Development/Plasticity/Repair

PP2B-Dependent Cerebellar Plasticity Sets the Amplitude of the Vestibulo-ocular Reflex during Juvenile Development

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Throughout life, the cerebellum plays a central role in the coordination and optimization of movements, using cellular plasticity to adapt a range of behaviors. Whether these plasticity processes establish a fixed setpoint during development, or continuously adjust behaviors throughout life, is currently unclear. Here, by spatiotemporally manipulating the activity of protein phosphatase 2B (PP2B), an enzyme critical for cerebellar plasticity in male and female mice, we examined the consequences of disrupted plasticity on the performance and adaptation of the vestibulo-ocular reflex (VOR). We find that, in contrast to Purkinje cell (PC)-specific deletion starting early postnatally, acute pharmacological as well as adult-onset genetic deletion of PP2B affects all forms of VOR adaptation but not the level of VOR itself. Next, we show that PC-specific genetic deletion of PP2B in juvenile mice leads to a progressive loss of the protein PP2B and a concurrent change in the VOR, in addition to the loss of adaptive abilities. Finally, re-expressing PP2B in adult mice that lack PP2B expression from early development rescues VOR adaptation but does not affect the performance of the reflex. Together, our results indicate that chronic or acute, genetic, or pharmacological block of PP2B disrupts the adaptation of the VOR. In contrast, only the absence of plasticity during cerebellar development affects the setpoint of VOR, an effect that cannot be corrected after maturation of the cerebellum. These findings suggest that PP2B-dependent cerebellar plasticity is required during a specific period to achieve the correct setpoint of the VOR.

Key words: **cerebellar development; compensatory eye movements; plasticity; protein phosphatase 2B; vestibulo-ocular reflex**

Significance Statement

Early damage to motor adaptation structures, such as the cerebellum, has been linked to neurodevelopmental disorders persisting into adulthood. Understanding these long-term effects requires disentangling the persistent effects of disrupted development from the acute effects directly caused by the continuous presence of the disruption. Here, we use the vestibulo-ocular reflex (VOR) to demonstrate that disruptions during early development affect both basal level and adaptation, whereas adult-onset disruption of cerebellar plasticity only affects the ability to adapt, not the setpoint of the reflex. Our finding that the setpoint of the VOR is specifically affected by the absence of plasticity during cerebellar development and cannot be corrected by reinstating plasticity after maturation, supports the concept of a sensitive developmental period for setting cerebellum-controlled reflexes.

Introduction

The cerebellum plays a crucial role in motor control and adaptation by integrating sensory and motor information (Ito, 2002; De Zeeuw and Yeo, 2005). Formation of the adult cerebellar cytoarchitecture, through neurogenesis, migration, morphological maturation, and circuitry fine-tuning, is initiated prenatally but completed in the postnatal period in both humans and mice (Marzban et al., 2014; Beekhof et al., 2021). In line with the fact that it is well-conserved throughout phylogenesis and that it develops relatively late during ontogeny, the cerebellum has a highly organized anatomical structure that can subserve the control of various behavioral and cognitive functions (Larsell, 1952; Bayer et al., 1993; Hatten and Heintz, 1995; Sillitoe et al., 2005). Accordingly, the cerebellum is often used to investigate the interactions between development and motor performance and motor learning (Manto and Jissendi, 2012; Martinez, 2014). Peri- and postnatal damage of the cerebellum has been linked to neurodevelopmental disorders with functional

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deficits that can persist well into adulthood, highlighting the possibility that the cerebellum may contribute to more widespread neurotypical brain maturation (S. S. Wang et al., 2014; Leto et al., 2016; R. Wang et al., 2018). Understanding these long-term effects requires disentangling the persistent, long-term effects of disrupted development from the acute, ongoing effects directly caused by the disruption. Based on the premise that cerebellar damage early in life can be linked to problems at later stages, we hypothesize that there is a differential effect of early versus late disruption of cerebellar function, with more basal functions affected by developmental disruption.

To quantitatively evaluate the influence of postnatal development on both motor performance and motor learning across the entire life span, it will be advantageous to study a cerebellum-dependent behavior that allows for measuring both behavioral components that shows minimal variation among subjects and that can be reliably recorded over a wide range of ages. Compensatory eye movements perfectly meet these criteria as they consist of adaptable reflexes induced by vestibular and optokinetic inputs (Miles and Lisberger, 1981; Katoh et al., 1998; Faulstich et al., 2004; Stahl, 2004). Vestibular input through head rotation activates the semicircular canals that in turn drive the vestibulo-ocular reflex (VOR) through the three-neuron arc (Lorente De Nó, 1933). Rotations of the visual field activate the visual systems and drive the optokinetic reflex (OKR). Lesions of the flocculus, the part of the cerebellum related to compensatory eye movements, abolish VOR plasticity, impair OKR and OKR plasticity, but have minimal effect on the VOR itself (Nagao, 1983). These findings indicate that the OKR and its plasticity also depend on other brain regions, for example, visual cortex (Liu et al., 2016). In contrast, the VOR operates independently but can be modulated by the cerebellum, making it suitable to study cerebellar impact on reflex development. Compensatory eye movement reflexes and their adaptation can be rapidly probed in a highly reproducible manner from birth in humans (Weissman et al., 1989) and shortly after opening of the eye in mice (Faulstich et al., 2004; Beekhof et al., 2021), making it an ideal system to study the acute and long-term effects of interventions at various stages in life. The expression of protein phosphatase 2B (PP2B), also known as calcineurin, starts early postnatally (Lin et al., 2021) and is critical for cerebellar plasticity, including parallel fiber-to-Purkinje cell (PC) long-term potentiation and plasticity of intrinsic excitability of PCs (Belmeguenai and Hansel, 2005; Belmeguenai et al., 2010; Schonewille et al., 2010). Mice harboring a PC-specific knock-out of PP2B (L7-PP2B KO) from early on display severe deficits in a broad range of cerebellum-dependent behaviors including performance and adaptation of compensatory eye movements (Schonewille et al., 2010; Rahmati et al., 2014; Vinueza Veloz et al., 2015; Lefort et al., 2019), including a lower OKR gain and a higher VOR gain. Whether these changes are due to the absence of PP2B at the adult stage or disrupted cerebellar development remains unclear.

Here, using comparative behavioral analysis in L7-PP2B KO mice across various ages, we demonstrate that, in contrast to early postnatal loss of function, acute pharmacological blockage of PP2B in adult mice affected the adaptation of the VOR but not the reflex itself. Moreover, we found that adult-onset but prolonged deletion of PP2B in adult mice, using a tamoxifen-dependent–inducible model, affects all forms of plasticity and the OKR but again not the VOR itself. In contrast, juvenile L7-PP2B KO mice exhibit a progressive loss of PP2B and concurrent changes in the VOR, in addition to the loss of adaptive abilities. Together, our results suggest that adult-onset loss of plasticity affects adaptation, whereas a loss during development results in an alternative setpoint of the basal reflex.

Materials and Methods

All experiments were approved by the Dutch Ethical Committee for animal experiments and in accordance with the Institutional Animal Care and Use Committee.

Animals. For all experiments, we used adult male and female mice with a C57Bl/6 background that were, unless stated otherwise, group housed until the first surgery and then individually housed. All mice had food *ad libitum* and were on a 12/12 h light/dark cycle. In all experiments, the experimenters were blind to mouse genotypes. Mice with PC-specific knock-out of PP2B were used previously (Schonewille et al., 2010; Lin et al., 2021). In short, the regulatory subunit of the calcium-/calmodulin-activated PP2B was flanked with loxP sites [Mouse Genome Informatics (MGI), C57BL/6-Ppp3r1^{tm1Stl}/J, also referred to as Ppp3r1$^{fl/fl}$], which can be selectively deleted from PCs by crossing this line with the pcp2 promoter–driven Cre recombinase expression [MGI, Tg(Pcp2-cre)1Amc/J, also known as L7^{cre}]. Mice of the following genotypes were used for the experiments: L7-cre$^{+/-}$/PPP3R1$^{fl/fl}$ (referred to as L7-PP2B KO) and L7-cre$^{-/-}$/PPP3R1$^{fl/fl}$, L7-cre$^{+/-}$/PPP3R1$^{+/+}$, and L7-cre$^{+/-}$/PPP3R1$^{+/+}$ (all littermate controls or L7-PP2B Ctrl). Eye movement recordings were performed in adult mice aged between 10 and 30 weeks and in two juvenile cohorts: aged postnatal 18–21 d (P18–21) and aged postnatal 26–30 d (P26–30). Inducible PC-specific PP2B knock-outs were generated by crossbreeding mice carrying the floxed PPP3R1 with mice expressing the tamoxifen-sensitive Cre recombinase Cre-ERT2 under the control of the L7 promoter [MGI, Tg(Pcp2-cre/ERT2)17.8Ics], generating experimental mice, L7$^{Cre-ERT2/-}$; PPP3R1$^{fl/fl}$, referred to as iL7-PP2B cKO + TAM. Tamoxifen was dissolved in corn oil to obtain a 20 mg/ml solution and intraperitoneally injected into all mice, mutants and controls, for 5 consecutive days, 4 weeks prior to eye movement recordings. Injections were performed in adults between 24 and 40 weeks of age. Experimental cohorts were always injected at the same time during the day. Littermate controls were mice without L7$^{Cre-ERT2}$ expression but with tamoxifen injections (L7$^{-/-}$; PPP3R1$^{fl/fl}$, referred to as iL7-PP2B Ctrl + Tam) or mice with L7$^{Cre-ERT2}$ but without vehicle instead of tamoxifen injections (L7$^{Cre-ERT2+/-}$; PPP3R1$^{fl/fl}$, referred to as iL7-PP2B cKO + Veh).

Immunohistochemistry. Anesthetized mice were perfused with 4% paraformaldehyde (PFA) in 0.12 M phosphate buffer (PB). The brains were taken out and postfixed for 1 h in 4% PFA at room temperature and then transferred in 10% sucrose overnight at 4°C. The next day, the solution was changed for 30% sucrose and left overnight at 4°C. Nonembedded brains were sectioned either sagittally or transversally at 40 µm thickness with freezing microtome. Free-floating sections were rinsed with 0.1 M PB and incubated 2 h in 10 mM sodium citrate at 80°C for 2 h, for antigen retrieval. For immunofluorescence, sections were rinsed with 0.1 M PB, followed by 30 min in phosphate-buffered saline (PBS). Sections were incubated for 90 min at room temperature in a solution of PBS/0.5% Triton-X100/10% normal horse serum to block nonspecific protein-binding sites and incubated 48 h at 4°C in a solution of PBS/0.4% Triton-X100/2% normal horse serum, with primary antibodies as follows: aldolase C (1:500, goat polyclonal, SC-12065), calbindin (1:7000, mouse monoclonal, Sigma-Aldrich, #C9848), and PP2B (1:500, rabbit polyclonal, Proteintech Group). After rinsing in PBS, sections were incubated for 2 h at room temperature in PBS/0.4% Triton-X100/2% normal horse serum solution with secondary antibodies coupled with Alexa Fluor 488, Cy3, or Cy5 (Jackson ImmunoResearch Laboratories), at a concentration of 1:200. Sections were mounted on coverslip in chrome alum (gelatin/chromate) and covered with Mowiol (Polysciences). Images were acquired with an upright LSM 700 confocal microscope (Zeiss). Overview images in

Figure 4*A–H* are maximum-intensity projections from short Z-stacks (5–10 images); all other images are taken from a single optical plane.

Western blot. Cerebellar tissue from mice was dissected and immediately frozen in liquid nitrogen. Samples were homogenized with a Dounce homogenizer in lysis buffer containing 50 mM Tris–HCl, pH 8, 150 mM NaCl, 1% Triton X-100%, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail. Protein concentrations were measured using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Samples were denatured, and proteins were separated by SDS-PAGE in Criterion TGX Stain-Free Gels (Bio-Rad Laboratories) and transferred onto nitrocellulose membranes with the Trans-Blot Turbo Blotting System (Bio-Rad Laboratories). Membranes were blocked with 5% BSA (Sigma-Aldrich) in TBST (20 mM Tris–HCl, pH7.5, 150 mM NaCl and 0.1%, Tween20) for 1 h and probed with the primary antibodies Dyn1-S778 (1:1000, sheep monoclonal, Abcam) and Dyn1 (1:1000, mouse monoclonal, Santa Cruz Biotechnology) and with secondary antibodies subsequently. Membranes were scanned by Odyssey DLx Imager (LI-COR Biosciences) and quantified using Image Studio Lite (LI-COR Biosciences).

Stereotaxic AAV injections. To re-express PP2B, we performed stereotaxic injection of the AAVs containing floxed, cre-dependent native PP2B (AAV-CAG-eGFP-PP2B) or enzyme-dead H151A mutant PP2B constructs (AAV-CAG-eGFP-PP2B/H151A) 2 weeks prior to eye movement measurements in the bilateral flocculi of adult L7-PP2B KO mice following a lateral-dorso-ventral direct approach (1 mm depth; 100–150 nl; titers, $1.0–1.2 \times 10^{13}$ vg ml^{-1}). After injection, the pipette was left in place for >10 min before being slowly withdrawn. All the process was done under isoflurane (4% induction, 1.5–2% maintenance) anesthesia. The quantification of the injection volume in each individual mouse was calculated using the "Calculate Ejection Fraction" plugin in the ImageJ software. Only mice with at least 30% but typically ≥50% of the left flocculus covered by the injection were included in the results.

Compensatory eye movement recordings. Mice were subjected to compensatory eye movement recordings as previously described in detail (Schonewille et al., 2010). In short, mice were equipped with an immobilizing construct, or "pedestal," under general anesthesia with isoflurane/O_2. After 2–3 d of recovery, mice were head-fixed with the body loosely restrained in a custom-made restrainer and placed in the center of a turntable (diameter, 60 cm) in the experimental setup (Fig. 1). A round screen (diameter, 63 cm) with a random dotted pattern ("drum") surrounded the mouse during the experiment to provide visual input. All stimulations occur around the vertical axis, evoking horizontal (left–right) eye movements. Compensatory eye movements, aimed at reducing retinal slip, were induced by sinusoidal rotation of the drum in the light (OKR), rotation of the table in the dark (VOR), or the rotation of the table in the light (visually enhanced VOR, VVOR) with an amplitude of 5° at 0.1–1 Hz. Whereas visual stimulation induces a reflexive eye movement in the same direction, table rotation-induced head movements drive an eye movement in the opposite direction (head left, eyes right), to minimize retinal slip (Fig. 1). Motor performance in response to these stimulations was evaluated by fitting the averaged stimulus and responses with a sine and calculating the gain (fitted amplitude eye velocity/stimulus velocity) and phase (timing of the eye velocity relative to the stimulus velocity in degrees). Motor learning was studied by subjecting mice to mismatched visual and vestibular input. Rotating the drum (visual) and table (vestibular) together in the same direction (in-phase, at 0.6 Hz) and with the same amplitude of (5°) for 5 × 10 min in the light will render the VOR obsolete and thus induce a decrease of the gain of the VOR (in the dark). Subsequently, VOR phase reversal was induced by rotating the visual and vestibular stimulus in the same direction but now with a larger amplitude of the visual stimulus (Fig. 1). Effectively, this stimulation will cause the VOR in the dark to reverse from a compensatory reflex that in response to head rotation turns the eyes in the opposite direction (head left, eyes right) to a reflex that turns the eye in the same direction to that of the head rotation (head left, eyes left). This was achieved by continuing the days following VOR gain decrease (Days 2–5, keeping mice in the dark in between experiments) with in-phase stimulation but now with drum amplitudes of 7.5° (Day 2) and 10° (Days 3, 4, and 5), while the amplitude of the turntable remained 5°. VOR gain increase was evoked by subjecting mice to out-of-phase drum and table stimulation at 1.0 Hz (both with an amplitude of 1.6°). A charge-coupled device (CCD) camera was fixed to the turntable in order to monitor the left eye of the mice. Eye movements were recorded with an eye-tracking software (ETL-200, iScan System). For eye illumination during the experiments, two infrared (IR) emitters (output 600 mW, dispersion angle 7°, peak wavelength 880 nm) were fixed to the table, and a third emitter, which produced the tracked corneal reflection as a reference point, was mounted to the camera and aligned horizontally with the optical axis of the camera. Eye movements were calibrated by moving the camera left–right (peak-to-peak 20°) during periods that the eye did not move. Gain and phase values of eye movements were calculated using custom-made MATLAB routines (MathWorks, https://github.com/MSchonewille/iMove).

Statistics. All values are shown as mean ± SEM. As described previously (Wu et al., 2019), the behavioral experiments' group sizes were estimated a priori using sample size calculations based on minimal relevant differences and expected variation in control cells or mice. For compensatory eye movements across stimulation frequencies and adaptation over time, an ANOVA for repeated measures was used to determine statistical significance, followed by post hoc tests for individual comparisons. For the complete dataset, see Extended Data Table 1-6. All statistical analyses were performed using the SPSS 20.0 software. Data were considered statistically significant if $p < 0.05$ (* indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$).

Results

Previous work has shown that chronic genetic ablation of native PP2B from PCs in L7-PP2B KO mice affects both the performance of compensatory eye movement reflexes and the plasticity-dependent adaptation thereof (Schonewille et al., 2010; Fig. 1). Recent work has indicated that PP2B facilitates motor learning predominantly through its enzymatic activity (Lin et al., 2021). To disentangle the contribution of the acute absence of the gene during the experiment from the long-term effects of genetic deletion, we first set out to acutely block PP2B by inhibiting the enzymatic activity using pharmacological intervention.

Acute pharmacological blocking of PP2B partially affects the adaptation of VOR but not the reflex itself

To test the effect of acutely blocking the enzymatic activity of PP2B, we administered FK506, a selective PP2B inhibitor (Butcher et al., 1997; Pardo et al., 2006). L7-PP2B control littermate mice (L7-PP2B Ctrl) were injected intraperitoneally 15 min before the start of the experiment with 10 mg/kg FK506 or vehicle (10% DMSO, 10% ethanol in 0.9% saline). For comparison, L7-PP2B mutant mice (L7-PP2B KO) were also injected with vehicle solution (Fig. 2*A*). In line with previous studies (Clayton et al., 2009; Cottrell et al., 2013), the pharmacological inhibition of PP2B by FK506 resulted in a similar hyperphosphorylation of Ser778 in Dynamin1, a presynaptic GTPase dephosphorylated by PP2B, to that of genetic deletion of PP2B, indicating a comparable blockage of the enzymatic activity of PP2B (Fig. 2*B*). L7-PP2B KO mice injected with vehicle exhibited a significantly lower gain of the OKR ($p = 0.042$; red vs black; repeated-measure ANOVA with Bonferroni's correction; Fig. 1) and correlating increase in the VOR gain ($p = 0.013$; red vs green) across different frequencies, while injecting control mice with FK506 did not significantly affect motor performance in the gain of OKR, VOR, or VVOR (all $p = 1.000$; green vs black, Fig. 2*C–E*). Similarly, OKR, VOR, and VVOR phases were not

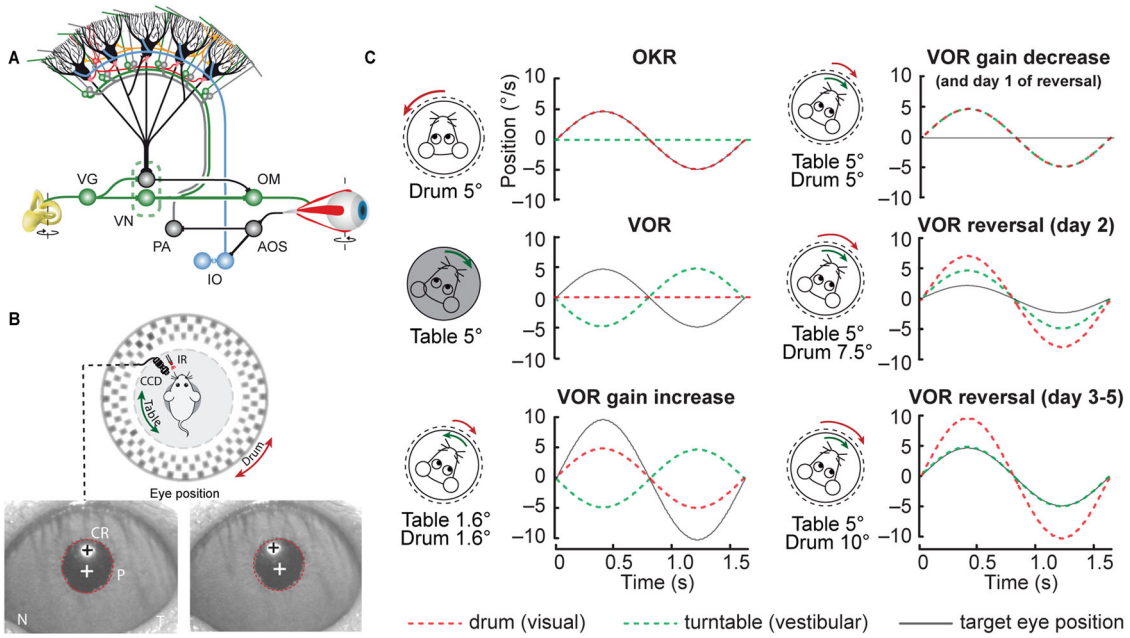

Figure 1. Schematic illustration of the methodology for recording compensatory eye movement and its adaptation. ***A***, Cerebellar circuitry controlling compensatory eye movements and their adaptation. The VOR is the results of a three-neuron arc taking the input to the semicircular canals (left) via the vestibular ganglion (VG) and the vestibular nuclei (VN) neurons, to the oculomotor nuclei (OMN) and then the eye muscles (right). The OKR requires visual input to be converted via the accessory optic systems and using the visual and cerebellar cortices, to drive eye movements via the VN to OMN as well. VOR plasticity uses visual feedback to adjust the properties of the VOR via similar circuits. PN, pontine nuclei; GC, granule cell. ***B***, Eye movement recording setup. Mice are head-fixed in the center of a turntable for vestibular stimulation and surrounded by a random dotted pattern ("drum") for visual stimulation. A CCD camera was used for IR videotracking of the left eye. Bottom, examples of nasal and temporal eye positions. Red circles, pupil fit; black cross, corneal reflection; white cross, pupil center. ***C***, Graphical explanation of the trajectory relationship between the turntable (equivalent to vestibular stimulation, green dashed line), drum (equivalent to visual stimulation, red dashed line), and target eye trace (black line) for basal compensatory eye movements (OKR and VOR) and VOR adaption paradigms (gain–increase, gain–decrease, and phase reversal).

affected in mice injected with FK506 (OKR, $p = 1.000$; VOR, $p = 0.243$; VVOR, $p = 1.000$; Extended Data Table 2). While wild-type mice injected with FK506 and mice injected with vehicle learned equally well in the gain–decrease paradigm ($p = 1.000$, green vs black, Fig. 2*F*), FK506 caused a significant impairment in the phase change induced by VOR phase-reversal training over 5 consecutive days ($p < 0.001$, green vs black, Fig. 2*G*). In line with previous results, L7-PP2B KO mutant mice injected with vehicle showed learning deficits in both paradigms (all $p < 0.01$, red vs black or red vs green, Fig. 2*F*,*G*). Thus, acute pharmacological blocking of the enzymatic function of PP2B only partially affects VOR adaptation while leaving compensatory eye reflexes intact.

Deletion of PP2B in adulthood affects OKR performance and VOR adaptation but not basal VOR

In addition to deficits in VOR adaptation, deleting PP2B from PCs early in the development resulted in a lower OKR and higher VOR gain (Schonewille et al., 2010). As acute interventions were not able to replicate this phenotype of early postnatal loss of PP2B, we next asked if delayed genetic PP2B deletion, starting from adulthood instead of early development, could affect the behavior of compensatory eye movements. To test this, we crossed the *loxP*-flanked PP2B mice with tamoxifen-dependent $L7^{Cre\text{-}ERT2}$ to generate conditional iL7-PP2B KO mice (Fig. 3*A*). When mice were >3 months old, we injected iL7-PP2B cKO mutant mice and iL7-PP2B Ctrl mice with tamoxifen as well as iL7-PP2B cKO mice with vehicle alone and waited for >4 weeks before starting the experiments. If the deficits of eye movement baseline reflexes and plasticity were completely or in part of developmental origin, we should observe no or less changes in adult conditional L7-PP2B KO mice after tamoxifen injections. Delayed genetic deletion of PP2B had profound effects on performance and learning. First, after tamoxifen injection, OKR and VVOR gain was impaired in iL7-PP2B cKO + TAM mice compared with iL7-PP2B Ctrl + TAM mice (Fig. 3*B–D*, $p = 0.020$ and $p < 0.001$, respectively, red vs black). In contrast, phase values were not affected by the deletion (OKR, $p = 0.106$; VOR, $p = 0.832$; VVOR, $p = 0.434$; Extended Data Table 3-2). Delayed PP2B deletion affected the eye movement adaptation in gain–increase ($p = 0.047$), gain–decrease ($p = 0.001$), and VOR phase reversal (Day 4, $p < 0.001$; Fig. 3*E–G*, black vs red; see Materials and Methods, Compensatory eye movement recordings, and Fig. 1 for description of the paradigm). In contrast to OKR, VVOR, and VOR adaptation, however, the VOR gain of iL7-PP2B cKO + TAM mice was not affected (Fig. 3*C*, $p = 0.375$, black vs red), the only deficit not replicated after adult-onset deletion. As expected, iL7-PP2B cKO + TAM mice did not differ from vehicle-injected mice in any of the OKR, VVOR, and VOR (adaptation) tests (Fig. 3*B*,*E–G*, blue vs black; Extended Data Table 3-1). Finally, we confirmed that 4 weeks after the tamoxifen treatment, PP2B is ablated from PCs in iL7-PP2B cKO + TAM mice (Fig. 3*H*), compared with iL7-PP2B Ctrl + TAM mice injected with tamoxifen (Fig. 3*I*) or iL7-PP2B cKO + VEH mice injected with vehicle (Fig. 3*J*).

Taken together, we find that prolonged PP2B deletion after cerebellar maturation is able to reproduce the deficits observed in OKR and VOR adaptation following developmental deletion of PP2B in L7-PP2B KO mice but not the higher VOR gain.

PP2B ablation affects eye movement behavior during early development

To investigate the behavioral consequences in relation to the onset and temporal profile of PP2B deletion, we examined eye movements in juvenile L7-PP2B KO mice during the first 4

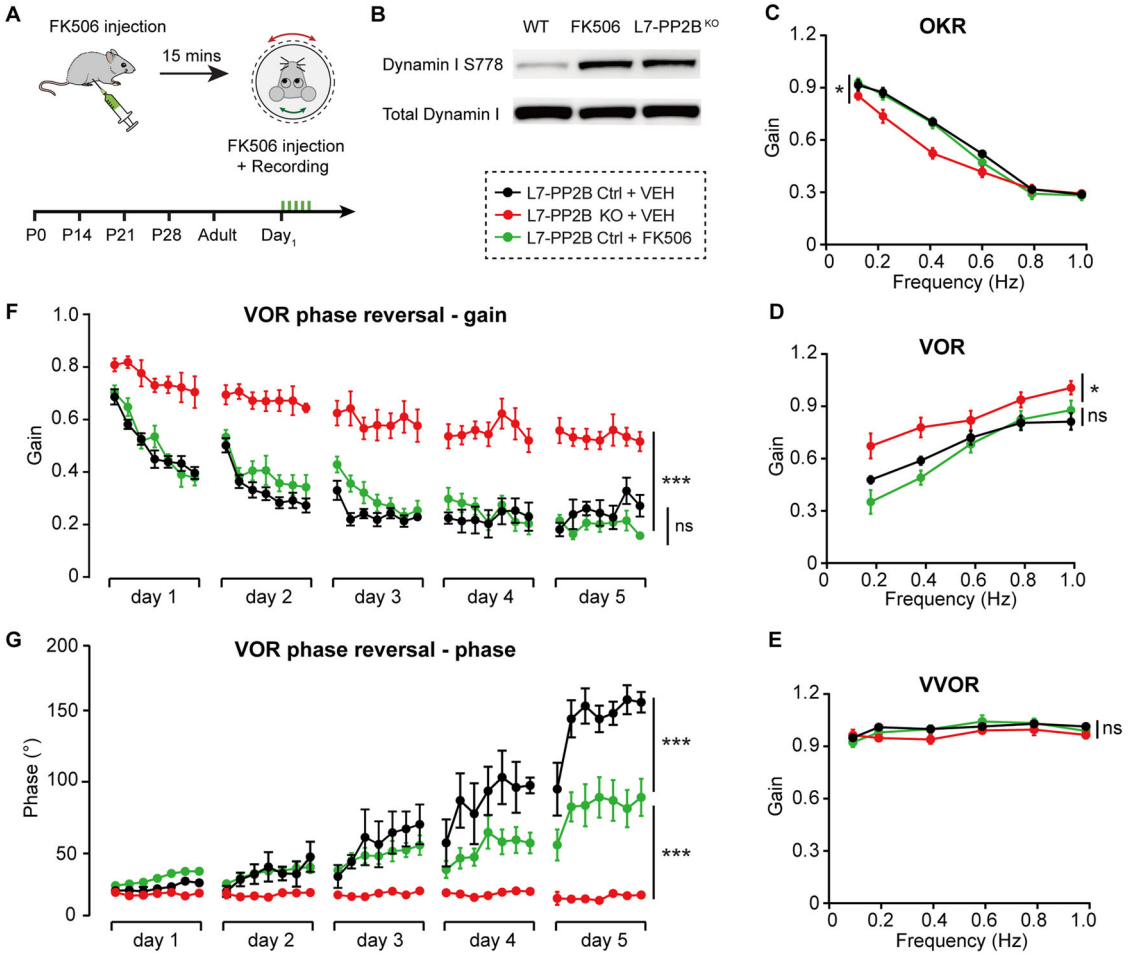

Figure 2. Effects of acute pharmacological blocking of PP2B on reflex and reflex adaptation. ***A***, Schematic strategy of eye movement recording following intraperitoneal FK506 injection. ***B***, Western blot of Ser778 in Dynamin1 validating FK506 inhibition efficiency. Cerebellar cortical tissue was collected from mice after the behavioral experiments. ***C–G***, Recorded OKR gain (***C***), VOR gain (***D***), VVOR gain (***E***), VOR gain decrease learning (***F***), and VOR phase-reversal training across 5 consecutive days (***G***) in L7-PP2B KO wild-type littermate mice injected with FK506 (green, $n = 8$ mice) and vehicle (black, $n = 7$ mice) and L7-PP2B KO mutant mice injected with vehicle (red, $n = 6$ mice), respectively. Panels ***F*** and ***G*** are reused from Lin et al. (2021; Fig. 1C) but replotted in gain and phase plots to facilitate comparison with later figures. Data are represented as mean ± SEM. ns, nonsignificant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. See Extended Data Table 2-1–2-3 for phase values and more details on statistics.

postnatal weeks. Given that the L7-Cre promotor starts to be expressed from the first postnatal week, with a complete expression in all PCs by the end of postnatal Week 2 (Barski et al., 2000), we examined the expression level of the PP2B protein in the flocculus of L7-PP2B KO mice on various days at the juvenile age of Postnatal (P) 14, 18, 20, and 24 d. At the age of P14, when mice started to open their eyes, a small proportion of floccular PCs no longer expressed PP2B (Fig. 4*A*,*B*). At P18, PP2B could no longer be detected in about half of the PCs (Fig. 4*C*,*D*). From P20 to P24, almost all PCs lacked PP2B, approaching the virtually complete deletion observed in adult L7-PP2B KO mice (Fig. 4*E–H*).

Next, we tested the compensatory eye movement in juvenile L7-PP2B KO and control mice at P18–21 and P26–30, respectively, and compared the results to adult mice. At P18–21, L7-PP2B KO mice showed intact baseline performance of OKR, VOR, and VVOR gain and phase, as well as normal VOR gain increase and gain decrease learning, compared with their littermate controls (OKR gain, $p = 0.965$; VOR gain, $p = 0.461$; VVOR gain, $p = 0.283$; phase, OKR, $p = 0.824$; VOR, $p = 0.827$; VVOR, 0.198; VOR gain increase, $p = 0.636$; VOR gain decrease, $p = 0.106$; Extended Data Table 5-1 and 5-2; squares, Fig. 5*A–E*). Note that in our histological analysis, we found that about half of the floccular PC PP2B was deleted at this stage, yet there is no evidence for an effect on the compensatory eye movement adaptation, possibly indicating that there is sufficient remaining circuit plasticity despite a substantial reduction of PP2B. In contrast, at P26–30, L7-PP2B KO mice showed evident impairments not only in OKR ($p = 0.001$) and VOR ($p = 0.013$) performance (triangles; VVOR, $p = 0.069$, Fig. 5*A–C*; VOR phase, $p = 0.029$; Extended Data Table 5-4 and 5-5) but also in VOR gain increase ($p = 0.014$) and VOR gain decrease ($p = 0.026$) learning as well as VOR phase reversal ($p < 0.001$; triangles lines, Fig. 5*D–F*). Behavior at P26–30 is similar to that observed in adult L7-PP2B KO mice (data from Schonewille et al., 2010; here shown in open circles, Fig. 5*A–F*) with the most prominent difference being that learning rates are lower in adult mice (Beekhof et al., 2021). Note that some capacity for learning was still observed in the juvenile L7-PP2B KO mice aged P26–30 (red triangles, Fig. 5*F*). Together, these findings indicate a direct correlation between the absence of PP2B and behavioral deficits. Moreover, at 4 weeks of age, there is a virtually complete deletion of PP2B that affects all recorded reflexes and adaptation, including the

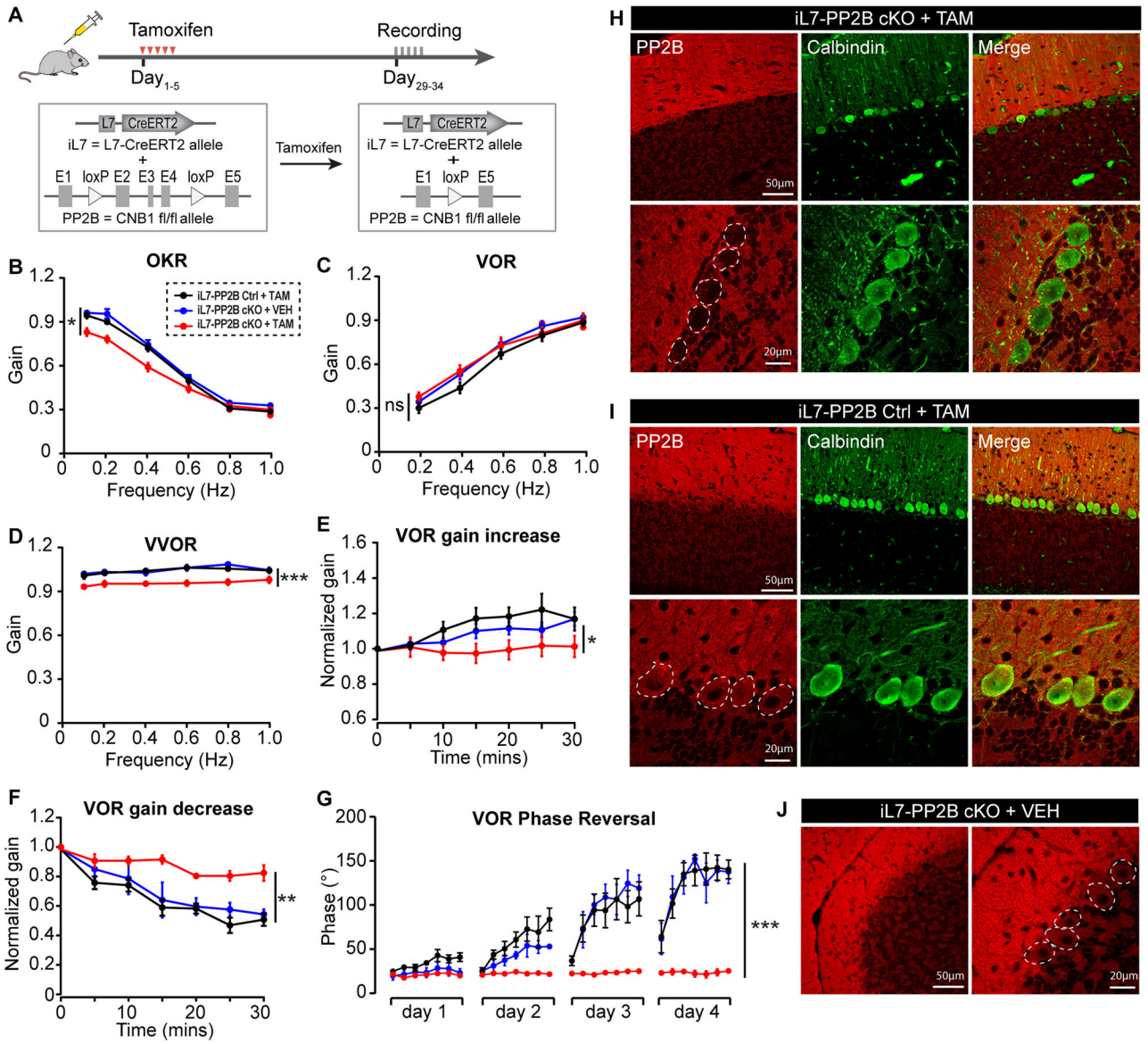

Figure 3. Effects of PC-specific adult-onset genetic ablation of PP2B. ***A***, Schematic strategy for generation of a PC-specific PP2B deletion triggered by tamoxifen. ***B–G***, Recorded OKR gain (***B***), VOR gain (***C***), VVOR gain (***D***), VOR gain increase learning (***E***), VOR gain decrease learning (***F***), and VOR phas- reversal training across 4 consecutive days (***G***) in iL7-PP2B cKO + TAM mice (red), iL7-PP2B Ctrl + TAM mice (black) injected with tamoxifen, and iL7-PP2B cKO + VEH mice injected with vehicle only (blue). ***H–J***, Corresponding immunofluorescent images of the three experimental groups (red, PP2B; green, calbindin; yellow, merge). White dotted lines indicate the somata of PCs. Data are represented as mean ± SEM. ns, nonsignificant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. See Extended Data Table 3-1–3-3 for phase values and more details on statistics.

VOR, indicating that the size of this reflex is set early in development.

Restoring PP2B expression in adult L7-PP2B KO mice rescues VOR adaptation and OKR but not VOR

Finally, if the adult-onset deletion of PP2B does not result in an increased VOR gain, the inverse should also hold the following: juvenile deletion followed by re-expressing PP2B in adult mice should rescue adaptation, while VOR gain remains impaired. To test this prediction, we injected restored PP2B expression in L7-PP2B KO mice by injecting AAV1-CAG-eGFP-PP2B and tested baseline compensatory eye movements and VOR adaptation. For comparison, in another group of L7-PP2B KO mice with AAV1-CAG-eGFP-PP2B/H151A, which lacks its enzymatic function due to the H151A mutation (Baksh et al., 2000; Lin et al., 2021), AAV injections were made bilaterally in the flocculi, and the location of injection was confirmed by postmortem analysis (Fig. 6*A*,*B*); only mice with at least 30% of the left flocculus covered by the injection were included in the results (Fig. 6*C*; Extended Data Table 6-4). Re-expression of functional PP2B in L7-PP2B KO mice increased the OKR and VVOR gain compared with that of PP2B/H151A, resulting in levels that are comparable to controls (both $p = 1.000$; purple vs black, repeated-measure ANOVA with Bonferroni's correction, Fig. 6*D–F*). Although in VOR phase reversal the improvement in phase was not significant (e.g., $p = 0.498$ for Day 5; purple vs red, Fig. 6*G*), the ability to decrease the gain, as a first step in the reversal, is significantly improved (all $p < 0.05$ for Days 1–5 purple vs red, Fig. 6*H*). The expression of PP2B lacking its enzymatic function had virtually no effect on OKR, VVOR, or VOR gain or VOR adaptation (compared with red curves, Fig. 2*C–G*; Lin et al., 2021). Re-expression of either of

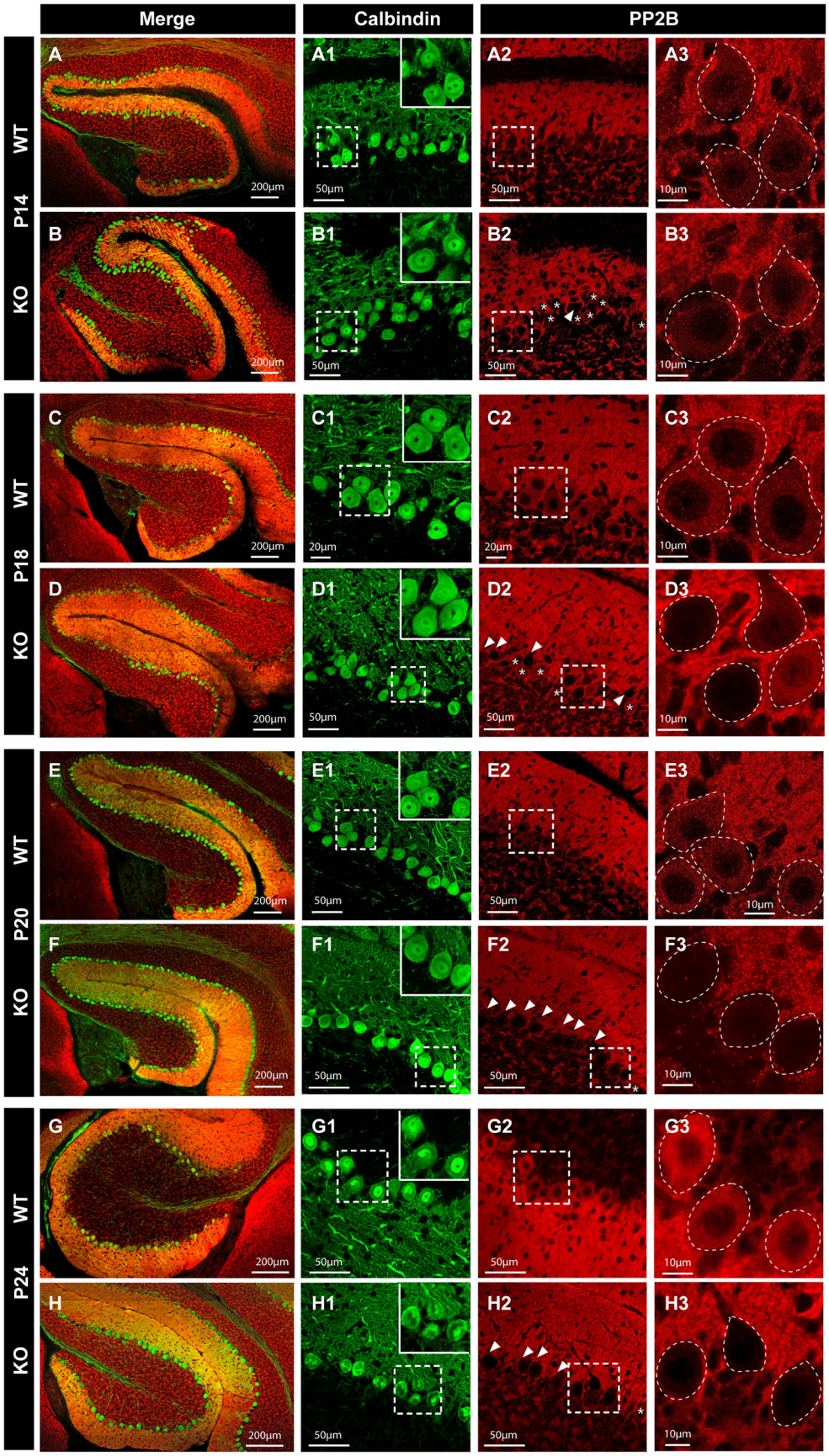

Figure 4. Floccular PP2B expression across development in juvenile L7-PP2B KO mice. ***A–D***, Immunofluorescent images of PP2B expression in the flocculus of L7-PP2B KO mice at P14 (***A,B***), 18 (***C,D***), 20 (***E,F***), and 24 (***G,H***). Left column, overview staining of flocculi (maximum-intensity projection of Z-stack); ***A1–H1*** images from a single optical plane for calbindin (green) and ***A2–H2*** for PP2B (red). ***A3–H3***, magnification of boxed area in ***A2–H2***. White dotted lines indicate the somata of PCs. White triangles, PC somata without PP2B; white asterisks, PC somata with PP2B. Scale bars: 200 μm for merge images, 50 μm for images in column 2 (***A1–H1***) and column 3 (***A2–H2***), except 20 μm for those in C1 and C2, and 10 μm for images in column 4 (***A3–H3***).

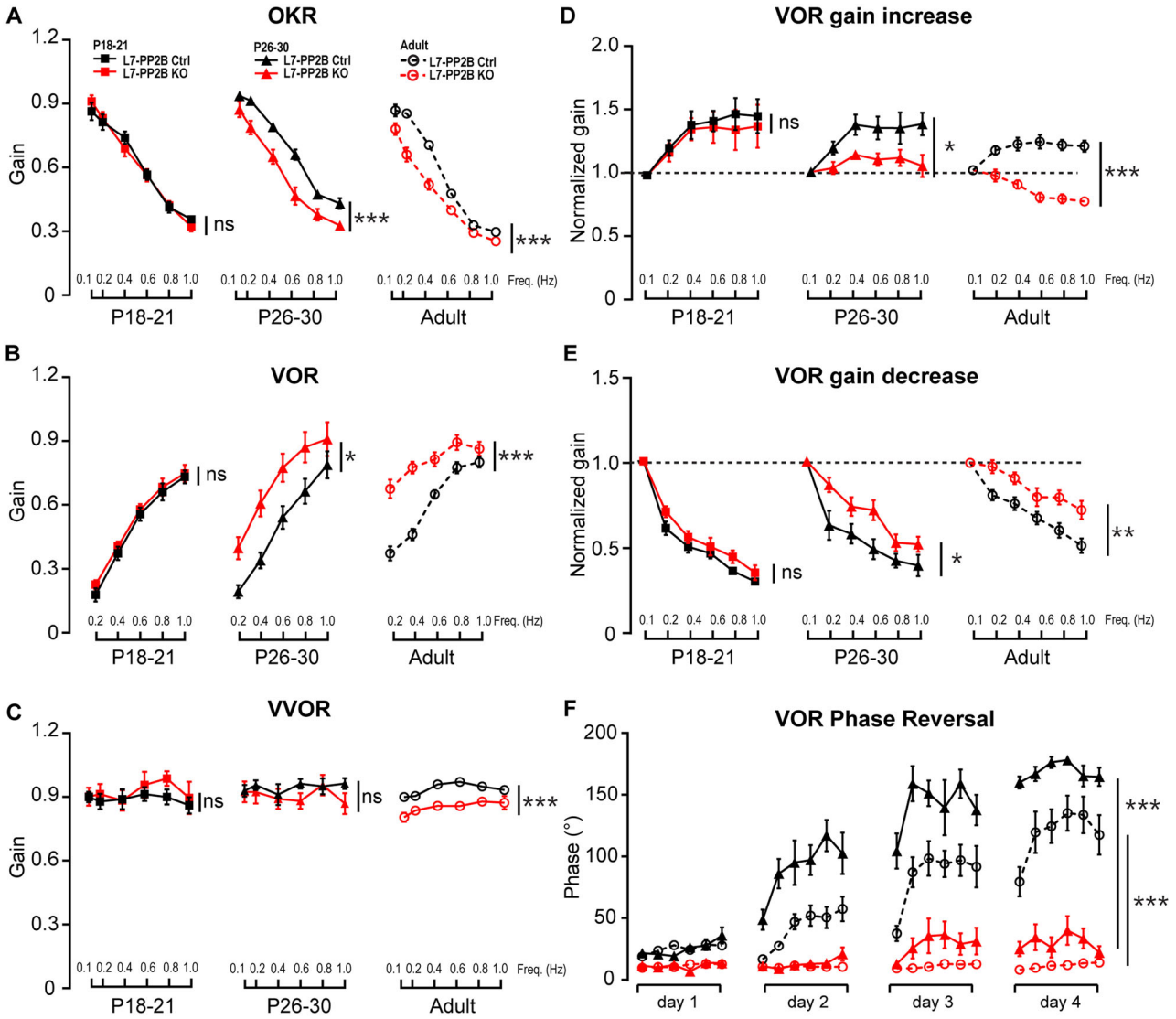

Figure 5. Comparison of reflex and reflex adaptation in L7-PP2B KO mice at different developmental ages. ***A***, OKR gain in L7-PP2B KO and their littermate control mice at the age of P18–21 (KO, $n = 9$ mice; WT, $n = 11$ mice; squares), P26–30 (KO, $n = 10$ mice; WT, $n = 13$ mice; triangles), and adulthood [KO, $n = 15$ mice; WT, $n = 23$ mice; open circles, data from Schonewille et al. (2010)]. ***B***, VOR gain in mice aged P18–21 (KO, $n = 8$ mice; WT, $n = 11$ mice; squares), P26–30 (KO, $n = 7$ mice; WT, $n = 8$ mice; triangles), and adulthood (KO, $n = 14$ mice; WT, $n = 23$ mice; open circles). ***C*** same as ***A*** but for VVOR gain. ***D***, VOR gain increase adaptation in L7-PP2B KO and their littermate control mice at the age of P18–21 (KO, $n = 9$ mice; WT, $n = 10$ mice; squared lines), P26–30 (KO, $n = 9$ mice; WT, $n = 7$ mice; triangles), and adulthood (KO, $n = 10$ mice; WT, $n = 13$ mice; open circles). ***E*** same as ***D*** but for VOR gain decrease adaptation in mice aged P18–21 (KO, $n = 8$ mice; WT, $n = 7$ mice; squares), P26–30 (KO, $n = 7$ mice; WT, $n = 8$ mice; triangles), and adulthood (KO, $n = 13$ mice; WT, $n = 13$ mice; open circles). ***F***, Phase-reversal training in mice aged P26–30 (KO, $n = 10$ mice; WT, $n = 11$ mice; triangles) and adulthood (KO, $n = 8$ mice; WT, $n = 8$ mice; open circles). Littermate controls in black; mutant mice in red. Data are represented as mean ± SEM. ns, nonsignificant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. See Extended Data Table 5-1–5-9 for phase values and more details on statistics.

the two types of PP2B did not affect the phase of the OKR, VOR, or VVOR (all $p > 0.05$; Extended Data Table 6). Unlike the effect on VOR adaptation and OKR gain and in line with our prediction, the re-expression of fully functional PP2B in L7-PP2B KO mice did not significantly change the VOR gain. As a result, VOR gain differs from that in L7-PP2B Ctrl ($p = 0.029$; purple vs black, Fig. 6*E*) and not from that in L7-PP2B KO mice injected with PP2B/H151A ($p = 0.973$; purple vs red, Fig. 6*E*).

Taken together, we find that reinstating PP2B in the majority of floccular PCs of adult L7-PP2B KO mice is sufficient to rescue OKR gain and improve VOR adaptation but does not significantly affect the VOR gain. These results confirm that the setpoint of the VOR is largely, if not completely, set in the first weeks after opening of the eye.

Discussion

The present study probed the contribution of the cerebellar cortex, in particular PC plasticity, to the formation and maintenance of a reflexive behavior. We found that an acute block of PP2B function, an enzyme required for synaptic and nonsynaptic plasticity, did not affect the baseline reflexes of compensatory eye movements, which is in clear contrast with what happens after permanent genetic deletion at early development. Prolonged PC-specific genetic PP2B ablation, starting from the adult stage, selectively affected VOR adaptation and basal OKR, but not basal VOR, performance. In contrast, at the age of P26–30, shortly after the complete loss of PP2B from their PCs, juvenile L7-PP2B KO mice exhibited impairments in OKR, VOR, and VOR phase reversal comparable with their adult mutant counterparts. Finally, re-expressing PP2B in adult L7-PP2B KO mice

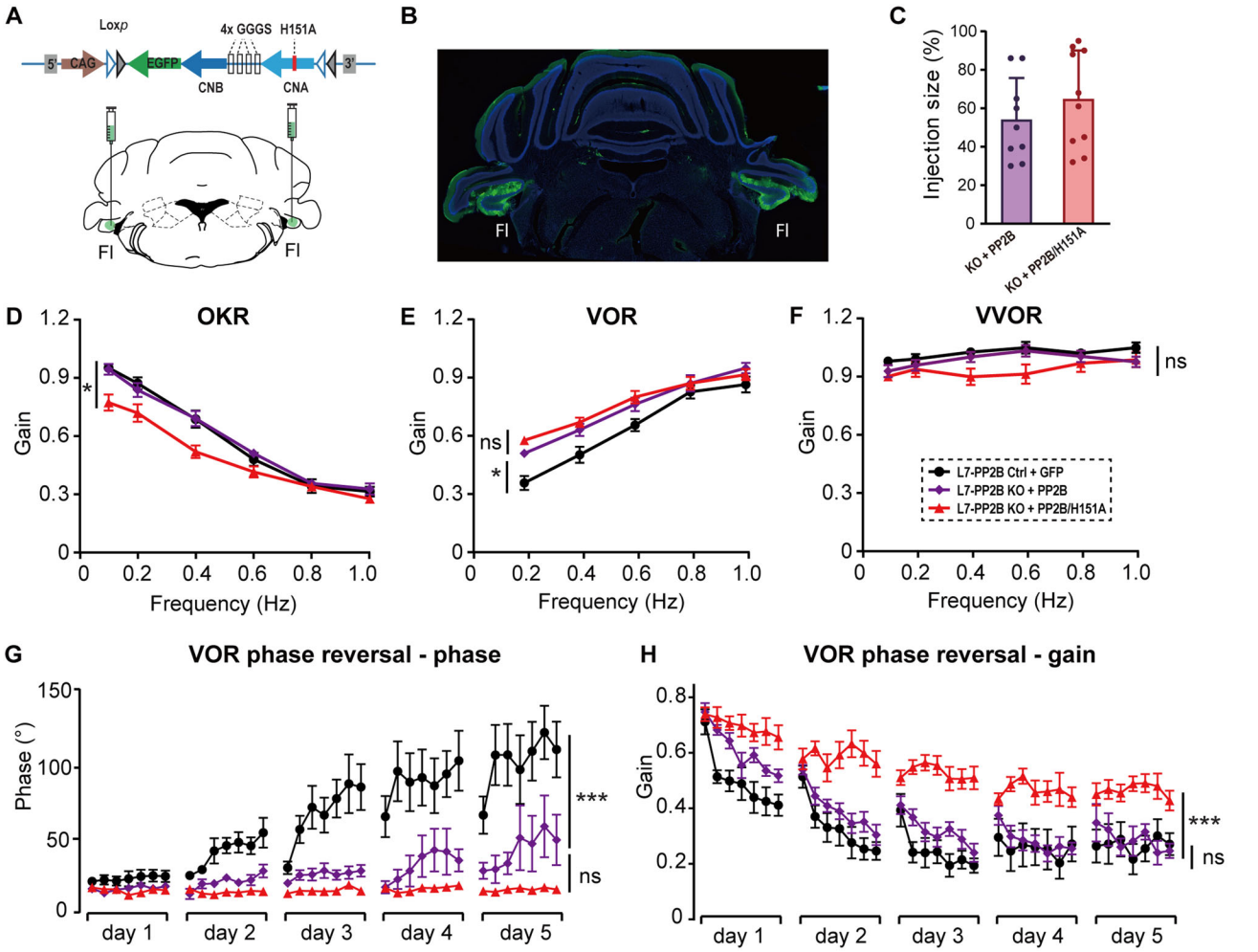

Figure 6. Re-expressing functional PP2B and PP2B lacking enzymatic function in adult L7-PP2B KO mice. ***A***, Schematic depiction of the AAVs carrying Cre-dependent functional PP2B and enzyme-dead PP2B due to the H151A mutation (PP2B/H151A) and the strategy of AAV injections made bilaterally in the flocculi. ***B***, Example of AAVs injections with either functional PP2B or enzyme-dead PP2B/H151A in bilateral flocculi (visualized by the coexpression of EGFP). ***C***, Quantification of the injection volume of AAVs carrying PP2B and enzyme-dead PP2B. ***D–H***, Eye movement recording of OKR gain (***D***), VOR gain (***E***), VVOR gain (***F***), VOR phase reversal with the change in phase (***G***) and gain (***H***) across 5 consecutive days in L7-PP2B control mice injected with AAVs with GFP (black, $n = 9$ mice) and L7-PP2B KO mutant mice injected with AAVs with PP2B (purple, $n = 9$ mice) or PP2B/H151A (red, $n = 10$ mice), respectively. Data are represented as mean ± SEM. ns, nonsignificant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. See Extended Data Table 6-1–6-4 for phase values, injections volumes, and more details on statistics.

rescued OKR gain and VOR adaptation but did not rescue the VOR gain (see summary in Fig. 7). Thus, using temporal variations in PP2B ablations, we demonstrate that disruption or absence of PP2B always directly impairs adaptation, while the setpoint of the VOR is exclusively determined during cerebellar development in the juvenile stage.

Compensatory eye movements form the ideal substrate to study the ontogenetic steps related to the onset and maturation of reflexes as well as their adaptations. Two processes together assure adequate compensatory eye movements throughout life: (1) the reflexes and related circuit need to be configurated during development, and (2) the adaptive, plastic process needs to maintain optimal performance by continuously calibrating the response to reduce retinal slip during head perturbations (Roucoux et al., 1983; Finocchio et al., 1991; Charpiot et al., 2010; Alahyane et al., 2016). The flocculus of the cerebellum is required for proper setup and adaptation of the VOR (Gauthier and Robinson, 1975; Roucoux et al., 1983; Sherman and Keller, 1986; Finocchio et al., 1991; Charpiot et al., 2010). Previously, we found that deleting PP2B from PCs not only impairs cerebellum-dependent learning in different tasks but also affects the performance of basic compensatory eye movement reflexes: the gain of the OKR was lower than in control mice, whereas that of the VOR was higher, particularly at the lower frequencies (Schonewille et al., 2010). The acute effect of PP2B deletion on OKR gain could be caused by loss of plasticity of excitatory synapses, but PP2B contributes to other cellular processes as well (Hirano and Kawaguchi, 2012; Lee et al., 2013; Lin et al., 2021), so effects on, for example, intrinsic activity or synaptic release cannot be excluded. The, mostly long-term, effects on VOR gain in turn are likely the result of cumulative plasticity, at the PC or in downstream target neurons in the vestibular nucleus (McElvain et al., 2010). The relatively larger increase in the gain of the lower frequencies could be related to the fact that the gain at higher frequencies is already close to the value of 1, or perfect compensation, but could also be linked to the factors indicated above. Interestingly, a higher VOR and lower OKR gain were also found following selective disruption of PC inhibition (Wulff et al., 2009) and in a mouse model for PC degeneration (Van Alphen et al., 2002). Moreover, experiments comparing juvenile and adult mice (Faulstich et al., 2004) have demonstrated that VOR gain is higher early in

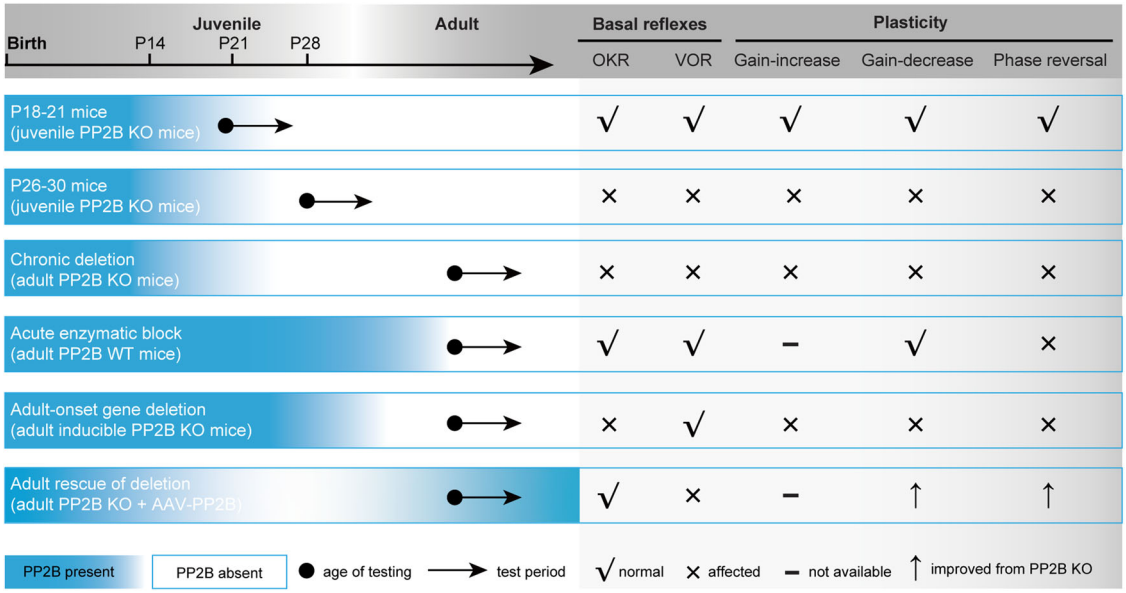

Figure 7. Summary of various impacts of PP2B manipulation strategies on both basal reflexes and plasticity of compensatory eye movement in juvenile or adult mouse. Table depicting the presence/absence of PP2B (in blue) and the age of compensatory eye movement recording (black dot), related to the experimental outcome. Note that all behavioral tests are normal in juvenile mice at P18–21. However, at P26–30, juvenile PP2B KO mice showed evident deficits in all basal reflexes and plasticity measurements, similar to adult PP2B KO mice. Conversely, when PP2B was acutely or chronically blocked, but with adult-onset, all reflexes and reflex adaptations, except for the VOR gain, were affected. Moreover, the re-expression of functional PP2B improved OKR gain and elements of VOR adaptation but not VOR gain. Hence, the presence of PP2B (in blue) between P21 and P28 correlates directly with a normal VOR gain, while the absence of PP2B in that period is linked to an affected (increased) VOR gain.

development than in adult mice, suggesting a reduction in gain toward the setpoint. The persistence of this difference in cerebellar mutants supports the idea that the VOR gain in early life is higher and subsequently reduced through cerebellum-dependent plasticity. Here, we provide further evidence for this concept by showing that eliminating PC-dependent plasticity in adult mice does not affect VOR gain, whereas eliminating PP2B in juvenile mice does, suggesting that there is a sensitive period for this cerebellum-dependent plasticity after which the setpoint can no longer be changed.

PCs in the flocculus reach mature levels in terms of activity level as well as dendritic and axonal morphology at 3 weeks after birth, and both the VOR and its adaptation already function virtually optimally at this early stage (Faulstich et al., 2004, Vis Res; Beekhof et al., 2021). In fact, the VOR adapts even faster in 3-week-old mice than in adult mice (Beekhof et al., 2021), suggesting the presence of additional or optimized plasticity mechanisms early in development. Whether adult plasticity and the enhanced developmental plasticity that generates the setpoint are indeed based on exactly the same mechanisms remains to be established. Although the possibility that PP2B is involved in additional mechanisms cannot be excluded, the data presented here support a central role of PP2B in both juvenile and adult plasticity, suggesting that a general mechanism underlies both forms of plasticity. The setpoint of VOR gain, on the other hand, is affected by deletion of PP2B during development but not during adulthood when compared at a similar time point after deletion. In the L7-PP2B KO mice, PP2B deletion from pups starts in the first 2 weeks after birth (Barski et al., 2000) and affected VOR gain when tested ~4 weeks later, while in adult mice, VOR gain was not affected 4 weeks after tamoxifen injections. This indicates that the same period of PP2B absence in juvenile mice has a distinctly different effect and thus a different mechanism or pathway, requiring PP2B, is present in early development. In line with an early completion of cerebellar development, here too we found that even at P18–20, <1 week after opening their eyes, both control and PC-specific PP2B knock-out mice exhibited normal eye movement performance and learning. One week later, at age of P26–30, both basal reflexes, the VOR and the OKR, are affected by a deletion of PP2B. In contrast, in adult mice neither acute block nor adult-onset prolonged deletion of PP2B affected the VOR gain, indicating the importance of development in setting the reflex level. Based on these findings, we postulate that the third to fourth postnatal weeks are the critical phases for the priming, or setting, of basal VOR, also referred to as the VOR baseline.

Why does the onset and fine-tuning of the compensatory eye movements take place at the third to fourth postnatal week? First, it should be noted that although the expression of Cre in PCs starts in the first postnatal week (Barski et al., 2000), our data indicate that PP2B is a stable protein that remains partially present after deletion of the gene for at least 2 more weeks. Given that opening of the eye occurs at postnatal 12–14 d in mice (Ko et al., 2013), it is possible that our method precluded the possibility to observe effects of PP2B deletion at earlier stages, when VOR can be observed in humans (Weissman et al., 1989; Goode et al., 2001). In humans, it has been suggested that early visual experience and the maturation of visual pathways are important to establish a setpoint for the VOR (Goode et al., 2001). In mice the morphological ontogenesis of the cerebellum (Beekhof et al., 2021), which is considered to be cerebellum-intrinsic and not input-dependent (Leto et al., 2016), could match that timeline. The cerebellum undergoes its major growth in the third month and infant stage in humans, and the first 2 weeks after birth in mice, primarily due to the expansion of granule cell progenitors (Rakic and Sidman, 1970; Dobbing and Sands, 1973). In mice, although some parameters continue to change into adulthood, the circuit and its activity are largely established by the end of the third week (van Welie et al., 2011; Leto et al., 2016; Beekhof et al., 2021). For example, in terms of morphology,

PCs are initially innervated by multiple climbing fibers with similar strengths in the first postnatal week, but from P9 to P17, climbing fibers successively undergo functional differentiation, dendritic translocation, and elimination (Watanabe and Kano, 2011; Hashimoto and Kano, 2013). Moreover, in vivo electrophysiological studies in anesthetized and awake mice found that the firing rate of complex spikes increased sharply at 3 weeks of age, whereas the firing rate of simple spikes gradually increased until 4 weeks of age (Arancillo et al., 2015; Beekhof et al., 2021), which matches with the onset timing of the behavioral phenotypes in the present study.

Given that cerebellar development is more protracted than that of other brain regions (Bayer et al., 1993), it appears to be more vulnerable for genetic or environmental disruptions, which could ultimately increase the risk for cerebellum-dependent behavior. That could be why many developmental disorders, such as autism, attention-deficit/hyperactivity disorder, and developmental dyslexia, have been suggested to have cerebellar deficiencies (Manto and Jissendi, 2012; S. S. Wang et al., 2014; Leto et al., 2016; R. Wang et al., 2018; Sathyanesan et al., 2018). Disruptions in PC development can lead to deficits later on in life (Badura et al., 2018; Sathyanesan et al., 2018), but the underlying principles are likely complex and currently far from clear. Therefore, it is key to elucidate how cerebellar development contributes to motor control in a systematic and comprehensive manner. Although numerous human studies have been performed to explore the developmental role of the cerebellum in eye movements (Finocchio et al., 1991; Charpiot et al., 2010; Alahyane et al., 2016; Krishna et al., 2018), rodent studies are overtly lacking. By directly examining the relationship between cerebellar development and compensatory eye movement at both the performance and adaptive functionalities, we were able to demonstrate that loss of cerebellar plasticity early in development has significant implications for basal motor behaviors at later stages, in that the third and fourth postnatal weeks seem particularly critical for the development of normal basal VOR function in mice.

Taken together, we here present a comprehensive, quantitative study of the effects of acute and (semi)chronic disruption and recovery of plasticity at various life stages on the setpoint and adaptation of a cerebellum-controlled reflex. The concept of a developmental, critical period to establish the baseline level of reflexes probably has important implications for sensorimotor functions in general. Interestingly, our results only pertain to the VOR not the OKR. The OKR was affected by both adult deletion and adult reintroduction of PP2B, suggesting that its setpoint remains adaptive throughout life or that OKR does not have a setpoint. The latter could be in line with the fact that OKR gain is not only under the control of the cerebellum but also that of, for example, the visual cortex (Liu et al., 2016) and thus has a more extensive control circuit. More work is needed to determine if and how our results can be converted to and possibly generalized across other modalities, including potential nonmotor functions, and, if so, whether it is possible to reset a deficit of developmental origin to normal levels through intervention during later stages.

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