animals.

Eyeblink conditioning: Surgical procedures were the same as described previously [7]. Four Teflon-coated stainless steel wires 140 μm in diameter were implanted in the left upper eyelid: two for recording eyelid electromyograms (EMG) and the other two for delivery of the US. Two to four days after the surgery, the frequency of spontaneous eyeblinking was measured for two days, and then the conditioning began. A daily conditioning session consisted of 100 trials grouped into 10 blocks, which included 9 CS-US paired trials followed by one CS-alone trial with a pseudorandomized inter-trial interval of between 20 and 40 s. The CS was a 350-ms tone (1 kHz, 85dB) with a rise- and fall-time of 5 ms and the US was a 100-ms periorbital shock (100 Hz square pulses) that elicited an eyeblink/head-turn response. In the delay paradigm, the CS preceded and coterminated with the US. In the trace paradigm, a stimulus-free trace interval of 500 ms was interposed between the CS and the US. The CR was monitored through EMG activity. The average + s.d. of the amplitudes of the EMG signals for 300 ms before the CS in 100 trials was defined as the threshold and was used in the analyses described below. In each trial, average values of EMG amplitudes above the threshold were calculated for 300 ms before the CS onset (pre-value), for 30 ms after the CS onset (startle value), and for 200 ms before the US onset (CR value). If the pre-values and startle values were <10% of

the threshold, the trial was regarded as a valid trial. The number of valid trials was 75.6 ± 0.4 (s.e.m) in the present study. Among the valid trials, a trial was assumed to contain the CR if the CR value was larger than 1% of the threshold value and exceeded two times the pre-value. In CS-alone trials, the period for CR value calculation was extended to the expected time of the end of the US. The frequency of the CR trials over the valid trials (CR%) was expressed as mean ± s.e.m. The frequency of the startle trials over the valid + startle trials was also calculated. To show the temporal pattern of the CRs, the EMG amplitude data for each mouse were averaged over the valid trials for each day. These trial-averaged EMG amplitude data were normalized to the time-averaged value for 300 ms before the CS onset.

Auditory brainstem response (ABR): The ABR was recorded in mice anaesthetized with ketamine (33.5 μg/body, i.p.**)** and xylazine (6.60 μg/body, i.p.), with the body temperature maintained at 37°C as reported previously [18]. Needle electrodes were inserted subcutaneously at the vertex (active), over the midline on the occipital bone (reference) and into the animal's back (ground). Sound stimuli (1 kHz, tone bursts) were at a rate of 10 Hz, including 1 ms each rise and fall. Physiological signals were filtered (50-3000 Hz band-pass) and each measurement was based on an average of 500 sweeps. Thresholds were determined by reducing the stimulus in 10-dB steps until the ABR disappeared, then by raising and lowering the stimulus intensity in 5-dB steps.

RESULTS

We examined delay eyeblink conditioning, which depends mainly on the cerebellum and the brainstem, but marginally on the forebrain. Our GluRε2 heterozygous mutant (+/–) mice exhibited a lower CR% than did wild-type GluRε2 (+/+) mice during the acquisition sessions (Fig. 1a). This was confirmed by a two-way repeated measures ANOVA; there was a significant interaction between genotypes and sessions ($F_{9, 162}$ = 5.11, $P < 0.001$). To investigate the temporal pattern of the CR, we compared the averaged EMG amplitude data over valid trials in the 10th session (Fig. 1b). Although the EMG amplitude of the GluRε2 (+/–) mice was lower than that of GluRε2 (+/+) mice, there was no apparent difference between the genotypes in the temporal pattern of the CR.

We next examined trace eyeblink conditioning with a trace interval of 500 ms, which is so long that successful CR acquisition critically depends on the forebrain. As expected, the GluRε2 (+/–) mice exhibited no trend at all toward an increasing CR% during the 10 days of acquisition sessions (Fig. 2a). A two-way repeated measures ANOVA revealed a highly significant interaction between genotypes and sessions ($F_{9, 126}$ = 4.21, $P < 0.001$). Figure 2b shows the temporal pattern of the CR in the wild-type and GluRε2 (+/–) mice in the 10th session. Consistent with their poor CR acquisition, there was little increase in the EMG amplitude after the CS onset in the GluRε2 (+/–) mice.

We also analyzed the sensory input and motor output involved in this conditioning. There was not a significant difference between the two genotypes in the US intensity required to elicit eyeblink/head-turn responses (data not shown) (two-way repeated measures ANOVA, $F_{1, 34}$ = 1.29, $P > 0.05$), or in the frequency of startle eyeblink responses to the CS (Fig. 3a) (*t* test, $P > 0.05$). Furthermore, the ABR threshold of the GluRε2 (+/–) mice for 1-kHz tone bursts was comparable to that of the wild-type mice (Fig. 3b) (*t* test, $P > 0.05$), indicating that the auditory response in the brainstem did not differ between the genotypes. These results suggest that GluRε2 (+/–) mice are not significantly different from GluRε2 (+/+) mice in their responsiveness to either the CS or the US used in the present conditioning. It is to be noted that GluRε2 (+/–) mice have been reported to show enhanced nociceptive and startle responses [18 21]. However, even if GluRε2 (+/–) mice had been more sensitive to the CS and US, it could not have explained the impairment observed in these mice, because enhanced sensitivity to the CS and US should facilitate CR

acquisition. When we looked at motor output, no significant differences were detected in the frequency of spontaneous eyeblinking (*t* test, $P > 0.05$) (Fig. 3c). We concluded that there was no serious disturbance in either sensory or motor functions that could explain the impairment observed in the GluRε2 (+/–) mice.

DISCUSSION

The present study investigated the involvement of the NMDA receptor GluRε2 in classical eyeblink conditioning using heterozygous mutant mice. We found that GluRε2 (+/–) mice exhibited severe impairment in both the delay and trace paradigms. These results suggest the critical involvement of GluRε2 in classical eyeblink conditioning, and provide further support for the importance of GluRε2 in learning and memory.

GluRε2 heterozygous mutant mice were severely impaired in CR acquisition during trace eyeblink conditioning. Because GluRε2 is strongly expressed in the forebrain [22], their poor performance in the trace paradigm, which depends on the forebrain, is not unexpected. And this result is also consistent with previous reports of pharmacological blockade of NMDA receptors [15, 20] and GluRε1-deficient mice [6, 7].

However, in the delay paradigm the impairment is more severe in our GluRε2 (+/–) mice than in the other two cases, in which the experimental groups acquired the CR more slowly but could eventually attain an asymptotic performance comparable to that of the control groups [6, 7, 15, 20]. This contrasts with the present result that GluRε2 (+/–) mice never acquired the CR, even after 10 days of training. Considering that the delay paradigm depends on the cerebellum and brainstem [3, 11], while the forebrain is not essential for CR acquisition [8], there are three possible interpretations of this impairment, which are not mutually exclusive. First, several reports suggest that altered forebrain activity retards CR acquisition in the delay paradigm. For example, the cholinergic antagonist scopolamine delays CR acquisition in intact rabbits, but not in those rabbits with hippocampal lesions [14]. Similar results have been obtained using rabbits with medial septal lesions [2]. These results suggest that the forebrain is involved in delay CR acquisition by regulating the essential circuitry in the cerebellum and the brainstem. Considering that expression of the GluRε2 subunit mRNA in the mature brain is mainly restricted to the forebrain [22], it is possible that a reduction in GluRε2 proteins affects normal forebrain function and that this causes the failure of the proper modulation of CR acquisition. In addition, GluRε2 (+/–) mice have exhibited an enhancement of the acoustic startle response [18] and nociceptive reflex [21], whose primary circuits exist in the brainstem, in which GluRε2 mRNA is undetectable [9, 24]. Taken together, these results suggest that

the NMDA receptor GluRε2 is important for the modulatory function of the forebrain to the cerebellum and brainstem. Second, whereas GluRε2 mRNA is undetectable in the cerebellar cortex and the deep cerebellar nuclei [23], it is slightly expressed in the pontine nuclei and the inferior olive [24], which mediate the CS and the US pathways, respectively. It is noteworthy that GluRε1 is also expressed in these areas [24]. Thus, GluRε2 heterozygous mutant mice may have some deficiency in these nuclei and, if this is the case, then GluRε2 would play a more important role in these nuclei than GluRε1 does. A difference in function between these two NMDA receptor subunits has already been revealed in hippocampus CA3 pyramidal neurons [5]. Third, since GluRε2 mRNA is expressed throughout the entire embryonic brain [22], the possibility exists that any reduction in GluRε2 affects normal formation of the essential circuitry in the cerebellum and brainstem during development. Future studies using conditional knockout mice, in which spatiotemporally restricted gene-expression of GluRε2 would be possible, may reveal the exact role of this subunit in classical eyeblink conditioning.

In conclusion, mutant mice heterozygous for the NMDA receptor GluRε2 exhibited severe impairment in both delay and trace eyeblink conditioning. With its expression pattern in the brain, these results suggest that GluRε2 plays critical roles in the brain circuitry involved in these paradigms.

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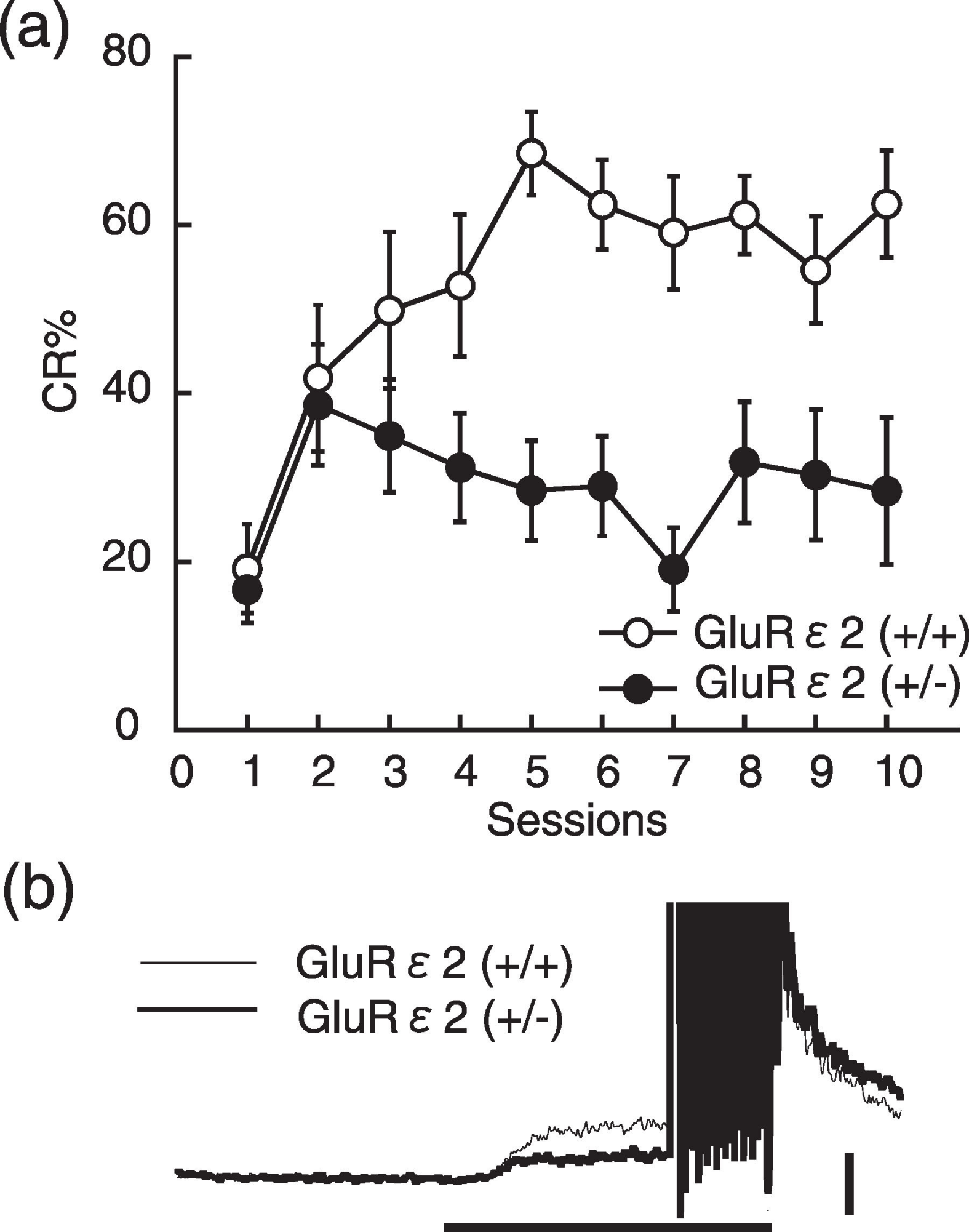
FIGURE LEGENDS

Fig. 1. Delay eyeblink conditioning. (a) Average CR% during the delay paradigm in wild-type mice (n=10, empty circle) and GluRε2 (+/–) mice (n=10, filled circle). Error bar indicates the standard error of the mean. (b) The temporal pattern of the CR of the wild-type mice (thin line) and the GluRε2 (+/–) mice (thick line) in the 10th session. The EMG amplitude data of each mouse were averaged over valid trials. This trial-averaged trace of each mouse in the 10th session was normalized to the time-averaged value over the pre-CS period. Then, this normalized trace was averaged over mice in each group. The solid line under the trace indicates the timing of the 350-ms CS. The vertical scale indicates the time-averaged value over the pre-CS period (100%).

Fig. 2. Trace eyeblink conditioning. (a) Average CR% of the wild-type mice (n=8, empty circle) and GluRε2 (+/–) mice (n=8, filled circle) during the trace paradigm with a trace interval of 500 ms. Error bar indicates the standard error of the mean. (b) The temporal pattern of the CR of the wild-type mice (thin line) and the GluRε2 (+/–) mice (thick line) in the 10th session. The traces show the group-average EMG pattern after normalization in each mouse, as in Fig. 1b. The solid line under the trace indicates the timing of the 350-ms CS. The vertical scale indicates the time-averaged value over the pre-CS period (100 %).

Fig. 3. Basic sensory input and motor performance. (a) Frequency of the startle response to the tone CS during the 1st acquisition session. (b) Threshold of the acoustic brainstem response (ABR) to a 1-kHz tone. (c) The spontaneous eyeblink frequency. All error bars indicate the standard error of the mean.


(a)
80
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CR%
0 1 2 3 4 5 6 7 8 9 10
Sessions
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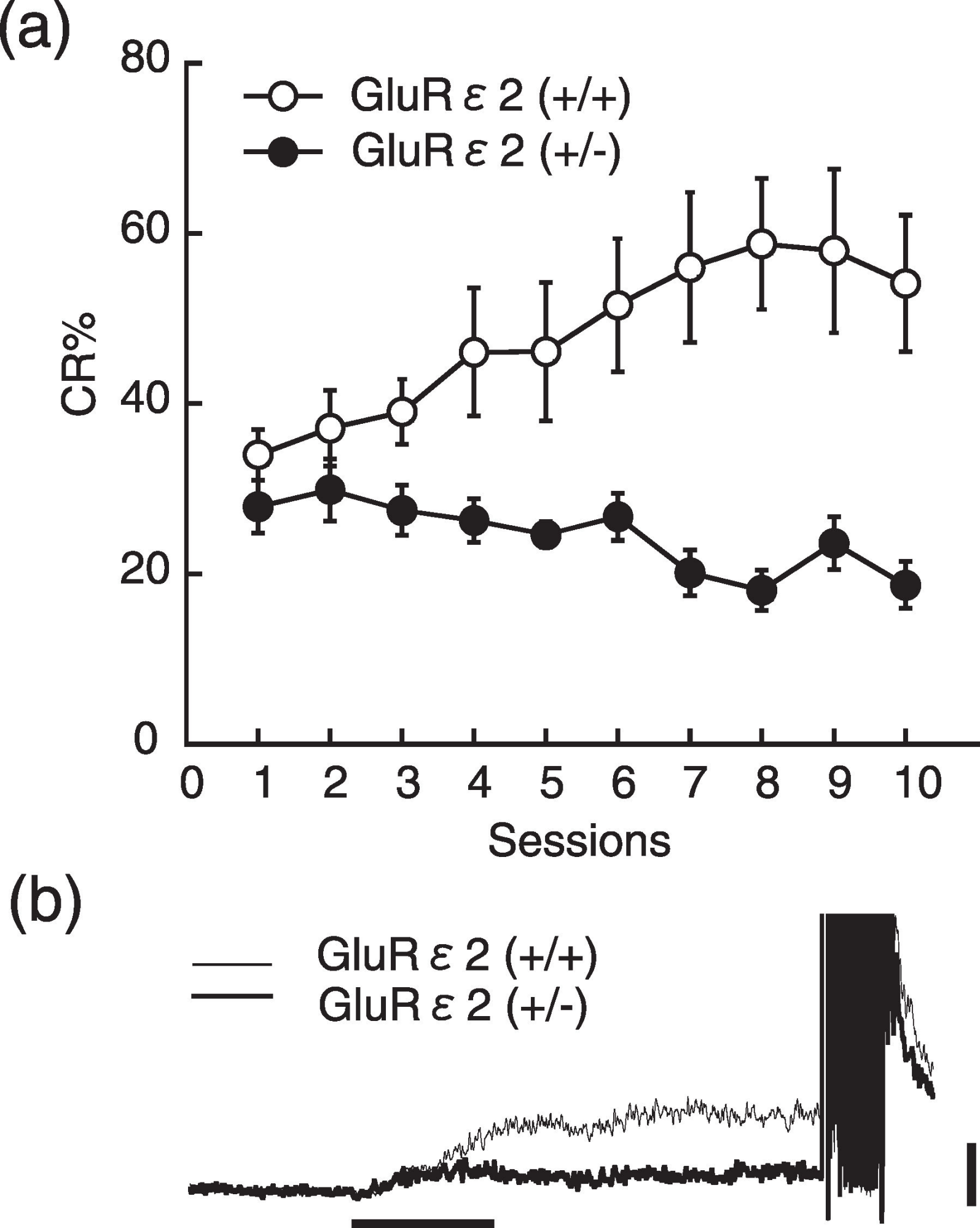
GluR ε 2 (+/-)


(a)
80
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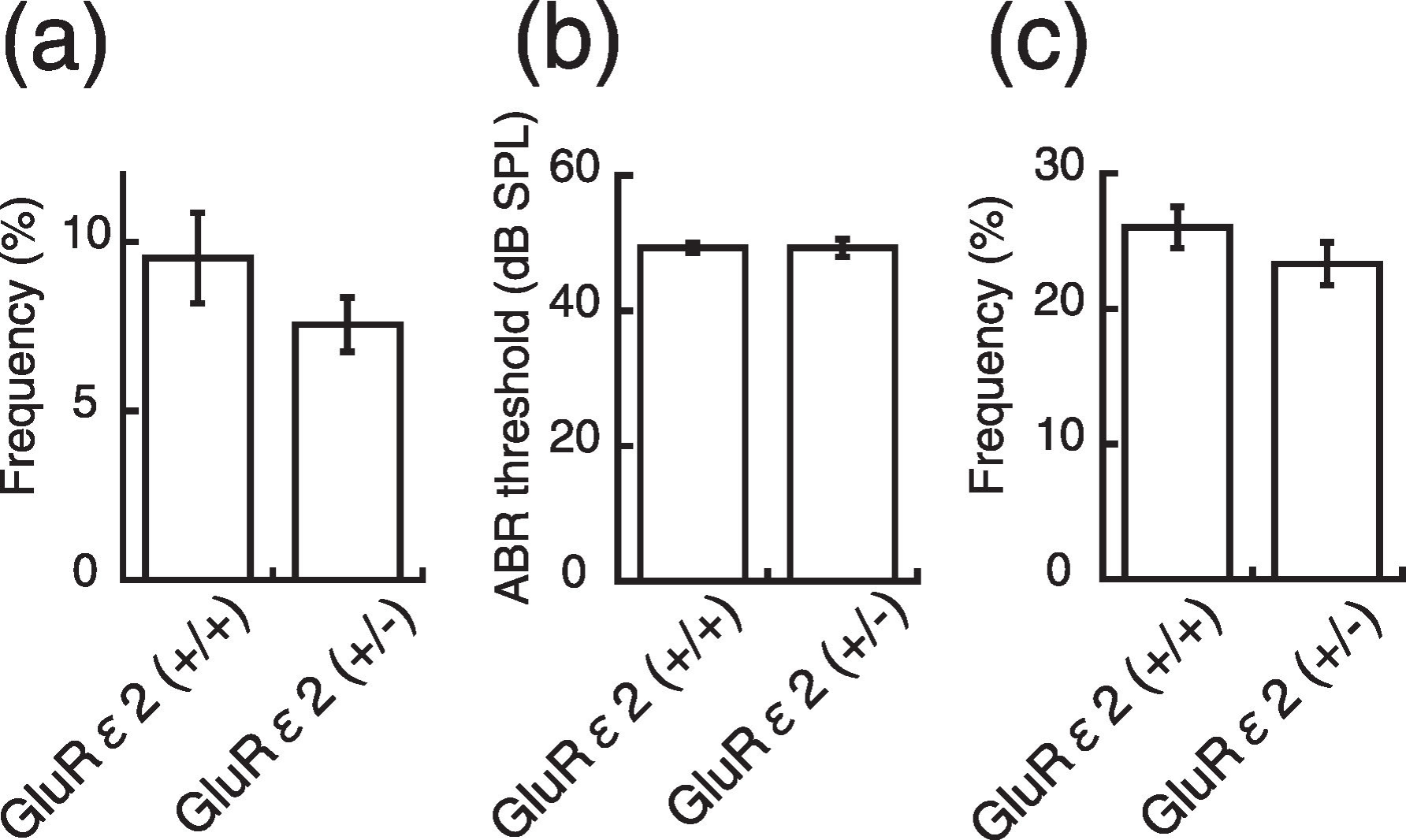
(a)
Frequency (%)
10
5
0
GluRε2 (+/+)
GluRε2 (+/-)
(b)
ABR threshold (dB SPL)
60
40
20
0
GluRε2 (+/+)
GluRε2 (+/-)
(c)
Frequency (%)
30
20
10
0
GluRε2 (+/+)
GluRε2 (+/-)