<table>
<thead>
<tr>
<th>Title</th>
<th>Age-Dependent Impairment of Eyeblink Conditioning in Prion Protein-Deficient Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Kishimoto, Yasushi; Hirono, Moritoshi; Atarashi, Ryuichiro; Sakaguchi, Suehiro; Yoshioka, Tohru; Katamine, Shigeru; Kirino, Yutaka</td>
</tr>
<tr>
<td>Citation</td>
<td>PLoS ONE, 8(4), e60627; 2013</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2013-04-10</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10069/32384">http://hdl.handle.net/10069/32384</a></td>
</tr>
<tr>
<td>Rights</td>
<td>© 2013 Kishimoto et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.</td>
</tr>
</tbody>
</table>
Age-Dependent Impairment of Eyeblink Conditioning in Prion Protein-Deficient Mice

Yasushi Kishimoto1,2a, Moritoshi Hirono2b, Ryuichiro Atarashi3, Suehiro Sakaguchi4, Tohru Yoshioka5, Shigeru Katamine3,6, Yutaka Kirino1,6a

1 Laboratory of Neurobiophysics, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan, 2 Laboratory for Motor Learning Control, RIKEN Brain Science Institute, Wako, Japan, 3 Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan, 4 Division of Molecular Neurobiology, The Institute for Enzyme Research, The University of Tokushima, Tokushima, Japan, 5 Center for Excellence for Environmental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, 6 Center for International Collaborative Research, Nagasaki University, Nagasaki, Japan

Abstract

Mice lacking the prion protein (PrPC) gene (Prnp), Ngsk Prnp0/0 mice, show late-onset cerebellar Purkinje cell (PC) degeneration because of ectopic overexpression of PrPC-like protein (PrPLP/Dpl). Because PrPC is highly expressed in cerebellar neurons (including PCs and granule cells), it may be involved in cerebellar synaptic function and cerebellar cognitive function. However, no studies have been conducted to investigate the possible involvement of PrPC and/or PrPLP/Dpl in cerebellum-dependent motor learning. Therefore, the present cross-sectional study was designed to examine cerebellum-dependent delay eyeblink conditioning in Ngsk Prnp0/0 mice in adulthood (16, 40, and 60 weeks of age). The aims of the present study were two-fold: (1) to examine the role of PrPC and/or PrPLP/Dpl in cerebellum-dependent motor learning and (2) to confirm the age-related deterioration of eyeblink conditioning in Ngsk Prnp0/0 mice as an animal model of progressive cerebellar degeneration. Ngsk Prnp0/0 mice aged 16 weeks exhibited intact acquisition of conditioned eyeblink responses (CRs), although the CR timing was altered. The same result was observed in another line of PrPC-deficient mice, ZrchI Prnp0/0 mice. However, at 40 weeks of age, CR incidence impairment was observed in Ngsk Prnp0/0 mice. Furthermore, Ngsk Prnp0/0 mice aged 60 weeks showed more significantly impaired CR acquisition than Ngsk Prnp0/0 mice aged 40 weeks, indicating the temporal correlation between cerebellar PC degeneration and motor learning deficits. Our findings indicate the importance of the cerebellar cortex in delay eyeblink conditioning and suggest an important physiological role of prion protein in cerebellar motor learning.

Introduction

Sheep scrapie, bovine spongiform encephalopathy (BSE), and Creutzfeldt–Jacob disease (CJD) in humans are variants of prion diseases, which are caused by infectious agents named prions [1–5]. Prion diseases may be expressed as genetic, infectious, or sporadic disorders, all of which involve modification of the normal cellular form of the prion protein (PrPc). The predominant symptoms are progressive dementia and motor dysfunction such as ataxia [6–7]. To investigate the physiological role of PrPC, several independent PrPC-deficient mouse lines have been developed [8–12].

PrPC-deficient mice generated by some of us (Ngsk Prnp0/0 mice) in which the functional PrP gene (Prnp), including the entire PrP-coding sequence of exon 3 and a part of intron 2 (900 bp), was deleted showed progressive symptoms of ataxic gait and hindquarter tremors late in life [13]. Late-onset ataxia is also observed in 2 other independently derived lines of Prnp0/0 mice [9,11]. In Ngsk Prnp0/0 mice, Purkinje cell (PC) death began at approximately 30–40 weeks of age [13–15]. The other 2 lines of PrPC-deficient mice, including ZrchI Prnp0/0 mice, do not exhibit cerebellar degeneration [8,10]. Ectopic expression of the novel locus Prm, which is 16 kb downstream of Prnp and encodes a 179-residue PrP-like protein Doppel (PrPLP/Dpl), has been observed in the brain of Ngsk Prnp0/0 mice, but not in ZrchI Prnp0/0 brains, and has been implicated in the cerebellar degeneration of Ngsk Prnp0/0 mice [11,16,17]. However, PrPC may be partially involved in long-term survival of PCs because Ngsk Prnp0/0 mice were rescued from PC degeneration by introduction of a transgene encoding wild-type mouse PrPC [15]. Cerebellar degeneration thus appears to require both functional loss of PrPC and cerebellar overexpression of PrPLP/Dpl [18].

PrPLP/Dpl is a putative membrane glycoprotein sharing 23% identity with the PrPC primary amino acid sequence structure [11,19,20]. The function and expression pattern of PrPLP/Dpl have been investigated in the past decade [17,21–26]. However, the molecular mechanism underlying the neuronal degeneration...
induced by ectopic expression of PrP^Lepr/Dpl is unclear, although some hypotheses have been proposed [27–29].

Eyeblink conditioning is one of the best-characterized behavioral models of associative learning in mammals [30–32]. In particular, the standard delay paradigm in which the conditioned stimulus (CS) and unconditioned stimulus (US) are continuous has been used for assessing cerebellum-dependent motor learning in a variety of mammalian species. The neural circuit involved in the response is well defined. The memory trace is considered to be localized in the cerebellar cortex and/or cerebellar deep nuclei, ipsilateral to the trained eye [30,33–36], even if the relative importance of the cerebellar cortex versus that of the cerebellar deep nuclei remains controversial [37,39–45].

Because PrP^Lepr is highly expressed in cerebellar neurons including PCs, granule cells, and deep nuclei [46–48], it may be involved in cerebellar synaptic function and cerebellum-dependent cognitive function. Therefore, investigation of the cerebellar motor learning in protein-deficient mice is important. However, no studies have been conducted to investigate the possible involvement of PrP^Lepr and/or PrP^Lepr/Dpl in cerebellum-dependent discrete motor learning such as adaptation of the vestibulo-ocular reflex (VOR) or eyelid conditioning. Thus, the present study was designed to test delay eyelid conditioning, in which the cerebellum is believed to play important roles for both acquisition and timing of the conditioned response (CR) [31,32,40,49–53]. In Ngsk Prnp^+/0 mice in adulthood (16, 40, and 60 weeks of age). The aims of the present study were two-fold: (1) to examine the role of PrP^Lepr and/or PrP^Lepr/Dpl in cerebellum-dependent motor learning and (2) to confirm the age-related deterioration of eyelid conditioning in Ngsk Prnp^+/0 mice as an animal model of progressive cerebellar degeneration.

Materials and Methods

Ethics Statement

All animal protocols were approved by the Animal Experiment Ethics Committee at the University of Tokyo, and the mice were cared for in accordance with the University of Tokyo Guidelines for the Care and Use of Laboratory Animals. We used the minimum number of animals for these experiments, and care was taken to minimize pain.

Subjects

Ngsk Prnp^+/0 mice were generated as described previously [12,13]. Male F3 Ngsk Prnp^+/0 mice were crossed with female C57BL/6j mice (purchased from CLEA Japan, Tokyo, Japan), producing F4 heterozygous mice (Prnp^+/+) mice. Mutant mice (Ngsk Prnp^+/0) and their littermate controls (Ngsk Prnp^+/+) were derived by intercrossing F4 Ngsk Prnp^+/0 males and females. Genotypes were confirmed by polymerase chain reaction (PCR) amplification of tail-extracted genomic DNA from each mouse. The specific primers for the mouse Prnp gene are 5’-CCGCTACCTACCAACATGTT-3’ and 5’-CCTAGACCCAC-GAGAATGC-3’ (generates a 346-bp PCR fragment). The neomycin-resistant gene primers are 5’-GGTGCCCTGAAT-GAATGCGA-3’ and 5’-GGTGACGGGAATGCAATGATTT-3’, resulting in a 227-bp PCR fragment.

We obtained 46 Ngsk Prnp^+/+ (32.6%), 61 Ngsk Prnp^+/0 (43.3%) and 34 Ngsk Prnp^+/0 (24.1%) mice. Ngsk Prnp^+/+ and Ngsk Prnp^+/0 mice were used for behavioral tests. They were maintained as described until the age of use (16, 40, or 60 weeks). Prior to each session were processed offline as follows: (i) The maximum amplitude of the EMG signals during a time period of 1±1 ms was calculated and denoted as the EMG amplitude at t. (ii) One-hundred EMG amplitude values for 300 ms were averaged (before CS onset), and the standard deviation (SD) was calculated. (iii) The average value obtained from (ii) +SD was defined as the threshold. (iv) For each trial, EMG amplitude data for 300 ms over the threshold were averaged (before CS onset) and called the Pre value. The Startle value was calculated in the same manner for 30 ms after CS onset. The CR value was calculated from the data for the period 52–252 ms after CS onset in the CS-US paired trials. The time window was extended by 100 ms to obtain the CR value in the CS-only trials. (v) Valid trials were defined as those with Pre and Startle values of less than 10% and 30% of the threshold, respectively. (vi) A trial in which the CR value exceeded 1% of the threshold and was double the Pre value was regarded as a successful CR trial. (vii) The ratio of successful CR trials to valid trials was calculated and denoted as the CR%.
In addition to the CR incidence analyses, the temporal pattern of eyeblink responses were also analyzed by averaged EMG and peak latency histogram analyses. In the EMG amplitude analyses, all 100 trials (including invalid trials) were used on day 3 or 7 [54], whereas only CR trials were used for the peak latency histogram analyses [55]. In the histogram analyses, the relative frequencies of the CR peak were plotted as a function of its latency. The CR on day 7 was binned into time windows of 52.1–102.0, 102.1–152.0, 152.1–202.0, 202.1–252.0, 252.1–302.0, and 302.1–352.0 ms, measured from the CS onset.

Statistical Analyses

Data were statistically analyzed in Microsoft Excel using a two-tailed Student’s t-test (for histogram latency analyses [55]), or repeated-measures ANOVA following a post hoc Scheffé’s test (for CR% analyses [55–57]). Differences were considered statistically significant when \( p < 0.05 \).

Results

**CR Acquisition in 16-week-old Ngsk Prnp \(^{0/0}\) Mice**

First, delay eyeblink conditioning with the CS and US simultaneously terminated was performed in 16-week-old Ngsk Prnp \(^{0/0}\) mice (without apparent histological and motor abnormalities) during 7 consecutive days. Figure 1A shows the development of the averaged percentage of the acquired conditioned response (CR%, an index of learning), which was calculated using 100 trials/day for each animal, in control (Prnp \(^{+/+}\)) and experimental (Ngsk Prnp \(^{0/0}\)) mice. The CR% for the 2 genotypic groups progressively increased to approximately 60% during the 7-day acquisition session, although the CR acquisition in experimental mice was moderately higher than in the control mice. A repeated-measures ANOVA revealed no significant group \( \times \) session interaction effect \( (F_{6,156} = 1.299, \ p = 0.26) \) or group effect \( (F_{1,26} = 3.14, \ p = 0.088) \) during the acquisition session. The top inset in Figure 1A shows individual EMG topographies averaged by 100 trials on days 3 and 7 in both groups. On day 3, experimental mice displayed somewhat higher CR amplitudes than the control mice did. Therefore, we conclude that CR acquisition of eyeblink conditioning was not impaired in experimental 16-week-old mice aged.

No difference was detected between control and experimental mice in the spontaneous eyeblink frequency (13.1 ± 2.1% for control mice and 13.3 ± 2.8% for experimental mice). Furthermore, the relative number of valid trials \( (81.8\% \pm 4.4\% \) for control mice and 79.9% ± 2.6% for experimental mice) and the frequency of startle responses \( (3.3\% \pm 1.0\% \) for control mice and 6.2% ± 1.8% for experimental mice) were not significantly different. In addition, no differences were noted between 16-week-old control mice and experimental mice in pseudoconditioning (see Figure S1A and Text S1) or responses to CS (see Figure S2A and Text S1). The experimental mice at the age of 16 weeks exhibited normal performance in rota-rod and fixed-bar tests, indicating that motor coordination was normal in this mutant at this age (data not shown).

**Altered Timing of CR in Ngsk Prnp \(^{0/0}\) Mice Aged 16 Weeks**

Figure 1B depicts the averaged electromyographic (EMG) amplitudes obtained from control and experimental mice aged 16 weeks on day 7 of the acquisition session. The data suggest that CRs in experimental mice are altered in their timing. In Figure 1B, however, CR components in the range of 250–350 ms from CS onset were masked by US artifacts. Therefore, we re-evaluated the averaged EMG amplitudes by using the 10 CS-only trials on day 7 in the acquisition session (Figure 1C). These analyses enabled us to investigate EMG patterns throughout the CS period without the US artifacts. As shown in Figure 1C, the peak CR amplitude for control mice is expressed within the expected US period. However, the experimental mice express a weaker, transient response with its peak before US onset.

In addition, we quantitatively analyzed the topography of CR expression in experimental mice at 16 weeks in 2 ways. Figure 2A shows the relative frequency of the CR peak until US onset in 100 trials on day 7, expressed in each time window (52–102, 102–152, 152–202, and 202–252 ms from CS onset). The normalized frequency of CR peak expression in the last time window (202–252 ms) is significantly higher in experimental mice than in control mice \( (p < 0.05) \). Next, the relative frequency of the CR peak on day 7 was re-analyzed using 10 CS-only trials in time windows extended to the US end (52–102, 102–152, 152–202, 202–252, 252–302, and 302–352 ms from CS onset; Figure 2B). Figure 2B shows that the normalized frequency of CR peak expression during the expected US time window (252–302 ms) from CS onset) is significantly lower in experimental mice than in control mice \( (p < 0.05) \). Thus, the CR peak latency within the UR period decreased in young Ngsk Prnp \(^{0/0}\) mice than in control mice, with a shifted CR peak in experimental mice approximately 100 ms earlier than in control mice.

**Eyeblink Conditioning in Prion Protein KO Mice**

Previous studies [13,15] have reported that 40-week-old Ngsk Prnp \(^{0/0}\) mice begin to exhibit histological abnormalities such as PC axonal swelling and demyelination in the spinal cord and peripheral nervous system, but exhibit no behavioral irregularities. However, as shown in Figure 3A, the CR% for the experimental mice at this age was 31.0% ± 8.0% on the 7th day of training, whereas the CR% for the age-matched control mice progressively increased to 63.2% ± 9.9% during the 7-day acquisition period. The results of repeated-measures ANOVA revealed significant effects in the interaction between sessions and genotypes \( (F_{6,100} = 2.28, \ p = 0.041) \). The top inset in Figure 3A shows individual EMG topographies averaged by 100 trials on day 7 in both groups. In contrast to 16-week-old mice, these data indicate impaired eyeblink conditioning in the 40-week-old experimental mice. However, they exhibited normal spontaneous eyeblink frequencies comparable to that of the control mice \( (12.6% \pm 3.2% \) for control mice and 10.2% ± 2.3% for experimental mice). Furthermore, the relative number of valid trials \( (78.8% \pm 8.7% \) for control mice and 75.8% ± 7.7% for experimental mice) and startle responses \( (5.1% \pm 1.9% \) for control mice and 4.1% ± 0.9% for experimental mice) did not differ between genotypes. In addition, no differences were detected between control and Ngsk Prnp \(^{0/0}\) mice aged 40 weeks in pseudoconditioning or auditory responses (see Figures S1B and S2A). Motor coordination, evaluated using the rota-rod and fixed-bar tests, was still normal in this mutant at this age (data not shown). These results indicate that 40-week-old experimental mice have intact eyeblink motor output and sensitivity to the CS.

Next, we investigated the CR temporal pattern in 40-week-old experimental mice. Figure 3B shows the averaged EMG amplitudes obtained from 40-week-old control and experimental mice on day 7 of the acquisition sessions. The shorter components of the CR peak latency were larger in the experimental mice, whereas all components were comparable in the control mice. Quantitative analyses of the CR temporal properties (Figure 3C) also indicated a significantly higher CR peak expression in the earliest time.
window (52–102 ms) in experimental mice (p, 0.05), whereas the relative frequency of CR peak expression in the last time window (202–252 ms) was much smaller than the control mice (p, 0.05). Thus, CR peak latency was significantly shortened in 40-week-old experimental mice. This aberrant CR timing is more prominent than that observed in 16-week-old experimental mice (Figure 2A and 2B).

Next, we investigated eyeblink conditioning in the 60-week-old Ngsk Prnp0/0 mice. Sixty-week-old Ngsk Prnp0/0 (experimental) mice began to exhibit dramatic loss of PCs throughout the cerebellar vermis [13,15]. Figure 4A shows the time course of the CR% in 60-week-old controls and experimental mice. The CR% for the wild-type mice gradually increased to 42.9%±6.8% on day 7. This comparatively low value is likely due to normal aging effects [58], although ANOVA revealed no statistically significant difference in the CR performance between 16- and 60-week-old control mice (statistical data not shown). However, CR acquisition was profoundly impaired in experimental mice, even when the age-related impairment was taken into account. The CR% was only 17.6%±2.2% on the 7th day of training. Although found no significant effects in the interaction between the sessions and genotypes (F(6,24) = 0.46, p = 0.86), we did find significant effects due to genotype (F(1,14) = 6.93, p = 0.019). The top inset in Figure 4A shows individual EMG topographies averaged by 100 trials/day for each animal during a 7-day session in 16-week-old control (open circle, n = 14) and Ngsk Prnp0/0 (experimental mice; closed circle, n = 14) mice. The top inset shows individual response topographies of averaged electromyographs (EMGs) until US onset in the control and experimental mice on days 3 and 7. (B) The averaged EMG amplitudes on day 7 of the acquisition session in control (n = 14) and experimental (n = 14) mice. All EMG amplitudes obtained in 1 day (100 trials) were summed, representing the average of the eyelid responses. The CR components of 250–350 ms from CS onset were masked by artifacts caused by the US. (C) The averaged EMG amplitudes were evaluated using only 10 CS-only trials on day 7 of the acquisition session. These analyses enable us to indicate EMG patterns in the whole CS period without the US artifacts. Values are mean ± SEM. *p<0.05. doi:10.1371/journal.pone.0060627.g001

Figure 1. Eyeblink conditioning in 16-week-old Ngsk Prnp0/0 mice. (A) CR development was calculated using 100 trials/day for each animal during a 7-day session in 16-week-old control (open circle, n = 14) and Ngsk Prnp0/0 (experimental mice; closed circle, n = 14) mice. The top inset shows individual response topographies of averaged electromyographs (EMGs) until US onset in the control and experimental mice on days 3 and 7. (B) The averaged EMG amplitudes on day 7 of the acquisition session in control (n = 14) and experimental (n = 14) mice. All EMG amplitudes obtained in 1 day (100 trials) were summed, representing the average of the eyelid responses. The CR components of 250–350 ms from CS onset were masked by artifacts caused by the US. (C) The averaged EMG amplitudes were evaluated using only 10 CS-only trials on day 7 of the acquisition session. These analyses enable us to indicate EMG patterns in the whole CS period without the US artifacts. Values are mean ± SEM. *p<0.05.

Eyeblink Conditioning in Prion Protein KO Mice
indicated that the normalized frequency of CR peak expression during the US time window (252–302 and 302–352 ms from CS onset) was significantly lower in the experimental mice than in the 3 control strains (p<0.01). Thus, the shift of CR peak latency to approximately 100 ms earlier was common to the 2 lines of Prnp0/0 mice. We found no differences between control groups and experimental mice in spontaneous eyelink frequency (12.4±2.7% for control mice and 16.1±3.5% for experimental mice), startle response frequency (3.1±0.7% for control mice and 4.6±0.7% for experimental mice), pseudoconditioning (See Figure S1D), or eyelink frequencies in the presence of CS (See Figure S2B). Motor coordination, evaluated by rota-rod and fixed-bar tests, was normal in the experimental mice (data not shown).

Discussion

In the present study, we investigated cerebellum-dependent eyelink conditioning in PrPFl-deficient Ngsk Prnp0/0 mice. Eyeblink conditioning has been used as an evaluation system for human dementias, including Alzheimer’s disease [56,57,61–63] and aging-related learning deficit [58,62,64,65]. Therefore, it represents a useful system for evaluating the learning ability of mutant animals and elucidating the mechanisms underlying human neurodegenerative diseases. This initially prompted us to examine eyelink conditioning in Ngsk Prnp0/0 mice, which exhibit late-onset PC degeneration, and are an animal model of progressive cerebellar degeneration disorder. Our results revealed that Ngsk Prnp0/0 mice exhibit significant CR incidence deficits in eyelink conditioning as early as at 40 weeks of age. The impairment became more pronounced in 60-week-old Ngsk Prnp0/0 mice, indicating an age-dependent deterioration of CR acquisition in eyelink conditioning. We also analyzed the learning-dependent timing of eyelink CRs. The CR timing was altered in advanced aged Ngsk Prnp0/0 mice as well as, unexpectedly, in 16-week-old Ngsk Prnp0/0 mice, before any apparent neurological abnormalities emerged. The altered CR timing was also observed in another line of PrPFl KO mice, Zrch1 Prnp0/0 mice. These results suggest that PrPFl is involved in cerebellar function for CR timing during eyelink conditioning.

Impaired Acquisition of Conditioned Eyelink Response in Aged Ngsk Prnp0/0 Mice

Sixteen-week-old Ngsk Prnp0/0 mice, which show no apparent behavioral or histological abnormalities [13], exhibited intact CR acquisition (Figure 1A). Zrch1 Prnp0/0 mice aged 18–22 weeks also exhibited normal CR incidence during the acquisition secession (Figure 5A). However, 40-week-old and 60-week-old Ngsk Prnp0/0 mice showed significant CR acquisition impairment (Figures 3 and 4). It should be noted that the control mice aged 60 weeks also exhibited a deterioration tendency related to normal aging effects [58]. Because histological changes of the CNS (including abnormal myelination in the spinal cord) occur at approximately 40 weeks of age [15], it is most likely that impaired CR acquisition is due to these neurological abnormalities, particularly cerebellar PC loss [24,28,66,67]. This possibility could be supported by previous results obtained using pcd (Purkinje cell degenerated) mutant mice [68], in which the postnatal death of virtually all cerebellar PCs occurred during the third and fourth postnatal week [69]. The pcd mutant mice exhibited severely impaired CR acquisition during delay eyelink conditioning, indicating the necessity of an intact cerebellar cortex, particularly preserved cerebellar PCs, for acquisition in delay eyelink conditioning. In addition, experiments using globally depleted cerebellar PCs with the immunotoxin OX7-saporin and investigations of the individual differences

Altered Timing but Normal CR Acquisition in Young Zrch1 Prnp0/0 Mice

We next investigated whether these behavioral abnormalities in Ngsk Prnp0/0 mice were also observed in another line of PrPFl-deficient mice, Zrch1 Prnp0/0 mice, which show neither neurodegeneration nor PrPLP/Dpl ectopic expression [8,59]. Figure 5A shows the CR% during the 7-day acquisition session in Zrch1 Prnp0/0 (experimental) mice and 3 wild-type control strains (C57BL/6J, 129/SvJ, and FVB/Nj) at 18–22 weeks of age. Although eyelink conditioning in mice depends on the genetic background [60], we observed no significant difference in CR% among the 3 control strains. Figure 5A shows development of the CR%. Experimental mice, as well as young Ngsk Prnp0/0 mice (Figure 1A), showed normal CR incidence during the acquisition secession (Figure 5A). However, 40-week-old and 60-week-old Ngsk Prnp0/0 mice showed significant CR acquisition impairment (Figures 3 and 4). It should be noted that the control mice aged 60 weeks also exhibited a deterioration tendency related to normal aging effects [58]. Because histological changes of the CNS (including abnormal myelination in the spinal cord) occur at approximately 40 weeks of age [15], it is most likely that impaired CR acquisition is due to these neurological abnormalities, particularly cerebellar PC loss [24,28,66,67]. This possibility could be supported by previous results obtained using pcd (Purkinje cell degenerated) mutant mice [68], in which the postnatal death of virtually all cerebellar PCs occurred during the third and fourth postnatal week [69]. The pcd mutant mice exhibited severely impaired CR acquisition during delay eyelink conditioning, indicating the necessity of an intact cerebellar cortex, particularly preserved cerebellar PCs, for acquisition in delay eyelink conditioning. In addition, experiments using globally depleted cerebellar PCs with the immunotoxin OX7-saporin and investigations of the individual differences...
of PCs have shown that the degree of PC loss was well correlated with the magnitude of CR acquisition impairments in rats or rabbits [70,71]. Furthermore, patients with cerebellar neurodegenerative diseases are reported to exhibit severe impairment of CR incidence during delay eyeblink conditioning [72–76]. Our results support the notion that delay eyeblink conditioning is highly sensitive for detecting cerebellar PCs neurodegenerative changes and deficits in both humans and rodents. However, in the present study, we did not examine delay eyeblink conditioning in aged Zrch Prnp0/0 mice (at the ages of 40 and 60 weeks). Therefore, we cannot deny the possibility that other undiscovered, Prnp deficiency-induced, age-dependent abnormalities might have impaired eyeblink conditioning in aged Ngsk Prnp0/0 mice.

We found no difference between controls and Prnp0/0 mice in spontaneous eyeblink frequency, sensitivity to the tone, and electrical shock (data not shown). The auditory responses to tone CS were not altered in Ngsk and Zrch Prnp0/0 mice during the age periods examined, despite the trend of higher eyelink frequencies in the mutants at 16 and 40 weeks of age (Figure S2 and Text S1). Pseudoconditioning was also not altered in Prnp0/0 mice (Figure S1 and Text S1). These data confirm that the impairments in older Ngsk Prnp0/0 mice did not originate from basic performance disturbances (including sensitivity to the US and CS). Nevertheless, Ngsk Prnp0/0 mice aged 60 weeks exhibited considerable ataxia and motor dysfunction [13]; therefore, at this age, we cannot exclude the possibility that the eyeblink CR deficit was due to general ataxia rather than a specific learning deficit in Ngsk Prnp0/0 mice. However, several examples of intact eyeblink conditioning despite severe ataxia have been reported in previous studies on knockout mice [77,78]. Thus, eyeblink conditioning deficits and motor function deficits may not necessarily be closely correlated. We observed apparently higher CR acquisition in 16- and 40-week-old Ngsk and Zrch Prnp0/0 mice at the early phase of delay eyeblink conditioning (Figures 1A, 3A, and 5A). We do not deny that the hyperactivity might be due to higher base line of eyelink frequencies under tone CS in the Prnp0/0 mice (Figure S2). However, even if this is the case, the important point is that 40-week-old Ngsk Prnp0/0 mice exhibited lower CR% in the attained level, despite the higher eyeblink frequency baseline.

Recently, Zrch Prnp0/0 mice were reported to have more excitability as well as larger and longer long-term potentiation (LTP) at the hippocampal CA1 synapse in vivo than their littermate controls [79]. The hippocampus is also important for simple delay eyeblink conditioning [80–82]; that is, the hippocampus affects the rate of CR acquisition during the delay conditioning [83]. Indeed, we previously showed that mutant mice lacking the NMDA receptor e1 subunit, which have impaired hippocampal CA1-LTP, exhibited slower CR acquisition but maintained an intact attained level of CRs during a 7-day acquisition session on delay eyeblink conditioning [84,85]. Taken together, our results suggest that the rapid acquisition, but normal attained level, of CRs during delay eyeblink conditioning observed in young Ngsk and Zrch Prnp0/0
mice (Figures 1A and 5A) might be explained by the larger hippocampal LTP in these strains.

Altered Timing of Conditioned Eyeblink Response in Ngsk and ZrchI Prnp0/0 Mice

In eyeblink conditioning, the CR is viewed as a prediction of the imminence of the US, and the peak CR amplitude coincides with the timing of the US. The results of lesion experiments have suggested the involvement of the cerebellar cortical circuit in CR timing [53], although it has been recently indicated that electrotonic coupling among olivary neurons by gap junctions is important for the proper timing of eyelid CRs [86]. Previous theoretical and simulation studies have suggested that the interactions between populations of granule cells and Golgi cells are engaged in the regulation of CR timing [49,52,87,88]. We have shown that young Ngsk Prnp0/0 mice exhibit altered CR timing (Figure 2A and 2B). ZrchI Prnp0/0 mice aged 18–22 weeks, which also exhibit no histological abnormalities [8,10], reproducibly exhibited altered timing of the CR (Figure 5B and 5C); these mutant mice had apparently longer CR peak latencies than control mice, as evident in the results of a conventional latency analysis (Figures 2A and 5B). However, taking into account of the CR components during US period, CR peaks of these mutant mice were substantially shifted earlier than those of control mice (Figures 2B and 5C). This temporal pattern resembles that observed in waggler and stargazer mutant mice, which have dysfunctional cerebellar granule cells and deficits in brain-derived neurotrophic factor [89–92]. In addition, intracellular calcium homeostasis was disturbed in cultured cerebellar granule cells from ZrchI Prnp0/0 mice [93]. This is also similar to the phenotype of waggler mutant mice, in which a putative neuronal Ca2+ channel γ subunit is disrupted in cerebellar granule cells. Thus, some abnormal granule cell signaling cascades could affect CR timing in both young Ngsk and ZrchI Prnp0/0 mice. Indeed, previous immunohistochemical studies have suggested that PrPC is highly expressed in the axon terminals of cerebellar granule cells [94]. Furthermore, ZrchI Prnp0/0 mice exhibited abnormal granule cell excitability and altered synaptic plasticity at synapses between mossy fibers and granule cells [95]. Therefore, examination of the neurophysiological properties of granule cells from Ngsk Prnp0/0 mice would be important. Recently, the Delgado-García group proposed a reinforcing-modulating role of posterior interpositus neurons in the proper performance of eyeblink CRs [42,43]. Thus, it would be interesting to investigate neurophysiological properties from the interpositus neurons of Prnp0/0 mice because PrPC is highly expressed in cerebellar deep nuclei [46].

On the other hand, in Ngsk Prnp0/0 mice aged 40 weeks, the early components of the CR occurring immediately after CS onset (52–102 ms) dominate, whereas later components (202–252 ms) of the CR are much decreased even by conventional timing analyses (Figure 3C). Ngsk Prnp0/0 mice aged 60 weeks exhibited a similar tendency, i.e., shortened CR peak timing, although no significant

Figure 4. Impaired eyeblink conditioning in 60-week-old Ngsk Prnp0/0 mice. (A) Eyeblink conditioning performance during the 7-day acquisition training in Ngsk Prnp0/0 mice (experimental mice; closed circle, n = 8) and control mice (open circle, n = 8) aged 60 weeks. The top inset shows individual response topographies of averaged EMGs until US onset in control and experimental mice on day 7. (B) Averaged EMG amplitudes during the acquisition session on day 7. All EMG amplitudes obtained in 1 day were summed, representing the average of the eyelid responses. (C) Frequency histogram showing the peak CR latencies. The CR on day 7 was binned into time windows of 52–102, 102–152, 152–202, and 202–252 ms measured from CS onset in experimental mice (closed column) and control mice (open column). Data points represent the mean ± SEM. Data points represent the mean ± SEM. *p<0.05, **p<0.01.

doi:10.1371/journal.pone.0060627.g004
differences were found in any component between Ngsk Prnp<sup>0/0</sup> and control mice (Figure 4C). We consider that analyses of the temporal pattern of CR expression were not applicable in Ngsk Prnp<sup>0/0</sup> mice aged 60 weeks, because only a few CRs were observed in the 60-week-old Ngsk Prnp<sup>0/0</sup> mice. This shortened aberrant CR timing pattern observed in 40-week-old and 60-week-old Ngsk Prnp<sup>0/0</sup> mice is similar to that observed in animals with a lesioned cerebellar cortex [33] and in pcd mutant mice, which lose nearly all of their PCs during development [68]. Additionally, a shortened CR peak timing was also observed in Lurcher mutant mice, which lack PCs and granule cells in the cerebellar cortex due to a mutation in the GluRδ2 receptor [96]. Furthermore, patients with cortical cerebellar degeneration also show similar timing deficits of eyelink CRs, suggesting that some areas of the superior cerebellar cortex are important for the formation of appropriately timed conditioned eyelink responses in humans [51,76]. Thus, the shorter latency of the CR in 60-week-old Ngsk Prnp<sup>0/0</sup> mice might also be explained by cerebellar cortical degeneration. In wild-type mice, the short-latency responses are not expressed, possibly because interpositus nuclei are strongly inhibited by PC activity during the early CS phases, whereas in older Ngsk Prnp<sup>0/0</sup> mice, the short-latency response pathway is unmasked because of the loss of cerebellar cortex output [52].

Conclusions
In this cross-sectional study, we investigated eyelink conditioning in Ngsk Prnp<sup>0/0</sup> mice, which exhibit cerebellar neurodegenerative symptoms late in life. We found age-dependent alterations of eyelink conditioning in 2 indices: (i) CR incidence and (ii) the timing of CR expression. The CR incidence impairment in older Ngsk Prnp<sup>0/0</sup> mice could be attributed to progressive degeneration of PCs, indicating the importance of the cerebellar cortex in acquisition of eyelink conditioning. Intriguingly, the CR timing was altered in both young Ngsk Prnp<sup>0/0</sup> mice and older Ngsk Prnp<sup>0/0</sup> mice. The shortened latency observed in young and older Ngsk Prnp<sup>0/0</sup> mice is consistent with the CR temporal pattern observed in cerebellar cortex-lesioned animals [53,68,96] and cerebellar patients [51,76], suggesting that cerebellar degeneration affects aberrant CR timing. Finally, the altered CR timing was also observed in young ZrchI Prnp<sup>0/0</sup> mice, suggesting that PrP<sup>C</sup> is involved in cerebellar function for CR timing during eyelink conditioning.

Supporting Information

Figure S1 Pseudoconditioning in prion knockout mice. (A) Pseudoconditioning in Ngsk Prnp<sup>0/0</sup> (n = 4) and their control (n = 4) mice at 16 weeks old. (B) Pseudoconditioning in Ngsk Prnp<sup>0/0</sup> (n = 8) and their control mice (n = 9) mice at 40 weeks old. (C) Pseudoconditioning in Ngsk Prnp<sup>0/0</sup> (n = 8) and their control (n = 8) mice at 60 weeks old. (D) Pseudoconditioning in Zrch Prnp<sup>0/0</sup> (n = 10) and their control (n = 9) mice at 60 weeks old. The data points represent the mean ± SEM.

Figure S2 Normal auditory response to tone CS in prion knockout mice. (A) Frequency of eyelink response during CS in Ngsk Prnp<sup>0/0</sup> (KO) and their control (CT) mice at the ages of 16, 40, and 60 weeks. (B) Frequency of eyelink response during CS in Zrch Prnp<sup>0/0</sup> (KO) and their control mice (CT). The data points represent the mean ± SEM. The values in parentheses above the column indicate the number of mice used.
Text S1 Supplemental Methods and Supplemental Results.

Acknowledgments

We are grateful to Dr. Charles Weissmann for generously providing the Zcrh Pnp<sup>−/−</sup> mice. We thank Dr. Tadashi Nakaya for technical assistance.

References


Author Contributions

Generation of mouse line: SS SK. Conceived and designed the experiments: Y. Kishimoto Y. Kirino. Performed the experiments: Y. Kishimoto. Analyzed the data: Y. Kishimoto MH. Contributed reagents/materials/analysis tools: RA SS TY. Wrote the paper: Y. Kishimoto Y. Kirino.
Eyeblink Conditioning in Prion Protein KO Mice


