In vivo analysis of Purkinje cell firing properties during postnatal mouse development

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ABSTRACT

Purkinje cell activity is essential for controlling motor behavior. During motor behavior Purkinje cells fire two types of action potentials: simple spikes that are generated intrinsically, and complex spikes that are induced by climbing fiber inputs. And although the functions of these spikes are becoming clear, how they are established is still poorly understood. Here, we used in vivo electrophysiology approaches conducted in anesthetized and awake mice to record Purkinje cell activity starting from the second postnatal week of development through to adulthood. We found that the rate of complex spike firing increases sharply at three weeks of age whereas the rate of simple spike firing gradually increases until four weeks of age. We also found that compared to adult, the pattern of simple spike firing during development is more irregular as the cells tend to fire in bursts that are interrupted by long pauses. The regularity in simple spike firing only reached maturity at four weeks of age. In contrast, the adult complex spike pattern was already evident by the second week of life, remaining consistent across all ages. Analyses of Purkinje cells in alert behaving mice suggested that the adult patterns are attained more than a week after the completion of key morphogenetic processes such as migration, lamination, and foliation. Purkinje cell activity is therefore dynamically sculpted throughout postnatal development, traversing several critical events that are required for circuit formation. Overall we show that simple spike and complex spike firing develop with unique developmental trajectories.
INTRODUCTION

The cerebellum controls motor behaviors such as coordination, learning, balance, and posture. The execution of these behaviors requires proper Purkinje cell function. Purkinje cells are the central components of all cerebellar circuits. They receive direct excitatory input from climbing fibers and granule cell parallel fiber axons, and inhibitory inputs from stellate and basket cell interneurons. Purkinje cells are the only output of the cerebellar cortex – they project their axons to inhibit neurons in the cerebellar and vestibular nuclei. The cerebellar nuclei are located in the inner core of the cerebellum and they provide the main output of the cerebellum. Purkinje cells, together with their afferent and efferent connections, constitute the basic circuit that controls cerebellar dependent behaviors (Oberdick and Sillitoe 2011; Reeber et al. 2012). This study is concerned with how postnatal cerebellar circuit activity arises during a key time window of development to establish Purkinje cell function (Hensch 2004; Letellier et al. 2009).

Mature Purkinje cells fire two types of action potentials. The first, called the simple spike, is generated intrinsically within the Purkinje cells (Cerminara and Rawson 2004). Even though the basal properties of simple spikes are determined intrinsically, sensory-induced afferent activity can influence their ultimate firing characteristics (Armstrong and Rawson 1979; Thach 1967; Wise et al. 2010). They are characterized by a relatively regular pattern and high frequency of firing (~40-50 Hz) (Ruigrok 2011; Shin et al. 2007), although they can fire over a wide range of frequencies in both anesthetized (Bosman et al. 2010; de Solages et al. 2008; White et al. 2014) and awake preparations (Chaumont et al. 2013; Cheron et al. 2009; Goossens et al. 2004; Shin et al. 2007; Witter et al. 2013; Zhou et al. 2014). The second type of spike is called the “complex” spike. Complex spikes occur at a low frequency (~0.5-1 Hz) and are elicited by climbing fiber input. Complex spikes are distinguished from the simple spikes by their unique waveform consisting of a single large spike followed by 3-5 smaller spikelets (Schmolesky et al. 2002). Complex spike firing is thought to modulate simple spike activity (Barmack and Yakhnitsa 2011). This is important for motor behavior because Purkinje cell firing influences cerebellar nuclear output (Person and Raman 2012). Thus, Purkinje cell activity is central to cerebellar function and the control of movement (Badura et al. 2013; Chaumont et al. 2013; Medina and Lisberger 2008; Popa et al. 2013; Witter et al. 2013). However, a poorly understood aspect of
this activity is how it arises during development. Therefore, the main goal of this study was to shed light on the functional characteristics of Purkinje cell behavior during cerebellar development. In particular, because Purkinje cells go through a protracted period of circuit and morphological plasticity during postnatal development (Cesa and Strata 2009; Sotelo and Dusart 2009), we found it interesting to consider the postnatal ontogenic program that generates the precise firing behavior of simple spikes and complex spikes as the cerebellum is forming.

The functional architecture of the cerebellum is established through multiple morphogenetic, patterning, and remodeling processes (White and Sillitoe 2013a). These processes include lobule formation, zonal patterning of zebra-striped compartments, and climbing fiber synaptogenesis. In mice, climbing fiber and mossy fiber afferents have already invaded the cerebellum by around embryonic day 13/14 (Grishkat and Eisenman 1995; Paradies and Eisenman 1993). Later, in the cerebellum of older embryos and newborn pups, multiple climbing fibers innervate a single Purkinje cell. During late postnatal development, these climbing fibers are pruned away by a process of competitive rewiring until only one climbing fiber forms functional synapses with only one Purkinje cell (Bosman and Konnerth 2009; Hashimoto et al. 2009; Kano and Hashimoto 2012). Immature mossy fiber afferents also synapse directly onto Purkinje cells during late embryonic and early postnatal development (Manzini et al. 2006; Mason and Gregory 1984; Takeda and Maekawa 1989), but they too are pruned away as they go through a major rearrangement during postnatal development when they detach from Purkinje cells to form direct contacts with granule cells (Kalinovsky et al. 2011). Thus, the cerebellum undergoes extensive rewiring and synapse formation during the mid-late postnatal stages, and the principal Purkinje cell microcircuit connections are not mature until about postnatal day 21. This study is concerned with understanding how Purkinje cells fire before and after their afferents have completed the remodeling process. We focused particularly on when the connections are rewiring and gene expression patterns and other intrinsic properties are maturing (White and Sillitoe 2013a; b). To address this problem we conducted a developmental analysis of Purkinje cell firing in mice.

We performed in vivo extracellular recordings in anesthetized and awake behaving mice from the second postnatal week through to early adulthood, respectively. The main objective was to investigate the functional changes that occur during Purkinje cell development in vivo. Our
experiments revealed three main findings: 1) Purkinje cell complex spike rate increases sharply at three weeks of age whereas simple spike firing rate increases gradually until it stabilizes at four weeks of age; 2) the pattern of Purkinje cell firing is highly irregular during development; and 3) the overall firing features of the Purkinje cell reach maturity at about four weeks of age. These findings provide a functional correlate to the well-studied anatomical and molecular plasticity that shapes cerebellar circuitry and a valuable resource for evaluating exactly how and when Purkinje cell function emerges during the final stages of brain development in mouse.
MATERIALS AND METHODS

Animal maintenance. Mouse husbandry and experiments were performed under an approved Institutional Animal Care and Use Committee (IACUC) protocol at Baylor College of Medicine. Male and female Swiss Webster mice were obtained from Taconic (Hudson, NY, USA) and a colony established and maintained in house. Noon on the day a vaginal plug was detected was considered embryonic day (E) 0.5. The day of birth was designated as postnatal day (P) 0. We analyzed developing mice daily from P14 to P31, and then examined adult mice from P60-P65. The number of mice analyzed per age was as follows: P14: n = 6; P15: n = 4; P16: n = 2; P17: n = 4; P18: n = 2; P19: n = 2; P20: n = 2; P21: n = 1; P22: n = 2; P23: n = 1; P24: n = 3; P25: n = 1; P26: n = 1; P27: n = 4; P28: n = 2; P29: n = 2; P30: n = 2; P31: n = 1; P60-64: n = 1 per day; P65: n = 3. The data were binned in groups that consisted of mice from three consecutive ages (P14-16, P17-P19, P20-P22, P23-P25, P26-P28, P29-P31, and P60-P65 for adults). This resulted in 5-12 mice per age group. Certain cells from specific mice were excluded because single-unit action potentials could not be clearly isolated from the recording trace during offline analysis.

Surgery. To deeply anesthetize mice during surgery and recordings, mice were first exposed to a slowly increasing concentration of the gas anesthetic isoflurane, followed by injection with a general anesthetic mixture of ketamine and dexmedetomidine. Pre-weaned mice were anesthetized in an oxygen perfusion chamber with isoflurane used to a maximum concentration of 2%. A cocktail of 50 mg/kg ketamine and 0.3 mg/kg dexmedetomidine was injected intraperitoneally once the mice became non-responsive to tactile stimuli, but showed signs of stable breathing. For post-weaned and adult mice, isoflurane was used to a maximum concentration of 3%, and ketamine and dexmedetomidine were injected at dosages of 75 mg/kg and 0.5 mg/kg, respectively. Mice were then transferred from the anesthesia chamber to a stereotaxic platform (David Kopf Instruments, Tujunga, CA, USA) that is integrated with an in vivo recording rig. Throughout the experiment, mice were connected to a breathing tube and isoflurane concentration was maintained at 0.15%-0.25%. This ensures a steady supply of oxygen for the mouse, and the constant, low dose of isoflurane supplements the ketamine and dexmedetomidine cocktail to stabilize breathing; we found that this small amount of isoflurane was essential for holding single units for at least 300 seconds (see electrophysiology below).
Isoflurane levels and oxygen flow were monitored and regulated using a tabletop anesthesia machine (V3000PK, Parkland Scientific, Coral Springs, FL, USA). The head was fixed with metal ear bars (rubber-tipped for young mice). Fur in the back of the head was removed using depilatory cream. An antero-posterior incision was made with a scalpel blade to expose the skull. Depending on the age, the muscle was cut and reflected away if the area for craniotomy was covered. All craniotomies were performed from 0-1.5 mm to the right of the midline with reference to Bregma (Paxinos and Franklin 2001), and above the region that approximately corresponds to lobules VI-VII of the vermis or CrusI and II of the hemispheres (Figs. 1A, 1B, 1C). A hole approximately 1mm in diameter was drilled into the skull using a Dremel handheld rotary drill (model 4000). Once the skull was drilled to translucence, the remaining bone and cartilage were etched with an 18-gauge needle until a circular flap could be removed to expose the brain. The craniotomy was performed carefully because even minimal tissue damage causes brain and blood vessel pulsations, which inhibit stable single unit recordings. Moisture within the craniotomy was maintained by keeping the opening full with drops of 0.9% w/v saline solution.

Electrophysiology. Single unit recordings were attained with 2-5 MΩ Tungsten electrodes (Thomas Recording, Germany) that are controlled by a motorized micromanipulator (MP-225, Sutter Instrument). The signals were band-pass filtered at 0.3-13 kHz, amplified with an ELC-03XS (NPI, Tamm, Germany) amplifier, and digitized into Spike2 (CED, Cambridge, UK). Analysis of the raw traces was performed with Spike2, Excel, and MATLAB. Purkinje cells were identified by the presence of both simple spikes and complex spikes, the latter of which are a characteristic action potential that result exclusively from the excitatory climbing fiber input and cause a subsequent pause of ~20ms in simple spikes (Figs. 1D and 1E). Simple spikes and complex spikes were sorted independently. We calculated the total number of simple and complex spikes over a pre-defined period of recording to obtain simple and complex spike firing frequency (Hz = spikes/second). Firing pattern variability, or regularity, is defined as a measure of the consistency of time intervals between spikes (inter-spike time interval or ISI = seconds). To quantify the average variability in firing pattern, coefficient of variance of the ISI (CV) was calculated as the ratio of the standard deviation of ISIs to the mean ISI of a given cell. To measure rhythmicity during burst firing, CV2 and the rhythm index were calculated. CV2 measures firing pattern variability within a short period of two interspike intervals (CV2 =
The rhythm index is a measure of the strength of oscillating patterns within a given period. To derive the rhythm index, autocorrelograms for simple spikes were first plotted in Spike2 for each Purkinje cell, with a width of 1.0 s, an offset of 0.5 s, and a bin size of 5 ms. Numerical values of the plot were then exported to Excel, and the rhythm index was calculated using previously published equations (Lang et al. 1997; Sugihara et al. 1995) that were integrated into our custom Excel macros. First, the baseline level (average bin count) was calculated using the formula:

\[
\text{baseline} = \frac{(\text{total spike number})^2}{(\text{recording time} / \text{bin width})}
\]

Random fluctuation about the baseline bin height was measured as the standard deviation (SD) of spike counts per bin, at time lags of -400 ms to -500 ms and 400 ms to 500 ms. The first peak was recognized as the highest bin within a time lag of 10 – 40 ms, and the first valley was recognized as the shortest bin within 15 – 45 ms. If bin height or depth exceeded the mean baseline level ± SD, or if the difference between a peak and the next valley is greater than twice the SD, then peaks and valleys during oscillations met the criteria to be included in the rhythm index calculation. The succeeding peaks and valleys were then detected within a time range of:

\[
t_n + t_1 \pm (t_1 / 2)
\]

in which \( t_n \) is the time lag of the \( n \)th peak or valley and \( t_1 \) is the time lag of the first peak. Oscillation frequency was calculated as the inverse of the time lag of the first peak \((1 / t_1)\). The rhythm index was calculated with the following formula:

\[
\text{rhythm index} = \frac{a_1}{z} + \frac{b_1}{z} + \frac{a_2}{z} + \frac{b_2}{z} + \ldots
\]

in which \( a_i \) \((i = 1, 2, \ldots)\) is the absolute value of the difference between the height of the \( i \)th peak and the baseline level in the autocorrelogram; \( b_i \) \((i = 1, 2, \ldots)\) is the absolute value of the difference between the depth of the \( i \)th valley and baseline level, and \( z \) is the absolute value of the difference between the total number of spikes (within the recording time) and the baseline level. Strong oscillatory activity is indicated by higher rhythm indices. A rhythm index of zero was assigned to autocorrelograms with no detectable peaks and valleys. Rhythm indices with values between 0 and 0.01 were also considered as non-oscillatory, and in these cases, oscillation frequency was not calculated. All numerical results are reported in the text as mean ± SD. For simple spike and complex spike firing frequencies, CV, CV2, and rhythm index among all age
groups, non-parametric statistical analyses were performed with the Kruskall-Wallis test (H), followed by a Dunn’s multiple comparison test for post hoc analyses between two groups (p). The values generated for comparisons of simple spike rates, CV, and CV2 between the different age groups are listed in Tables 1, 2, and 3. For comparison of Purkinje cell firing properties between anesthetized and awake, behaving mice, parametric statistical analyses were performed with the \( t \)-test. Significance is indicated in the graphs for \( p<0.05 \), \( p<0.01 \), \( p<0.001 \), or \( p<0.0001 \) with *, **, ***, and ****, respectively. Non-significance is indicated by “n.s.”. Error bars indicate standard error of the mean (SEM). Additional procedural details of our electrophysiology approach and technical details of our equipment and measurements can be found in White et al. 2014 (White et al. 2014).

Electrophysiology in awake mice. Two-month old mice were implanted with sterilized aluminum custom-made head plates. The mice as given subcutaneous injections of 0.75 mg/ml buprenorphine as an analgesic during the surgery and supplied with additional doses post-operatively twice a day for three days. We use triple antibiotic ointment (Walgreens, USA) to protect the recording holes from soiled bedding, fecal debris, etc. when the mice are in their home cages. During surgery a recording hole was made in the skull, and extracellular recordings performed (described above) after full recovery. After two days of recovery, mice are trained in our custom-built head mount. The head mount is attached to metal posts that fix a foam running wheel below the animal (Chettih et al. 2011; Heiney et al. 2014). We train the mice with practice runs in order for them to acclimate to moving while being head fixed above the wheel. We train them for three days, with 30 minutes per session. On the fourth day, the mice are placed into the head mount and recordings taken for 1-2 hours. We used 5 mice for the awake recordings.

Tissue preparation and cutting. For perfusion-fixation, animals were deeply anesthetized with avertin (2, 2, 2-Tribromoethanol), and then perfused through the heart with 0.1 M phosphate-buffered saline (PBS; pH 7.2), followed by 4% paraformaldehyde (4% PFA) diluted in PBS. The brains from the perfused mice were post-fixed for 24-48 hours in 4% PFA and then cryoprotected stepwise in PBS buffered sucrose solutions (15% and 30% each time until the brain sunk). Serial 40 µm thick coronal or sagittal sections were cut on a cryostat, and then they were collected in 24-well culture plates and processed free-floating in PBS.
Immunohistochemistry. Immunohistochemistry was carried out as described previously (Reeber et al. 2011; Sillitoe et al. 2003; Sillitoe et al. 2010; White and Sillitoe 2013b). Briefly, tissue sections were thoroughly washed, blocked with 10% normal goat serum (NGS; Sigma, St. Louis MO, USA) for 1 hour at room temperature and then incubated in 0.1M PBS containing 10% NGS, 0.1% Tween-20 and the anti-CAR8 primary antibodies (1:3,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 16-18 hours at room temperature, shaking gently. The tissue sections were then washed three times in PBS and then incubated in goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (1:200; DAKO, Carpinteria, CA, USA) for 2 hours at room temperature, again shaking gently. The tissue was rinsed again and immunoreactivity analyzed using SG substrate (Vector Laboratories, Burlingame, CA, USA) as a chromogen. After mounting them on glass slides the sections were coverslipped using Entellan mounting media (Electron Microscopy Sciences, Hatfield, PA, USA). We tested the specificity of the secondary antibodies by processing the tissue sections in the absence of primary antibodies. No signal was detected in such control experiments indicating that the staining we observed was not due to non-specific signals from the HRP antibodies (data not shown). The recording tracks were revealed using hematoxylin and eosin staining, which was performed on 10 μm-thick paraffin-embedded tissue sections as described in Sillitoe et al. (Sillitoe et al. 2008).

Imaging and data analysis. Photomicrographs of tissue sections were captured with a Zeiss AxioCam MRC5 camera mounted on a Zeiss Axio Imager.M2 microscope. Images of tissue sections were acquired with the Zeiss Apotome.2 system and analyzed using Zeiss ZEN software (2012 edition). After imaging, the raw data was imported into Adobe Photoshop CS5 and corrected for brightness and contrast levels. Schematics were drawn in Adobe Illustrator CS5.
RESULTS

We used outbred Swiss Webster mice in this study. There are several reasons for this choice. First, these mice have been the strain of choice for genetic inducible fate mapping and cell lineage tracing regimes (Sgaier et al. 2005; Sillitoe et al. 2009; Zervas et al. 2005). This is important because a major motivation for our study was to provide a fundamental knowledge base for understanding how genetic and developmental cell lineage data relate to cell function. Therefore, Swiss Webster mice were an ideal choice. Second, Swiss Webster mice are well suited for genetic and in vivo physiology studies because the dams regularly deliver larger litters, typically exceeding 10 pups. This is important for physiology because each pup may only yield a few single units that are clean enough for analysis. Third, Swiss Webster mice are very resilient to surgery (Sillitoe et al. 2010), which is a key component in electrophysiology studies because each mouse is often probed for single units over several hours. There are no major differences in the morphogenesis, developmental patterning, or adult architecture of the cerebellum in Swiss Webster compared to inbred C57BL/6 mice (although there are strain specific features), which indicated to us that because of the advantages listed above they would be a good model to assess developing neural function (Sillitoe et al. 2009; Sillitoe and Joyner 2007; Sillitoe et al. 2010).

As a starting point for understanding how Purkinje cell firing activity develops, we sought to identify an age that would be old enough to clearly reveal neuronal spikes, early enough that we could track whether developmental changes influence emerging function, and experimentally accessible for routine in vivo electrophysiology, which is invasive and requires that the general physiological state of the mouse be maintained at a stable level for several hours. We found that P14 satisfied each of these criteria. Therefore, in this study we measured Purkinje activity in vivo in developing mice starting from P14, and then we continued to analyze Purkinje cells every day thereafter until P31. We then aged an additional cohort of mice for one more month in order to record from adults at age P60-P65. We collected data from both males and females at each age.

Purkinje cell complex spikes are distinguishable from simple spikes in Swiss Webster mice

Purkinje cells have several physiological properties that can be used to distinguish them from all other types of cerebellar neurons. In adults, Purkinje cells are identified by extracellular
spontaneous signals based on 1) their 40-50 Hz firing rate, 2) the presence of a 0.5-1 Hz complex spike firing rate, with the complex comprised of a large positive spike followed by three to five smaller amplitude spikelets, 3) a short 25-50 ms pause in simple spikes that occur after the complex spike ends, and 4) the resurgence of simple spike firing after the pause (Ruigrok et al. 2011; Shin et al. 2007). No other known cerebellar cell type exhibits this combination of firing properties (Barmack and Yakhnitsa 2011). We found that P60-P65 adult Swiss Webster mice anesthetized with ketamine/dexamedetomidine and maintained with low isoflurane exhibited the expected Purkinje cell properties (Figs. 2A, 2C, 2D). They have an average simple spike rate of 37.2 ± 14.7 Hz, a complex spike rate of 0.52 ± 0.25 Hz (n = 23 cells), and when traces of at least 300 seconds are examined we found that they fire with a relatively regular firing pattern (Fig. 2A). But before examining developing Purkinje cells in detail, we compared the activity of Purkinje cells recorded under anesthesia with those recorded in awake mice to test whether there are any firing features that are affected by our anesthetic regime. This was critical to know because it is currently only feasible to cleanly isolate single developing Purkinje cells under anesthesia, although the awake recording technology is clearly advancing (Sokoloff et al. 2014).

A comparison of Purkinje cell firing in anesthetized versus awake Swiss Webster mice

We recorded from awake P60 Swiss Webster mice and found comparable firing characteristics to what has recently been reported (Witter et al. 2013; Zhou et al. 2014) (Figs. 2B, 2E, and 2F). However, simple spike and complex spike firing frequencies are faster in awake, behaving mice (n = 14 cells; 67.9 ± 27.8 Hz and 1.25 ± 0.48 Hz, respectively) compared to anesthetized P60-P65 mice (n = 23 cells; 37.2 ± 14.7 Hz and 0.52 ± 0.25 Hz, respectively; t_{simple}(35) = 4.4, p < 0.0001; t_{complex}(35) = 6.1, p < 0.0001) (Figs. 2G and 2J). This means that based on a pre-defined period of time, any given Purkinje cell will exhibit more spikes when the animal is awake compared to when it is anesthetized. Despite the difference in the overall average rate, we noticed a substantial overlap in the individual rates of anesthetized and awake recorded cells (Figs. 2G and 2J). In contrast to the rates, the patterns of firing were not different between the two preparations. The degree of regularity in Purkinje cell firing pattern is determined by their inter-spike interval CV and CV2 (see methods). In anesthetized adult mice, we found a simple spike CV of 0.83 ± 0.37 and CV2 of 0.63 ± 0.14, and a complex spike CV of 0.78 ± 0.13, which were not statistically different from awake mice, in which we found a simple
spike CV of 0.75 ± 0.19 and a CV2 of 0.56 ± 0.13, and a complex spike CV of 0.71 ± 0.10
(simple spike: tCV(35) = 0.69, p = 0.49; tCV2(35) = 1.4, p = 0.17; complex spike: tCV(35) = 1.7, p
= 0.10) (Figs. 2H, 2I, and 2K). These data indicate that our anesthetized preparation does not
cause unexpected alterations in the firing behavior of Purkinje cells. Specifically, our anesthetic
regime does not mask complex spikes, though simple spike and complex spike firing frequency
are different. This was important for us to establish because based on these data we were
confident that during development we could identify individual cells, and reliably recognize
them as Purkinje cells based on the presence of complex spikes and simple spikes. We had to use
the anesthetized approach for young mice because it is currently not feasible to perform the
awake recordings when the skull is not yet ossified. The lack of strong ossified bone makes it
challenging to implant our head plates onto young mice, making it impractical to keep the
electrode stable during a long recording session (see methods for details).

Complex spikes are a reliable feature for identifying developing Purkinje cells

In anesthetized developing mice, we found that Purkinje cells could be identified based on
the presence of complex spikes that each contained multiple spikelets (Figs. 3A and 3C). We
observed clear complex spikes at P14, and they were obvious at every age that we analyzed
(Figs. 3A and 3C). On the other hand, unlike adult mice that have a regular ~40-50 Hz simple
spike frequency, the rate in developing mice was more variable between ages and the firing
pattern was irregular (Figs. 3A, 3B). Therefore, we found that the complex spike is the one
reliable feature for distinguishing Purkinje cells from other cerebellar cell types at any age.

Throughout the study we used the unique features of the complex spike waveform not only to
identify cells as being Purkinje cells, but also to isolate single units. Isolating single units is a
critical obstacle when recording young mice because even though the Purkinje cells have formed
a monolayer by P5-P7 (Sudarov and Joyner 2007) (Fig. 1D), lamination is not complete, there
are many migrating granule cells, and the dendrites are not yet fully formed. In addition, Purkinje
cells are small and more clustered at this age, making it more difficult to isolate them (Sillitoe
and Joyner 2007). Because we found that the overall spike rates and spike patterns in developing
two-week old Purkinje cells appeared to be dramatically different from the adults, we decided to
examine both properties further to determine how they behave during different stages of postnatal cerebellar circuit formation.

**During development Purkinje cell complex spike rate increases sharply whereas simple spike rate increases gradually over time**

We analyzed complex spike and simple spike traces by binning the data that we collected into groups consisting of P14-P16 (n = 19 cells), P17-19 (n = 13 cells), P20-P22 (n = 18 cells), P23-P25 (n = 11 cells), P26-P28 (n = 17 cells), P29-P31 (n = 11 cells), and P60-P65 (n = 23 cells) (Figs. 4A and 4B). We started by analyzing complex spikes because they are a very reliable feature for positively identifying developing Purkinje cells. Within the P14-P16 age group, we found an average complex spike firing rate of $0.39 \pm 0.24$ Hz. Complex spike firing rate dramatically increased at P17 ($0.71 \pm 0.21$ Hz; P14-P16 vs. P17-P19: $p = 0.0046$). Although two-week old mice had a slower complex spike rate compared to mature mice, as the mice got a little older we found that even though complex rate rates varied within each age bracket, there were no statistical differences all the way through to adulthood (P20 $0.64 \pm 0.27$ Hz); P25 $0.55 \pm 0.20$ Hz); P28 $0.56 \pm 0.18$ Hz); P29-P31 Purkinje cells $0.50 \pm 0.30$ Hz); P60-P65 adults $0.52 \pm 0.25$ Hz; $H = 17.87, p = 0.0066$)) (Fig. 4A). Note also that the rates of firing are indistinguishable when we compare mice at ages P29-P31, which we consider to be juvenile but with a mature cerebellar structure, to mice at P60-65, which we consider to be the beginning of adulthood (P29-P31 vs. P60-P65: $p > 0.999$) (Fig. 4A).

Then we analyzed simple spike rate to determine how they behave as development proceeds. In contrast to complex spikes, simple spike dynamics were more protracted over development. P14-P16 Purkinje cells had an average simplex spike firing frequency of $24.5 \pm 9.0$ Hz. Over the next two weeks this frequency increased gradually. Mean simple spike firing rates were $27.5 \pm 8.9$ Hz during P17-P19, $33.4 \pm 9.2$ Hz during P20-P22, $34.1 \pm 11.0$ Hz during P23-P25, and $36.4 \pm 11.5$ Hz during P26-P28. By four weeks of age (P29-P31), the mean simple spike firing frequency was $37.5 \pm 9.9$ Hz, which was not significantly different from the P60-P65 adult firing rate ($37.2 \pm 14.7$ Hz; $H = 19.5; p = 0.0034$; P14-P16 vs. P60-P65: $p = 0.025$; P14-P16 vs. P29-P31: $p = 0.028$; P29-P31 vs. P60-P65: $p > 0.99$) (Fig. 4B). The increase in simple spike rate was therefore gradual over time (Table 1), which was reflected in the upward trend of the data.
These findings indicate that complex spike and simple spike firing frequencies have different trajectories during development. The data also show that in mice, the overall firing frequency of Purkinje cells reaches functional maturity at approximately four weeks of age.

**Purkinje cell complex spike firing is more regular and exhibits limited change over time, whereas simple spike firing is irregular and dynamically shaped during development**

We next wondered what the variability is in the inter-spike intervals. We found that complex spike firing patterns were remarkably consistent across all the ages that we analyzed. We found that complex spike CV was 0.77 ± 0.027 at P14-P16, 0.71 ± 0.094 at P17-P19, 0.73 ± 0.094 at P20-P22, 0.75 ± 0.062 at P23-P25, 0.072 ± 0.11 at P26-P28, and 0.782 ± 0.10 at P29-P31, and these values were not significantly different from the CV at P60-65, which was 0.78 ± 0.13 (H = 7.219, p = 0.31) (Fig. 5A). These data indicate that regardless of age, Purkinje cells have a consistent pattern of complex spike activity.

In contrast to the complex spike patterns, we found that the developing simple spike firing patterns were dynamic. We report an average simple spike CV of 0.83 ± 0.37 at P60-P65 (Fig. 5B). During development however, simple spike CV is higher, and thus decreases over time. Average CV was 1.47 ± 0.68 at P14-P16, 1.81 ± 1.0 at P17-P19, 1.51 ± 0.79 at P20-P22, 1.07 ± 0.71 at P23-P25, 1.23 ± 0.87 at P26-P28, and 0.91 ± 0.46 at P29-P31 (H = 22.39, p = 0.0010). We found a significant difference when we compared P14-P16 to P60-P65 (p = 0.036), but not P29-P31 to P60-P65 (p > 0.99) (Fig. 5B). This decreasing trend in CV during development indicates that young Purkinje cells fire at variable intervals (Table 2), which become more regular as they reach maturity. Taken together, these findings demonstrate that compared to mature Purkinje cells, developing Purkinje cells can have a comparable complex spike firing rate, a low simple spike firing rate, and an overall irregular pattern of simple spike firing.

**Developing Purkinje cells fire irregularly but with a rhythmic pattern of activity**

Although Purkinje cell firing *in vivo* is not highly rhythmic at maturity, their firing pattern is in fact relatively regular (P60-P65: CV = 0.83 ± 0.37) (Fig. 5B). Because we noticed a somewhat chronic pattern of spike clusters (which we refer to here as “bursts”) on low power views of the
raw traces (Fig. 3), in spite of a significantly higher CV at P14-P16 compared to P29-P31 (Fig. 5B), we wondered whether the irregular firing in developing Purkinje cells was highly rhythmic at short intervals. If this were the case, then we might expect immature Purkinje cells to have a lower CV2 compared to adult cells. Analysis of the intrinsic spike regularity by computing the simple spike CV2 (see methods) of the different groups indicated that indeed immature Purkinje cell activity is rhythmic (P14-P16: 0.41 ± 0.11; P17-P19: 0.54 ± 0.15; P20-P22: 0.50 ± 0.17; P23-P25: 0.56 ± 0.17; P26-P28: 0.51 ± 0.13; P29-P31: 0.53 ± 0.10) compared to P60-P65 adult Purkinje cells (0.63 ± 0.14; H = 24.3, p = 0.0005) (Fig. 6). Similar to our findings on simple spike rate and CV, the increase in CV2 between each age group showed an increasing trend (Table 3), though statistical significance is obvious when we compare the P14-P16 mice to the adult mice (P14-P16 vs. P60-P65: p < 0.0001) (Fig. 5). We next used an autocorrelation analysis (performed in Spike2) to further examine the developing Purkinje cells for spike trains that are interrupted by long pauses. The autocorrelation analysis and accompanying raster plot data confirmed the CV2 results, as we generated a relatively flat histogram for the spike train autocorrelation of P29-P31 and adult Purkinje cell firing (Figs. 7C and 7D), which was in contrast to the histograms generated for the younger mice that consisted of multiple side peaks for the spike trains of P14-P16 to P20-P22 Purkinje cells (Figs. 7A and 7B). We next used the autocorrelograms to quantify the strength of rhythmicity in developing and mature Purkinje cells (Fig. 8A). This measure is referred to as the rhythmicity index (see material and methods). The rhythmicity indices were significantly higher in developing mice (P14-P16: 0.92 ± 0.46; P20-22: 0.83 ± 0.43) compared to mature P29-P31 mice and P60-P65 adult mice (P29-31: 0.38 ± 0.91; P60-P65: 0.36 ± 0.16; H = 30.15, p < 0.0001) (Fig. 8B), indicating that developing Purkinje cells exhibit strongly repetitive burst firing patterns that are diminished at maturity (P14-16 vs. P29-31: p = 0.0011). The frequency of oscillation also changed over time with P14-P16 Purkinje cells showing significantly lower oscillation frequencies (45.46 ± 16.55 Hz) compared to Purkinje cells at P20-22 (60.63 ± 23.23 Hz), P29-P31 (58.72 ± 24.13 Hz), and P60-P65 (68.94 ± 23.17 Hz; H = 10.10, p = 0.018) (P14-P16 vs. P60-P65: p = 0.0097).
DISCUSSION

The goal of this study was to better understand the ontogenesis of Purkinje cell activity. We used *in vivo* electrophysiology to measure spike activity in the cerebellum. We found several unique features of immature Purkinje cells, and among these were temporal changes that occurred from P14 through P31. Our results revealed a sharp increase in complex spike firing rate before three weeks of age. Surprisingly, simple spike activity showed a gradual increase in firing rate, and a shift from an irregular to a more regular firing pattern, which didn’t occur until about P28. These differences in firing properties indicate that the maturation of simple spike and complex spike firing follow distinct developmental timetables, each with specific features that together define how Purkinje cells behave over time. We conclude that the ontogenesis of Purkinje cell firing occurs over a protracted period of postnatal cerebellar development, with the adult properties attained well after the basic structure of the cerebellar circuit has been formed.

Several previous studies have followed Purkinje cell firing in developing rodents. *In vitro* analysis in mice and rats demonstrated using slices, cell culture, and tissue explants that Purkinje cells can establish simple spike and complex spike firing activity in the absence of the highly structured architecture of the intact cerebellum (Bishop 2002; Gruol and Franklin 1987; Guan et al. 2006; Hockberger et al. 1989; Mariani and Changeux 1981; Sokoloff et al. 2014). Furthermore, in acute cerebellar slices a trimodal pattern of firing becomes prominent in the second week of life (Womack and Khodakhah 2002). Although we did not observe obvious trimodal patterns of activity in our anesthetized *in vivo* studies, our findings share the common idea that there are distinct firing patterns in developing Purkinje cells that evolve over time.

Previous *in vivo* studies have also followed developing Purkinje cells, showing that complex spike activity can be detected during the first week after birth in rats (Crepel 1971; Shimono et al. 1976; Woodward et al. 1969). More recently, whole-cell *in vivo* electrophysiology conducted in developing mice also demonstrated complex spike activity in the first week of life (Kawamura et al. 2013). Moreover, using an *in vivo* extracellular recording approach Lorenzetto and colleagues conducted an analysis of P11-P16 and P30 mice (Lorenzetto et al. 2009). Consistent with our findings they found that simple spike activity increases with age. However, in contrast
to our data they showed that P15 Purkinje cells have a relatively regular non-burst pattern and P30 Purkinje cells have clusters of spikes interrupted by periods of long quiescence (Lorenzetto et al. 2009). One possible explanation for the discrepancy with our data is that their study utilized higher impedance glass electrodes of up to 10 MΩ whereas we used 2-5 MΩ wire electrodes. This means that between the two studies different subsets of Purkinje cells could in fact have been selected for, based on the cells’ physiological properties. Recent work indicates that indeed Purkinje cell intrinsic properties can be different (Kim et al. 2012; Snow et al. 2014), and these differences are likely related to the specific zonal circuitry that they integrate into (Xiao et al. 2014; Zhou et al. 2014). Moreover, our observation of relatively regular firing at P30 may in fact co-exist with the irregular burst-like activity reported by Lorenzetto et al. (Lorenzetto et al. 2009) in light of recent data from an awake preparation that reported the presence of tonic, bursting, and even quiescent modes of Purkinje cell firing (Cheron et al. 2014). In the future, it would be interesting to use juxtacellular labeling (Pinault 1996) to anatomically identify recorded neurons that were developmentally “marked” based on their genetic lineage (Sillitoe et al. 2009), and then examine whether each class of Purkinje cell, based on its firing pattern, has a distinct location in the cerebellum, specific afferent connectivity, and unique developmental fate within the zonal “stripe” expression map (Sillitoe and Joyner 2007; White and Sillitoe 2013a). Indeed, the formation of zones is critically dependent on proper Purkinje cell firing (White et al. 2014).

In adult Purkinje cells, climbing fiber activity is thought to modulate simplex spike firing (Barmack and Yakhnitsa 2011; Cerminara and Rawson 2004). Although developing Purkinje may also have the same modulation, there is likely to be a weaker correlation between the spike properties because their rates reach maturity on different time scales (Figs. 4A and 4B). The time to maturation could potentially be shaped by the rewiring of climbing fibers and mossy fibers on Purkinje cells, as well as intrinsic gene expression changes within Purkinje cells. But how would the processes of innervation potentially influence the time scale of complex spike maturity? During embryonic and postnatal development multiple climbing fibers innervate a single Purkinje cell, but during the second and third week after birth the circuit is pruned to mono-innervation (Hashimoto et al. 2009; White and Sillitoe 2013a). It is possible that the complex spike rate increases suddenly because a single “winner” climbing fiber is selected by P17 (Carrillo et al. 2013; Hashimoto and Kano 2013). Furthermore, the potassium channel Kv3.3,
which is required for the generation of spikelets in a complex spike (Hurlock et al. 2008), is expressed in a mosaic pattern in Purkinje cells starting at P10 and its expression gradually increases before becoming uniformly expressed in adulthood (Goldman-Wohl et al. 1994). This suggests that by around P17, many Purkinje cells fire complex spikelets with a relatively mature profile, although there is likely still a population that has variable numbers of spikelets. This could mean that complex spikes may not have a significant influence on simple spike firing frequency until all the Purkinje cells exhibit mature spikelets. Developing Purkinje cells have also been shown to exhibit variability in the shape of the complex spike waveform (Lorenzetto et al. 2009). However, it is not clear how spike shape differences impact Purkinje cell function. Regardless, the overall variability in the composition of complex spikes underscores the idea that even though postnatal developing Purkinje cells already receive climbing fiber input, the complex spikes that are generated are diverse and age-specific. It also raises the possibility that the capacity of complex spikes to modulate simple spike firing only arises at a late stage of circuit development, perhaps coincident with the onset of adult firing or maybe even as a driving mechanism for mature circuit behaviors. Below we further consider how other extrinsic and intrinsic neuronal features might also influence the maturation of Purkinje cell firing in vivo.

We speculate that one stimulus for increasing the rate of simple spikes may be related to the wiring of the parallel fiber-to-Purkinje cell circuit. Although simple spikes are generated mainly intrinsically within the Purkinje cells, their overall rate does also depend on mossy fiber to granule cell inputs (Wise et al. 2010). But, what would cause simple spike rate to increase gradually and continue to rise until P28? It is possible that the gradual increase in simple spike rate is related to the progression of granule cell development combined with the switch in innervation of mossy fibers from Purkinje cells to granule cells (Kalinovsky et al. 2011). Granule cells migrate from the external granular layer past the Purkinje cells and into the internal granular layer. The number of cells that are migrating gradually falls off by ~P20 (Goldowitz and Hamre 1998; White and Sillitoe 2013a), but it likely takes several more days for the mossy fibers to find their target granule cells and make the functional contacts that will eventually deliver excitatory signals to Purkinje cells through the parallel fiber axons. Therefore, the process of rewiring itself may be directly related to the onset of mature Purkinje cell microcircuit function –
mossy fiber innervation is sculpted over a protracted period of development, and the whole process requires multiple stages to define the final functional wiring map.

In addition to potential changes in innervation, intrinsic developmental changes within the Purkinje cells may also play a role in increasing simple spike firing frequency. In the absence of synaptic inputs, an increase in simple spike firing frequency was previously observed in slices from developing rat cerebellum (McKay and Turner 2005). In accordance with our data, CV decreased with age in the rat slices, and this change occurred in parallel with changes in Purkinje cell soma size, dendritic size and complexity, and channel expression within these compartments (McKay and Turner 2005). In addition, the time-course of fast Na⁺ current after-hyperpolarization plateaued at P18 (~P16 in mouse), which may contribute to the ability of Purkinje cells to fire more rapidly at maturity (McKay and Turner 2005). We therefore propose that the establishment of complex spike and simple spike activities are in part set up by distinct developmental mechanisms, with the maturity of each spike profile uniquely controlled by its own circuit formation processes that include specific intrinsic changes within the Purkinje cells.

Cerebellar Purkinje cells and neurons in the cerebellar nuclei exhibit burst firing patterns in rodent models of ataxia (Gao et al. 2012), dystonia (Fremont et al. 2014; LeDoux 2011), and even multiple sclerosis (Saab et al. 2004). This irregular burst firing may be a contributing factor to the abnormal motor behavior seen in these animals (Alvina and Khodakhah 2010). It is interesting that we have revealed prominent burst activity in the developing cerebellum of normal mice (Figs. 7 and 8B). An interesting parallel can be drawn between the developing brain and conditions that affect circuitry. In both cases the circuit is incomplete, either because the circuit has yet to make proper contacts or because it has lost its contacts. Regardless of the reason, having an incomplete circuit could ultimately cause burst activity that translates into limited motor control. But the behaviors could also arise because of spike relationships. In the Kv3.3 mutants, loss of complex spike spikelets and an overall decrease in complex spike rate cause an increase in simple spike frequency (Hurlock et al. 2008). On the other hand, increasing complex spike rate with harmaline treatment abolishes simple spike firing (LeDoux and Lorden 2002). Importantly, both of these manipulations lead to motor impairments. Our data from normal developing mice raises a third scenario; that in specific cases a dynamic relationship may...
exist between simple spike and complex spike rates, and it may be that motor behavior is fine
tuned only when this relationship stabilizes (as discussed above). Unfortunately, we have a
limited understanding of how motor behavior arises during development (Jacquelin et al. 2012).
In the future, it would be interesting to test whether the changes in firing during development
indeed influence the relationship between spike types, and if an increased interdependence of
simple spikes and complex spikes correlates with improved motor control (Cerminara and

There are several limitations of our study. First, as we alluded to before, it is not yet
technically feasible to attain stable awake recordings in developing mice with our approach. The
skull is too soft to hold a head plate. However, our anesthetized recordings, which we conducted
on every day from P14 all the way through P31, is still a significant advance because we provide
the first day-by-day analysis of Purkinje cell simple spike and complex spike frequency and
pattern. Another limitation is that we did not analyze the firing of Purkinje cells in all lobules.
We concentrated on lobules VI-VII of the vermis and CrusI-II of the hemispheres because they
are easily accessible—although, because of the way we manipulate the electrode, adjacent deeper
lobules are sometimes penetrated (Fig. 1). In future studies it would be interesting to know what
other lobules reveal because the electrophysiological characteristic of the cells could be different
depending lobule identity (Snow et al. 2013; Zhang and Linden 2012), and perhaps even the
specific position within a lobule (Zhang and Linden 2012). Interestingly, the temporal changes in
Purkinje cell firing during development could also be different depending on the lobule because
subsets of lobules tend to develop at different rates (Altman and Bayer 1997). For instance,
morphogenesis of lobules VI-VII of the central cerebellum, which we analyzed here, develops
later than other regions. Therefore, the developing cerebellum may not only be
compartmentalized by the timing of its morphogenetic progression (Altman and Bayer 1997),
gene expression patterning (Ozol et al. 1999), and afferent connectivity (Ashwell and Zhang
1992), but also by the maturation of Purkinje cell firing activity. We conclude that Purkinje cell
spike firing may be a valuable measure for the routine analysis of genetically modified mice,
with a particularly strong relevance for examining the features of disease causing mutations that
cause motor deficits, and perhaps even non-motor problems as well (D'Angelo and Casali 2012).
**FIGURE LEGENDS**

Figure 1. *In vivo* extracellular single-unit recording of cerebellar Purkinje cells. **A**, Schematic diagram depicting a cerebellar *in vivo* extracellular recording. **B**, Sagittal section of a P30 cerebellum stained with hematoxylin and eosin. Roman numerals show the location of lobules and the schematic with the electrode shows the approximate trajectory during recording. Scale bar = 500 μm. **C**, Lesion (white arrows) shown in a high-power magnification view from the boxed region in **B**, indicating approximate recording sites. Scale bar = 200 μm. **D**, DIC image of a sagittal section of an adult cerebellum that was immunoperoxidase-stained for CAR8. CAR8 is heavily expressed in the cerebellum, and within the cerebellum its expression is restricted to Purkinje cells. The layers of the cerebellar cortex are indicated by *ml* (molecular layer), *pcl* (Purkinje cell layer), and *gl* (granular layer). Scale bars = 25 μm. **E**, A 200 ms raw trace showing simple spikes and a complex spike (asterisk). Scale bars: x = 10 ms, y = 5 mV.

Figure 2. Purkinje cell firing properties recorded extracellularly in anesthetized and awake adult Swiss Webster mice. **A**, A sample single-unit recording from an anesthetized adult mouse. Asterisks mark complex spikes (Scale bars: x = 1 sec, y = 5 mV). **B**, A sample single-unit recording from an awake, behaving, adult mouse. Asterisks mark complex spikes (Scale bars: x = 1 sec, y = 5 mV). **C**, Average simple spike waveform (black trace) from an anesthetized recording. Gray lines indicate standard deviation. (Scale bars: x = 0.2 ms, y = 2 mV). **D**, Average complex spike waveform (black trace) from an anesthetized recording. Gray lines indicate standard deviation. (Scale bars: x = 1.0 ms, y = 2 mV). **E**, Average simple spike waveform (black trace) from an awake recording. Gray lines indicate standard deviation. (Scale bars: x = 0.2 ms, y = 2 mV). **F**, Average complex spike waveform (black trace) from an awake recording. Gray lines indicate standard deviation. (Scale bars: x = 1.0 ms, y = 2 mV). **G**, Summary plot of simple spike firing frequency in anesthetized and awake mice. **H**, Summary plot of simple spike CV in anesthetized and awake mice. **I**, Summary plot of simple spike CV in anesthetized and awake mice. **J**, Summary plot of simple spike CV2 in anesthetized and awake mice. **K**, Summary plot of complex spike firing frequency in anesthetized and awake mice. **L**, Summary plot of complex spike CV in anesthetized and awake mice. For all plots: error bars = SEM, gray dots = individual data points.
Figure 3. General characteristics of Purkinje cell firing during postnatal development. A, Examples of raw recording traces from each age group. Scale bar = 0.5 sec. B, Examples of simple spikes in each age group. C, Examples of complex spikes recorded in each age group. Scale bars: x = 5 ms, y = 2 mV.

Figure 4. Complex spike and simple spike firing rate during postnatal cerebellar development. Purkinje cell complex spike firing rate decreases sharply after three weeks, whereas simple spike firing shows a gradual increase until P31. A, Summary plot of complex spike firing rate over time. B, Summary plot of simple spike firing frequency over time. For both plots: error bars = SEM, gray dots = individual data points.

Figure 5. Purkinje cell firing is irregular during development. The overall variability in simple spike firing intervals decreases over time, whereas variability in complex spike firing intervals remains constant during development. A, Summary plot of complex spike CV. B, Summary plot of simple spike CV. For both plots: error bars = SEM, gray dots = individual data points.

Figure 6. The lower short-interval regularity of developing Purkinje cells suggests rhythmic firing. Simple spike firing in developing Purkinje cells is more regular at short time intervals, compared to simple spike firing in mature cells. A, Summary plot of simple spike CV2. Error bars = SEM, gray dots = individual data points.

Figure 7. Autocorrelation analyses indicate that firing of developing Purkinje cells at short time intervals exhibits strong repetitive patterns. For A P15, B P21, C P30, and D P60: Raster plots (top) show the occurrence of simple spikes before and after every simple spike (Time = 0) in a recording. The autocorrelograms (bottom) were derived from the raster plot data.

Figure 8. Developing Purkinje cells have a high rhythmicity index, which declines at maturity. A, Schematic of an autocorrelogram from a P15 Purkinje cell (Fig. 7A) showing the specific measures used to calculate the rhythm index (as described by Sugihara et al. 1995; see methods). B, Summary plot of rhythm index. Error bars = SEM, gray dots = individual data points.
Table 1. Post-hoc statistical analysis of simple spike firing frequency between age groups. Significance is denoted by asterisk, non-significance is denoted by “ns”.

Table 2. Post-hoc statistical analysis of simple spike CV between age groups. Significance is denoted by asterisk, non-significance is denoted by “ns”.

Table 3. Post-hoc statistical analysis of simple spike CV2 between age groups. Significance is denoted by asterisks, non-significance is denoted by “ns”.
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