Developmentally unique cerebellar processing prioritizes self- over other-generated movements

Abbreviated title: Sensorimotor processing in the infant cerebellum

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ABSTRACT

To compute internal models of movement, the cerebellum must distinguish sensory input arising from self- and other-generated movements (reafference and exafference, respectively). This distinction is enabled by copies of motor commands (i.e., corollary discharges) that are sent to the cerebellum. The capacity to compute internal models emerges gradually through a process that is not yet understood. Previously, we demonstrated in 8-day-old (P8) rats that precerebellar nuclei—including the inferior olive and lateral reticular nucleus—convey corollary discharge and reafference to the cerebellum during active (REM) sleep when pups produce self-generated limb twitches. By P20, the cerebellum is able to compute an internal model and convey it to motor thalamus via the interpositus nucleus (IP). Here, recording from IP in P12 rats, we compare reafferent and exafferent responses to twitches and limb stimulations, respectively. As expected, most IP units show robust and reliable responses to twitches. However, in contrast with other sensory structures throughout the brain, exafferent responses in IP are neither robust nor reliable. Upon finding that exafferent responses occur in pups under urethane anesthesia, we hypothesized that urethane inhibits cerebellar cortical cells, thereby disinhibiting exafferent responses in IP. In support of this hypothesis, ablating cortical tissue dorsal to IP mimics the effects of urethane on exafference. Finally, twitch-related corollary discharge and reafference are conveyed to IP and cerebellar cortex in parallel. Based on these results, we propose that twitches provide opportunities for the nascent cerebellum to integrate somatotopically organized input, thereby enabling the development of closed-loop circuits and, subsequently, internal models.

SIGNIFICANCE STATEMENT

For adult animals to produce flexible and adaptive movements, they must distinguish self- from other-generated movements and learn to anticipate how their body moves through space. The computations required for this capacity occur within the cerebellar system. In early infancy, these computations are not yet established and must develop through sensorimotor experience. By comparing how the infant cerebellum processes sensory input, we found that it prioritizes input associated with self-generated movements. Such prioritization appears unique to the infant cerebellum and helps us understand how that structure establishes its adult neural circuity and functions.

INTRODUCTION

The cerebellum's ability to distinguish reafference from exafference rests on the fact that only reafference, because it arises from self-generated movement, is accompanied by corollary discharge (Blakemore et al., 2000; Crapse and Sommer, 2008; Cullen, 2004). Information from reafference and corollary discharge is used to compute internal models of movement that, in turn, allow animals to discriminate expected from unexpected stimuli (Miall et al., 1993; Wolpert et al., 1998; Bastin, 2006; Person, 2019). Given that the cerebellum exhibits protracted postnatal development (e.g., in rats and humans; Altman, 1969; Kano et al., 2011) and that internal models must accurately reflect the specific features of individual bodies as they grow and develop, it follows that internal models emerge through extensive sensorimotor experience. But what kinds of sensorimotor experiences are available to infant animals? Noting that active (or REM) sleep is the predominant behavioral state of early infancy (Blumberg et al., 2013), we proposed that the abundant self-generated limb twitches that characterize that state are uniquely suited to building internal models (Mukherjee et al., 2018; Dooley et al., 2021).

In early development, limb movements are produced by brainstem motor structures, including the red nucleus, that project to motor neurons in the spinal cord. In addition, these brainstem motor structures project to precerebellar nuclei, including the inferior olive (IO) and lateral reticular nucleus (LRN; Mukherjee et al., 2018; Alstermark and Ekerot, 2015). As shown in postnatal day (P) 8 rats, neurons in the IO and LRN increase their activity contemporaneously with the production of twitches, rather than preceding (i.e., motor) or following (i.e., sensory) them (Mukherjee et al., 2018). This observation, combined with the fact that the IO and LRN do not directly produce movement (Poulet and Hedwig, 2007; Sommer and Wurtz, 2002; Sommer and Wurtz, 2008), indicates that these structures process corollary discharges before conveying them to the cerebellar cortex and interpositus nucleus (IP) (Sokoloff et al., 2015a; Sokoloff et al., 2015b; Del Rio-Bermudez et al., 2016). However, in none of these earlier studies did we directly compare cerebellar activity between twitches and manual stimulations; such comparisons are needed to assess the functional significance of corollary discharge for the developing cerebellum before the onset of a functionally precise internal model.

Therefore, here we recorded extracellular activity in the IP of P12 rats, an age when IP exhibits robust responses to twitches (Del Rio-Bermudez, 2016), to assess its ability to distinguish input associated with twitches and stimulations. Because rats at similar ages also exhibit robust responses to stimulation from the medulla to the cerebral cortex (Gómez et al., 2023; Gómez et al., 2021; Tiriac and Blumberg, 2016; Del Rio-Bermudez et al., 2015), we expected to see similar responses in IP. Contrary to expectation, however, exafferent responses in IP were weak. Next, we discovered serendipitously that when pups were anesthetized with urethane, robust and reliable exafferent responses emerged. Based on this result, we hypothesized that urethane exerted its effect by disinhibiting the cerebellar cortex. When we tested this hypothesis by ablating the cerebellar cortex, we found that stimulations evoked strong IP responses and that the responses to twitches were unaffected. Finally, in a separate cohort of P12 rats, we recorded from units in cerebellar cortex and found that their twitch-related activity profiles matched those of IP units, thus suggesting that the two structures receive twitch-related input simultaneously and in parallel.

Altogether, these findings suggest a model of cerebellar function at this age in which input arising from self-generated movements is prioritized over that from other-generated movements. By providing opportunities for the infant cerebellum to receive converging input from corollary discharge and reafference, such prioritization may be necessary for the development of somatotopically organized closed-loop circuits (Najac and Raman, 2017).

MATERIALS AND METHODS

All experiments were conducted in accordance with the National Institutes of Health 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.

Subjects

Male and female Sprague-Dawley rats (48 total, 25 female) aged P12-P13 (hereafter P12;

body weight: 31.34 ± 3.6 g) were used. Pups were born to dams housed in standard laboratory cages (48 × 20 × 26 cm) with a 12-hr light/dark cycle. Food and water were available ad libitum. The day of birth was considered P0 and litters were culled to eight pups by P3. Pups were randomly assigned to different experimental groups and, to protect against litter effects, pups selected from the same litter were never assigned to the same experimental group (Abbey and Howard, 1973; Lazic and Essioux, 2013).

Surgery

Surgery was performed using established methods (Blumberg et al., 2015; Glanz et al., 2021). Briefly, on the day of testing a pup with a healthy body weight and a visible milk band was removed from the litter. Under isoflurane anesthesia (3.5–5%, Phoenix Pharmaceuticals, Burlingame, CA), bipolar electrodes (California Fine Wire, Grover Beach, CA) were inserted bilaterally into the nuchal muscle and secured with collodion (Avantor Performance Materials, LLC; Rachor, PA). For those pups receiving subcutaneous infusions of urethane (n = 8; 5 female) or saline (n = 8; 4 female), a small incision (~1 cm) was made in the skin near the base of the tail and surgical-grade silicon tubing (inner diameter: 0.020 in; outer diameter: 0.037 in; SAI Infusion Technologies, Lake Villa, IL) was inserted subcutaneously and secured with veterinary adhesive (Vetbond, 3M, St. Paul, MN).

In all surgeries, an anti-inflammatory agent (Carprofen, 0.1 mg/kg SC; Putney, Portland, ME) was administered and the torso of the pup was wrapped in soft surgical tape (Micropore, 3M). The scalp was sterilized with iodine and isopropyl alcohol, and a portion of the scalp was removed to reveal the skull; a topical analgesic (bupivacaine, 0.25%; Pfizer, New York, NY) was applied to the skull surface and surrounding skin, and Vetbond was used to secure the skin to the skull. A stainless-steel head-fix (Neurotar, Helsinki, Finland) was attached to the skull using cyanoacrylate adhesive (Loctite, Henkel Corporation, Westlake, OH) that was dried with accelerant (Insta-Set, Bob Smith Industries, Atascadero, CA).

Next, while still under anesthesia, the pup was then secured in a stereotaxic apparatus

(Kopf Instruments, Tujunga, CA) and a steel trephine (1.8 mm; Fine Science Tools, Foster City, CA) was used to drill openings in the skull. For interpositus recordings, a hole was drilled over right side of the cerebellum to record from the anterior interpositus nucleus (IP; coordinates from lambda: -1.8 mm AP; +2.0 mm ML). For those pups with cerebellar cortical ablations (n = 8; 4 female), the right portion of the interparietal bone was trephined to expose and disconnect the right cerebellar cortex from the deep cerebellar nuclei. This procedure entailed either ablation of cortical tissue using a surgical hook and forceps or disconnection (without tissue removal) using a 27G needle (BD Precision Glide; Franklin Lakes, NJ), bent at a 90-degree angle. For sham pups (n = 8; 3 female), only the interparietal bone was removed. Finally, for recordings in cerebellar cortex (12 pups; 7 female), the procedure was similar to that described above, but the electrode was placed in the cortex immediately dorsal to the IP (right crus I/II lobe coordinates from lambda: 2.3 +/- 0.3 mm AP; 2.2 +/- 0.1 mm ML).

Throughout surgery, the pup's respiration was monitored and the concentration of isoflurane was adjusted accordingly. All surgical procedures lasted 16-24 min. After surgery was complete, the pup was transported to the recording rig, where it was head-fixed in a warm environment for at least 1 h. Recording began only after regular sleep–wake cycles were observed and intracranial temperature was at least 36°C.

Data Acquisition

To record neural activity, a 16-site linear silicon electrode (100 μm between sites; NeuroNexus, Ann Arbor, MI) was lowered into IP (-3.3 +/- 0.3 mm DV) or cerebellar cortex (-2.5 +/- 0.5 mm). Before insertion, the electrode was coated with fluorescent DiI (Life Technologies, Carlsbad, CA) to enable histological confirmation of electrode location. Neurophysiological recordings were accomplished using a data acquisition system (Tucker-Davis Technologies, Alachua, FL). EMG and neural activity were acquired at 1 kHz and 25 kHz, respectively. Video was acquired at 100 frames/s using a BlackFly-S camera (FLIR Integrated Systems, Wilsonville, OR) and recorded using SpinView software (FLIR). Video and neural data were synchronized using a custom MATLAB script as described previously (Dooley et al., 2021).

IP activity preceding and following urethane injection. Neurophysiological, EMG, and video data were recorded for 60 min as the pup cycled freely between sleep and wake. After 60 min, ~50 manual stimulations of the right forelimb. were delivered using a cotton-tipped wooden dowel (Gómez et al., 2021). Stimulations were delivered at random intervals between 5 and 15 s apart. Stimulations were ipsilateral to the recording site and any stimulation that evoked a movement response was excluded from analysis. After the stimulations were delivered, urethane (1.0 mg/g b.w.; Sigma-Aldrich, St. Louis, MO) or an equivalent volume of sterile saline (1.0 ml/g b.w.; Hospira Inc, Lake Forest, IL) was administered via the subcutaneous cannula. After 15 min, another set of ~50 stimulations of the right forelimb were delivered as described above.

IP activity following cerebellar cortical ablation. Data were collected, and stimulations were delivered as described above. Following stimulations, recordings continued for an additional 45 min to collect data as pups cycled freely between sleep and wake.

Cerebellar cortical activity. In 16 pups (9 female; 4 pups excluded from analysis due to loss of units due to drift or lack of forelimb responsivity to stimulations), data were collected, and stimulations were delivered as described above.

Histology

At the end of the recording period, the pup was euthanized with 0.05 ml/kg of ketamine-xylazine (90:10; 0.002 ml/g b.w. IP Akorn, Inc.; Lake Forest, IL) and perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). The brain was extracted from the skull and placed in PFA for at least 24 h, after which it was transferred to phosphate-buffered sucrose for at least 48 h before sectioning. The cerebellum was sectioned coronally at 80 μm. Electrode locations were determined using fluorescent microscopy before Nissl staining.

Data Analysis

Spike sorting. Neurophysiological, EMG, and behavioral data were imported to MATLAB

and analyzed as described previously (Gómez et al., 2023). Briefly, using custom scripts, raw neurophysiological data were filtered for unit activity (band-pass: 500-5,000 Hz) and extracted using Kilosort (Pachitariu et al., 2016); subsequent unit templates were visualized in Phy2 (Rossant & Harris, 2022). Units in cerebellar cortex were analyzed using template matching in Spike2 (cerebellar cortex; Sokoloff et al., 2015a). In IP, only data from single units were analyzed; in cerebellar cortex, both single units and multi-units were analyzed.

Video analysis of movement and behavioral-state determination. Synchronized electrophysiological and behavioral data were imported into Spike2 (Cambridge Electronic Design, Cambridge, UK). Based on EMG activity and behavior (from video), Spike2 was used to define behavioral states, as described previously (Del Rio-Bermudez et al., 2016). Periods of high muscle tone and atonia were identified when nuchal EMG was above or below, respectively, an average threshold of 3x baseline (Gómez et al., 2023). Movements of the limb were detected using a custom MATLAB script that identified movement based on frame-by-frame changes in pixel intensity within regions-of-interest (ROIs; Dooley et al., 2020). Using an ROI encompassing the right forelimb, the number of pixels with >5% changes in pixel intensity were summed frame by frame, resulting in a trace of real-time movement. Using a threshold for displacement of the right forelimb, events were triggered at movement onset, and subsequently confirmed visually by the experimenter using video. Finally, the onset of a limb stimulation was scored manually based on the frame immediately preceding visual displacement of the limb. Manual stimulations that evoked sudden increases in nuchal EMG and large movements of multiple limbs were not included in the analysis.

Active wake was defined as a period of high muscle tone when the pup was also moving its limbs in a coordinated fashion. Active sleep was defined as a period of muscle atonia punctuated by sharp spikes in the EMG record and rapid changes in pixel intensity, indicative of myoclonic twitching. The remaining periods were defined as behavioral quiescence and were not analyzed further.

Determination of responsive and nonresponsive units. We characterized units in IP and cerebellar cortex either responsive or nonresponsive to twitch-related reafference or stimulation-related exafference. First, baseline firing rate was calculated for each unit based on its activity 250-500 ms before movement onset. Second, a unit was considered responsive to a twitch or stimulus if peak mean firing rate for the unit within 250 ms of movement onset exceeded 3.5 standard deviations above baseline firing rate.

Width at half-height. To determine width at half-height, neural data was normalized to baseline activity, as described above. Data were then smoothed using a 5-bin kernel before being interpolated from 10-ms bins to 1-ms bins (using the MATLAB interp1() function). The baseline corrected neural data were normalized such at the baseline was set to 0 and the peak 1. The width at half-height was measured as the duration (in ms) at the point halfway between baseline (0) and peak (1).

Statistical Analysis

To compare the proportion of responsive units before and after the injection of urethane or saline in the IP, a within-subjects a repeated-measures analysis of variance (ANOVA) was conducted. A Shapiro-Wilk test for normality and a Levene's test for homogeneity were performed on all comparisons before analysis—in the event a comparison failed either of these tests, the appropriate alternative test was run (i.e. Welch's in the event of Levene's test reaching significance). To compare the proportion of responsive units following cerebellar cortical ablation, a between-subjects one-way ANOVA was performed between twitch responsive units in the. Post-hoc analyses were performed when appropriate. All statistical analysis of the proportion of responsive and nonresponsive neurons was performed using an arc-sin transformation. A chi-squared test was used to determine if the proportion of neurons that responded to twitches in unanesthetized animals were the same as those that responded to exafference following urethane administration. Between-subject t tests were performed to compare average width-at-half-height, and peak response time, and amplitude between stimulus responsive units. Bonferroni corrections were implemented where appropriate.

All statistical tests were performed using SPSS (IBM; Chicago, IL). Unless otherwise noted, means are presented with standard errors and alpha was set at 0.05. Effect size was calculated using an adjusted partial eta-squared for ANOVAs (adj η_p^2) and adjusted eta-squared for t tests (adj η^2), adjusted against positive bias (Mordkoff, 2019). When appropriate, a Bonferroni correction was used to correct alpha for multiple comparisons.

RESULTS

To distinguish differences in IP responses to reafference and exafference, we recorded extracellular unit activity in head-fixed P12 rats (**Figure 1A**). For each pup, we recorded 60 min of spontaneous sleep-wake cycling, followed by ~50 manual stimulations of the forelimb ipsilateral to the recording site (36-48 stimulations/pup met criteria; **Figure 1B**). Next, urethane or saline was administered and, after 20 min, the forelimb was stimulated again (40-50 stimulations/pup met criteria). Electrode location in IP was confirmed histologically (**Figure 1C**). In pups injected with urethane, we recorded 78 units (n = 8 pups; 8-17 units/pup); in pups injected with saline, we recorded 86 units (n = 8 pups; 7-22 units/pup).

A representative recording of IP unit activity, right forelimb movements, scored twitches, and nuchal EMG during active sleep and wake is shown in **Figure 1D**. The recording illustrates four forelimb twitches and the IP activity surrounding those events. Two additional representative recordings during the stimulation period are shown for pups after administration with saline or urethane (**Figure 1E**). Three forelimb stimulations are shown for each condition; for the urethanized pup, the increase in IP activity after each stimulation is readily observed. Twitches and stimulations were used as triggers for perievent histograms of unit firing rate that, in turn, were used to assess whether a unit was responsive or nonresponsive (**Figure 1F**; see Methods).

IP units respond to reafference, but not exafference

As found previously (Del Rio-Bermudez et al., 2016), we observed robust and reliable IP responses to twitches in pups before the administration of saline or urethane, but relatively weak and unreliable IP responses to stimulations (**Figure 2A-B**). In fact, the

proportion of responsive units for twitches was approximately 3x that for stimulations (70-76% vs. 25-22%; *p*s < .001, binomial test).

IP units respond to exafference after urethane anesthesia

After recording IP responses to reafference and exafference, pups received either saline or urethane before the stimulation protocol was repeated (note that urethane suppresses twitches). There was no difference in the proportion of stimulation-responsive units before and after administration of saline (**Figure 2A**). In contrast, urethane administration significantly increased the proportion of stimulation-responsive units (20% vs. 61%; $p < .0001$, binomial test; **Figure 2B**). Repeated-measures ANOVA revealed a significant main effect of group (urethane vs. saline; $F_{(1,14)} = 15.93$, $p = 0.001$, adj $\eta_p^2 = 0.499$) but not time (pre vs. post; $F_{(1,14)} = 4.41$), as well as a significant group x time interaction ($F_{(1,14)} = 6.53$, $p = 0.023$, adj $\eta_p^2 = 0.269$; **Figure 2C**). A post hoc test confirmed that the proportion of responsive IP units was significantly higher in the Post-Urethane group ($F_{(1,14)} = 43.36$, $p < 0.001$, adj $\eta_p^2 = 0.738$).

The afferent pathways for twitches and stimulations are expected to be the same. Accordingly, we expected to see an association between those IP units that were responsive to twitches in the Pre-Urethane group and those units that were responsive to stimulations in the Post-Urethane group. Indeed, there was a significant association, with units exhibiting dual responsivity to twitches and stimulations being more prevalent than all other units combined ($X^2 = 35.03$, $p < 0.001$; **Figure 2D**).

Reafferent and exafference IP responses exhibit different response profiles

Why did reafference from twitches trigger reliable IP responses but exafference from stimulations did not? The most likely explanation relates to the fact that twitches, as self-generated movements, are accompanied by corollary discharges (Mukherjee et al., 2018). Accordingly, we sought to determine whether corollary discharge is an observable component of the twitch-related profile of IP units. To do this, we compared twitch-related unit responses (in the Pre-Urethane group) and stimulation-related unit responses (in the Post-Urethane group; **Figure 3A-B**); the latter presumably comprise only a sensory

response. In this comparison of response profiles, we focused on the width at half-height (a measure of the duration of the response profile) and the timing of the response peak (which provides insight into the whether the peak reflects sensory feedback, corollary discharge, or a combination of the two).

Compared with the IP unit response profiles for stimulations, the twitch-related profiles exhibit several distinct features: They are typically initiated earlier, have significantly longer-duration widths at half-height ($t_{(14)}$ = 2.49, p = 0.031, adj η^2 = 0.303; **Figure 3C**), and have a significantly earlier peak response ($t_{(14)}$ = 7.52, p < 0.001, adj η^2 = 0.900; **Figure 3D**). Together, these findings suggest that the combined inputs of corollary discharge and reafference are detectable in the response profiles of IP units at this age.

Ablation of cerebellar cortex reveals exafference in IP

Corollary discharge conveyed to the cerebellum may explain the presence of sensory responses to twitches and their absence to stimulations. One possibility is that corollary discharge directly activates cerebellar cortex to release cortical inhibition of reafference in IP (Davie et al., 2008). A second possibility is that corollary discharge acts on local inhibitory circuits within IP (Kebschull et al., 2020; Brinke et al., 2017), to diminish the inhibitory influence of cortex on reafference. Both possibilities hinge on the idea that cortical inhibition modulates IP at this age.

To test this idea directly, we ablated cerebellar cortex in P12 rats and subsequently recorded ipsilateral IP activity in relation to twitches and stimulations (**Figures 4A-B**). We recorded from 65 units in pups with cortical ablations (n = 8 pups; 6-11 units/pup) and 66 units in pups with sham ablations (n = 8 pups; 6-14 units/pup; **Figure 4C-D**). Whereas cortical ablation had no significant effect on the proportion of twitch-responsive units ($t_{(14)}$ = 1.29; **Figure 4E**), it significantly increased the proportion of stimulation-responsive units (from 25% to 80% of units; $t_{(14)}$ = 25.74, p < 0.001, adj η^2 = 0.623; **Figure 4F**). This substantial increase in exafferent responsivity after cortical ablation supports the notion that cortical inhibition of IP suppresses exafference.

Ablation of cerebellar cortex does not affect reafference

The fact that cortical ablation did not affect the ability of IP units to exhibit twitch-related responses indicates that the cortex is not necessary for those responses. However, it is possible that the cortex asserts more subtle effects on IP responding. We assessed this possibility by comparing the response profiles of IP units during twitching in pups with cortical ablations and sham ablations. Cortical ablation did not significantly affect width at half-height ($t_{(14)} = 0.10$; **Figure 5B**) or time of response peak (Welch's $t_{(9.28)} = 5.86$; **Figure 5C**). We did observe an increase in peak amplitude after cortical ablation (**Figure 5D**), but this effect was not significant (Welch's $t_{(1,8.55)} = 0.81$; **Figure 5E**). Cortical ablation did produce significant increases in the variance of the time of peak response (Levene's $F_{(1,14)} = 9.49$, $p = 0.008$, adj $\eta_p^2 = 0.361$) and peak amplitude (Levene's $F_{(1,14)} = 6.17$, $p = 0.026$, adj $\eta_p^2 = 0.256$). These last results suggest that the cerebellar cortex exerts a tonic modulatory influence on IP activity, even if it does not mediate the effect of corollary discharge on IP responsivity.

Parallel reafferent input to IP and cerebellar cortex

Having demonstrated that IP does not depend on cerebellar cortex for its reafferent response, the question arises as to whether cerebellar cortex receives twitch-related input before, after, or at the same time as IP. We addressed this question by recording activity in cerebellar cortex in a separate cohort of P12 rats (n = 66 twitch-responsive units from 12 pups; 2-10 units/pup; **Figure 6A**). We compared the response profiles of these twitch-responsive units to those of the 94 twitch-responsive units in IP (combined across the Pre-Saline and Pre-Urethane groups; n = 94 units from 16 pups; 7-22 units/pup; **Figure 6B**). There was no significant difference in either the width at half-height or the peak response time ($t_{(26)}$s = 0.55-2.07; **Figure 6C-D**). Overlaying the response profiles of twitch-responsive cortical and IP units highlights their similar onset and offset times (**Figure 6E**). These results suggest that the two structures receive parallel twitch-related input from precerebellar structures at this age.

DISCUSSION

The infant IP appears to be unique among the sensorimotor structures examined thus far in that it does not readily respond to exafferent input (Gómez et al., 2023; Gómez et al., 2021; Tiriac and Blumberg, 2016; Del Rio-Bermudez et al., 2015). Here we sought to understand the mechanisms responsible for this lack of response. Through serendipity, we discovered that urethane anesthesia permits the expression of exafference in IP. When we next discovered that cortical ablation mimics the effect of urethane, we concluded that the cortex tonically inhibits IP to prevent the expression of exafference (**Figure 7A**). But such an explanation does not address why twitches, unlike stimulations, exert such a powerful activational effect on IP.

How are twitches prioritized?

To explain the ability of twitches to preferentially activate the infant IP, we turn to the feature that distinguishes them from stimulations: the production of corollary discharge. After corollary discharge is received by the IO, it is conveyed to the cerebellum via climbing fibers (Mukherjee et al., 2018; **Figure 7B**; LRN also conveys corollary discharge, but via mossy fibers). In contrast, reafference from twitches and exafference from stimulations are conveyed to the cerebellum via mossy fibers arising from other precerebellar nuclei (e.g., LRN, external cuneate nucleus; Alstermark and Ekerot, 2013; Puro and Woodward, 1977). Critically, climbing fibers provide more powerful cerebellar input than mossy fibers at this age (Najac & Raman 2017). Accordingly, we propose that the early component of the twitch-related activation of IP—that is, the component associated with corollary discharge—reflects input from climbing fibers.

But what about the reafferent component of twitch-related IP activity? Based on what we know about the adult cerebellum, we had initially hypothesized that corollary discharge from IO activates complex spikes in Purkinje cells, thereby disinhibiting reafference in IP (Davie et al., 2008; Sato et al., 1993; Armstrong and Rawson, 1979). (It follows that the lack of corollary discharge during stimulations prevents exafference from activating IP.) However, this hypothesis is difficult to reconcile with the observation that twitch-related response profiles in IP—particularly their temporal features—were not affected by ablation of the cerebellar cortex: If the cortex effectively modulates reafference in IP, we

should have observed some evidence of that modulation in the pups with cortical ablations.

The notion that the reafferent component of twitch-related IP activity is gated by the cerebellar cortex has another problem: If corollary discharge causes cerebellar cortex to disinhibit IP activity to allow for the expression of reafference, then cortical activity should decrease as IP activity increases in the period immediately after a twitch. Instead, cortical and IP activity exhibited nearly identical twitch-related increases in activity (**Figure 6E**). This observation suggests that the two structures receive twitch-related input in parallel and simultaneously, inconsistent with one influencing the other.

The expression of reafference in IP thus requires that the activation can bypass tonic cortical inhibition. To achieve this, one possibility is that corollary discharge from the IO acts within local inihbitiory IP circuits to enable its responsivity to reafference (Kebschull et al., 2020; Brinke et al., 2017). Testing this and other possibilities remains for future investigation.

Why are twitches prioritized?

We are not the first investigators to show that IP receives w eak exafference in early development (Freeman and Muckler, 2003; Campolattaro and Freeman, 2008): In P20 but not P12 rats, IP supports classical eye-blink conditioning in which a tone is used as the conditioned stimulus (Campolattaro and Freeman, 2008; Stanton et al., 1998). However, IP can mediate learning at P12 when the tone is replaced by direct electrical stimulation of the precerebellar nucleus that carries tone information to IP—a manipulation that apparently works by overcoming the system's weak exafference. The investigators concluded that "cerebellar neurons in developing animals are therefore ready to learn before they have something to learn about" (Campolattaro and Freeman, 2008). However, the abundance of twitch-related corollary discharge and reafference processed by IP at P12 suggests that it has something to learn about after all; namely, the somatotopic relations between motor commands and their sensory consequences.

The projections from IO to IP appear to play a critical role in the development of cerebellar circuitry (Lang et al., 2017; Najac and Raman, 2017). These projections, which initially

arise from exuberant climbing-fiber collaterals, have a strong excitatory influence on IP that weakens with age (Najac and Raman, 2017; Lu et al., 2016). Consequently, climbing fibers drive much of IP's activity at a time when the cerebellar system has limited functional capacity and is still developing its basic functional organization. The current findings provide additional support for the notion that twitch-related corollary discharge and reafference are better-suited than exafference to building the cerebellar system (Mukherjee et al., 2018; Dooley et al., 2021; Sokoloff et al., 2015a).

Based on the present results, we propose that the corollary discharge signals conveyed by climbing fibers are sufficiently strong to overcome cortical inhibition and activate IP in ways that exafference alone cannot. Moreover, because climbing fibers convey input in parallel to the cerebellar cortex (**Figure 6E**), and because twitch-related reafference arrives contemporaneously from the sensory periphery via mossy fibers, a competitive context is established that involves coincident activity of somatotopically related neurons (**Figure 8**). In this way, functional closed-loop circuits can be built within cerebellar microzones (Sugihara, 2006; Ruigrok and Voogd, 2000).

BCE: Before cerebellar error

The present results do not align with what is known about the adult cerebellar system. This lack of alignment is particularly clear when we consider the IO: In adults, IP inhibits IO activity during the processing of expected stimuli (Hesslow & Ivarsson, 1996). Such inhibition is not likely at P12 when the inhibitory connection from IP to IO is underdeveloped (Nicholson and Freeman, 2003), and the cerebellum lacks the computational capacity to distinguish expected from unexpected stimuli (Dooley et al., 2021).

Thus, at an age when the cerebellar system is still acquiring very basic information about what to expect from self-generated movements, it is not yet ready to detect error. In adults, error signals—also called "teaching signals"—represent violations of expectation that are conveyed by the IO to the cerebellar cortex (Medina et al., 2002; Devor, 2002; Ito, 1984). Whereas the adult IO responds to unexpected exafference (Wu et al., 2010; Rushmer et al., 1976; Gellman et al., 1985), the infant IO does not (Mukherjee et al., 2018). Also, unlike the adult IO, the infant IO responds predominantly or exclusively to

corollary discharge. These substantial age-related differences highlight the need to understand the infant's cerebellar system within its appropriate developmental context. Just as the infant "motor cortex" functions like somatosensory cortex (Dooley et al., 2018), we should not expect the infant's cerebellar system to be a miniature version of the adult's.

Conclusions and Future Directions

Previous research from our lab documented twitch- and stimulation-related activity in precerebellar nuclei before P12 (Mukherjee et al., 2018; Tiriac and Blumberg, 2016), and the expression of a cerebellar-dependent internal model of movement in motor thalamus by P20 (Dooley et al., 2021). However, in our studies of neural activity within the cerebellum itself (Sokoloff et al., 2015b; Del Rio-Bermudez et al., 2016), we documented twitch-related activity but did not examine cerebellar responses to stimulation.

Thus, there was a gap in our understanding of how twitches—with their associated corollary discharge and reafference—might uniquely influence cerebellar development. The current study fills that gap by showing that at P12, neurons in IP exhibit robust and reliable responses to corollary discharge and reafference, but not exafference, indicating a prioritization of twitch-related input. This prioritization, coupled with the in-parallel activation of IP and cerebellar cortex (thus providing the opportunity for coincident activity in functionally related neurons), suggests a functional role for twitching in the development of somatotopically organized closed-loop circuits (Najac and Raman, 2017). Such circuits are undoubtedly necessary for the capacity to compute precise internal models.

Understanding the prioritization of reafference over exafference in the infant IP opens a host of new questions. For example, through what mechanism does twitch-related corollary discharge influence local processing within IP to enable the expression of reafference? Given that corollary discharge is conveyed to IP via both the IO and LRN (Mukherjee et al., 2018), is one more important than the other? In addition, understanding the functional development of the IO is critical to understanding how cerebellar internal models develop. For example, why does the adult IO, unlike the infant IO, readily respond to exafference? One possibility is that the development of inhibitory feedback from IP,

beginning around P17, alters the IO's responsivity to exafference (Nicholson and Freeman, 2003). Finally, questions remain concerning the necessity and sufficiency of twitches—as opposed to wake movements—for providing the corollary discharges that are needed to build internal models. Ultimately, answering these and other questions will help to explain why activity in the developing cerebellar system is so profoundly modulated by movements that occur specifically during sleep (Dooley et al., 2021).

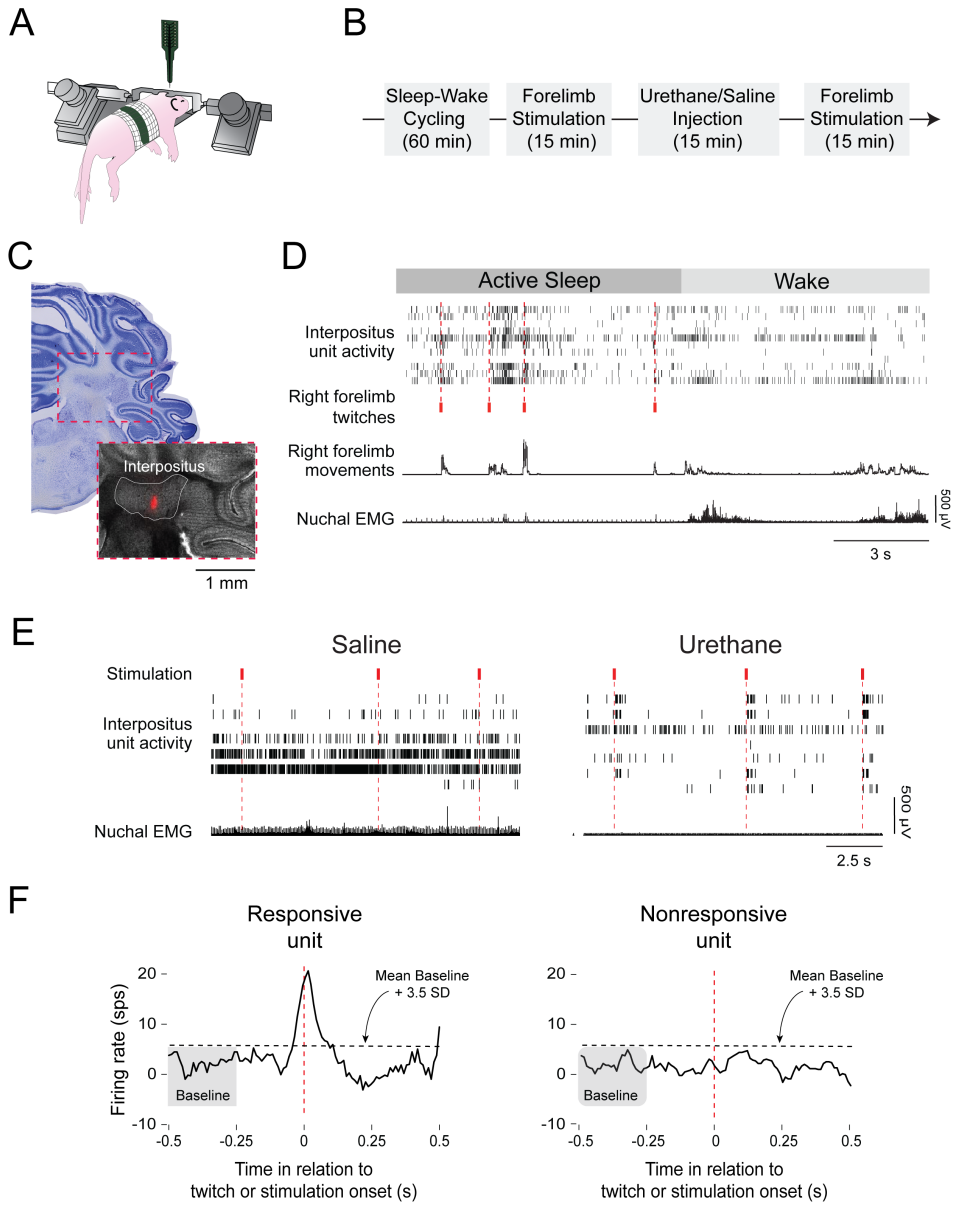

Figure 1. Experimental design and representative data. **A**, Illustration showing the head-fixed pup in the recording apparatus. **B**, Timeline of the experimental procedure. ***C***, Representative histology showing the red-florescent electrode track in interpositus (inset). ***D***, Representative data from recording in a P12 rat. From top: Behavioral states marked as active sleep (dark gray) or wake (light gray), black ticks showing interpositus spiking activity for individual units in each row, red ticks showing identified twitches of the right forelimb, movements of the right forelimb derived from video, and nuchal EMG activity. ***E***, Representative data from pups after saline (left) or urethane (right) injection. From top: Red ticks denoting the onset of a forelimb stimulations, black ticks showing interpositus spiking activity for individual units in each row, and nuchal EMG activity. ***F***, Representative units illustrating method for identifying units that were responsive (left) and nonresponsive (right) to twitches or stimulations. See Methods for additional detail.

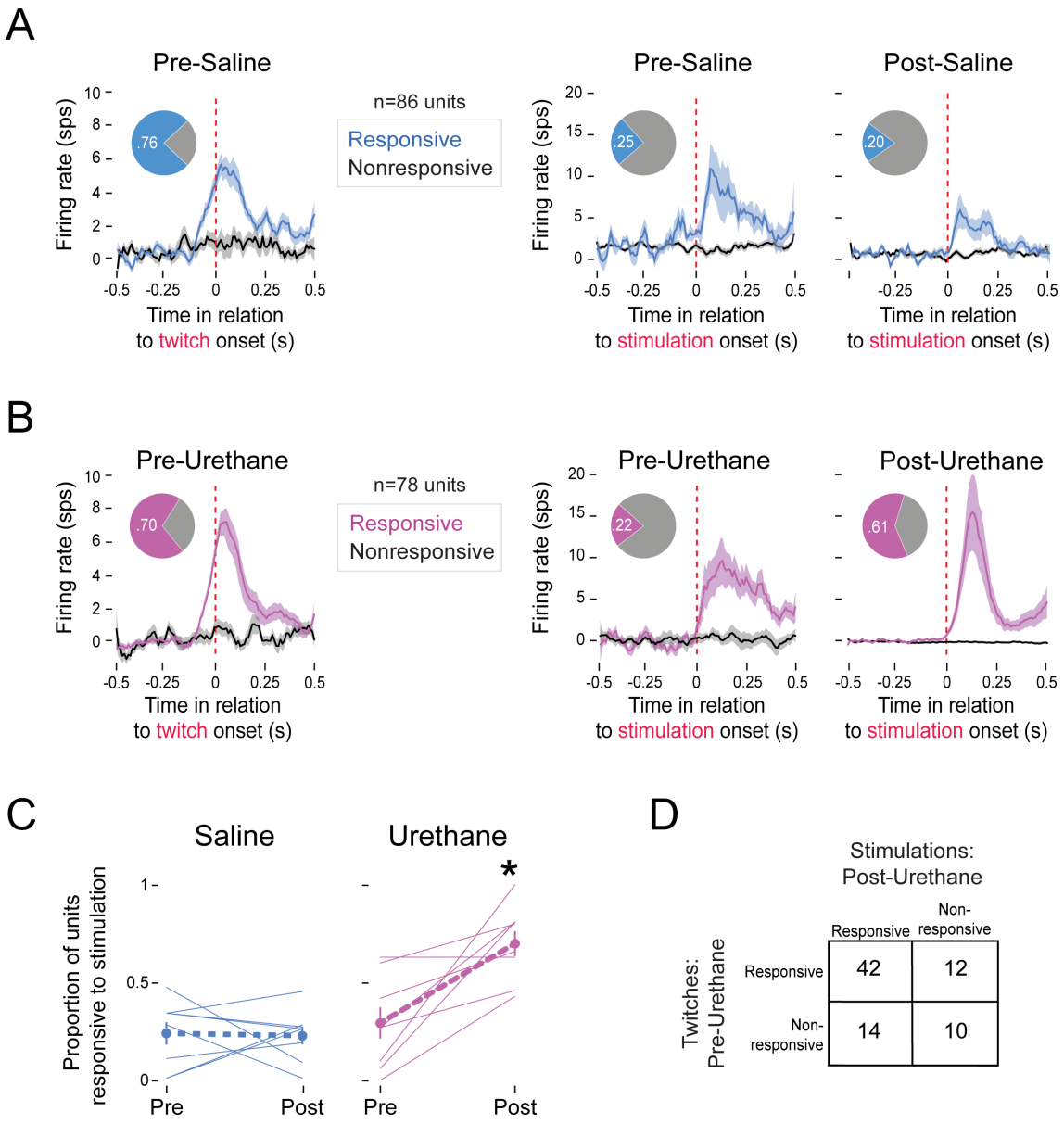

Figure 2. Responsiveness of interpositus units to twitches and stimulations before and after urethane or saline injection. ***A***, Perievent time histogram of unit firing rate for responsive (blue) and nonresponsive (black) units in the interpositus in relation to the onset of forelimb movements associated with twitches or stimulations (red dotted lines) before and after saline injection. Pie charts represent proportion of responsive and nonresponsive units. ***B***, Same as in ***A*** but for pups before and after urethane injections (responsive units: magenta). ***C***, Proportion of neurons responsive to stimulation before and after saline (left) or urethane (right) injection. Asterisk denotes significant difference in relation to the post-urethane pups. ***D***, Latin square showing the association between IP units that were or were not responsive to twitches (pre-urethane) and stimulations (post-urethane). Statistical analysis revealed that units that responded to twitches were also significantly more likely to respond to stimulations.

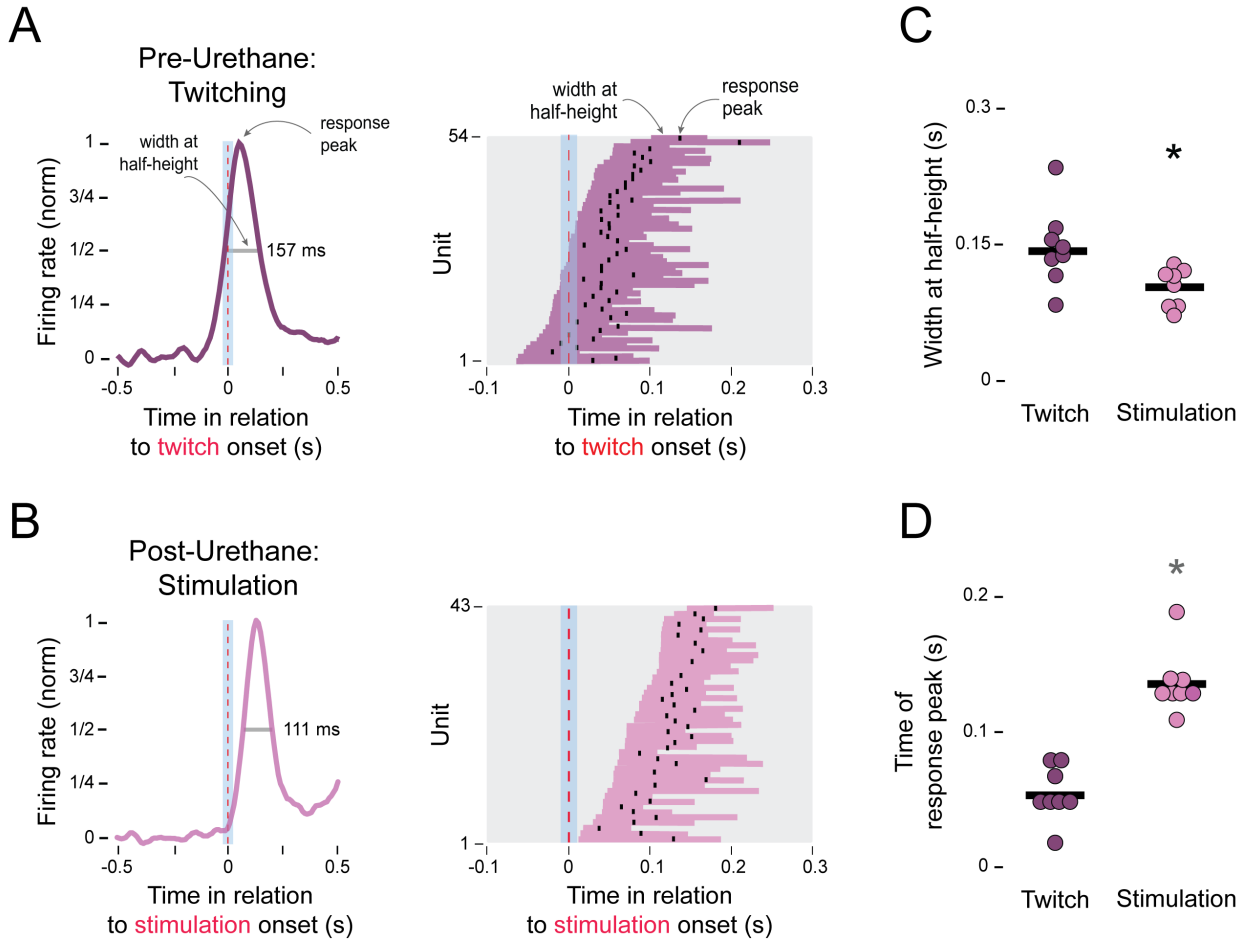

Figure 3. Interpositus response profiles for twitches and stimulations. ***A***, Left: Representative perievent time histogram for a twitch-responsive unit (pre-urethane). Right: Representations of the range of width at half-height for all individual twitch-responsive units. Response peaks are also shown as black ticks. ***B***, Same as in ***A*** but for stimulation-responsive units (post-urethane). ***C***, Mean width-at-half-height for twitch- and stimulation-responsive units. n = 8 pups/group. Asterisk denotes significant difference between groups. ***D***, Same as in ***C*** but for mean time of response peak.

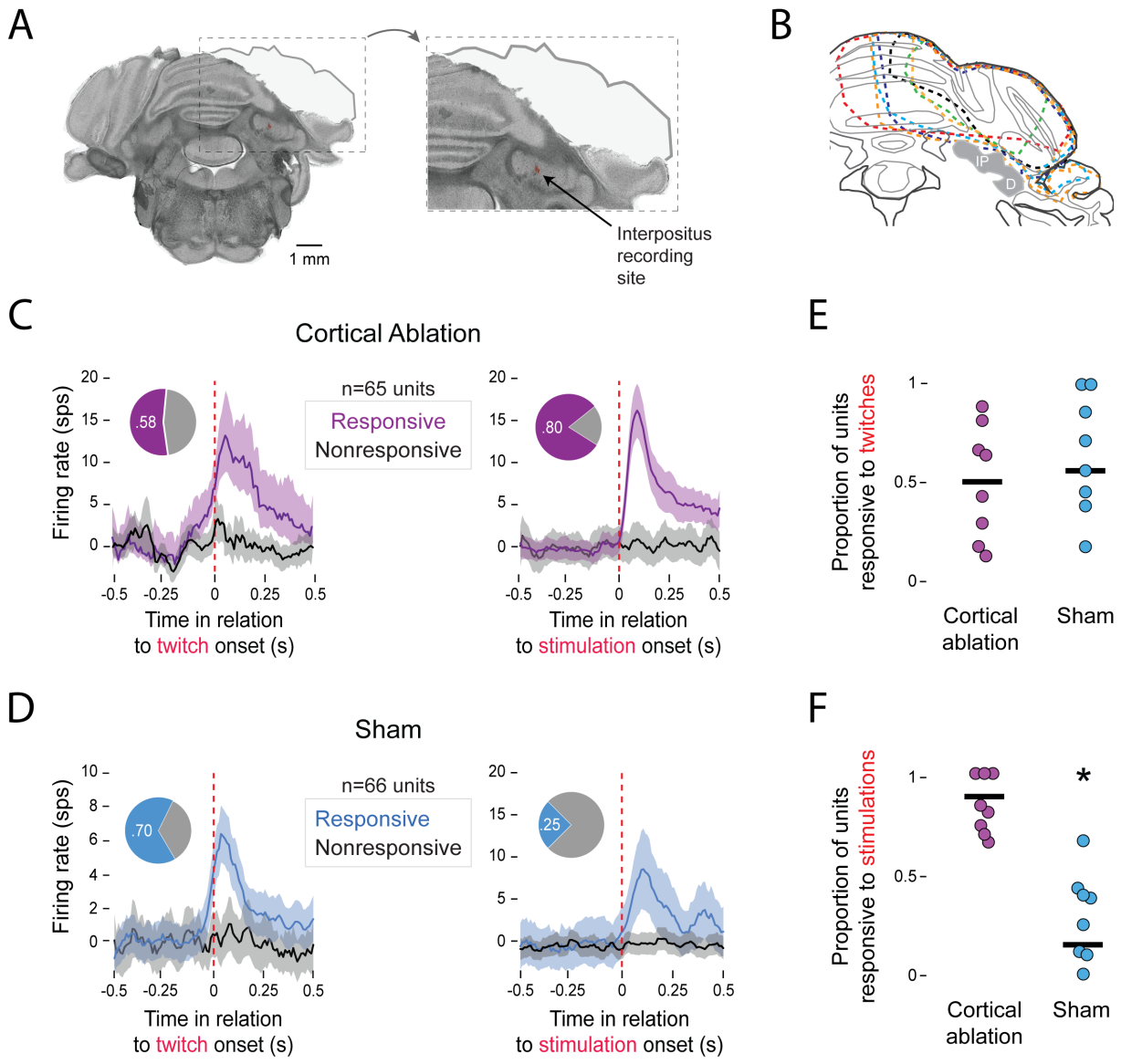

Figure 4. Effect of cortical ablation on responsiveness of interpositus units to twitches and stimulations. ***A***, Representative coronal section showing unilateral ablation of cerebellar cortex. Inset shows location of electrode in interpositus (red-fluorescent dot). ***B***, Extent of cerebellar cortical ablations in individual pups denoted by dotted lines of different colors. IP, interpositus nucleus; D, dentate nucleus. ***C***, Perievent time histogram of unit firing rate for responsive (violet) and nonresponsive (black) units in the interpositus in relation to the onset of forelimb movements associated with twitches or stimulations (red dotted lines) in pups with cortical ablations. Pie charts represent proportion of responsive and nonresponsive units. ***D***, Same as ***C*** but for pups with sham ablations. ***E***, Mean proportion of twitch-responsive units for pups with cortical ablations and sham ablations. n = 8 pup/group. ***F***, Same as ***E*** but for stimulation-responsive units. Asterisk denotes significant difference between groups.

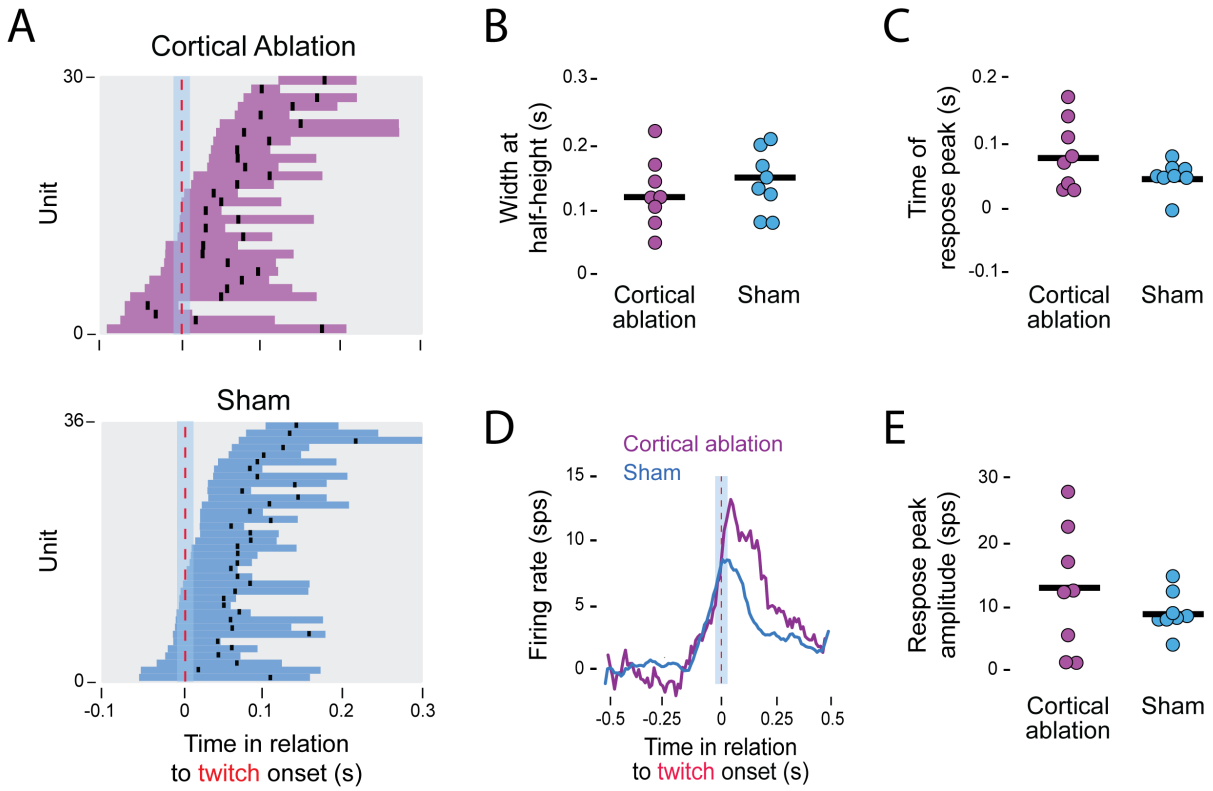

Figure 5. Effect of cortical ablation on interpositus response profiles for twitches. ***A***, Representations of the range of width at half-height for all individual twitch-responsive units for pups with cortical ablations (top) and sham ablations (bottom). Response peaks are also shown as black ticks. ***B***, Mean width-at-half-height for twitch-responsive units in pups with cortical ablations and sham ablations. n = 8 pups/group. ***C***, Same as in ***B*** but for mean time of response peak. ***D***, Perievent time histogram of mean unit firing rate for cortical ablation and sham units. ***E***, Same as in ***B*** but for mean response-peak amplitude.

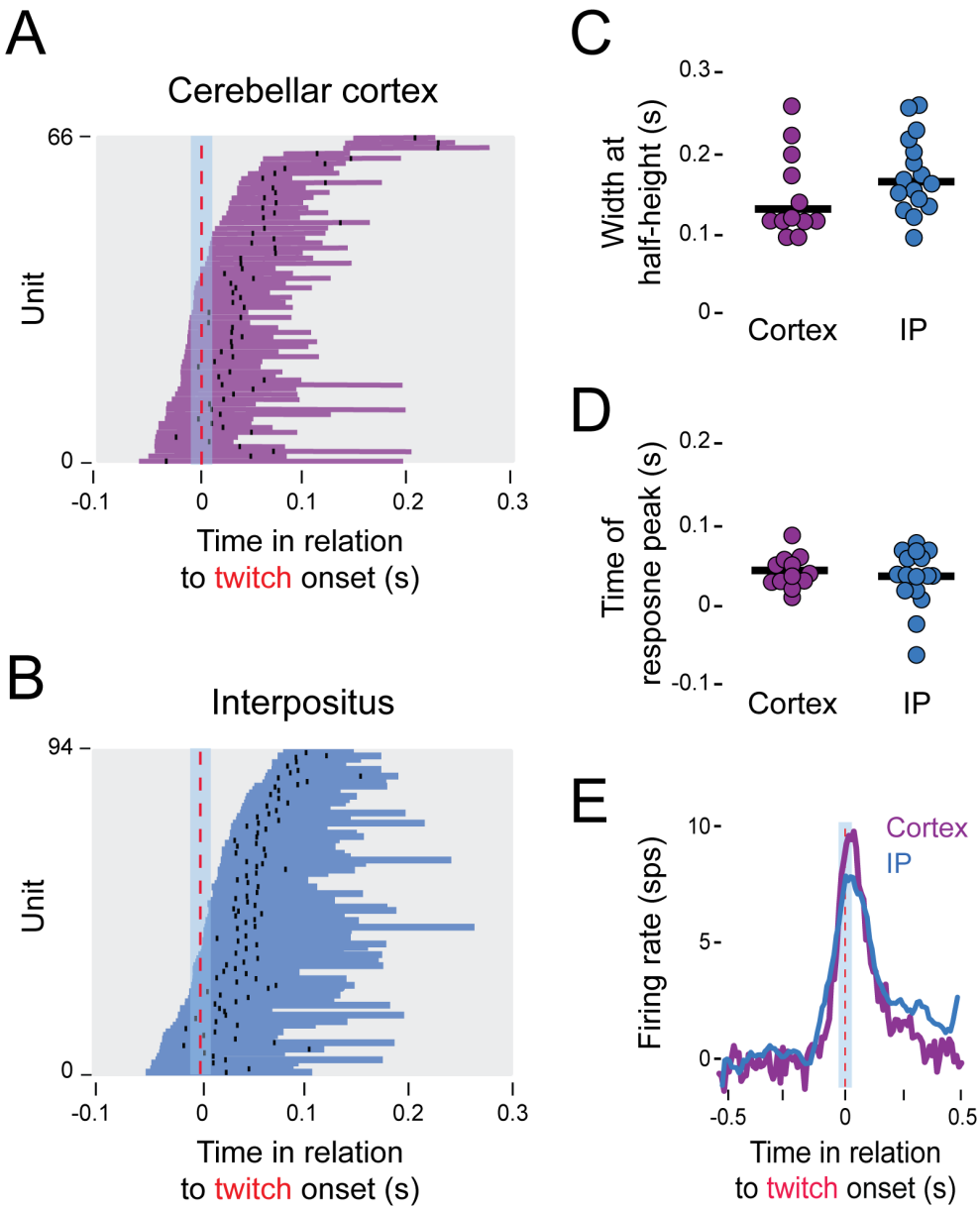

Figure 6. Response profiles for twitch-responsive units in cerebellar cortex and interpositus. ***A***, Representations of the range of width at half-height for all individual twitch-responsive units in cerebellar cortex. Response peaks are also shown as black ticks. ***B***, Same as in ***A*** but for IP (combined across the Pre-Saline and Pre-Urethane groups). ***C***, Mean width-at-half-height for twitch-responsive units in cerebellar cortex (n = 12 pups) and interpositus (n = 16 pups). ***D***, Same as in ***C*** but for mean time of response peak. ***E***, Mean firing rate for twitch-responsive units in cerebellar cortex and interpositus.

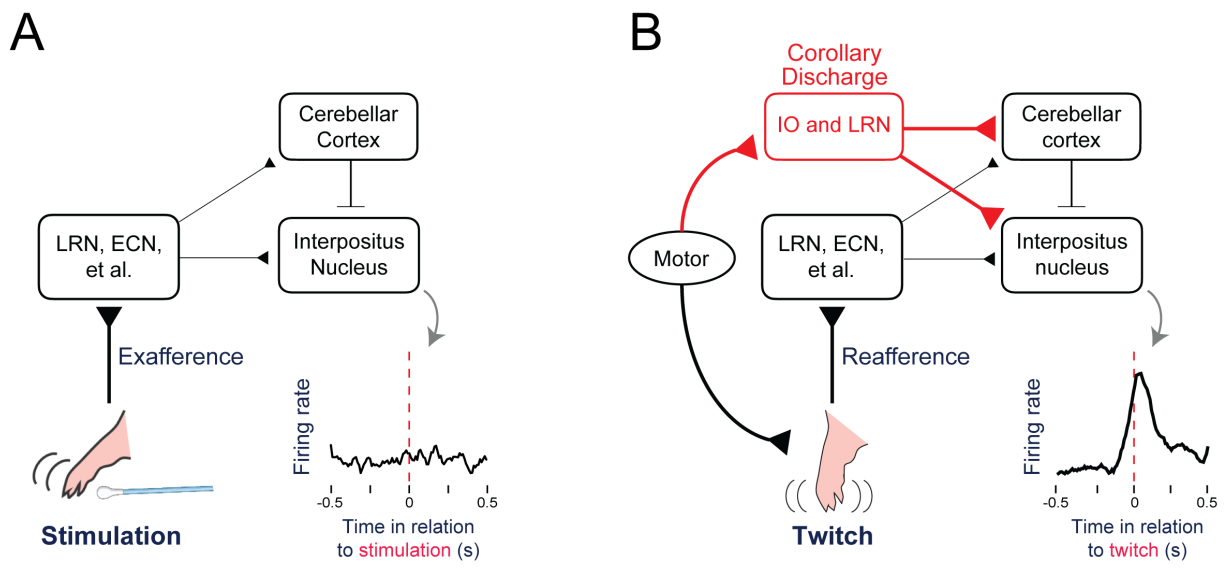

Figure 7. Summary diagrams to explain differential responses of interpositus units to twitches and stimulations in P12 rats. ***A***, When the forelimb is stimulated, exafference is processed in such precerebellar nuclei as the lateral reticular nucleus (LRN) and external cuneate nucleus (ECN) before being conveyed via mossy fibers to interpositus and cerebellar cortex. As shown here, exafference is not expressed in interpositus at this age. ***B***, Twitches are produced by motor commands arising from brainstem premotor nuclei. At the same time, a corollary discharge (red) is conveyed to the inferior olive (IO) or lateral reticular nucleus (LRN). Whereas signals from the IO are sent to the cerebellum via climbing fibers, signals from the LRN are sent via mossy fibers (it is not yet clear which of these two pathways is necessary or sufficient), resulting in a robust and reliable response in interpositus that reflects input from both corollary discharge and reafference.

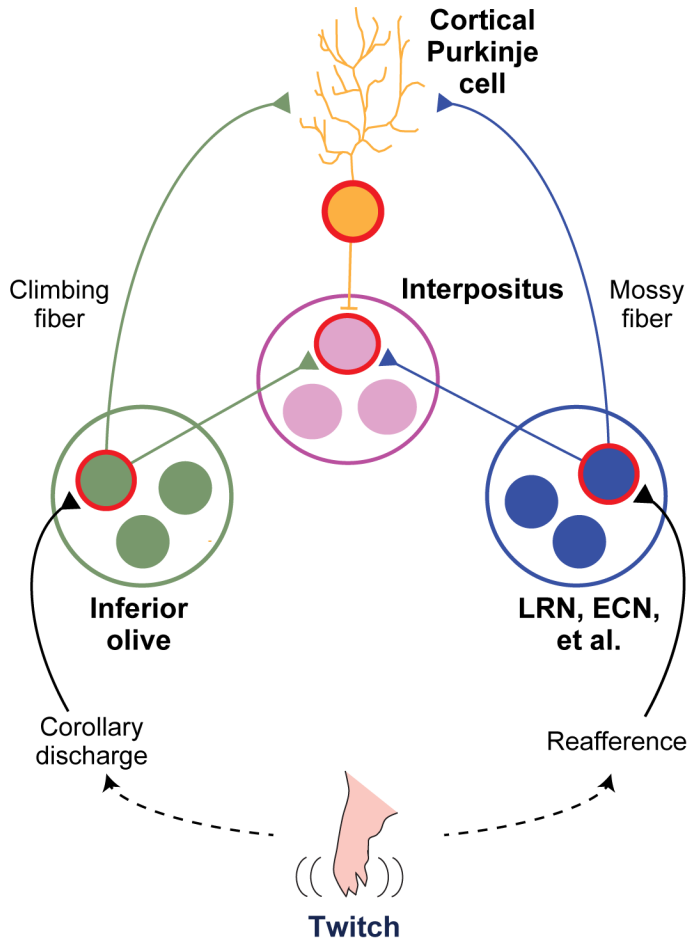

Figure 8. Developing a somatotopically organized closed-loop circuit in the cerebellar system. In early development, there are exuberant connections among key cerebellar and precerebellar components. Based on the current and previous findings, we propose that twitches, because they are associated with both corollary discharge and reafference, provide the opportunity for discrete activation of (for example) forelimb-associated neurons in the inferior olive and other precerebellar nuclei (e.g., LRN, lateral reticular nucleus; ECN, external cuneate nucleus). The resulting parallel coactivation of neurons in interpositus and cerebellar cortex enables the construction of somatotopically organized closed-loop circuits that will, over the next week, provide a foundation for the computation of internal models that allow for precise predictive control of movement.

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