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Research Report

The postnatal development of refractory periods and threshold potentials at cerebellar Purkinje neurons

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ABSTRACT

Cerebellum is involved in the motion coordination and working memory, to which spike programming at Purkinje neurons are essential. The development of Purkinje neurons in the embryonic stage has been well studied. However, it is not clear about the maturation of their intrinsic property related to spike programming during postnatal period. We developed the approach to quantify the intrinsic property of sequential spikes with whole-cell recording, and analyzed the postnatal development of Purkinje neurons in cerebellar slices. Our results demonstrate that the threshold potentials shift toward more negatively than resting membrane potential, refractory periods following each of spikes decrease, as well as the relationship between refractory periods and inter-spike intervals converts to be more linear during the postnatal maturation. This postnatal plasticity of neuronal intrinsic properties enhances the firing ability and spike capacity, in turn strengthens spike programming, at cerebellar Purkinje neurons.

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1. Introduction

The cerebellum regulates fine-tuning movement and balance, as well as plays an important role in working memory and higher cognition, in which GABAergic Purkinje neurons are presumably critical (Kleim et al., 1997; Mauk et al., 2000; Raymond et al., 1996; Wang and Zoghbi, 2001). In cerebellar cortex, Purkinje cells receive synaptic inputs from the glutamate-releasing inferior olivary neurons and granule cells, as well as GABA-releasing stellate and basket cells. They subsequently project to the deep nuclei in the cerebellum. Therefore, the precise and loyal programming of sequential spikes based on intrinsic properties at Purkinje cells is essential to fulfilling cerebellar functions, which requires cerebellar Purkinje cells to be well developed and matured.

In terms of development, the cerebellum achieves its matured configuration a few months after birth despite an early differentiation. Purkinje cells are born at embryonic day 13 and migrate along radial glial fiber to the cerebellar anlage (Hatten and Hentz, 1995), in which reelin pathway is involved (Myata et al., 1997). Their maturation needs extensive interactions with the neighbors through eliminating supernumerary synapses from climbing fibers, developing extensive dendritic arbors and making synapses onto granule neurons and deep cerebellar nuclei in late stage (Baptista et al., 1994; Goldowtz and Hamre, 1998; Hatten and Hentz, 1995; Wang and Zoghbi, 2001).

Compared to the structural development, the functional maturation, especially the intrinsic mechanisms underlying the maturation of spike programming, of cerebellar Purkinje cells has been less studied, except for the developmental

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profiles in spike patterns (McKay and Turner, 2005), calcium storage (Hockberger et al., 1989; Liljelund et al., 2000), Ca^{2+} channels (Gruol et al., 1992), Ca^{2+}-dependent potassium channels (Cingolani et al., 2002; Muller et al., 1998). The intrinsic properties of Purkinje cells influence analyzing synaptic inputs and programming spike patterns for the execution of cerebellar functions. Therefore, we investigated spike programming and intrinsic properties at Purkinje cells during postnatal development with whole-cell recording in cerebellar slices before and after rat's eyes open.

2. Results

2.1. *The developmental change in the thresholds of firing spikes*

The threshold of action potentials is one of indices presenting the ability of firing spikes at excitable cells. Several ways can be used to describe this intrinsic property, threshold stimulation, threshold potential (Aizenmann and Linden, 2000; Armano et al., 2000; Desai et al., 1999; Zhang et al., 2004), as well as the gap between resting membrane potential (Vr) and threshold potential (Vts). The reasons to use Vts–Vr are followings. The values of Vr and Vts vary among the cortical neurons (Zhang et al., 2004); Vts–Vr is an energy barrier to lift Vr toward Vts; and Vts–Vr is better to show how easily synaptic inputs drive neurons to fire spikes.

Waveforms in Fig. 1A show the membrane potentials (Vr and Vts) of cerebellar Purkinje cells from SD rats with PND 15–18 (left) and PND 7–10 (right), from which we read Vr, Vts and gap potential (ΔV = Vr–Vts). Fig. 1B shows the comparisons of average values for Vr and Vts at Purkinje cells from PND 15–18 (gray bars) and PND 7–10 rats (black bars). Vr values at Purkinje cells from PND 15–18 and PND 7–10 rats are −62.54 ± 0.93 (n = 17) and −58.52 ± 0.62 mV (n = 19), respectively (P < 0.01). Vts values at Purkinje cells from PND 15–18 and PND 7–10 rats are −48.87 ± 1.28 and −39.75 ± 1.38 mV, respectively (P < 0.01). Fig. 1C shows a comparison of Vr–Vts values at Purkinje cells from PND 15–18 (gray bars, 13.67 ± 0.73 mV) and PND 7–10 rats (black bars, 18.77 ± 1.03 mV; P < 0.01). These results indicate that cerebellar Purkinje cells in PND 15–18 rats are more easily driven to be excited by synaptic inputs, i.e., the ability of firing spikes is higher, than those in PND 7–10 rats.

2.2. *The developmental change in spike capacity*

Spike capacity is another index presenting the ability of firing spikes in excitable cells, which denotes the ability of encoding digital signals at neurons. The high spike capacity, i.e., high frequency or short inter-spike intervals (ISI), at the central neurons supports a wide spectrum of digital codes that program various biological events. In this regard, the spike capacity is presumably enhanced with a postnatal development of fine-tuning movement and balance. We have tested this assumption by measuring ISI at cerebellar Purkinje cells from PND 15–18 and PND 7–10 rats.

Fig. 2A shows the repetitive spikes at cerebellar Purkinje cells from PND 15–18 and PND 7–10 rats in the response to a depolarization current pulse (100 ms). In addition to the differences in Vr and Vts, similar to Fig. 1, the number of spikes at Purkinje cells is lower in PND 7–10 rats (trace with dash line) than PND 15–18 (solid line). In order to quantify the differences in spike capacity, we measured ISI between spikes one and two (ISI_{1-2}) up to four and five (ISI_{4-5}). The values of ISI_{1-2} to ISI_{4-5} at Purkinje cells in PND 7–10 rats are 14.26 ± 1.4, 20.54 ± 1.15, 21.84 ± 1.17 and 21.88 ± 1.17 ms (filled circles in Fig. 2B, n = 19); whereas the values from ISI_{1-2} to ISI_{4-5} at Purkinje cells in PND 15–18 rats are 8.82 ± 0.86, 14.73 ± 1.35, 16.23 ± 1.32 and 17.09 ± 1.24 ms (open circles in Fig. 2B, n = 17), respectively. ISI values relevant to the same number in the

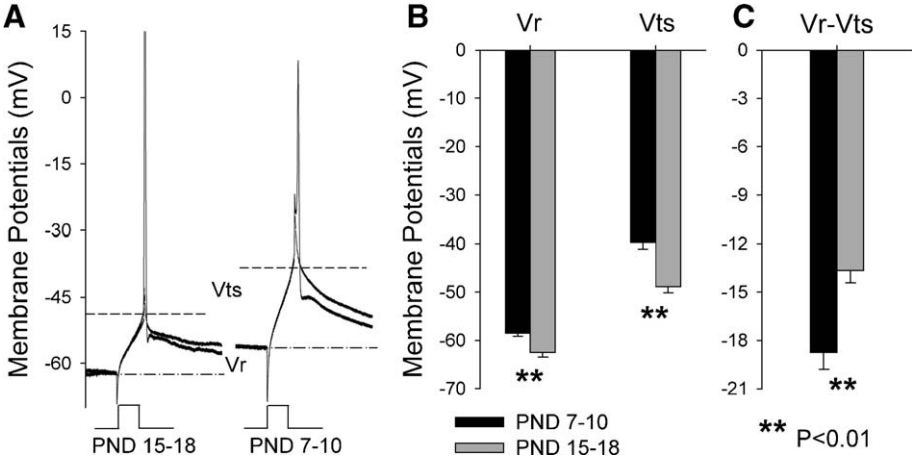

Fig. 1 – The postnatal maturation of the resting membrane potential (Vr) and spike threshold potential (Vts) at cerebellar Purkinje cells. (A) The represented waveforms of the membrane potentials and spikes. Threshold is defined as spike firings at 50% of probability, at which the intensity of depolarization pulses (10 ms) is threshold stimuli, the initial point of rising phase of spikes is Vts (dot line), and gap potential is Vts–Vr. Left waveform is recorded at Purkinje neurons from PND 15–18, and the right is from PND 7–10 rats. (B) The comparisons of Vr and Vts at Purkinje neurons between PND 15–18 (gray bars) and PND 7–10 rats (black bars). (C) The comparisons of Vr–Vts at Purkinje neurons between PND 15–18 (gray bars) and PND 7–10 rats (black bars). Two asterisks present a statistical difference (P < 0.01).

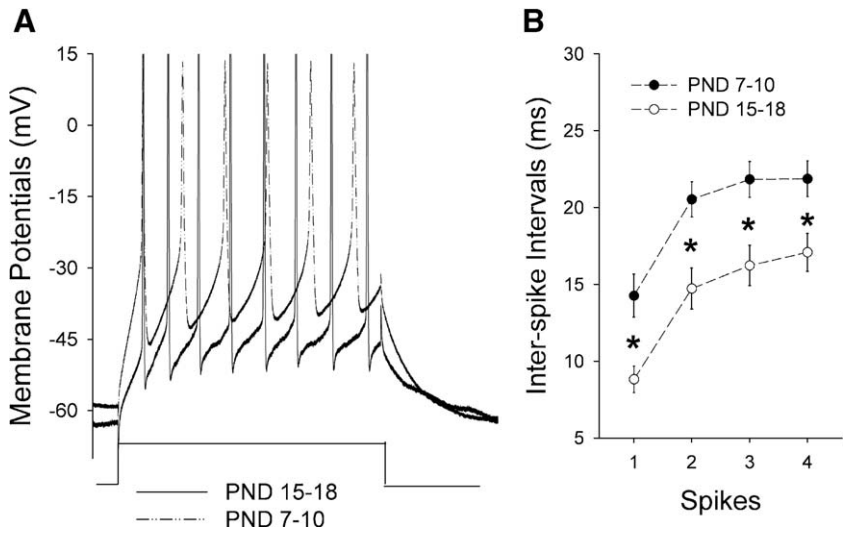

Fig. 2 – The comparisons of inter-spike intervals (ISI) at cerebellar Purkinje neurons between PND 15–18 and PND 7–10 rats. (A) Waveforms show the burst spikes evoked by depolarization pulses with an intensity that is the same as threshold stimuli in figure one at Purkinje neurons from PND 15–18 (solid line) and PND 7–10 rats (dash line). (B) The plot shows the inter-spike intervals of sequential spikes at Purkinje neurons from PND 15–18 (hollowed symbols) and PND 7–10 rats (filled symbols). The asterisks indicate the statistical difference ($P < 0.01$).

sequence of spikes at Purkinje cells between PND 7–10 and PND 15–18 rats are statistically difference ($P < 0.01$).

The results in Fig. 2 imply that excitatory synaptic inputs on cerebellar Purkinje cells are able to drive them to fire more spikes (the high ability of firing sequential spikes) after rat's eyes open. Together Figs. 1 and 2, we suggest that rat's PND 15–18 during postnatal development is a critical period for the cerebellar Purkinje cells to be matured in the ability of firing sequential spikes by having the lowered spike threshold and the enhanced spike capacity.

2.3. *The developmental change in refractory periods subsequent to each spike*

Inter-spike intervals are presumably determined by refractory periods following each of spikes. With a shortening of inter-spike intervals during the development, we predicted that refractory periods (RP) would be reduced. To test this prediction, we developed an approach to measure the absolute and relative RP of sequential spikes with whole-cell current-clamp recording, in which single depolarization current pulses (3 ms), whose intensity was just above threshold, were injected into Purkinje neurons immediately after each of complete spikes. By changing inter-pulse intervals, we defined ARP as the duration from a complete spike to a subsequent spike at 50% of firing probability, and RRP as the duration to a complete spike to a subsequent spike that has 100% of firing probability and initially reaches to the amplitude of natural spikes (Fig. 3A).

Figs. 3B–C shows the comparisons of ARP and RRP at cerebellar Purkinje cells from PND 15–18 and PND 7–10 rats. ARP values of spikes 1–4 are 4.15 ± 0.18, 4.8 ± 0.22, 5.48 ± 0.3 and 5.87 ± 0.32 ms in PND 15–18 rats (open circles), whereas the values are 5.65 ± 0.37, 6.38 ± 0.39, 6.86 ± 0.52 and 8.59 ± 1.15 ms in PND 7–10 (filled circles in Fig. 3B). RRP values for spikes 1–4 are 5.1 ± 0.19, 6.1 ± 0.33, 7.1 ± 0.51 and 7.97 ± 0.65 ms in PND 15–18 rats (open circles), whereas values are 6.84 ± 0.52, 8.84 ± 1.14, 10.35 ± 1.17 and 13.25 ± 2.4 ms in PND 7–10 rats (filled circles in Fig. 3C). The refractory periods relevant to the same number in the sequence of spikes at cerebellar Purkinje cells from PND 7–10 and PND 15–18 rats are statistically different ($P < 0.01$). These results imply that the refractory periods subsequent to each of spikes decrease during postnatal development, which allows inter-spike intervals to be shorten.

Interestingly, the differences between RRP and ARP (ΔRP) of spikes one to four at Purkinje cells are 0.9 ± 0.14, 1.26 ± 0.2, 1.59 ± 0.34 and 2.1 ± 0.44 ms in PND 15–18 (open circles); and ΔRP values are 1.42 ± 0.29, 2.61 ± 0.71, 3.64 ± 0.69 and 4.74 ± 1.06 ms in PND 7–10 rats (filled circles in Fig. 3D). Such differences relevant to the same number in the sequence of spikes 2–4 in Purkinje cells from PND 15–18 and PND 7–10 rats are statistically significant ($P < 0.01$), indicating that decreases in refractory periods are mainly RRP, especially in the late phase of sequential spikes. In other words, an increase in spike capacity during postnatal period occurs in the late phase of long-term spike bursts. Moreover, ΔRP from spikes one to four gradually increase, indicating that RRP and ARP are not tightly associated in the late phase of spikes.

In addition, we plotted the correlations between ARP and RRP of spikes 1–4 in Purkinje cells from PND 15–18 and PND 7–10 rats. As shown in Fig. 3E, ARP and RRP are linearly correlated before ($r^2 = 0.95$) and after rat's eyes open ($r^2 = 0.98$, $P < 0.01$), supporting a notion that ARP and RRP are under the control of the kinetics of voltage-gated sodium channels. It is noteworthy that the linear slopes in the Purkinje cells from PND 15–18 and PND 7–10 rats are 1.65 and 2.12, respectively, i.e., larger than 1. The unsymmetrical increases in RRP and ARP during sequential spikes imply that these two parameters are controlled by different functional domains.

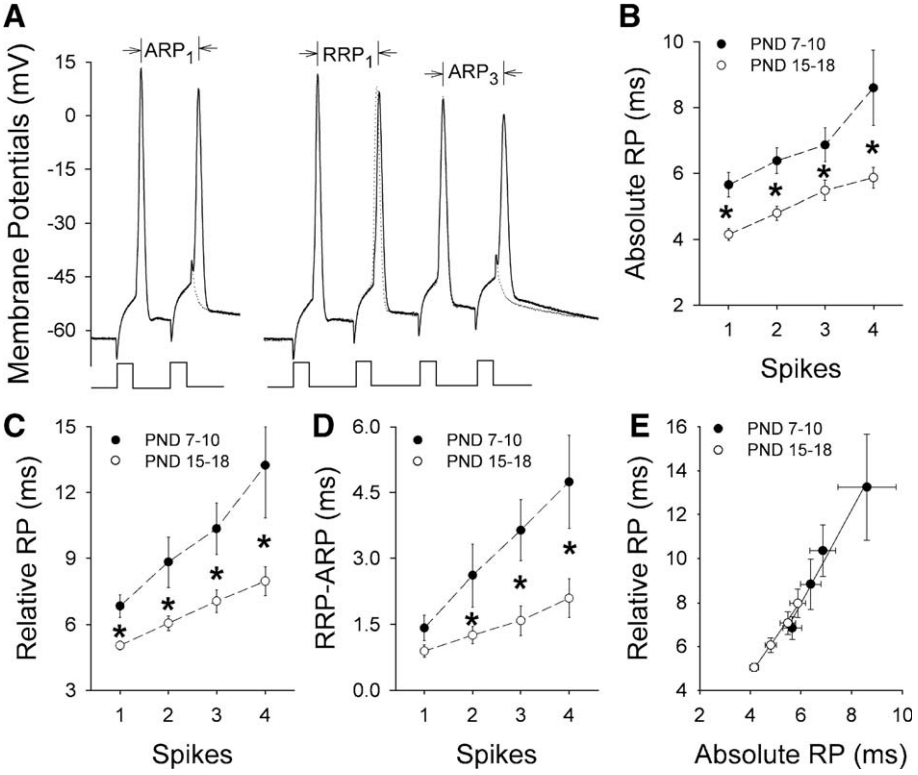

Fig. 3 – The developmental changes of refractory period (RP) at cerebellar Purkinje neurons. (A) The measurement of RP of multiple sequential spikes by depolarization pulses (3 ms) added immediately after each of spikes. Absolute RP (ARP) is defined as a duration from a complete spike to firing a subsequent spike at 50% of probability, and relative RP (RRP) as a duration from a complete spike to a subsequent spike that has 100% of firing probability and initially reaches to the amplitude of natural spikes. Panels B and C show the plot of ARP and RRP subsequent to each of spikes at Purkinje neurons from PND 15–18 (opened symbols) and PND 7–10 rats (filled symbols), respectively. (D) The comparisons of the difference between the RRP and ARP of sequential spikes at the two groups of Purkinje cells. Panel E shows the linear correlation between ARP and RRP before (filled symbols, $r^2 = 0.96$) and after eyes open (opened symbols, $r^2 = 0.99$). The asterisks indicate the statistical difference ($P < 0.01$).

2.4. *Developmental change in the correlation between refractory periods and inter-spike intervals*

Based on an assumption that the refractory periods subsequent to each of spikes determine inter-spike intervals, we expect to see a linear correlation between them. If it is a case, we are also interested in knowing whether the linear correlation develops during postnatal period. With the measured RP and ISI of sequential spikes, we plot inter-spike intervals vs. refractory periods. ISI and ARP in cerebellar Purkinje neurons change from less linear correlation in PND 7–10 rats ($r^2 = 0.53$, filled circles in Fig. 4A) to linear correlation in PND 15–18 rats ($r^2 = 0.88$, open circles in Fig. 4A; $P < 0.01$). Similarly, the correlations between ISI and RRP are converted to be more linear in PND 15–18 rats ($r^2 = 0.86$, open circles in Fig. 4B) from in PND 7–10 rats ($r^2 = 0.64$, filled circles in Fig. 4B). This result implies that the dependence of inter-spike intervals on the refractory periods of sequential spikes develops postnatally.

3. Discussion

With measuring the generation of sequential spikes at Purkinje cells in cerebellar slices from PND 7–10 and PND 15–18 rats, we first provide the experimental evidence for the developmental regulation of their intrinsic properties (e.g., refractory periods and threshold potentials). Compared to the resting membrane potential (Vr), spike threshold potentials (Vts) shift toward more negatively during postnatal development, which causes the gap between Vts and Vr to be shorten. Moreover, during the postnatal development, inter-spike intervals (ISI) and refractory periods (RP) reduce; and a correlation between ISI and RP converts to be more linear. Such developmental changes in the threshold potentials and refractory periods of sequential spikes raise the ability of generating spikes at Purkinje cells. For example, the decrease in Vts–Vr allows neurons to be more sensitive to excitatory synaptic inputs after rat's eyes open. A postnatal converted linear correlation between RP and ISI as well as the reduction of spikes' RP make inter-spike intervals to be shortened, i.e., an enhanced spike capacity driven by excitatory inputs.

The differences between the RRP and ARP of spikes 1–4 in cerebellar Purkinje cells reduce postnatally (Fig. 3D), implying that the determinant of inter-spike intervals hand over from relative refractory period toward absolute refractory period dominant. In addition, RP reduction occurs predominantly in the late phase of sequential spikes (Fig. 3), which allows the late phase of spikes moving closely to

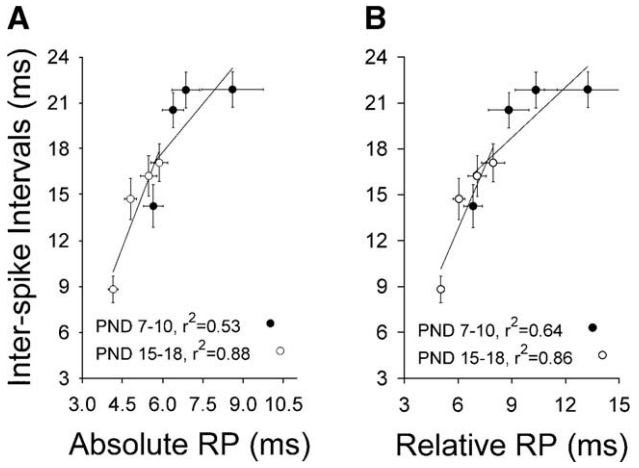

Fig. 4 – Developmental regulation in the relationship between refractory periods (RP) and inter-spike intervals (ISI). (A) The relationship between ISI and ARP at cerebellar Purkinje cells before (filled symbols, r^2 = 0.53) and after eyes open (opened symbols, r^2 = 0.88). (B) The relationship between ISI and RRP at cerebellar Purkinje cells before (filled symbols, r^2 = 0.64) and after eyes open (opened symbols, r^2 = 0.86).

initial ones. If spikes represent digital "1" and inter-spike interval denotes digital "0" (London et al., 2002), the reduction of RP and ISI will mainly lower the number of digital "0" between "1" in the late phase of sequential spike programming.

The changes in the intrinsic properties (spike threshold and refractory periods) of cerebellar Purkinje cells during postnatal period facilitate excitatory synaptic inputs on these cells to drive them firing more spikes after rat's eyes open. Together these data with a developmental regulation in the plasticity of neuronal excitability (Zhang et al., 2004), we suggest that the time window between PND 7–10 and 15–18 is a critical period for the cerebellar Purkinje cells to be matured in the ability of firing spikes by expressing a low threshold and a short refractory periods in the response to excitatory synaptic inputs.

The advantages of the approaches used in our studies are the followings. Instead of experimenting in the cultured Purkinje cells (Gruol et al., 1992; Hockberger et al., 1989; Liljelund et al., 2000; Muller et al., 1998), we investigated the development profiles of the intrinsic properties of cerebellar Purkinje cells in acute isolated slices, in which a more natural environment is provided for cell survivals. We developed the approach to measure the refractory periods after each of sequential spikes, which helps to address the mechanisms underlying the quantitative study of spike capacity and timing precision.

Our study demonstrate the developmental profiles of the intrinsic properties (e.g., refractory periods and threshold potentials of sequential spikes) at cerebellar Purkinje cells for understanding the maturation of the cerebellum in programming the spectrum of neural signals, i.e., cerebellar language, to manage motion coordination, balance, working memory and cognition as well.

4. Experimental procedures

4.1. *Brain slices and neurons studied*

Cerebellar slices (400 μm) were prepared from Sprague–Dawley rats with either eyes-opened or eyes-unopened. Rats in postnatal day (PND) 7–10 and 15–18 were anesthetized by inhaling isoflurane and decapitated with a guillotine. Sagittal cerebellar slices were cut with a Vibratome in the oxygenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF) in the concentration of mM (124 NaCl, 3 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 4 $MgSO_4$, 10 dextrose and 5 HEPES; pH 7.35) at 4 °C. The slices were held in (95% O_2 and 5% CO_2) ACSF (124 NaCl, 3 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 10 dextrose and 5 HEPES; pH 7.35) at 25 °C ACSF for 2 h. A slice was transferred to a submersion chamber (Warner RC-26G) that was perfused with the oxygenated ACSF at 31 °C for whole-cell recording (Wang, 2003; Wang and Kelly, 2001). Chemicals were from Sigma. The procedures were approved by IACUC in Anhui, China.

Purkinje cells in the cerebellar cortex, which show a round or ovary-like soma and tree branch-like dendrites under DIC optics (Nikon FN-600), were recorded. Purkinje cells show responses to hyper- and depolarization pulses similarly to previous report (Wang, 2003).

4.2. *Whole-cell recordings*

The recordings were conducted in current-clamp model with an Axoclamp-2B amplifier (Axon Instrument Inc., Foster CA, USA); and electrical signals were inputted into pClamp 9 (Axon Instrument, Inc) for data acquisition and analysis. Output bandwidth in amplifiers was 3 kHz. The spike patterns were evoked by depolarization current pulses, in which the amplitude and duration were based on the aim of experiments. Pipettes for whole-cell recordings were filled with the standard solution that contained (mM) 150 K-gluconate, 5 NaCl, 5 HEPES, 0.4 EGTA, 4 Mg-ATP, 0.5 Tris–GTP and 5 phosphocreatine (pH 7.35 adjusted by 2 M KOH). Fresh pipette solution was filtered with centrifuge filters (0.1μm pores) before use, and the osmolarity was 295-305 mosMol. Pipette resistance was 5–6 MΩ.

The intrinsic properties of cerebellar Purkinje cells in our investigation include the thresholds of firing spike and refractory periods following each spike. Spike thresholds were measured by two approaches, threshold stimulation [injecting a depolarization current into soma (10 ms)] to evoke single spikes with 50% probability and threshold potentials that is a start point of the rising phase of spikes, which were used in our previous study (Zhang et al., 2004). The thresholds were detected by changing the intensity of current pulses throughout each of experiments, in which stimulus intervals were 10 s. The absolute and relative refractory periods of sequential spikes were measured by injecting multiple depolarization current pulses (3 ms) whose intensity were just above the threshold into Purkinje cells following each of spikes (see Fig. 3A). By changing inter-pulse intervals, we defined absolute refractory period (ARP) as a duration from a complete spike to a subsequent spike at 50% probability, and

relative refractory period (RRP) as a duration from a complete spike to firing a subsequent spike that has 100% of firing probability as well as initially reaches to the amplitudes of natural spikes (Chen et al., 2006).

Data were analyzed if the recorded neurons had the resting membrane potentials negatively more than –55 mV. The criteria for the acceptation of each experiment also included less than 5% changes in resting membrane potential, spike magnitude and input resistance throughout each of experiments. Input resistance was monitored by measuring cell responses to the hyperpolarization pulses at the same values as the depolarization that evoked spikes. Thresholds and refractory periods are presented as mean ± SE. The comparisons before and after PND 15–18 are done by *t* test.

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