Brain folding is initiated by mechanical constraints without a cellular pre-pattern

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Summary

During human brain development the cerebellum and cerebral cortex fold into robust patterns that increase and compartmentalize neural circuits. Although differential expansion of elastic materials has been proposed to explain brain folding, the cellular and physical processes responsible at the time of folding have not been defined. Here we used the murine cerebellum, with 8-10 folds, as a tractable model to study brain folding. At folding initiation we considered the cerebellum as a bilayer system with a fluid-like outer layer of proliferating precursors and an incompressible core. We discovered that there is no obvious cellular pre-pattern for folding, since when folding initiates, the precursors within the outer layer have uniform sizes, shapes and proliferation, as well as a distribution of glial fibers. Furthermore, although differential expansion is created by the outer layer expanding faster than the core at folding initiation, thickness variations arise in the outer layer that are inconsistent with elastic material models. A multiphase model was applied that includes radial and circumferential tension and mechanical constraints derived from in vivo measurements. Our results demonstrate that cerebellar folding emerges from mechanical forces generated by uniform cell behaviors. We discuss how our findings apply to human cerebral cortex folding.
Introduction

Recent work in brain folding has primarily focused on the cerebral cortex and involved models of differential expansion of elastic materials to drive mechanical instabilities that lead to folding. Current models are able to create three dimensional shapes strikingly similar to the folds seen in the human cortex. However, the cell and tissue level mechanics actually present at the initiation of folding have not been considered or defined. The simple alignment of folds along the anterior-posterior axis of the murine cerebellum and the genetic tools available in mouse allow for precise developmental interrogation, to identify and analyze the cellular behaviors driving growth that could create differential expansion, and to reveal additional critical forces.

The developing cerebellum is distinct from the cerebral cortex, as it has an external granule cell layer (EGL) of proliferating precursors that covers the surface and generates growth primary in the anterior-posterior (AP) direction. A bilayer system is therefore an excellent starting approximation for cerebellar folding. Here we show that cerebellar folding emerges from differential expansion between an un-patterned uniformly expanding fluid-like EGL and an incompressible underlying core. Additionally, we demonstrate that thickness variation in the EGL at the base of the forming fissures, termed anchoring centers (AC), are inconsistent with traditional elastic wrinkling models driven by differential growth. We constrained a recent model that takes into consideration that tension could play an import role in the developing brain and used our in depth developmental data to capture the initiation of cerebellar folding.

No cellular pre-pattern at initiation of folding

To systematically determine the cellular behaviors underlying the initiation of cerebellar folding, we first tested whether regional differences in EGL proliferation rates are present that could influence the folding pattern of the cerebellum, since the EGL drives the majority of cerebellar growth. Proliferation rates (S phase index) were measured in the EGL during folding initiation (E16.5 and E17.5) in the inbred FVB/N strain to reduce variation between samples. First we asked if the regions that will give rise to distinct sets
of lobules have different rates of proliferation that could contribute to the larger and smaller sizes that the lobules ultimately attain. We focused on the anterior cerebellum that divides into a larger region with lobules 1-3 (L123) and smaller region (L45), as well as the central area that comprises lobules 6-8 (L678) of the cerebellum (Figure 1a-b). The more posterior cerebellum does not consistently fold at this stage, thus measurements were not included. Interestingly, we found that the proliferation rates were the same in the three regions at E16.5 (Fig. 1c). The EGL proliferation rate in L678 was slightly reduced compared to the L123 region at E17.5, but no other differences were found (Fig. 1d). Thus proliferation is uniform just before initiation of folding and the small difference found during folding does not correlate with lobule size. This result indicates that lobule size is not determined by modulating the levels of proliferation at the onset of folding. Rather, lobule size could be set by the timing of invagination and the distance between ACs as granule cell precursors do not cross ACs 10.

Each AC is first detected by a regional inward thickening of the EGL 12. We measured the proliferation of the EGL specifically within the forming AC regions to test whether altered proliferation rates could explain the thickenings and therefore the initiation of an AC. We found the rate of proliferation within each forming AC region at E16.5 and E17.5 was the same as in the surrounding EGL (Fig. 1e,f), thus proliferation within all regions of the EGL at the initiation of folding is uniform. Furthermore, regional modulation of proliferation does not form or position the ACs.

At E18.5, after the initiation of folding, we found that the rate of proliferation was significantly lower in the L678 region compared with the L123 and L45 regions (Supplemental Fig. 1a). However, proliferation within the ACs at E18.5 remained uniform with the surrounding regions (Supplemental Fig. 1b). Therefore, as ACs compartmentalize the EGL, regional differences in proliferation rates can arise in lobule regions, which thus could be important for determining the ultimate shapes of the folds.
Changes in cell size and shape have been shown to induce morphological changes\textsuperscript{14-17}. To test if regionally specific regulation in cell shape or size directs folding, we fluorescently labeled cell membranes of scattered granule cell precursors (GCPs) in the EGL using genetics (\textit{Atoh1-CreER\textsuperscript{+/+}; R2\textsuperscript{MTMG/\textsuperscript{+}} mice injected with tamoxifen two days prior to analysis). We then segmented the cells in 3D and quantified their sphericity (Fig. 1g). We discovered that GCPs in the EGL take on a large variation of shapes and sizes at E16.5 and E18.5. However, we found no difference in cell shape in the different lobule regions of the EGL or between the AC areas and the surrounding EGL at either age (Fig. 1h,i). Cell size was uniform at both stages except for a slight reduction in L678 at E16.5 when compared with L123 and the AC regions. However, cell size is reduced at E18.5 (Fig 1 j,k and Supplemental Fig. 1c). Thus, the proliferating GCPs that drive expansion of the EGL have similar shapes and sizes across the lobule regions defined by the first three ACs at folding initiation.

The EGL is traversed by fibers of Bergmann glial and radial glial cells\textsuperscript{18-20}. We tested whether the fibers are distributed in patterns that could locally change the physical properties of the EGL and induce invaginations. Genetics was used to fluorescently label cell membranes of scattered glial cells (\textit{nestin-creER\textsuperscript{+/+}; R2\textsuperscript{MTMG/\textsuperscript{+}} mice injected with tamoxifen at E14.5) (Fig 2a). Fibers crossing the EGL at E16.5 were counted in sagittal slices and aligned relative to the ACs (Fig. 2b). This analysis showed that the Bergmann glial and radial glial fibers are distributed evenly along the AP axis of the EGL, and therefore are not directing the positions where folding initiates based on an uneven regional distribution.

Taken together, our results show that at the initiation of folding, the EGL has uniform cell behaviors and distribution of glial fibers, and thus has no evidence of any pre-patterning in the EGL. Other factors therefore must be guiding cerebellar folding. We chose to test whether there is an emergent tissue-level regulation of folding.

\textbf{Tissue level mechanical forces drive folding}
It is well known that differentially expanding bilayer systems can wrinkle to relax building stress\textsuperscript{21-25}. We reasoned that in the cerebellum the EGL could behave as a quickly expanding outer layer and its attachment to a more slowly growing core could generate forces that result in a wrinkling-like phenotype. To test whether the cerebellum has differential expansion between the two layers, we measured the expansion of the EGL and the core during the time of initiation of folding from midline sagittal sections (Fig 3a-d). The length of the surface of the EGL was used as a measure of the cerebellum surface area and the area of the core as an approximation of cerebellum volume (Fig. 3d), and measurements were made each day from E16.5 through P0. In cross-section the unfolded cerebellum approximates a semicircle, therefore we reasoned that if the cerebellum were to remain unfolded then the ratio of expansion between the length of the EGL and the area of the core should approximate the ratio of the circumference of a semi-circle to its area. Of significance, we found that at E16.5 and E17.5 the ratios of growth between the EGL and core closely approximated the expansion of a semi-circle. However, at E18.5 and P0 the expansion rate of the EGL was greater than the rate of core expansion (Fig. 3e). Thus we uncovered that the cerebellum does indeed go through a phase of differential expansion. We next determined whether differential expansion correlates with folding by calculating a folding index (the convex curvature of the EGL divided by the length of the EGL) at each stage\textsuperscript{26}. Indeed, we found that the cerebellum remains unfolded during the initial proportional expansion between the EGL and core and only folds when the differential expansion is initiated (Fig. 3f). These results provide physical evidence that cerebellar folding is derived from tissue level mechanical forces arising from differential expansion.

\section*{Elastic bilayer models do not account for shape change}

Since cerebellar folding correlates with differential expansion, we tested an elastic bilayer model against our data for how the EGL grows faster than the core\textsuperscript{22}. The resulting wrinkling instability should set up a characteristic lengthscale of the distance between folds as the initial sinusoidal undulations increase in amplitude to ultimately turn into lobules. The folding wavelength will depend on the thickness of the external layer (EGL) and the ratio of the stiffness of the two layers (EGL/core). In particular, for a planar
geometry, with the stiffness of the external layer defined as $E_o$, the stiffness of the core as $E_i$, and the
thickness of the external layer denoted as $t$, the folding wavelength $\lambda$ is given by

$$\lambda = 2\pi t \left( \frac{1}{3} \frac{E_o}{E_i} \right)^{1/3}.$$

If the length of the system is $l$, then the number of folds is

$$n = \frac{l}{\lambda} \propto \frac{l}{t} \left( \frac{E_i}{E_o} \right)^{1/3}.$$

In other words, the number of folds is inversely proportional to the thickness of the EGL.

To more closely model the geometry of the cerebellum, we explored a standard elastic bilayer model in a
circular geometry using the observed ratio of thickness of the EGL to radius of the cerebellum near the
onset of shape change (smooth to wrinkled after E16.5) and invoking a neo-Hookian elastic solid for both
layers. Previous work applied a tri-layer elastic model to the cerebellum, incorporating the molecular
layer. We have not included the molecular layer in our model since it is not present when folding
initiates. The resulting shape change was studied as a function of the ratio of the layer stiffness values
(Fig. 4a). We found that to induce the observed number of folds at initiation of folding through wrinkling
based models constrained by our measurements of the embryonic cerebellum, a large stiffness ratio was
required of around 50. To map the stiffness contrast in the cerebellum we used scanning acoustic
microscopy to measure the bulk modulus of the cerebellum daily from E16.5 to P18.5 (Fig. 4b-c, Supplemental Fig. 2). For small deformations, we expect the instantaneous bulk modulus to be linearly
related to the stiffness and, therefore, the ratio of the instantaneous bulk moduli should scale similarly to
the ratio of stiffnesses (assuming the same Poisson’s ratio). We found that although the EGL has a higher
instantaneous bulk modulus than the core at all stages measured, the ratio was not sufficient to produce a
folding wavelength similar to that in the cerebellum (Fig. 4d). Small modulus contrasts have been
reported for other brain regions with other test modes.
Wrinkling models predict compressive forces in the outer layer. Consistent with this prediction, simulations of cuts through the outer layer and into the inner layer predict that the outer layer should not open (Fig. 4e). Using surgical dissection blades we made radial cuts across the meninges, EGL, and into the core of live E16.5 tissue slices. Time-lapse imaging revealed that unlike the prediction, the EGL opens as well as part of the underlying cut in the core (Fig. 4f-h, Supplemental Fig. 4a-c, and Supplemental Movie 1). This result is consistent with there being circumferential tension within the outer layers of the cerebellum.

An additional aspect of the model that does not fit the biology of the developing cerebellum is that it requires the EGL to be thinnest at the base of each AC, which are the lowest parts of the cerebellar surface, and thus to have an “in-phase” thickness variation. Without this feature, a purely elastic model cannot be in mechanical equilibrium (in the quasistatic limit). However, we previously noted that the EGL is thickest in the ACs when folding initiates, i.e., it has an “out-of-phase” thickness variation. To validate this observation, we quantified the thickness of the EGL and found it to be around 1.2 – 1.4 times thicker in the ACs than in the surrounding EGL at E16.5 and 17.5 when folding initiates (Fig. 5a-d and Supplemental Fig. 3), and the thickness ratio increased to 1.7 times at E18.5 (Fig 5d). It is of interest to note that the final thicknesses of the layers of the cortex of the mature cerebellum, just as in the cerebral cortex, are in-phase. These results further show that traditional wrinkling models cannot capture the initiation of cerebellum folding, and highlight the importance of making measurements at the time of folding rather than when it is complete.

A multiphase model approximates folding

We recently developed a model for folding from differentially expanding bi-layer brain tissues that takes into account the possible contribution of mechanical constraints present in the developing brain. We made five primary assumptions based on the measurements presented above. First, the core is an incompressible material (\( \mu \)), as indicated by our instantaneous bulk modulus measurements. Second we...
utilize a uniform expansion of the EGL ($k_t$) as indicated by the proliferation rate. Third, we do not assume that the EGL is an elastic material. Forth, we assume an elastic component radially and circumferentially to the entire cerebellum ($k_r$), possibly mediated by fibers spanning the cerebellum including those of the Bergmann glia and radial glia as well as the pial surface and meninges covering the cerebellum. And fifth, we posit that the EGL is constrained towards a uniform thickness by fibers spanning the EGL ($\beta$), possibly the Bergmann glia. Given the interplay between incompressible material, compressible fibrous material, and a proliferating fluid-like EGL, this model is multiphase.

We constructed an energy functional parameterized by both the inner and outer boundary of the EGL and incorporating the above five assumptions into three dimensionless parameters ($\mu/k_r$, $k_t/k_r$, $k_t/\beta$). We also constrained the parameters with our developmental data. Minimization of the energy functional yields an equation for a driven harmonic oscillator yielding sinusoidal shapes for both the inner and outer boundary of the EGL. In contrast with the elastic bilayer wrinkling model, EGL thickness oscillations are found to be out-of-phase with the surface height (radius) oscillations when $0 < \mu/k_r < 1$. The ratio of these two quantities, the measured surface height amplitude ($A_r$) and the EGL thickness amplitude ($A_t$) is given by

$$\frac{A_r}{A_t} = \frac{\mu}{k_t} \left(1 - \frac{\mu}{k_r}\right),$$

which need not be $\gg 1$ as is typical of elastic bilayer wrinkling, and the number of initial folds at E16.5 is determined by

$$n = \sqrt{\frac{k_t}{\beta}} \sqrt{1 + \frac{\mu}{k_t}} \frac{1 - \frac{\mu}{k_r}}{1 - \frac{\mu}{k_r}}.$$

Note that in contrast with elastic wrinkling, the number of initial folds does not depend on the thickness (a length scale) of the EGL, but only on material properties.
To test the model, we used the observed EGL thickness amplitude, average EGL thickness, average cerebellum radius, and the number of initial folds at E16.5 to constrain 3 of the 5 model parameters. A 4th parameter \((u/k_r, \text{denoted as } \epsilon)\) was assumed to scale linearly with time and thus generate predictions of cerebellum shapes at later developmental stages (E17.5 and E18.5). We indeed found that the model well-approximates the phase and amplitude behavior of EGL thickness and radius oscillations, during these stages. (Fig. 5e-g, Supplemental Fig. 6).

As the model requires radial tension in addition to the circumferential tension demonstrated above, we examined evidence of radial tension between the EGL and the ventricular zone (VZ) at the initiation of folding. Horizontal cuts were made across the core of live E16.5 tissue slices below the EGL and above the VZ that would cut across anterior radial fibers (Fig 5h). As predicted, after cutting, the tissue relaxed revealing tension directed radially within the cerebellum (Fig. 5i,j and Supplemental Fig. 4 and Supplemental Movie 2). Interestingly, quantification of how the radial and horizontal cuts open revealed that only the horizontal cuts opened along the full length of the cut although they opened more slowly than radial cutes (Supplemental Fig. 4g-j), indicating different stress profiles in the two orientations.

Hierarchical folding involves differential growth

The cerebellum has hierarchical folding in which the initial folds become subdivided. Given that ACs hold their position during development and compartmentalize granule cells within lobules of the EGL\(^{10}\) we reasoned that the ACs could be acting as mechanical boundaries enabling similar mechanics to drive the secondary folding. To test this possibility we measured the expansion of the EGL and the core of the individual lobule regions from E18.5 to P3. We found that indeed in the lobule regions that undergo folding there is a temporal correlation between the onset of sub-folding and differential expansion occurs (Fig. 6a-d). In contrast, the region (L45) that does not fold during the same time period has a different, more rectangular shape, and the ratio of EGL growth to core growth is proportional for a rectangle during the time measured (Supplemental Fig. 5). We propose that ACs create mechanical boundaries that divide
the cerebellum into fractal-like domains with similar physical properties to the initial unfolded cerebellum. Additionally, since ACs compartmentalize granule cells within the EGL lobule regions, once separated the lobule regions can develop distinct characteristics, like the observed differential proliferation rates. We speculate, therefore, that the folding patterns seen across cerebella in different species evolved by adjustment of global as well as regional levels of differential expansion and tension which ultimately mold the functionality of the cerebellum.

Discussion

Here we have provided the first evidence that brain folding emerges from a uniform expansion of cells without obvious pre-patterning. Thus, traditional morphometric cellular behaviors such as changes in cell shape, size and proliferation do not direct where cerebellar folding initiates. Furthermore, our developmental interrogation revealed that thickness variations arise in the EGL that are fundamentally inconsistent with traditional elastic bilayer wrinkling models. By applying a novel multiphase model based on the cellular structure of the cerebellum at the time of folding initiation we were able to capture the shape variations with the correct number of folds. The model accounts for: 1) a rapidly expanding fluid-like EGL, whose thickness is regulated by Bergmann glial fibers, 2) a slower growing incompressible core, and 3) fibrous material in the form of glial fibers (and possibly axons) as well as the meninges that provide radial and circumferential tension (Supplemental Fig. 6). One prediction of the model is that adjusting the amount of tension spanning the cerebellum will change the degree of folding. Indeed, alterations of the cells that likely create tension-based forces could explain the dramatically disrupted folding seen in mouse mutants in which radial glia do not produce Bergmann glia. Without Bergmann glia, the EGL would be expected to not form a layer with regular thickness and it should be more sensitive to variations in radial glial tension. Consistent with this prediction, mutants without Bergmann glia have more localized and less regular folds. Our combination of experimental studies and modeling thus provide new insights into brain folding, including an underappreciated role for tension.
For our multiphase model to predict the observed shape changes in the murine cerebellum from E17.5 to E18.5 the ratio of the core stiffness over the radial tension must increase. As the measured bulk modulus of the core shows no increase during development, our model predicts that the radial tension must decrease during development. While the cerebellum is crossed by many fibers at folding initiation, radial glial fibers are an attractive candidate to mediate this change in radial tension\textsuperscript{31,32}. First, they span from the VZ to the surface of the cerebellum at E16.5. Additionally, during folding initiation the radial glia undergo a transition into Bergmann glia where they release their basal connection to the VZ and the cell body migrates towards the surface\textsuperscript{19}. This transition could lead to a reduction in the global radial tension and thus would be consistent with our model prediction.

Finally it is interesting to note the similarities between the developing cerebellum and the cerebral cortex. Radial glia span the entire cerebral cortex just as in the cerebellum\textsuperscript{33}. Furthermore, species with folded cerebrums have evolved outer radial glial cells with cell bodies near the surface and fibers that are anchored on the surface, similar to Bergmann glia in the cerebellum\textsuperscript{18,34}. Additionally outer radial glial cells proliferate, much like the EGL, to drive the expansion of the outer regions of the cerebral cortex at the time of initiation of folding\textsuperscript{35-37}. Moving the zone of proliferation out from the VZ gives more space for the increased proliferation required in folding systems. The cerebellum, housing 80% of the neurons in the human may be an extreme example requiring the region of proliferation to be completely on the outer surface\textsuperscript{38}. While we have emphasized the notion of tension via glial fibers in the developing cerebellum, axonal tension has been discussed in the context of shaping the developing cerebrum\textsuperscript{39}. Our work calls for a revival of the notion of how tension affects the shape of the developing cerebrum in model mammals like the ferret where experimental testing at the cellular and tissue level are possible. By constraining models with developmental data we will bring about a quantitative understanding of the shaping of the developing brain.
Materials and Methods

Animals.

The inbred FVB/N stain was used for all proliferation rate, area, length, and expansion rate measurements. *Atoh1-CreER*<sup>40</sup>, *Nestin-CreER*<sup>41</sup>, *Rosa26<sup>MTMG</sup>*<sup>42</sup>, were used to quantify cell shape and size as well as fiber distribution and were maintained on the outbred Swiss Webster background. The Swiss Webster strain was used for scanning acoustic microscopy. Both sexes were used for the analysis.

Animals were kept on a 12 hour light/dark cycle and food and water were supplied ad libitum. All experiments were performed following protocols approved by Memorial Sloan Kettering Cancer Center’s Institutional Animal Care and Use Committee.

The appearance of a vaginal plug set noon of the day as Embryonic day 0.5 (E0.5). All animals were collected within two hours of noon on the day of collection. Tamoxifen (Tm, Sigma-Aldrich) was dissolved in corn oil (Sigma-Aldrich) at 20 mg/mL. Pregnant females carrying litters with *Atoh1-CreER/+;R26<sup>MTMG/MTMG</sup>* or *NestinCER/+;R26<sup>MTMG/MTMG</sup>* embryos were given one 20 µg/g dose of TM via subcutaneous injection two days prior to analysis. 25 µg/g of 5-ethynyl-2-deoxyruidine (EDU; Invitrogen) was administered via subcutaneous injection one hour prior to collection.

Tissue processing, immunohistochemistry, and Imaging

For embryonic stages heads were fixed in 4% paraformaldehyde overnight at 4°C. For postnatal animals, the brain was dissected out first before fixation. Tissues were stored in 30% sucrose. For all proliferation, area, length, and thickness measurements brains were embedded in optimal cutting temperature (OCT) compound. Parasagittal sections were collect with a Leica cryostat (CM3050s) at 10µm.

Prior to IHC, EdU was detected using a commercial kit (Invitrogen, C10340). Following EdU reaction the following primary antibodies were used either overnight at 4°C or 4 hours at room temperature: mouse anti-P27 (BD Pharmingen, 610241), rabbit anti-GFP (Life Technologies, A11122), rat anti-GFP (Nacalai
Tesque, 04404-84). All antibodies were diluted to 1:500 in 2% milk (American Bioanalytical) and 0.2% Triton X-100 (Fisher Scientific). Alexa Fluor secondary antibodies (1:500; Invitrogen) were used: Alexa Fluor 488 donkey anti-rabbit, A21206, Alexa Fluor 488 donkey anti-rat, A21208, Alexa Fluor 488 donkey anti-mouse, A21202, Alexa Fluor 647 donkey anti-mouse, A31571. EdU was detected using a commercial kit (Invitrogen, C10340).

For cell size, shape and fiber density analysis 60 μm parasagittal sections were collected on a Leica vibratome (VT100S). Primary and secondary antibodies were diluted 1:500 in 2% milk and incubated overnight at 4°C.

For scanning acoustic microscopy brains were processed for paraffin embedding and parasagittal sections of 10 μm thick were collected on a microtome (Leica RM2255). Structured illumination and confocal Imaging was done with Zeiss Observer Z.1 with Apatome or Zeiss LSM 880 respectively.

Quantification of Proliferation, Length, Area, Folding Index and Thickness

Measurements for all analysis were taken from the three most midline sagittal sections and averaged. The most midline section was determined by dividing the distance in half between the lateral edges where the third ventrical and the mesencephalic vesicle are no longer connected. Quantifications were made using Imaris (Bitplane) and Matlab (Mathworks) software.

EGL Proliferation rate was calculated as EDU+/[(Dapi+;P27-) cells. All cells were counted within the lobule region to the midpoint of the Anchoring Centers. For proliferation measurements through the ACs and the surrounding EGL at E16.5 and E17.5 a 50 μm window measured from the outer surface of the EGL was centered at the AC. The measuring window was centered at every 25 μm anterior and posterior to the EGL for a total distance of 250 μM anterior and posterior to the AC. At E18.5, when the AC is fully formed, everything proximal to the centroid of the cerebellum under the midpoint of the AC was counted.
as the AC. Non-overlapping regions of 50 µm also were measured in either direction for a total of 200 µM anterior and posterior to the AC. Proliferation was measured in 3 cerebella at E16.5 and E17.5 and in 4 cerebella at E18.5.

EGL length was measured from the outer surface of the EGL following the curvature of the EGL. Cerebellar area was calculated as the area within the outer surface of the EGL and the ventricular zone. A short straight edge was made perpendicular to the ventricular zone to close the area back up to the anterior end of the EGL. The convex curvature of the cerebellum was measured by following only the positive curvature of the EGL. The folding index was determined as $FI = 1 - (\text{Positive curvature}/\text{EGL length})$.

Data collected for E16.5, E17.5, E18.5 and P0 came from 6, 8, 7, and 9 cerebella respectively.

EGL thickness was measured by defining the outer and inner curvature of the EGL. The shortest distance lines were drawn to the outer curvature from discrete points distributed at every 12.5 µm along the inner curvature of the EGL. Nine ACs and surrounding regions from five cerebella were quantified at E16.5 and 13 ACs from five cerebellar were analyzed at E17.5. At E18.5 six ACs from two cerebellar were quantified.

Quantification of Cell Shape

Midline sections were imaged with a Zeiss LSM 880. Serial images were taken to cover the entire EGL of lobule regions L123, L45, and L678 and the ACs. Manual cell masks were created with Imaris software defining the curvature at every z-slice. Every cell that was completely included in the imaging window and that was distinguishable from surrounding cells was counted to reduce sampling bias. Cells from three brains were measured at each stage for a total of 131 at E16.5 and 201 at E18.5. Shape was defined via sphericity, which is the surface area of a sphere having the same volume as the cell of interest divided by the surface area of the cell of interest.

Quantification of Fibers within the EGL
Midline sections were imaged with a Zeiss LSM 880. Image tiling was used to cover the EGL. Using Python, a 4th or 5th order polynomial was fitted to the outer edge of the EGL in each image, and five scan lines were positioned at 12.2 \( \mu m \) intervals beneath the surface, and parallel to it. A bin width of 50 \( \mu m \) as measured along the polynomial contour was centered at the AC. Bins of equal distance were extended both anteriorly and posteriorly. Staining intensity was counted along each scan line at every z-slice of the confocal stack. Each image was normalized to the mean intensity and smoothed with a Gaussian filter. Peak counting was done using minimum and maximum filters, keeping neighborhood size and threshold parameters constant for all datasets. The results from the 5 scan lines were averaged.

**Tissue cutting**

Live cerebella of E16.5 FVB/N mice were collected in dissection buffer as previously described\(^{43}\) and embedded in low-melting point agarose (Invitrogen). Sagittal slices at a thickness of 250 microns were collected. Slices were removed from the agarose and placed in petri-dishes coated with Poly(2-hydroxyethyl methacrylate)(Sigma-Aldrich). Tissue cuts were made with a 30° Premier Edge stab knife (Oasis Medical). Slices were allowed to relax for 10 minutes. Time-lapse images were acquired on a Leica MZ75 dissection scope.

**Scanning Acoustic Microscopy**

Mechanical tissue properties were analyzed using a 250 MHz Scanning Acoustic Microscope (SAM), described previously\(^{44,45}\). Briefly, 12 \( \mu m \) paraffin sections of mouse embryonic brains were de-paraffinized, hydrated in de-ionized water and scanned on the SAM to generate maps of amplitude, sample thickness, speed of sound, acoustic impedance, attenuation, bulk modulus, and mass density. Co-registered histology and SAM amplitude images were used to identify regions-of-interest (ROIs) corresponding to the EGL layer and underlying core of the cerebellum in each sample. Bulk modulus was analyzed as a measure of tissue stiffness: ROI measurements were acquired from 3 sections from 3 embryos at each developmental stage.
**Finite element simulations**

The wrinkle of a circular bilayer structure in Fig. 3a was simulated with commercial software ABAQUS. Both film and substrate were modeled as incompressible neo-Hookean materials. The ratio between shear moduli of the film and substrate was 50 and the initial radius of the simulated structure was 16 times that of the film thickness. The differential growth of the EGL and core was modeled by an isotropic expansion of the film in the bilayer structure.

To test the elastic wrinkling model, we conducted finite element (FE) simulations for bilayer structures with a film bonded on a substrate, which represents the EGL layer and core structure, respectively. The structures were assumed to be under 2D plane strain deformation to mimic the quasi-2D nature of cerebellum wrinkles. Neo-Hookean model was adopted to describe the elastic properties of both film and substrate, whose strain energy can be expressed as

\[ U = \frac{1}{2} \mu (I_1 - 3) \]

where \( \mu \) is the shear modulus and \( I_1 \) represents the first invariant of the deformation gradient tensor \( F \).

The Poisson’s ratios for the film and substrate were set to be 0.5, based on experimental observations that the bulk modulus of EGL and core are in the order of GPa, much larger than the shear modulus of soft tissues (~ kPa).

We carried out FE simulations through commercial software ABAQUS. A second order 6 node hybrid element (CPE6MH) was utilized to discretize the film and substrate. Very fine FE meshes were used to make sure the results independent of mesh size. To incorporate differential growth in real EGL layer and core, an isotropic growth deformation tension was applied to the modeled film by decoupling the deformation tensor \( F \) into elastic deformation part \( A \) and growth part \( G \).

\[ F = A \cdot G \]
For simplicity, we assume the growth part is isotropic and controlled by a scalar variable $g$

$$G = g \begin{bmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{bmatrix}$$

where $g > 1$ represents a faster growth in EGL than the core. To trigger instabilities in numerical simulations, random perturbations (e.g., White Gaussian noise with $0.001\sigma$ mean magnitude) were applied to the nodal positions at the top surface of the film and the interface between the film and substrate.

To qualitatively understand the cut experiments we ran a FE simulation of a pre-cut circular bilayer structure and then assigned swelling strain to the film. This neglected the dynamical process in the real cut experiments and only focused on the final equilibrium of the cerebellum after long time relaxation. All the simulation parameters were the same as those in the wrinkling simulation. The initial cut length $a$ is equal to $8t$. The minimum in-plane principal stress corresponds to the hoop stress in the film.

**Methods for modeling initiation of cerebellar folds**

We considered the midsagittal section of the cerebellum and, therefore, formulated a two-dimensional model. The distance of the outer edge of the EGL and, hence, the outer edge of the cerebellum from the center of the cerebellum was defined as $r(\theta)$ with $\theta$ as the angular coordinate. We assumed that $r(\theta)$ was single-valued. The thickness of the EGL was defined as $t(\theta)$. See model schematic below.

Taking into account the four assumptions discussed in the main text, we constructed the following energy functional to be minimized

$$E \left[ r, t, \frac{dt}{d\theta} \right] = \int d\theta \left\{ k_r (r - r_0)^2 - k_t (t - t_0)^2 + \beta \left( \frac{dt}{d\theta} \right)^2 \right\},$$

with $k_r$ as the stiffness modulus (a spring constant in one-dimension) of the radial glial fibers and the pial surface contained in the meninges surrounding the cerebellum since the cerebellar radius is proportional
to its perimeter, $r_0$ as the preferred radius of the cerebellum, $k_t$ denoting a growth potential due to cell proliferation, $t_0$ as thickness of the EGL (cortex), and, $\beta$ quantified the mechanical resistance to changing the thickness of the EGL. Given our first assumption of an incompressible cerebellar core, we imposed the constraint

$$\frac{1}{2} \int d\theta (r - t)^2 = A_0,$$

with $A_0$ as a preferred cerebellar area. We applied the variational principle to minimize the energy functional subject to the core constraint, i.e.

$$\delta (E - \mu \int d\theta (r - t)^2) = 0,$$

where $\mu$ is a Lagrange multiplier. Assuming the preferred radius of the cerebellum is constant and the thickness of the EGL/cortex is also constant, then the preferred cerebellar shape was a circle and the EGL an annulus.

The variational analysis yielded the following equation of shape for $t(\theta)$;

$$\frac{d^2 t}{d\theta^2} + q^2 t(\theta) = \frac{k_t}{\beta} \left( t_0 + \frac{\mu r_0}{k_t \frac{k_t}{1 - \frac{k_t}{k_r}}} \right),$$

with $q^2 = \frac{k_t}{\beta} \left( 1 + \frac{\mu}{k_t \frac{k_t}{1 - \frac{k_t}{k_r}}} \right)$. The solution to the equation of shape was

$$t(\theta) = A_t \sin(q\theta + \phi) + C_1(r_0, t_0, k_r, k_t, \mu),$$
with $C_1$ independent of $\theta$ and $A_t = \sqrt{2} \left(1 - \frac{\mu}{k_r}\right) \frac{A_0}{\pi} - C_2 (r_0, t_0, k_r, k_t, \mu)$ such that $A_0 > \pi C_2$. There was an additional equation of shape for $r(\theta)$ from the variational principle that depended on $t(\theta)$ and so was determined

$$r(\theta) = -\frac{\mu}{1 - \frac{\mu}{k_r}} A_t \sin(q\theta + \phi) + C_3 (r_0, k_r, \mu).$$

We used the measured data at E16.5 to set the parameters to make predictions for the shape of both the EGL and core (and so the relationship between the two) at later times. Plots assumed a circular preferred shape, and with other parameters as follows: $\epsilon = \mu/k_r$ is shown in Fig. 5f, $c = k_r/k_t = 0.06/\epsilon$,

$$A_t/r_0 = \epsilon/9.6, \ t_0/r_0 = \epsilon/4.8, \text{ and } q = 6. \text{ Note that for } \epsilon = 0.3, \text{ these parameters are numerically consistent with our E16.5 measurements: } A_t/t_0 = 0.5 \text{ and } r_0/t_0 = 16, \text{ as well as the observed number of invaginations in the half circle: } q/2 = 3. \text{ All of these parameters are either constant or depend on the time-like parameter } \epsilon. \text{ One of these dependencies has a functional form that is physically justifiable } (A_t \sim \epsilon), \text{ another has a form that is biologically justifiable } (c \sim 1/\epsilon), \text{ owing to the decrease in the number of radial glia over time.}

We defined a dimensionless “shape factor” as half of the perimeter divided by the square root of half of the area as appropriate for a semi-circle. To compare the model’s predictive deviation of this quantity form the semi-circular value we assumed a linear relationship between $\epsilon$ and time $t$ measured in embryonic days: $\epsilon(t) = 0.3(t-15.5)$.

**Statistical analyses**

Statistical analyses were performed using Matlab software. Significance was determined at $P<0.05$. Two-way ANOVA was used for proliferation analysis as two variables were tracked, mouse and region. Cell shape, volume, fiber distribution, EGL thickness and bulk modulus were run under a standard ANOVA.

After ANOVA analysis a multiple comparison was run with Tukey’s honestly significant difference
criterion. F-test for variance and two-tailed student’s paired t-test were used for slice cutting and relaxation quantifications. The degrees of freedom, test statistics, and P values, are given in the figure legends, where appropriate. All error bars are standard deviations. No statistical methods were used to predetermine the sample sizes. We used sample sizes aligned with the standard in the field. No randomization was used nor was data collection or analysis performed blind.

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Author Contributions
A.K.L. and A.L.J. conceived the project; T.E., T.Z. and J.M.S. designed and performed the modeling, A.K.L and A.L.J. designed the experimental research; A.K.L performed the experiments; D.R., M.O., J.M. and D.T. performed the acoustic microscopy experiments and analyzed the data; all authors discussed the data; A.K.L. and A.L.J. wrote the manuscript with contributions from all authors.

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References

1. Tallinen, T., Chung, J. Y., Biggins, J. S. & Mahadevan, L. Gyrification from constrained cortical expansion. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 12667-12672, doi:10.1073/pnas.1406015111 (2014).

2. Ronan, L. *et al.* Differential tangential expansion as a mechanism for cortical gyration. *Cerebral cortex* **24**, 2219-2228, doi:10.1093/cercor/bht082 (2014).

3. Bayly, P. V., Okamoto, R. J., Xu, G., Shi, Y. & Taber, L. A. A cortical folding model incorporating stress-dependent growth explains gyral wavelengths and stress patterns in the developing brain. *Physical biology* **10**, 016005, doi:10.1088/1478-3975/10/1/016005 (2013).

4. Xu, G. *et al.* Axons pull on the brain, but tension does not drive cortical folding. *J Biomech Eng* **132**, 071013, doi:10.1115/1.4001683 (2010).

5. Hohlfeld, E. & Mahadevan, L. Unfolding the sulcus. *Physical review letters* **106**, 105702, doi:10.1103/PhysRevLett.106.105702 (2011).

6. Bayly, P. V., Taber, L. A. & Kroenke, C. D. Mechanical forces in cerebral cortical folding: a review of measurements and models. *Journal of the mechanical behavior of biomedical materials* **29**, 568-581, doi:10.1016/j.jmbbm.2013.02.018 (2014).

7. Lejeune, E., Javili, A., Weickenmeier, J., Kuhl, E. & Linder, C. Tri-layer wrinkling as a mechanism for anchoring center initiation in the developing cerebellum. *Soft matter* **12**, 5613-5620, doi:10.1039/c6sm00526h (2016).

8. Tallinen, T. *et al.* On the growth and form of cortical convolutions. *Nature Physics* **12**, 588-593, doi:10.1038/nphys3632 (2016).

9. Leto, K. *et al.* Consensus Paper: Cerebellar Development. *Cerebellum* **15**, 789-828, doi:10.1007/s12311-015-0724-2 (2016).

10. Legue, E., Riedel, E. & Joyner, A. L. Clonal analysis reveals granule cell behaviors and compartmentalization that determine the folded morphology of the cerebellum. *Development* **142**, 1661-1671, doi:10.1242/dev.120287 (2015).

11. Legue, E. *et al.* Differential timing of granule cell production during cerebellum development underlies generation of the foliation pattern. *Neural development* **11**, 17, doi:10.1186/s13064-016-0072-z (2016).

12. Sudarov, A. & Joyner, A. L. Cerebellum morphogenesis: the foliation pattern is orchestrated by multi-cellular anchoring centers. *Neural development* **2**, 26, doi:10.1186/1749-8104-2-26 (2007).

13. T. A. Engstrom, T. Z., A.K. Lawton, A.L. Joyner, and J.M. Schwarz. Buckling without bending: A new paradigm in morphogenesis. *arXiv* **1806.06961** (2018).

14. Mammoto, T. & Ingber, D. E. Mechanical control of tissue and organ development. *Development* **137**, 1407-1420, doi:10.1242/dev.024166 (2010).

15. Harding, M. J., McGraw, H. F. & Nechiporuk, A. The roles and regulation of multicellular rosette structures during morphogenesis. *Development* **141**, 2549-2558, doi:10.1242/dev.101444 (2014).

16. Stemple, D. L. Structure and function of the notochord: an essential organ for chordate development. *Development* **132**, 2503-2512, doi:10.1242/dev.01812 (2005).

17. He, B., Doubrovinski, K., Polyakov, O. & Wieschaus, E. Apical constriction drives tissue-scale hydrodynamic flow to mediate cell elongation. *Nature* **508**, 392-396, doi:10.1038/nature13070 (2014).

18. Leung, A. W. & Li, J. Y. H. The Molecular Pathway Regulating Bergmann Glia and Folia Generation in the Cerebellum. *Cerebellum* **17**, 42-48, doi:10.1007/s12311-017-0904-3 (2018).

19. Yuasa, S. Bergmann glial development in the mouse cerebellum as revealed by tenascin expression. *Anat Embryol (Berl)* **194**, 223-234 (1996).

20. Yamada, K. & Watanabe, M. Cytodifferentiation of Bergmann glia and its relationship with Purkinje cells. *Anat Sci Int* **77**, 94-108, doi:10.1046/j.0022-7722.2002.00021.x (2002).

21. Richman, D. P., Stewart, R. M., Hutchinson, J. W. & Caviness, V. S., Jr. Mechanical model of brain convolutional development. *Science* **189**, 18-21 (1975).
Nelson, C. M. On Buckling Morphogenesis. *J Biomech Eng* **138**, 021005, doi:10.1115/1.4032128 (2016).

Hannezo, E., Prost, J. & Joanny, J.-F. *Mechanical Instabilities of Biological Tubes*. Vol. 109 (2012).

Shyer, A. E. *et al.* Villification: How the Gut Gets Its Villi. *Science (New York, N.Y.)* **342**, 212-218, doi:10.1126/science.1238842 (2013).

Wiggs, B. R., Hrousis, C. A., Drazen, J. M. & Kamm, R. D. On the mechanism of mucosal folding in normal and asthmatic airways. *J Appl Physiol (1985)* **83**, 1814-1821, doi:10.1152/jappl.1997.83.6.1814 (1997).

Mota, B. & Herculano-Houzel, S. BRAIN STRUCTURE. Cortical folding scales universally with surface area and thickness, not number of neurons. *Science* **349**, 74-77, doi:10.1126/science.aat9101 (2015).

Allen, H. G. *Analysis and design of structural sandwich panels*. 1st edn, (Pergamon Press, 1969).

Zhao, R. & Zhao, X. *Multimodal Surface Instabilities in Curved Film-Substrate Structures*. Vol. 84 (2017).

Budday, S. *et al.* Rheological characterization of human brain tissue. *Acta Biomater* **60**, 315-329, doi:10.1016/j.actbio.2017.06.024 (2017).

Li, K., Leung, A. W., Guo, Q., Yang, W. & Li, J. Y. Shp2-dependent ERK signaling is essential for induction of Bergmann glia and foliation of the cerebellum. *The Journal of neuroscience: the official journal of the Society for Neuroscience* **34**, 922-931, doi:10.1523/JNEUROSCI.3476-13.2014 (2014).

Sillitoe, R. V. & Joyner, A. L. Morphology, molecular codes, and circuitry produce the three-dimensional complexity of the cerebellum. *Annu Rev Cell Dev Biol* **23**, 549-577, doi:10.1146/annurev.cellbio.23.090506.123237 (2007).

Rahimi-Balaei, M. *et al.* Embryonic stages in cerebellar afferent development. *Cerebellum Ataxias* **2**, 7, doi:10.1186/s40673-015-0026-y (2015).

Gotz, M., Hartfuss, E. & Malatesta, P. Radial glial cells as neuronal precursors: a new perspective on the correlation of morphology and lineage restriction in the developing cerebral cortex of mice. *Brain Res Bull* **57**, 777-788 (2002).

Reillo, I., de Juan Romero, C., Garcia-Cabezas, M. A. & Borrell, V. A role for intermediate radial glia in the tangential expansion of the mammalian cerebral cortex. *Cerebral cortex* **21**, 1674-1694, doi:10.1093/cercor/bhq238 (2011).

Hansen, D. V., Lui, J. H., Parker, P. R. & Kriegstein, A. R. Neurogenic radial glia in the outer subventricular zone of human neocortex. *Nature* **464**, 554-561, doi:10.1038/nature08845 (2010).

Heng, X., Guo, Q., Leung, A. W. & Li, J. Y. Analogous mechanism regulating formation of neocortical basal radial glia and cerebellar Bergmann glia. *eLife* **6**, doi:10.7554/eLife.23253 (2017).

Nowakowski, T. J., Pollen, A. A., Sandoval-Espinosa, C. & Kriegstein, A. R. Transformation of the Radial Glia Scaffold Demarcates Two Stages of Human Cerebral Cortex Development. *Neuron* **91**, 1219-1227, doi:10.1016/j.neuron.2016.09.005 (2016).

Andersen, B. B., Korbo, L. & Pakkenberg, B. A quantitative study of the human cerebellum with unbiased stereological techniques. *J Comp Neurol* **326**, 549-560, doi:10.1002/cne.903260405 (1992).

Van Essen, D. C. A tension-based theory of morphogenesis and compact wiring in the central nervous system. *Nature* **385**, 313-318, doi:10.1038/385313a0 (1997).

Machold, R. & Fishell, G. Math1 is expressed in temporally discrete pools of cerebellar rhombic- lip neural progenitors. *Neuron* **48**, 17-24, doi:10.1016/j.neuron.2005.08.028 (2005).

Isaka, F. *et al.* Ectopic expression of the bHLH gene Math1 disturbs neural development. *The European journal of neuroscience* **11**, 2582-2588 (1999).

Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L. & Luo, L. A global double-fluorescent Cre reporter mouse. *Genesis* **45**, 593-605, doi:10.1002/dvg.20335 (2007).
Wojcinski, A. et al. Cerebellar granule cell replenishment postinjury by adaptive reprogramming of Nestin(+) progenitors. *Nature neuroscience* **20**, 1361-1370, doi:10.1038/nn.4621 (2017).

Rohrbach, D., Lloyd, H. O., Silverman, R. H. & Mamou, J. Fine-resolution maps of acoustic properties at 250 MHz of unstained fixed murine retinal layers. *The Journal of the Acoustical Society of America* **137**, EL381-387, doi:10.1121/1.4916790 (2015).

Rohrbach, D. et al. Improved High-Frequency Ultrasound Corneal Biometric Accuracy by Micrometer-Resolution Acoustic-Property Maps of the Cornea. *Translational vision science & technology* **7**, 21, doi:10.1167/tvst.7.2.21 (2018).
Fig. 1: At folding initiation the EGL has uniform proliferation, cell size, and cell shape. 

a, b Low and high power images of immuno-histochemical (IHC) staining of sagittal cerebellar sections to measure proliferation in the lobule (L) indicated at 25 μm windows surrounding the ACs (stars). Scale bar: 200 μm. 

c, d, EGL Proliferation is uniform before and during the onset of invagination (Two-way anova: df = 2. c P = 0.10, F = 4.36 d P = 0.03, F = 10.31). 

e, f, Proliferation in the AC is uniform with the surrounding EGL (Two-way anova: df = 18. e P = 0.03, F = 2.15 f P = 2.1e-3 F = 3.06). 

g, Section of Atoh1-CreER/+; R26MTMG/+ E16.5 cerebellum showing masked labeled cells. Scale bar: 20 μm. 

h, i, Cell shape is uniform before and during folding (anova df = 3. h P = 0.34, F = 1.13 i P = 0.61, F = 0.61). 

j, k, Cell size is uniform (anova df = 3. j P = 3.6e-3 F = 4.75, k P = 0.85, F = 0.26). Stars indicate statistical differences. Error bars: S.D.
Fig. 2: At folding initiation the EGL has a uniform distribution of crossing fibers. a, E16.5
nestin-creER/+; R26\textsuperscript{MTMG}/+ cerebellum section showing IHV labeling of radial and Bergmann glial fibers. Stars: AC. Dotted lines denote EGL. Scale bar: 50 \textmu m. b, Fiber density in the ACs is uniform with the surrounding EGL (anova df = 10; P = 0.76, F = 0.66). Error bars: S.D.
Fig. 3: Cerebellar folding correlates with differential expansion. a-d, H&E stained midline sagittal sections of FVB/N mice. Stars: ACs. Brackets: anterior/posterior ends of the EGL. Black line and red line in (d): EGL and convex length, respectively. Shaded area: core. e, At E16.5 and E17.5 expansion of EGL length and cerebellar area fit the proportional expansion of a semi-circle (curve). At E18.5 and P0 EGL expansion is greater than core area growth creating differential expansion. f, Folding index [1 - (convex length/EGL length) x 100] reveals folding initiates during differential expansion. Scale bars: 200 µm.
**Fig. 4:** Measured tissue stiffness and stress at folding initiation are inconsistent with wrinkling models. 

- **a,** Inducing the correct number of folds through a wrinkling model require a stiffness differential between the layers of 50 fold ($\mu_f/\mu_s = 50, g = 1.05$).

- **b,** Acoustic mapping of cerebellar slices show a stiffer EGL than core at each stage (anova df = 5; $P = 1.0e^{-4} F = 13.59$), but not the required differential. Stars indicate statistical differences. Error bars: S.D.

- **d,** Wrinkling simulations constrained by developmental data produce wavelengths inconsistent with the cerebellum.

- **e,** Simulations predict the EGL remains closed after cutting.

- **f,g,** Images of a live cerebellar slice before and after cutting, and images from time lapse movie, **h,** show the EGL opens, revealing circumferential tension along the EGL. Red and yellow dots: cut edges. Lines: relaxation angle.
**Fig. 5:** A multiphase model with radial and circumferential constraints and liquid-fibrous EGL composition approximates cerebellar folding.  

**a.** Staining of nuclei with EGL outlined and lines used to measure thickness.  
**b-d.** EGL thickness at the ACs increases during folding initiation (anova E16.5 df = 29, $P = 8.2 \times 10^{-20}$ $F = 12.59$. E17.5 df = 29, $P = 3.4 \times 10^{-116}$ $F = 62.78$, E18.5 df = 57, $P = 6.8 \times 10^{-67}$ $F = 13.28$). At E16.5 only brains with visible ACs were included. Error bars: S.D.  
**e.** Schematic of multiphase model showing types of tension.  
**f.** Thickness variations arise concomitant with folding.  
**g.** Shape factor analysis: semicircle (red), model shape prediction (blue) measured shape (black). Assumed linear relationship between $\varepsilon$ and time, $\varepsilon(t) = 0.3(t - 15.5)$.  
**h-j.** Tissue cutting reveals radial tension between the EGL and the VZ. Red and yellow dots: cut edges at $t = 0$ and $t = 10$. Lines: relaxation angle.
Fig. 6: Progressive folding occurs during regional differential expansion. 

a, b, H&E stained midline sagittal sections of FVB/N cerebella at P1 and P3. 

c, Expansion of lobule length and lobule area approximate the proportional expansion of a semi-circle (curve) at E18.5 and P0. After P0 the EGL expansion in both regions increases more than the underlying area creating differential expansion. 

d, Folding initiates during regional differential expansion. Scale bars: 200 µm.
Supplemental Fig. 1: Proliferation rate is reduced in the central zone of the cerebellum after folding initiation. 

a, Proliferation is reduced in lobule region L678 compared to other lobules (anova: df = 2; P = 0.01, F = 9.24). 

b, Proliferation through the AC regions is uniform with the surrounding EGL (anova: df = 10; P = 0.17, F = 1.64). 

c, At E18.5 cells within the EGL are smaller than at earlier stages (two-tailed t-test df = 330 P = 2.92e-20, T = 9.85). Stars: statistical differences. Error bars: S.D.
Supplemental Fig. 2: Examples of the regions measured regions by acoustic microscopy. a-f, Examples of the Region of Interest (ROI) measured for each for core and EGL. h-j, Representative bulk modulus maps.
Supplemental Fig 3: EGL thickness increases in the ACs during the initiation of folding. Only E16.5 cerebella that showed regional thickening in the geometry where ACs normally arise were used for the measurements, and one embryo did not yet have an AC3. a-c, Thickness variation in and surrounding AC1 (anova a, df = 17, P = 0.13, F = 1.55 b, df = 29, P = 7.0e−14 F = 6.82 c, df = 57, P = 9.1e−11 F = 4.05). d-f, Thickness variation in and surrounding AC2 (anova d, df = 17, P = 0.08 F = 1.74 e, df = 29 P = 3.9e−22 F = 11.88 f, df = 57 P = 2.9e−35 F = 16.35). g-i, Thickness variation in and surrounding AC3 (anova g, df = 17, P = 0.59 F = 0.89 h, df = 29, P = 2.4e−17 F = 9.81 i, df = 57, P = 7.6e−33 F = 14.57). Error bars are S.D.
Supplemental Fig. 4: The stress patterns within the cerebellum are different between the EGL and the VZ. a-c, Example of a live cerebellar slice before a and after b a radial cut through the EGL, and still images from a time-lapse c. Time = 0 minutes is at the time it takes to remove the knife and start the imaging, therefore the cut has already begun opening. d-f, Example of a live cerebellar slice before d and after e a horizontal cut through between the EGL and ventricular zone (VZ), and still images from a movie f. g-i, radial cuts through the EGL open more quickly initially than horizontal cuts between the EGL and the Ventricular zone, but the latter continue to relax for longer (g, f-test for unequal variance $P = 0.09$, two tailed t-test $df = 16$, $p = 0.03$, $T = -2.43$; h, f-test $P = 0.04$ and unequal variance two-tailed t-test $df = 12.8$, $P = 0.16$, $T = -1.48$; i, f-test $P = 0.49$ and two tailed t-test $df = 16$, $P = 0.03$, $T = 2.43$). j The degree of opening is tightly related to the length of the opening in horizontal cuts but not in radial cuts (f-test $P = 0.02$, unequal variance two tailed t-test $df = 11.89$, $P = 0.02$, $T = -2.80$). Stars: statistical differences. Error bars: S.D.
Supplemental Fig. 5: L45 region has a columnar starting shape, proportional expansion, and does not undergo folding from E18.5 to P3. a,b, Low and high power images, respectively, of H&E stained cerebellar sections of FVB/N showing L45 is columnar, whereas L123 and L678 are semi-circular. Scale bar is 200 µm. c, Lobule length and area expansion of L45 are proportional to the expansion of a rectangle with a semi-circular top (cartoon). The height of the rectangle expands 5 times as much as the width. d, The folding index shows no folding during proportional expansion. Scale Bars: 200 µm
Supplemental Fig. 6: Uniform cell behaviors within a fluid-like EGL create differential expansion based folding approximated by a multiphase model with radial and circumferential tension. 

**a**, Cartoon of E16.5 cerebellum showing the EGL (dark purple) overlying the incompressible core (light purple) with fibers (lines) of radial glia (closed ovals) and Bergmann glia (open ovals) crossing the cerebellum and the EGL. 

**b**, Map of stress within the cerebellum at the onset of foliation. 

**c**, Schematics showing that an AC is first detected as a regional inward thickening of the EGL (left). The constraining tensions shape the fluid-like EGL such that the EGL becomes thicker at the AC (middle). As radial glia transition to Bergmann glia, modeling predicts a reduction in radial tension (right). 

**d**, Since ACs hold their position in space, and compartmentalize the cells within the EGL, we propose that they behave as mechanical boundaries allowing local domains of differential expansion to arise and progressive folding to occur.
**Supplementary Movie 1: Live slice cutting and relaxation reveals**

circumferential tension along the EGL. Time-lapse movie shows relaxation of live tissue slice after cutting radially through the EGL and into the underlying core. Images were acquired every 10 seconds for 10 minutes. The time-lapse was started moments after the tissue was collected in frame after the cut. The slice shown in the video is the same as in Fig. 4f-h.

**Supplementary Movie 2: Live slice cutting and relaxation reveals radial tension between the EGL and the VZ.** Time-lapse movie shows relaxation of live tissue slice after cutting horizontally into the core between the EGL and the VZ. Images were acquired every 10 seconds for 10 minutes. The time-lapse was started moments after the tissue was collected in frame after the cut. The slice shown in the video is the same as in Fig. 5h-j.