Impairment of cerebellar long-term depression and GABAergic transmission in prion protein deficient mice ectopically expressing PrPLP/Dpl

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Prion protein (PrPơ) knockout mice, named as the "Ngsk" strain (Ngsk Prnp0/0 mice), show late-onset cerebellar Purkinje cell (PC) degeneration because of ectopic overexpression of PrPơ-like protein (PrPLP/Dpl). Our previous study indicated that the mutant mice also exhibited alterations in cerebellum-dependent delay eyelblink conditioning, even at a young age (16 weeks of age) when neurological changes had not occurred. Thus, this electrophysiological study was designed to examine the synaptic function of the cerebellar cortex in juvenile Ngsk Prnp0/0 mice. We showed that Ngsk Prnp0/0 mice exhibited normal paired-pulse facilitation but impaired long-term depression of excitatory synaptic transmission at synapses between parallel fibres and PCs. GABA$_A$-mediated inhibitory postsynaptic currents recorded from PCs were also weakened in Ngsk Prnp0/0 mice. Furthermore, we confirmed that Ngsk Prnp0/0 mice (7–8-week-old) exhibited abnormalities in delay eyelink conditioning. Our findings suggest that these alterations in both excitatory and inhibitory synaptic transmission to PCs caused deficits in delay eyelink conditioning of Ngsk Prnp0/0 mice. Therefore, the Ngsk Prnp0/0 mouse model can contribute to study underlying mechanisms for impairments of synaptic transmission and neural plasticity, and cognitive deficits in the central nervous system.

Over the past few decades, various independent lines of mice lacking prion protein (PrPơ) have been generated to evaluate the role of this protein1–3. Most lines of PrPơ KO mouse show neuronal dysfunction, such as impaired long-term potentiation, and motor incoordination, and altered circadian rhythm4. Among the PrPơ KO mouse lines, the locus Prnd, which is 16 kb downstream of Prnp and encodes the 179 residue PrP-like protein Doppe (PrPLP/Dpl), were ectopically expressed in the brain of Ngsk Prnp0/0 mice4,5, but not in the brain of ZrchI Prnp0/0 mice. Ngsk Prnp0/0 mice exhibited drastic neuronal changes of late-onset cerebellar Purkinje cell (PC) degeneration6, possibly because of both the functional loss of PrPơ and/or overexpression of PrPLP/Dpl in the cerebellum7,8. Indeed, PrPơ is thought to have neuroprotective properties against oxidative stress9. Our previous study demonstrated that the Ngsk Prnp0/0 mice exhibited age-dependent alterations in cerebellum-dependent eyelink conditioning in 2 indices: the conditioned response (CR) probability and timing of CR expression5,10. Ngsk Prnp0/0 mice at the age of 16 weeks exhibited apparently faster CR acquisition but a lower CR amplitude and impaired adaptive CR timing10.

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PrPC is highly expressed in normal cerebellar Purkinje cells (PCs) and granule cells, indicating that the protein plays a role in normal cerebellar synaptic function and neuronal plasticity. Although electrophysiological studies of cerebellar function have been already performed in ZrchI Prnp0/0 mice, the physiological properties of cerebellar PCs in Ngsk Prnp0/0 mice have not been characterized. Therefore, in the present study, we examined whether a deficiency in PrPC and ectopic expression of PrPLP/Dpl in Ngsk Prnp0/0 mice affect cerebellar physiological functions by evaluating the basic excitatory and inhibitory synaptic transmission to PCs and long-term depression (LTD) of excitatory synaptic transmission at parallel fibre (PF)-PC synapses. We found that Ngsk Prnp0/0 mice showed not only weakened GABA-mediated inhibitory postsynaptic currents in PCs but also impaired LTD, suggesting that PrPLP/Dpl expression can induce cerebellar dysfunctions by impairing cerebellar synaptic transmission.

Results

Normal synaptic transmission and altered IPSCs in Ngsk Prnp0/0 PCs. First, to examine synaptic function at PF-PC synapses in Ngsk Prnp0/0 mice, we measured PF-excitatory postsynaptic currents (EPSCs) and obtained their rise and decay time constants and paired-pulse facilitation (PPF). It is noted that PF-induced EPSCs are measured in the presence of bicuculline (10 μM) in ACSF to abolish IPSCs. The mean rise time constant of EPSCs, calculated using a single exponential fit, was 2.04 ± 0.37 ms (n = 13) and 1.69 ± 0.33 ms (n = 12) in cerebellar PCs from Ngsk Prnp+/+ (control) and Ngsk Prnp0/0 mice, respectively. The mean decay time constant was 15.5 ± 1.1 ms (n = 13) in the control slices vs. 18.4 ± 2.1 ms (n = 12) in the Ngsk Prnp0/0 slices. There was no significant difference in either the rise or decay time constants between cerebellar PCs from control and Ngsk Prnp0/0 slices (p = 0.74, 0.52, respectively). The PF-evoked responses exhibited PPF, which is considered to arise from increased transmitter release from PF terminals. The PPF decreased with interpulse intervals in a similar manner as in control and Ngsk Prnp0/0 mice (p > 0.05, Fig. 1a,b). Thus, the short-term synaptic plasticity of PF-PC synapses appeared to be normal in Ngsk Prnp0/0 mice. Furthermore, no significant difference was found in the resting membrane potentials (–51.5 ± 1.1 mV, n = 13 for control slices vs –55.4 ± 1.5 mV, n = 12 for Ngsk Prnp0/0 slices, p = 0.74). However, monosynaptic GABA-A-mediated inhibitory postsynaptic currents (IPSCs) elicited by extracellular stimulation of inhibitory interneurons within the molecular layer were significantly altered in Ngsk Prnp0/0 PCs (Fig. 2). The rise time constant was significantly larger (i.e., IPSC rise was slower, p < 0.001) in Ngsk Prnp0/0 mice (3.09 ± 0.20 ms, n = 10) than in control mice (1.98 ± 0.19 ms, n = 10), while the decay-phase time constant did not differ significantly between the two groups (21.89 ± 2.32 ms, n = 10 for control slices vs 28.45 ± 2.34 ms, n = 10 for Ngsk Prnp0/0 slices, p = 0.062). Additionally, the averaged amplitude of IPSCs in Ngsk Prnp0/0 PCs was smaller than that in controls (80.1 ± 9.0 pA, n = 10 for control slices vs 44.1 ± 6.4 ms, n = 10 for Ngsk Prnp0/0 slices, p = 0.023) without a different in the paired-pulse ratio (PPR) (1.00 ± 0.07, n = 10 for control slices vs 1.15 ± 0.11, n = 10 for Ngsk Prnp0/0 slices, p = 0.74). Thus, GABA-A-mediated IPSC was slower and weaker in Ngsk Prnp0/0 PCs.
Reduced cerebellar LTD in Ngsk Prnp<sup>0/0</sup> PCs. Next, to test the cerebellar LTD of synaptic transmission between PFs and PCs in Ngsk Prnp<sup>0/0</sup> mice, we recorded PF-EPSCs from cerebellar PCs in the control and Ngsk Prnp<sup>0/0</sup> mice and induced the LTD of PF-EPSCs by a conjunctive stimulation (CJ-train) protocol composed of 300 PF stimuli in conjunction with a depolarizing pulse to PCs (200 ms, –60 to +20 mV) repeated at 1 Hz<sup>21–23</sup>. In 12 of 13 PCs from control mice, CJ stimulation reduced the amplitude of PF-EPSCs; this depression (>10% reduction) persisted for more than 30 min after the onset of stimulation (Fig. 3a). The mean percentage amplitude of PF-EPSCs measured at 25–30 min after CJ stimulation was 69.9 ± 4.3% of the original baseline (n = 13 from 8 mice, three cells studied blind). By contrast, PCs in Ngsk Prnp<sup>0/0</sup> mice exhibited attenuated LTD of PF-EPSCs after CJ stimulation (Fig. 3b). Indeed, in 6 of 12 PCs from Ngsk Prnp<sup>0/0</sup> mice, no significant depression of the EPSC was observed. The mean percentage amplitude of PF-EPSCs recorded at 25–30 min after CJ stimulation was 82.9 ± 6.9% (n = 12 from 7 mice, two cells studied blind). There was a significant difference in the magnitude of LTD between control and Ngsk Prnp<sup>0/0</sup> mice (Mann–Whitney U test, p < 0.05), indicating that LTD-induction is impaired in Ngsk Prnp<sup>0/0</sup> mice (Fig. 3c).

Altered delay eyeblink conditioning in Ngsk Prnp<sup>0/0</sup> mice at 7–8 weeks of age. Finally, we examined delay eyeblink conditioning, a form of cerebellum-dependent discrete motor learning, in Ngsk Prnp<sup>0/0</sup> mice aged 7–8 weeks, which were almost the age of mice used for electrophysiological studies. With conditioning, the animals learn the adaptive timing of eye blinking; in our study, the conditioned response frequency (CR%) for the control mice progressively and significantly increased to over 70% on day 7 (Fig. 4a). On the contrary, CR% for Ngsk Prnp<sup>0/0</sup> mice did not reach 60%. However, repeated measures ANOVA failed to reveal significant differences between the two groups (session × group interaction, F(6, 84) = 0.58, p = 0.57, a genotypic effect, F(1, 14) = 2.75, p = 0.12). The normalized electromyographic (EMG) amplitude on day 7 for Ngsk Prnp<sup>0/0</sup> mice seemed to be lower than that for the control mice (Fig. 4b). Indeed, CR amplitude on day 7 was significantly reduced in Ngsk Prnp<sup>0/0</sup> mice (p = 0.023, Fig. 4c, right panel). Peak latency was also significantly decreased in Ngsk Prnp<sup>0/0</sup> mice compared to that in the control mice (p = 0.031, Fig. 4c, left panel). These results confirmed...
that the timing and amplitude of conditioned eyeblink response were altered in juvenile Ngsk Prnp°/° mice that had not yet undergone PC degeneration.

**Discussion**

Several Prnp null mutant mouse strains, including Ngsk, have been questioned in the field as to whether their phenotype is physiological. Indeed, given that many different PrP knockout mouse strains produce diverse physiological phenotypes, most of the PrP knockout mouse strains have disparate findings because the Prnp°/° locus is surrounded by genes other than Prnp itself ("flanking genes"), which it is thought to be indicative of. Nevertheless, the present study aimed to specifically characterize the electrophysiological properties of Ngsk Prnp°/° mice strain, which exhibits cerebellar PC degeneration and motor learning deficits.

The present electrophysiological study shows that cerebellar LTD is significantly impaired in Ngsk Prnp°/° mice (Fig. 3). Furthermore, our behavioral study indicated that Ngsk Prnp°/° mice (7–8-week-old) exhibited abnormalities in CR amplitude and CR timing, without a significant difference in CR probabilities (Fig. 4). These behavioral results replicate those obtained in experiments with Ngsk Prnp°/° mice at 16 weeks old; however, the extent of their learning disability appears to be more pronounced. The reason for the severe impairments of 7–8-week-old mice is unclear. In Ngsk Prnp°/° mice, GFAP begins to increase gradually from 7 to 8 weeks of age. GFAP is thought to be a significant factor needed for proper communication between Bergmann glia and PC, enabling occurrence of LTD. Indeed, GFAP KO mice exhibited the impairments of cerebellar LTD and eyeblink conditioning. Hence, one possibility is that in 16-week-old Ngsk mutants, improved LTD due to increased GFAP may have had a milder impact on the motor learning disabilities. Regardless, the present results are consistent with those of previous studies suggesting parallelism between impaired cerebellar LTD and altered delay eyeblink conditioning; although the extent of impairment in both conditioned eyeblink response and cerebellar LTD appeared to be less than that observed in the previous report. As in the past, the results of the present study do not demonstrate a direct causal relationship between LTD and eyeblink conditioning, but suggest that there is a common molecular basis for both.

In addition, the effects of molecular layer interneuron-PC feed-forward inhibition (FFI) and absence of FFI on LTD formation have been studied in mice that are genetically deficient in inhibitory synaptic inputs to the PC. The excitatory/inhibitory (E/I) ratio of PCs in these mice appears to be imbalanced, resulting in smaller action potential variability and loss of temporal fidelity of PC responses to parallel fiber stimulation. Furthermore, although LTD formation was normal in these mice, the vestibulo ocular reflex was impaired. Eyeblink conditioning was also found to be impaired in the same mice. Ngsk Prnp°/° mice exhibited not only impaired LTD but also weakened GABA_A receptor-mediated inhibition in the cerebellum. Thus, alternatively, the dysfunction of inhibitory synaptic transmission in the molecular layer could be also responsible for the impairment of CR acquisition or timing. IPSCs and long-term potentiation in hippocampal CA1 pyramidal cells of ZrchI Prnp°/° mice have been reported to be abnormalities, but the cerebellar LTD has not been clarified in the ZrchI Prnp°/° mice. Previous studies suggested PrP-mediated several possible mechanisms underlying the regulation of cerebellar LTD and cellular toxicity, e.g., PrP can bind to mGluR1 and modulate its function to prevent irregular Ca^2+ signalling. Because mGluR1 expressed in PCs is essential for both LTD induction and eyeblink...
conditioning\textsuperscript{28,40,41}, the deficiency in PrP-mediated regulation of mGluR1 may be responsible for the impaired cerebellar plasticity in Ngsk \textit{Prnp}\textsuperscript{0/0} mice.

Histological changes in Ngsk \textit{Prnp}\textsuperscript{0/0} mice occur at an age of approximately 40 weeks\textsuperscript{42}, and a molecular mechanism underlying neuronal degeneration induced by ectopic expression of PrPLP/Dpl has not been identified, although some hypotheses have been suggested\textsuperscript{43–46}. In the present study, we found that IPSCs in cerebellar PCs were altered in Ngsk \textit{Prnp}\textsuperscript{0/0} mice, whereas a previous report showed different results using the \textit{Zrch1} \textit{Prnp}\textsuperscript{0/0} mouse cerebellum\textsuperscript{15}. The discrepancies in IPSCs between the two types of the mutant mice can be explained by ectopic expression of PrPLP/Dpl in Ngsk \textit{Prnp}\textsuperscript{0/0} mice. Our result is rather similar to that the \textit{Zrch1} \textit{Prnp}\textsuperscript{0/0} mouse hippocampus exhibits a reduction in GABAA receptor-mediated fast inhibition, suggesting that PrP\textsuperscript{C} plays a key role in normal inhibitory postsynaptic function\textsuperscript{14} and implies that excessive excitation of PCs induced by suppressing inhibitory inputs via ectopic overexpression of PrPLP/Dpl inhibits the maintenance of PCs in these Ngsk \textit{Prnp}\textsuperscript{0/0} mice. Thus, impairment of CR acquisition in old Ngsk \textit{Prnp}\textsuperscript{0/0} mice could be caused by a
secondary effect of PrPLP/Dpl overexpression, particularly the loss of cerebellar PCs. Figure 5 illustrates such a schematic model for age-dependent alterations of delay eyeblink conditioning in Ngsk Prnp<sup>0/0</sup> mice, from the present study and previous reports.<sup>10</sup>

There is at least one other possible explanation for alteration of eyeblink conditioning in young Ngsk Prnp<sup>0/0</sup> mice. Since normal prion protein is expressed on all neuronal cell types in the cerebellum<sup>11</sup>, alterations in GABA<sub>A</sub>-mediated IPSCs could be true in other types of cerebellar neurons other than Purkinje cells. Thus, GABAergic transmission at Golgi-granule cell synapses may be modified in PrPC-null mice. Considering the timing model described in previous studies<sup>47,50,51</sup>, functional alterations in synaptic transmission between Golgi cells and granule cells may be a factor causing changes in learning-dependent CR timing in young Ngsk and ZrcI<sup>Prnp</sup><sup>0/0</sup> mice. Their altered CR timing may be attributed to abnormal regulation of the granule cells by Golgi cells (Fig. 5b). This explanation is consistent with a previous immunohistochemical study indicating that PrP<sup>C</sup> is most highly expressed in the axon terminals of granule cells in the cerebellum<sup>52</sup>. Therefore, the physiological properties of granule cells in Ngsk Prnp<sup>0/0</sup> mice should be further examined. Furthermore, because aberrant timing of eyeblink conditioning was also observed in ZrcI<sup>I</sup> mice<sup>10</sup>, it is important to investigate the physiology of granule cells in ZrcI<sup>III</sup> mice on a pure C57BL/6J genetic background for future studies<sup>53</sup>.

An appropriate E/I balance is essential for normal adult brain function<sup>54,55</sup>. Thus E/I imbalance affects the normal function and disrupts synchronization between various circuit elements, and can cause autism spectrum disorder (ASD)<sup>96,97</sup>, schizophrenia<sup>58,59</sup>, and Alzheimer’s disease (AD)<sup>60</sup>. Indeed, several studies on their model mice have elaborated on the correlation between decreased IPSCs and neuronal death<sup>51,52</sup>. In most human prion diseases, including Creutzfeldt-Jakob disease (CJD), neuronal loss in the cerebellum and abnormal PrP deposition are major neuropathological findings. Furthermore, epileptic-like symptoms or abnormal waves in EEG are often observed in patients with CJD<sup>98,99</sup>. Therefore, the Ngsk Prnp<sup>0/0</sup> mouse model may help in studying the mechanisms underlying synaptic loss and neurodegeneration in the cerebellum resulting from the loss of PrP<sup>C</sup> and ectopic expression of PrPLP/Dpl.<sup>96</sup>

**Materials and methods**

**Subjects.** Ngsk Prnp<sup>0/0</sup> and Prnp<sup>+/0</sup> mice were obtained as described previously<sup>96</sup>. Male F3 Ngsk Prnp<sup>0/0</sup> mice were crossed with female C57BL/6J mice (purchased from CLEA Japan, Tokyo, Japan), producing F4 heterozygous (Prnp<sup>+/+</sup>) mice. The mutant mice (Ngsk Prnp<sup>0/0</sup>) and their littermate controls (Ngsk Prnp<sup>+/+</sup>) were derived by inter-crossing F4 Ngsk Prnp<sup>+/0</sup> male and female mice. Their genotypes were confirmed by polymerase chain reaction (PCR) amplification of genomic DNA extracted from the tail of each mouse using specific primers for the mouse PrP gene in a 346-base pair PCR fragment (5′-CCGCTACGCTAAACAAAGTGT-3′ and 5′-CTTGA CCAGGAAATGC-3′) and neomycin-resistant gene (5′-GGTGCCCTGAATGACTGA-3′ and 5′-GGTGCCT
AGCCGGATCAAGCGTAT-3'), resulting in a 227-base pair PCR fragment). All subjects were maintained on a 12-h:12-h dark:light cycle with food and water available ad libitum. All animal procedures were performed in accordance with the guidelines for animal experimentation from the ethical committee of The University of Tokyo and Tokushima Bunri University. The experimental protocol was approved by the guidelines for the care and use of experimental animals in the animal investigation committee at Tokushima Bunri University, and the animal welfare committees of The University of Tokyo. In addition, the minimum number of required animals was used for these experiments, and efforts were made to minimize pain.

**Electrophysiology.** Cerebellar slices from 3–6-week-old Ngsk Prnp<sup>−/−</sup> (control) and Ngsk Prnp<sup>0/0</sup> mice were prepared as described previously<sup>90</sup>. The mice were treated with CO<sub>2</sub> and decapitated. Sagittal slices (180–200-μm thick) of the cerebellar vermis were prepared with a microslicer (DTK-1000, Dosaka, Japan) in ice-cold extracellular solution containing (in mM) 252 sucrose, 3.35 KCl, 21 NaHCO<sub>3</sub>, 0.6 NaH<sub>2</sub>PO<sub>4</sub>, 9.9 glucose, 1 molar 1200-μm thick) of the cerebellar vermis were prepared with a microslicer (DTK-1000, Dosaka, Japan) in ice-cold extracellular solution containing (in mM) 252 sucrose, 3.35 KCl, 21 NaHCO<sub>3</sub>, 0.6 NaH<sub>2</sub>PO<sub>4</sub>, 9.9 glucose, 1 molar

For the behavioural study, 7–8-week-old Ngsk Prnp<sup>−/−</sup> (control) and Ngsk Prnp<sup>0/0</sup> male mice were used. The surgery was performed as described previously<sup>32,69</sup>. The mice were anesthetized with ketamine (80 mg/kg, i.p. Sankyo, Tokyo, Japan) and xylazine (20 mg/kg, i.p. Bayer, Tokyo, Japan). Four Teflon-coated stainless-steel wires (100 μm in diameter, A-M Systems, WA, USA) were implanted subcutaneously under the left eyelid. Two of the wires were used to deliver the US and the remaining two to record an electromyogram (EMG) from the musculus orbicularis oculi, which is responsible for eyelid closure. Here, we modified the conventional EMG procedure to improve the sensitivity for detecting MOO activities. The mice were trained in delay eyelink conditioning, in which the CS overlaps and coterminates with the US, for seven days. A tone of 352 ms duration (1 kHz, 80 dB) was used as CS and electrical shock with 100 ms duration (100 Hz square pulses) as US. The US intensity was carefully determined, and the minimal current amplitude required to elicit an eyelink response with constant amplitude was adjusted daily for each animal (less than 0.5 mA). Experiments were conducted during the light phase of the LD cycle in a container (10 cm in diameter) placed in a sound- and light-attenuating chamber. Daily acquisition training consisted of 100 trials grouped in 10 blocks. Conditioning sessions consisted of 10 CS-only (every 10th trial) and 90 CS-US paired trials. The CR amplitude was calculated as the average amplitude over the 50 ms period just before the US. Data were analyzed as described previously<sup>30,31</sup>.

**Statistical analysis.** All data and samples were analyzed by an individual blinded to the genotype. Unpaired t-tests or the Mann–Whitney test were used. Data for eyelink conditioning were analyzed by the two-way repeated measures ANOVA to assess the effects of genotype and/or session. The difference was considered significant when the P value was less than 0.05. Tests were performed using Excel or GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). All data are displayed as mean ± standard error of the mean (SEM).

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Y.Kishimoto and M.H. wrote the paper. Y.Kishimoto, M.H., R.A. and S.S. collected and analyzed the preliminary data. Y.Kishimoto, Y.Kirino, S.K. and T.Y. provided ideas and contributed to the interpretation of the results.

Competing interests
The authors declare no competing interests.

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