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Spontaneous activity and functional connectivity in the developing cerebellorubral system

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Del Rio-Bermudez C, Plumeau AM, Sattler NJ, Sokoloff G, Blumberg MS. Spontaneous activity and functional connectivity in the developing cerebellorubral system. J Neurophysiol 116: 1316-1327, 2016. First published July 6, 2016; doi:10.1152/jn.00461.2016.-The development of the cerebellar system depends in part on the emergence of functional connectivity in its input and output pathways. Characterization of spontaneous activity within these pathways provides insight into their functional status in early development. In the present study we recorded extracellular activity from the interpositus nucleus (IP) and its primary downstream target, the red nucleus (RN), in unanesthetized rats at postnatal days 8 (P8) and P12, a period of dramatic change in cerebellar circuitry. The two structures exhibited state-dependent activity patterns and agerelated changes in rhythmicity and overall firing rate. Importantly, sensory feedback (i.e., reafference) from myoclonic twitches (spontaneous, self-generated movements that are produced exclusively during active sleep) drove neural activity in the IP and RN at both ages. Additionally, anatomic tracing confirmed the presence of cerebellorubral connections as early as P8. Finally, inactivation of the IP and adjacent nuclei using the GABA_A receptor agonist muscimol caused a substantial decrease in neural activity in the contralateral RN at both ages, as well as the disappearance of rhythmicity; twitch-related activity in the RN, however, was preserved after IP inactivation, indicating that twitch-related reafference activates the two structures in parallel. Overall, the present findings point to the contributions of sleep-related spontaneous activity to the development of cerebellar networks.

red nucleus; cerebellum; sleep; myoclonic twitching; rhythmicity

NEW & NOTEWORTHY

Investigations of functional development in cerebellar networks can inform our understanding of the origins of neurodevelopmental disorders such as schizophrenia and autism. Critical to the function of these networks is the cerebellorubral pathway, which connects the cerebellum with one of its primary efferent targets, the red nucleus. For the first time, we characterize sleep- and wake-related

neural activity in this critical system in infant rats during a period of rapid developmental change.

THE CEREBELLUM plays an important role in sensorimotor processing (Apps and Garwicz 2005; Manto et al. 2012), including the learning of conditioned responses (Krupa et al. 2008; Pacheco-Calderón et al. 2012; Thompson 1988; Thompson and Steinmetz 2009) and the performance of fine motor skills (Bastian 2006; Cooper et al. 2000; Manto et al. 2012; Martin et al. 2000; Perciavalle et al. 2013; Thach et al. 1992). Additionally, dysfunction of cerebellar networks produces sensorimotor and cognitive deficits associated with such neurodevelopmental disorders as autism and schizophrenia (Ito 2008; Shevelkin et al. 2014; Wang et al. 2014). Importantly, in humans, rats, and other mammals, the cerebellum undergoes extensive postnatal development (Altman 1972; Dobbing and Sands 1973; Ellis 1920; McKay and Turner 2005; Shimono et al. 1976) that is shaped by activity-dependent mechanisms (Andjus et al. 2003; Kakizawa et al. 2000; Kalinovsky et al. 2011; Mikuni et al. 2013).

In rats, the early postnatal period is one of rapid cerebellar development (Altman 1972; Mason and Gregory 1984; McKay and Turner 2005; Shimono et al. 1976; Watanabe and Kano 2011). During this period, the cerebellum is sensitive to perturbations that can disrupt activity-dependent processes, including synaptic rearrangement (Kakizawa et al. 2000; Kalinovsky et al. 2011). One potential contributor to these activitydependent processes involves sensory feedback (i.e., reafference) from myoclonic twitches that occur during active sleep (AS), a state that predominates in early infancy (see Blumberg et al. 2013). Moreover, reafference from twitching limbs is a potent driver of neural activity throughout the infant brain, including Purkinje cells in cerebellar cortex (Sokoloff et al. 2015a, 2015b).

In addition to twitch-dependent activity, Purkinje cells of week-old rats exhibit rhythmic patterns of unit activity (Sokoloff et al. 2015a, 2015b). Spontaneous rhythmic neural activity occurs across sensory systems (Blankenship and Feller 2010; Lippe 1994; Watt et al. 2009; Yvert et al. 2004) and is thought to facilitate the formation of functional connectivity (Blankenship and Feller 2010; Kilb et al. 2011; Kirkby et al. 2013; Moody and Bosma 2005; O'Donovan 1999; Uhlhaas et al. 2010). Thus shared spontaneous rhythmic activity within

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the cerebellar cortex and downstream structures likely reflects the establishment of functional connections within this system.

Cerebellar outputs originate from the deep cerebellar nuclei (DCN), including the interpositus nucleus (IP; Dow 1942). In adults, the IP and its primary downstream target, the red nucleus (RN), support conditioned motor responses (Krupa et al. 2008; Pacheco-Calderón et al. 2012; Thompson 1988; Thompson and Steinmetz 2009) and skilled motor behavior (Cooper et al. 2000; Martin et al. 2000; Perciavalle et al. 2013; Soechting et al. 1978). In infants, little is known about the contributions of the IP to motor behavior. In contrast, the infant RN contributes to the production of twitches and wake-related movements as early as postnatal *day* 8 (P8). In addition to motor output, the RN processes reafference arising from twitches (Del Rio-Bermudez et al. 2015). Thus, although the RN is functional by P8, it is not known when its activity is modulated by the DCN.

In the present study, we recorded neural activity in the IP and RN of rats at P8 and P12, a period of rapid developmental change in the cerebellum (Mason and Gregory 1984; McKay and Turner 2005; Shimono et al. 1976; Sokoloff et al. 2015a; Watanabe and Kano 2011). In addition to age-related increases in firing rate, we found that activity in both structures was characterized by state dependency, activation by twitch-related reafference, and rhythmicity. Inactivation of the DCN greatly attenuated overall firing and rhythmicity in the RN. In contrast, twitch-related reafference was spared after inactivation, thus indicating parallel reafference pathways to the two structures. Together, these results demonstrate that the IP and RN are functionally connected during the period that immediately precedes the onset of cerebellar-dependent learning (Campolattaro and Freeman 2008; Freeman 2014).

MATERIALS AND METHODS

All experiments were conducted in accordance with the National Institutes of Health (NIH) "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 80-23) and were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

Spontaneous Neural Activity in the RN and DCN

Subjects. We used male and female Sprague-Dawley Norway rats (*Rattus norvegicus*) at P7–P9 (n = 20; hereafter designated as P8) and P11–P13 (n = 22; hereafter designated as P12). For all experiments, mothers and litters were housed in standard laboratory cages ($48 \times 20 \times 26$ cm). Animals were maintained on a 12:12 light-dark schedule with lights on at 0700 and with water and food available ad libitum. At P3, litters were culled to eight pups. Littermates were never assigned to the same experimental group.

Surgery. On the day of testing, a pup with a visible milk band was removed from the litter and anesthetized with 2–5% isoflurane. Stainless steel bipolar hook electrodes (50- μ m diameter; California Fine Wire, Grover Beach, CA) were implanted into the nuchal, forelimb, and hindlimb muscles, and a ground wire was implanted transdermally on the back. Flexible collodion was used to secure electromyography (EMG) electrodes and ground wire. The skull was exposed and dried. A custom-built head-fix device was then attached to the skull with cyanoacrylate adhesive (Blumberg et al. 2015). Next, the pup was lightly wrapped with gauze and allowed to recover for at least 1 h in a humidified incubator maintained at 35–36°C. The pup was then briefly reanesthetized and secured in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). A small hole was drilled in the skull to allow for later insertion of the electrode into the IP [P8 (n = 9): anteroposterior (AP) = -2.5 to -2.9 mm, mediolateral (ML) = \pm 2.0 mm, dorsoventral (DV) = -3.2 to -3.8 mm, 10–12° angle; P12 (n = 7): AP = -3.7 mm, ML = \pm 2.0 mm, DV = -3.5 to -4.5 mm] or RN [coordinates relative to bregma; P8 (n = 12): AP = -4.8 to -5.0 mm, ML = \pm 0.4 to 0.6 mm, DV = 4.3 to 4.8 mm; P12 (n = 15): AP = -5.3 to -5.5 mm, ML = \pm 0.4 to 0.6 mm, DV = -5.0 to -5.5 mm]. Two additional holes were drilled above the cerebral cortex for insertion of a ground wire and thermocouple.

Procedure. As described previously (Blumberg et al. 2015), electrophysiological recordings were performed in a stereotaxic apparatus with the animal's torso secured to a platform and the limbs dangling freely. Brain temperature was monitored using a fine-wire thermocouple (Omega Engineering, Stamford, CT) and maintained at $36-37^{\circ}$ C. Testing began after a 1- to 2-h acclimation period and only when organized sleep-wake cycles were observed.

The EMG bipolar electrodes were connected to a differential amplifier (A-M Systems, Carlsborg, WA; amplification: 10,000×; filter setting: 300-5,000 Hz). A ground electrode (Ag-AgCl, 0.25-mm diameter; Medwire, Mt. Vernon, NY) and thermocouple were inserted into the cerebral cortex. Neurophysiological recordings were acquired using 16-channel silicon depth electrodes or 4-channel linear probes (A1×16-Poly2-5 mm-50-177, A1×16-10 mm-100-177, Q1×4-10 mm-50-177; NeuroNexus, Ann Arbor, MI) connected to a data acquisition system (Tucker-Davis Technologies, Alachua, FL) that amplified $(10,000\times)$ the signals. Multiunit recordings were bandpass filtered (500-5,000 Hz), and a 60-Hz notch filter was applied; for some recordings, the signals were filtered after the experiment. A digital interface and Spike2 software (Cambridge Electronic Design, Cambridge, UK) were used to record neural and EMG signals at 25 and 1 kHz, respectively. Before insertion of electrodes into the brain, the recording electrode was coated with fluorescent DiI (Vybrant DiI cell-labeling solution; Life Technologies, Grand Island, NY) for histological verification of placement.

When DCN or RN activity was detected, spontaneous neural and EMG activity during sleep and wake were recorded continuously over a period of 30 min. For IP recordings, data collection began when action potentials were detected that were distinct from those typically observed when recording from infant Purkinje cells (Sokoloff et al. 2015a, 2015b). Similarly, RN recordings began when neural activity resembled that typically observed from neurons in this structure (Del Rio-Bermudez et al. 2015). During each recording session, the experimenter was blind to the electrophysiological record as he or she manually scored the pup's sleep and wake behavior.

For DCN inactivation, we first recorded RN activity for 30 min. A $0.5-\mu$ l microsyringe (Hamilton, Reno, NV) was then lowered stereotaxically into the DCN (coordinates relative to lambda; P8: AP = -3.4 mm, ML = $\pm 2.9 \text{ mm}$, DV = -3.2 mm, 20° angle; P12: AP = -3.7 mm, ML = \pm 3 mm, DV = -3.2 mm, 20° angle). Pups received a 0.2-µl unilateral infusion into the DCN of either fluorophore-conjugated muscimol (1.6 mM, dissolved in 60% dimethyl sulfoxide; Sigma-Aldrich; n = 6 at P8, n = 9 at P12) or 0.9% saline (n = 6 at P8 and P12) at a rate of 0.1 µl/min. Previous studies using similar concentrations of fluorophore-conjugated muscimol have shown that its behavioral effects are similar to those caused by nonconjugated muscimol, with the additional advantage of corroborating the extent of drug diffusion (Allen et al. 2008). As tested in rat pups' perirhinal neurons in vitro, the maximum inhibitory effect of muscimol is observed within the first 15 min after infusion onset (Allen et al. 2008). Thus in the present study the 30-min postinfusion session began 10-15 min after infusion onset.

Anatomical Tracing

An additional four pups were used at P7 (n = 2) and P11 (n = 2). Under 2–5% isoflurane anesthesia, the pup was secured in a stereotaxic apparatus and the skull was exposed. A small hole was drilled in the skull and, with the use of the coordinates described above, a $0.5-\mu$ l microsyringe was lowered stereotaxically into the RN. After a period of 10 min, the pup received a $0.025-\mu$ l unilateral infusion over 1 min of 2% wheat germ agglutinin conjugated to Alexa Fluor 555 (WGA-555; Invitrogen Life Technologies, Carlsbad, CA). WGA-555 is rapidly transported and has been used to study cerebellar projections in developing animals (Reeber et al. 2011). A 10-min postinfusion period allowed for diffusion of the tracer. After the infusion was complete and the microsyringe withdrawn, the scalp incision was closed using Vetbond (3M, Maplewood, MN), and the pup was returned to its home cage. Twenty-four hours later, the pup was perfused and its brain prepared for histology.

Data analysis

Spike sorting. Template matching was used to perform spike sorting as described previously (Sokoloff et al. 2015b). For RN recordings, we distinguished between simple and complex spikes (Del Rio-Bermudez et al. 2015; Lisman 1997). As defined by Lisman (1997), a "complex spike" is composed of a burst of at least two action potentials with short interspike intervals (ISIs); they also are often characterized by spikes with progressively decreasing amplitudes. (Complex spikes in the RN should not be confused with the complex spikes recorded from cerebellar Purkinje cells.) To define complex in the RN, we used a Spike2 script that identifies bursts on the basis of ISI; complex spikes were defined as bursts of two or more spikes with ISIs ≤ 8 ms, whereas simple spikes comprised all noncomplex spikes. Although data from complex spikes and simple spikes were analyzed, only data from complex spikes are presented in this report to simplify the communication of the results; in general, complex spikes provide more consistent and robust activity in relation to sleep-wake processes (see Del Rio-Bermudez et al. 2015).

Behavioral state. EMG signals and behavioral scoring were used to identify behavioral states (Blumberg et al. 2015). Periods of sleep and wakefulness were differentiated on the basis of high nuchal muscle tone (indicative of wakefulness) or muscle atonia (indicative of sleep). EMG signals were rectified, and the mean of 5 representative 1-s segments of high tone and atonia were used to calculate a midpoint that was used to dichotomize behavioral states. Furthermore, active wake (AW) was characterized by high-amplitude and often coordinated limb movements against a background of high muscle tone; active sleep (AS) was characterized by bouts of myoclonic twitches against a background of atonia (Blumberg and Seelke 2010; Seelke and Blumberg 2008). Twitches were defined as spikes of EMG activity with amplitudes at least three times greater than background atonia. Additionally, behavioral quiescence (BQ) was characterized by periods of low muscle tone, typically interposed between bouts of AW and AS.

State-dependent neural activity. For each unit, we determined mean firing rates across all behavioral states. On rare occasions, bouts of AW, BQ, and AS were excluded when firing rates exceeded three times the standard deviation. Pairwise comparisons of firing rates across states were tested using the Wilcoxon matched-pairs signed-ranks test (SPSS; IBM, Armonk, NY). Individual units were categorized as AW-On (AW > BQ = AS), AS-On (AS > AW = BQ), AS-On/AW-On (AS = AW > BQ), state independent (AS = AW = BQ), or other. Average firing rates for each unit were pooled across behavioral states and tested for significance; age-related changes in firing rate were tested using the Kolmogorov-Smirnov test for independent samples.

Twitch-related neural activity. The temporal relationships between twitches and unit activity in the RN and IP were examined. First, for each individual unit, using nuchal or forelimb muscle twitches as trigger events, we generated perievent histograms (10-ms bins, 1-s windows). To test for statistical significance, we jittered twitch events 1,000 times within a 500-ms window using PatternJitter (Amarasingham et al. 2012; Harrison and Geman 2009) implemented in MATLAB (The MathWorks, Natick, MA). We corrected for multiple comparisons by generating upper and lower acceptance bands for each event correlation (P < 0.01 for each band; Amarasingham et al. 2012). Only units that surpassed the established 99% threshold for statistical significance were pooled for further analysis. (For each unit, the EMG record for the muscle that yielded the strongest twitch-dependent activity was used in subsequent analyses.) Using the pooled data, we performed event correlations and statistical tests as described above.

Rhythmic neural activity. We calculated unit variability and rhythmicity using the coefficient of variance (CV_2) and rhythmicity index (RI), respectively (see Arancillo et al. 2015; Sokoloff et al. 2015a). CV_2 measures variability by comparing differences in consecutive ISIs at times *t* and *t* + 1:

$$CV_2 = \frac{SD(ISI_{t+1}, ISI_t)}{mean(ISI_{t+1}, ISI_t)} \times \sqrt{2}$$

Thus this index provides a measure of the regularity of firing, from burstlike (low values) to random (high values; Holt et al. 1996).

To calculate RI (Arancillo et al. 2015; Sokoloff et al. 2015a), we first performed autocorrelations (5-ms bins; 1-s window) for the entire recording period using Spike2. Baseline activity over the autocorrelation window was then calculated using the equation:

Baseline =
$$\frac{\text{(total number of spikes)}^2}{\text{(total time/bin duration)}}$$

Peaks and valleys in the autocorrelogram were identified on the basis of when the bin values fell below or above the lower and upper thresholds, respectively. Thresholds were calculated by adding and subtracting from baseline the standard deviation of the total count of spikes within the first and last 100 ms in the autocorrelogram window. In addition, subsequent peaks and valleys had to occur within the following time range:

$$\mathbf{t_n} + \mathbf{t_1} \pm \mathbf{t_1}/2,$$

where t_n is the bin (in ms) of the *n*th valley or peak and t_1 is the bin for the first peak in the autocorrelation. The RI was calculated as follows:

Rhythmicity index =
$$\Sigma \frac{|\text{bin count} - \text{baseline}|}{|\text{total number of spikes} - \text{baseline}|}$$

where bin count is the spike count of either a peak or valley.

Finally, oscillation frequency (OF) was calculated using the inverse of the time value (in ms) of the bin corresponding to the first peak in the autocorrelation (t_1^{-1}) . CV_2 and OF were not calculated for nonrhythmic units (RI < 0.01). Differences in CV_2 , RI, and OF were assessed using the Kolmogorov-Smirnov test. Post hoc pairwise comparisons were tested across age.

Pharmacological inactivation of the DCN. We quantified mean time spent in AS and rate of twitching for each subject during the preand postinfusion recording sessions. To calculate twitching rates, the total number of forelimb twitches for each pup was divided by the total time in AS. To assess group effects, we performed independent pairwise comparisons (pre- and postinactivation measures) using the Wilcoxon matched-pairs signed-rank tests for each experimental group at both ages.

Changes in state-dependent firing rates in the RN before and after DCN inactivation were determined by calculating the median percent change in firing rates between the pre- and postinfusion periods across behavioral state and age. On rare occasions, units with percent changes that exceeded ± 2.5 SD were classified as outliers and excluded from further analyses; accordingly, 3 units in the saline group and 2 units in the muscimol group were excluded, all at P12. The Wilcoxon matched-pairs signed-ranks test was used to test for

differences in firing rates before and after inactivation within each behavioral state. Unless otherwise stated, alpha was set at 0.05 and Bonferroni corrections were used when appropriate.

Histology

At the end of the recording session, the pup was overdosed with ketamine-xylazine (0.08 mg/g ip) and transcardially perfused with phosphate-buffered saline and 4% paraformaldehyde. For recording studies, brain sections were sliced at 80 μ m using a freezing microtome (Leica Microsystems, Buffalo Grove, IL). Electrode placements within the IP or RN and drug diffusion in the DCN were verified by visualizing the DiI track or drug diffusion at ×2.5–5 magnification using a fluorescent microscope and digital camera (Leica Microsystems). Sections were subsequently stained with cresyl violet, and electrode placements and infusions sites were confirmed. For tracing, sections were sliced at 50 μ m. Alternating slices were stained with cresyl violet or processed for WGA-555 visualization. Images were acquired at ×5–20 magnification.

RESULTS

Spontaneous Activity in the IP and RN

State-dependent activity in the IP and RN. Extracellular activity was recorded from the IP (P8: n = 36 units; P12: n = 24 units) and the RN (P8: n = 43 units; P12: n = 54 units) of infant rats (2–6 units per pup). Histology confirmed electrode placement in the IP (Fig. 1A, top) and RN (Fig. 1A, bottom).

 Table 1.
 Classification of IP and RN neurons on the basis of state dependency at P8 and P12

	AS	AW	AS and AW	Other	State Independent
IP					
P8	31% (11)	25% (9)	17% (6)	11% (4)	17% (6)
P12	59% (14)	4% (1)	13% (3)	8% (2)	17% (4)
RN					
P8	49% (21)	28% (12)	16% (7)	5% (2)	2% (1)
P12	20% (11)	35% (19)	31% (17)	6% (3)	7% (4)

Values are percentage (total number) of units for each state within each age.

Figure 1*B* depicts a representative recording from a neuron in the IP exhibiting state-dependent activity. Overall, we observed state- and age-dependent modulation of firing rates in the IP and RN at both ages (Fig. 1*C*). Specifically, neurons in both structures were predominantly active during behavioral states characterized by movement (i.e., AS and AW). IP neurons increased their firing rates during AS and AW in relation to BQ (all P < 0.001) at P8. At P12, IP firing rates were also significantly higher during AS than BQ and AW (P < 0.01). Similarly, RN neurons at P8 exhibited increased firing rates during AS and AW in relation to BQ (all P < 0.001). At P12, RN firing rates during both AS and AW were higher than BQ (all P < 0.001) and firing rates were higher during AW than AS (P < 0.01). Table 1 summarizes



Fig. 1. Recording neural activity in the IP and RN. *A*: coronal sections stained with cresyl violet showing electrode placement in the IP (*top*) and RN (*bottom*). Red lines denote electrode location based on fluorescent Dil tracks in the same coronal sections. IP, interpositus nucleus; RN, red nucleus; Lat, lateral nucleus; Med, medial nucleus; D, dorsal; L, lateral. *B*: representative recording from the IP of a P12 rat showing multiunit activity (MUA), behavioral scoring (twitches denoted by vertical ticks; wake movements denoted by horizontal lines), and rectified EMGs from the ipsilateral forelimb and nucleal muscles across behavioral states for neurons in the IP (*left*) and RN (*right*). Within each behavioral state, firing rates at P12 were significantly higher than other states at that age. †P < 0.01, firing rate significantly higher than other states at that age.

the classification of IP and RN neurons based on state dependency at both ages. Regardless of state dependency, firing rates in both structures markedly increased as a function of age (all P < 0.001).

Activity in the IP and RN during AS is associated with twitching. We previously showed that reafference from twitching during AS triggers neural activity in the RN (Del Rio-Bermudez et al. 2015) and cerebellar cortex (Sokoloff et al. 2015a, 2015b). In the present study we asked whether the IP also exhibits twitch-related activity. Figure 2A shows that the IP exhibited robust twitch-related activity at P8 (n = 29, 81%of units, P < 0.01) and P12 (n = 16, 66% of units, P < 0.01) after twitch onset (P8: 41 ± 6 ms; P12: 43 ± 6 ms). Consistent with previous results (Del Rio-Bermudez et al. 2015), RN neurons at P8 (n = 24, 55% of units, P < 0.01) and P12 (n =38, 70% of units, P < 0.01) showed significant twitch-related activity before (P8: -20 ± 3 ms; P12: -18 ± 3 ms) and after (P8: $35 \pm 8 \text{ ms}$; P12: 26, $\pm 3 \text{ ms}$) twitch onset (Fig. 2*B*). These data further support the proposed role of the RN in both sensory and motor processing during AS in early development. In addition, the latencies observed in the RN were noticeably shorter than those in the IP, suggesting that twitch-related reafference does not reach the RN via the cerebellorubral pathway.

Rhythmicity in IP and RN unit activity is age dependent. Consistent with our earlier findings in cerebellar Purkinje cells (Sokoloff et al. 2015a, 2015b), rhythmic activity was prominent in the IP at P8 (n = 32, 89% of units; Fig. 3A, top left). Although fewer units were rhythmic at P12 than at P8, a high proportion of IP neurons at P12 still exhibited rhythmic firing (n = 18, 75% of units, Fig. 3A, top right). Measures of rhythmicity are shown in Fig. 3A, bottom left and bottom center. A significant decrease in CV_2 was observed across ages (P < 0.001), indicating a shift toward more regular firing (Arancillo et al. 2015). However, there were no significant age-related differences in the rhythmicity index. Similar to previous findings in cerebellar cortex (Sokoloff et al. 2015a), we found a large increase in the oscillation frequency between P8 and P12 (median = 17.4 and 40.0 Hz, respectively; P < 0.01, Fig. 3A, *bottom right*).

In the RN, rhythmic activity was also prominent at P8 (n = 33, 77% of units; Fig. 3B, top left) but not at P12 (n = 10, 18% of units; Fig. 3B, top right). As with IP neurons, there was an age-dependent decrease in CV₂ (P < 0.05, Fig. 3B, bottom left) but no change in rhythmicity index (Fig. 3B, bottom center). In addition, for rhythmic neurons, oscillation frequency increased significantly across ages (median = 8.3 and 15.4 Hz, respectively; P < 0.05, Fig. 3B, bottom right).

Anatomical Connectivity Between the DCN and RN

As a prelude to determining the effects of IP inactivation on RN activity, we assessed their anatomical connectivity by injecting the tracer WGA-555 into the RN (P8: n = 2; P12: n = 2). We found retrograde labeling cell bodies in the DCN (IP and lateral nucleus) at both P8 and P12. Representative examples of diffusion of the tracer and labeled areas of the DCN are shown in Fig. 4. These results indicate that the RN receives afferent input from the DCN (both IP and lateral nuclei) as early as P8.

Effect of Pharmacological Inactivation of the DCN on RN Spontaneous Activity

Having established anatomical connectivity between the RN and DCN at both ages, we next asked whether spontaneous activity in the RN is modulated by the DCN. To address this question, we recorded spontaneous activity in the RN before and after inactivation of the DCN using the GABA_A receptor agonist muscimol (Fig. 5A). A representative example of drug diffusion in the DCN is shown in Fig. 5B. After histological inspection, data from one animal at P8 were excluded due to lack of drug diffusion in the DCN. Pups infused with either saline or muscimol cycled similarly between sleep and wake (Table 2). *Inactivation of DCN produced a state-dependent decrease*

in RN firing rate. Figure 5C depicts representative examples

of state-dependent RN activity at P8 (top) and P12 (bottom)

Fig. 2. Twitch-related activity in the IP and RN of infant rats. Perievent histograms (10-ms bins) for spike activity in relation to twitches in the IP (*A*) and RN (*B*) at P8 (*left*) and P12 (*right*). Vertical dashed lines denote twitch onset. For each histogram, data were pooled across all units that exhibited significant twitch-dependent activity. Upper and lower acceptance bands (P < 0.01 for each band) are indicated by dashed lines. Stacked plots indicate percentage of all units in that group that were significantly twitch dependent.





Fig. 3. Patterns of rhythmic activity in the IP and RN of infant rats. *A*, *top*: representative autocorrelograms (5-ms bins) in the IP for P8 (*left*) and P12 (*right*) rats. Stacked plots depict percentage of rhythmic units in each group. *Bottom*, boxplots depicting median values for measures of rhythmicity (CV_2 and rhythmicity index, RI) and oscillation frequency (OF). *B*: same as depicted in *A* except showing RN data. **P* < 0.05, significant difference from P8.

before and after DCN inactivation. Compared with baseline, RN firing rates were markedly reduced after DCN inactivation across the three behavioral states at both ages (all P < 0.05; Fig. 6A). However, this reduction in activity was significantly greater for BQ at both ages (all P < 0.05; Fig. 6B).

Twitch-related RN activity. After DCN inactivation at both ages, significant twitch-related increases in RN activity were still observed both before and after twitch onset (all P < 0.01; Fig. 7). Recall also the shorter mean latencies between twitch onset and neural activation for the RN than the IP. All together,

these findings indicate that twitch-related reafference to the RN does not pass first through the DCN.

Rhythmic RN activity. Finally, to test whether rhythmic activity in the RN is modulated by the DCN, we analyzed rhythmicity before and after DCN inactivation in those RN neurons that exhibited rhythmicity during the preinfusion period. Because we found few rhythmic neurons in the RN at P12, we focused our analyses at P8. Figure 8*A* shows a representative autocorrelogram for a RN unit at P8, showing the loss of rhythmicity after DCN inactivation. Overall, rhythmic RN activity was abolished in most of the rhythmic neuron

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Fig. 4. Retrograde labeling of the deep cerebellar nuclei (DCN) after infusion of WGA-555 into the RN. *A*: coronal sections from rats at P8 (*top*) and P12 (*bottom*) depicting WGA-555 diffusion in the RN at $\times 2.5$ (*left*) and $\times 5$ magnification (*right*). Adjacent Nissl-stained sections are also shown. *B*: coronal sections labeled with WGA-555 in the DCN at P8 ($\times 10$ magnification) and P12 ($\times 5$ magnification). *Insets*: WGA-labeled cell bodies in the DCN at $\times 20$ magnification. IP, interpositus nucleus; RN, red nucleus; Lat, lateral nucleus; Med, medial nucleus; D, dorsal; L, lateral.

rons, as indicated by the significant reduction in the rhythmicity index (P < 0.01; Fig. 8*B*). No differences in CV₂ or oscillation frequency were observed after DCN inactivation (data not shown).

DISCUSSION

The RN plays a major role in motor control early in development before corticospinal connections have fully developed (Williams et al. 2014). Moreover, the RN is well connected with structures that process sensory and motor information, including the IP (Flumerfelt 1980; Naus et al. 1985). In the present study, we provide evidence for the first time that the cerebellorubral pathway is functionally connected in rats as early as P8.

State- and Twitch-Dependent Activity in the IP-RN Pathway

Neurons in the IP and RN exhibited higher firing rates during periods of AS and AW compared with BQ. Similar to previous findings in cerebellar cortex (Sokoloff et al. 2015a, 2015b), we observed a pronounced age-related increase in overall firing rate in both the IP and RN. Despite this increase, state-dependent firing patterns were preserved in the IP and RN at both ages. State-dependent activity profiles also occur in the IP and RN of adult cats (Gassel et al. 1966; Palmer 1979), suggesting that preferential firing during AS and AW is maintained across development.

In addition to state dependency, robust twitch-related activity occurred in the IP and RN at both ages. We

previously suggested that these twitch-related reafferent signals could reach the RN via two possible pathways (Del Rio-Bermudez et al. 2015): through direct ascending spinorubral projections (Padel and Jeneskog 1981; Padel et al. 1986) or indirectly through the cerebellum. The present findings support the involvement of a direct pathway: first, the latencies for twitch-related reafference in the RN were shorter than for those in the IP; and second, twitch-related reafference in the RN was preserved after pharmacological inactivation of the contralateral DCN. Therefore, it appears that twitch-related reafference activates the IP and RN in parallel, not in series. Consistent with this view, hemicerebelloctomy in adult cats does not alter the latency of evoked sensory responses in the RN after limb stimulation (Tarnecki 2003).

Twitch-related activity was previously reported in the IP of adult cats (Palmer 1979). Given the role of the adult IP in motor control, it was suggested that it is involved in the generation of twitching. In our study, however, the majority of IP neurons showed twitch-related increases in activity only after twitch onset. Moreover, we did not observe any significant effect of DCN inactivation on forelimb twitching. Therefore, our data do not support a role for the IP in the generation of twitches in early development. In contrast, inactivation of the RN substantially reduced forelimb twitches at P8, indicating that the RN contributes to the production of twitches independently of DCN input (Del Rio-Bermudez et al. 2015).

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Fig. 5. Spontaneous activity in the RN across behavioral states before and after DCN inactivation A: depiction of electrode placement in the RN (*left*) and microsyringe placement in the DCN (*right*). B: coronal section showing diffusion of fluorescent muscimol in the DCN (*left*) and corresponding section Nissl-stained with cresyl violet (*right*). Asterisk denotes infusion site. IP, interpositus nucleus; Lat, lateral nucleus; Med, medial nucleus. C: representative recordings from the RN at P8 (*top*) and P12 (*bottom*) before (blue) and after (red) inactivation of the DCN. Spontaneous behavior (twitches denoted by vertical ticks; wake movements denoted by horizontal lines), MUA, unit activity, complex spikes, and contralateral forelimb and nucleal EMG activity are shown across behavioral states.

State-Dependent Modulation of RN Spontaneous Activity by the DCN

Although twitch-related activity in the RN persisted after DCN inactivation, spontaneous RN activity was reduced by at least 50% at both ages. Therefore, DCN neurons clearly modulate RN activity at these ages in an excitatory manner, as

previously observed in adult animals (Tarnecki 1988, 2003; Toyama et al. 1968). It should be noted that DCN inactivation affected the IP and adjacent nuclei. Thus inactivation of the lateral nucleus, in addition to the IP, could have partially contributed to the marked reduction of firing rates observed in the RN.

Table 2. Total time in AS and forelimb twitching rates (single limb only) before and after inactivation of the DCN

	Muscimol		Sa	Saline	
	Pre	Post	Pre	Post	
Total time in AS, s					
P8	1,146 (±93)	1,091 (±102)	945 (±65)	1,035 (±103)	
P12	777 (±131)	667 (±110)	670 (±127)	689 (±175)	
Twitching rates, forelimb twitches/min					
P8	$12(\pm 2.4)$	12 (±0.6)	14 (±2.4)	17 (±3.0)	
P12	11 (±2.4)	8 (±0.6)	13 (±1.8)	11 (±1.8)	

Values are mean (±SE) time in AS and twitching rates before (pre) and after (post) infusion of muscimol or saline in the DCN at P8 and P12.



Fig. 6. Effect of DCN inactivation on RN firing rate at P8 and P12. A: boxplots depicting median firing rate before (gray) and after (black) DCN inactivation during active wake (AW), behavioral quiescence (BQ), and active sleep (AS) in P8 (top) and P12 rats (bottom). MUS, muscimol group; SAL, saline group. *P < 0.01, significant difference from preinfusion. B: percent change in RN firing rate after DCN inactivation at P8 (top) and P12 (bottom) for each behavioral state. *P < 0.05, significant difference from AW and AS. $\dagger P < 0.05$, significant difference from AW.

Although DCN inactivation reduced RN activity across all behavioral states, we observed the greatest percent change in activity during periods of behavioral quiescence (see Figs. 5 and 6). Indeed, activity during quiescence was often completely abolished after DCN inactivation. Thus non-movement-related activity in the RN is almost exclusively driven by cerebellar input. Why would DCN modulation of RN neurons also occur during periods of quiescence? In the absence of motor behavior, sustained excitatory input from

the DCN may provide RN neurons with a minimum of excitability in preparation for subsequent motor output. Support for this idea comes from studies examining the effect of excitability of motor systems on the amplitude of evoked motor responses (Bestmann and Krakauer 2015). In general, the amplitude of evoked motor responses is positively correlated with excitability in the motor system at the moment of stimulation (Schneider et al. 2004; Taylor 2006). Therefore, the DCN may provide basal excitability to RN

Fig. 7. Twitch-related activity in the RN before and after inactivation of the DCN. Perievent line histograms show spike activity in relation to twitches in P8 (top) and P12 rats (bottom) in the muscimol (left) and saline groups (right). Twitchrelated activity is presented for the pre- (blue) and postinfusion (red) recording sessions. Vertical dashed lines denote twitch onset. For each histogram, data were pooled across all units that exhibited significant twitch-dependent activity before inactivation. Upper and lower acceptance bands (P < 0.01 for each band) are indicated by the dashed lines.



Time in relation to twitch (ms)

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Fig. 8. Patterns of rhythmic activity in the RN before and after inactivation of the DCN at P8. A: representative autocorrelogram (5-ms bins) for before (PRE; gray) and after (POST; black) muscimol infusion into the DCN. B: boxplots depicting median rhythmicity index (RI) before (gray) and after (black) infusion of either muscimol (MUS) or saline (SAL) into the DCN. *P < 0.05, significant difference from preinfusion period.

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neurons so that motor commands can be rapidly produced in response to changing circumstances.

Activity-Dependent Development in Sensorimotor Systems

In the present study, rhythmic neuronal activity was observed in both the IP and RN at P8. By P12, however, we observed considerably fewer rhythmic neurons in both structures. Interestingly, this developmental reduction in rhythmicity was also observed in Purkinje cells across the same ages (Kalinovsky et al. 2011; Manzini 2006; Mason and Gregory 1984; Sillitoe 2016; Sokoloff et al. 2015a). It was proposed that the loss of Purkinje cell rhythmicity could reflect developmental changes in cerebellar circuitry, including the elimination of direct connections between mossy fibers and Purkinje cells (Sokoloff et al. 2015a). In turn, the developmental decrease in rhythmic activity in Purkinje cells could result in the change in the rhythmicity of IP neurons, because synchronicity among Purkinje cell populations can influence the temporal organization of DCN activity (De Zeeuw et al. 2011; Gauck and Jaeger 2000; Person and Raman 2012). Regardless of the mechanisms responsible for rhythmicity in the IP, here we found that inactivation of the DCN eliminates rhythmic firing in the RN. To the extent that correlated spontaneous activity within neural networks contributes to the formation and strengthening of developing circuits (Blankenship and Feller 2010; Kirkby et al. 2013; Moody and Bosma 2005; O'Donovan 1999; Uhlhaas et al. 2010), we suggest that shared spontaneous rhythmicity along the cerebellorubral pathway contributes to the development of functional connectivity in this system.

The development of sensorimotor systems entails functional integration of sensory and motor maps (Blumberg et al. 2013). Because twitches are abundant during early development and drive substantial reafferent activity in structures throughout the neuraxis (Del Rio-Bermudez et al. 2015; Khazipov et al. 2004; Mohns and Blumberg 2008; Sokoloff et al. 2015a, 2015b; Tiriac et al. 2012, 2014), they provide the opportunity to learn about the relationship between self-generated motor commands and subsequent associated reafference. We have previously shown that twitches activate reafference in cerebellar Purkinje cells (Sokoloff et al. 2015a, 2015b), and in the present study we extend those findings to the output of the cerebellum and one of its primary downstream targets.

Overall, the present findings support the hypothesis that the period between P8 and P12, characterized by rapid developmental changes in cerebellar circuitry and activity, is a sensitive period for cerebellar development (see Sokoloff et al. 2015a). If it is a sensitive period, we would expect the cerebellum during this period to be relatively vulnerable to endogenous or exogenous perturbation. Indeed, any challenge to normal cerebellar development could disrupt the functional integration of the cerebellum with other structures, including the neocortex (Wang et al. 2014). The present findings in the IP and RN, as well as previous findings in cerebellar cortex (Sokoloff et al. 2015a, 2015b), point to a possible contribution of sleep-related spontaneous activity to the activity-dependent development of cerebellar networks. Accordingly, sleep restriction or deprivation, by disrupting the quantity and patterning of spontaneous activity, could be a risk factor for atypical cerebellar development. Atypical development of cerebellar networks has been associated with sensorimotor and cognitive deficits in such neurodevelopmental disorders as schizophrenia and autism (Baumann et al. 2015; Ito 2008; Shevelkin et al. 2014; Wang et al. 2014). In addition, anatomical alterations and functional deficits in the developing cerebellorubral pathway have been implicated in rodent models of dystonia (Brown and Lorden 1989; LeDoux and Lorden 1998; Liang et al. 2014). Therefore, characterizing the patterns of activity in developing cerebellar network structures may prove crucial for understanding the mechanisms underlying a variety of neurodevelopmental disorders with links to cerebellar function (Arancillo et al. 2015; Manto and Jissendi 2012).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.D.R.-B., A.M.P., G.S., and M.S.B. conception and design of research; C.D.R.-B., A.M.P., and N.J.S. performed experiments; C.D.R.-B., A.M.P., and CEREBELLORUBRAL INTERACTIONS IN DEVELOPING RATS

N.J.S. analyzed data; C.D.R.-B., A.M.P., G.S., and M.S.B. interpreted results of experiments; C.D.R.-B., A.M.P., N.J.S., and M.S.B. prepared figures; C.D.R.-B., A.M.P., and M.S.B. drafted manuscript; C.D.R.-B., A.M.P., G.S., and M.S.B. edited and revised manuscript; C.D.R.-B., A.M.P., G.S., and M.S.B. approved final version of manuscript.

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