Condensation of the *Drosophila* Nerve Cord is Oscillatory and depends on Coordinated Mechanical Interactions

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Summary

During development, organs reach precise shapes and sizes. Organ morphology is not always obtained through growth; a classic counterexample is condensation of the nervous system during *Drosophila* embryogenesis. The mechanics underlying such condensation remain poorly understood. Here, we characterize the condensation of the embryonic ventral nerve cord (VNC), at both subcellular and tissue scales. This analysis reveals that condensation is not a unidirectional continuous process, but instead occurs through oscillatory contractions. The VNC mechanical properties spatially and temporally vary, and forces along its longitudinal axis are spatially heterogeneous. We demonstrate that the process of VNC condensation is dependent on the coordinated mechanical activities of neurons and glia. These outcomes are consistent with a viscoelastic model of condensation, which incorporates time delays and effective frictional interactions. In summary, we have defined the progressive mechanics driving VNC condensation, providing insights into how a highly viscous tissue can autonomously change shape and size.

"What utilitarian goal has nature pursued in forcing nervous system differentiation to these lengths?

The refinement and enhancement of reflex activity, which protects the life of both the individual and the species" (Cajal, 1899).

INTRODUCTION

Morphogenesis proceeds as a result of changes in cells proliferation, adhesion, differentiation and survival, and it is also the subject of mechanical inputs (Heisenberg and Bellaïche, 2013; Hogan, 1999; Weber et al., 2011; Zhang and Labouesse, 2012). Further, during ontogenesis, all organs develop in synchrony to reach physiological optimization (Oliveira et al., 2014). In this scenario, how mechanics influences the final shape or size of an organ remains far from clear (Heisenberg and Bellaïche, 2013; LeGoff and Lecuit, 2015; Saunders and Ingham, 2019). A critical issue is that mechanical processes must be highly coordinated, while also accounting for geometric and scaling constraints (Amourda and Saunders, 2017).

Biological tissues display both elastic and viscous properties and are, in many cases, mechanically heterogeneous both in space and time (Serwane et al., 2017). They are constituted by active materials, and so standard equilibrium biophysical approaches are often insufficient to describe their behaviors. The material properties of tissues are thus key for the development of the organism (Mammoto and Ingber, 2010; Miller and Davidson, 2013; Mongera et al., 2018). However, understanding how the material properties of tissues impact the building and shaping of organs during development remains an open question.

Precise tissue organization is especially relevant when considering the functional complexity of the Central Nervous System (CNS) (Redies and Puelles, 2001). The complex architecture of the mature CNS is achieved through a well-known sequence of cellular events (Roig-Puiggros et al., 2020; Tessier-Lavigne and Goodman, 1996). At the local level, tension forces contribute to the formation and maintenance of active synapses and the stabilization of neurites (Anava et al., 2009; Kilinc, 2018). They also influence the shortening of neuronal processes, thus contributing to circuitry compactness (Franze, 2013). However, it is unknown which mechanical processes, at the tissue-scale, are involved in the spatial organization of neural architecture.

Here we fill this knowledge gap by determining how mechanical forces translate into tissue level sculpting of the entire *Drosophila* embryonic ventral nerve cord (VNC) during its condensation. The embryonic CNS is built stepwise by neuroblasts that delaminate from the neurectoderm in an invariant pattern, generating a diverse population of neurons and glia (Hartenstein and Wodarz, 2013). Neurons are unipolar and project their axons towards the neuropil. Cohesive axon bundles travel together and branch in the same or closely adjacent neuropil compartments, creating stereotyped segmental structures (Landgraf et al., 2003; Technau, 2008). Axon tracts include three longitudinal connectives that pioneer the neuropil of the VNC, and transverse pioneer commissures establishing contralateral connections (Lin et al., 1994). Neurons are supported by a complex scaffold of glia, which builds a meshwork of cortex processes required for stabilizing neurons' positions (Beckervordersandforth et al., 2008). Macroscopically, the VNC exhibits a dramatic late shortening that further progresses in larval stages (Campos-Ortega and Hartenstein, 1985; Olofsson and Page, 2005; Page and Olofsson, 2008). It is worth noting that changes in embryo length do not substantially alter VNC condensation (Tiwari et al., 2021). From an architectural viewpoint, the mechanisms modulating how the CNS gets shaped and how its composing elements are brought together into a mechanically stable functional structure are unknown.

To analyze the VNC condensation dynamics across scales, we used four-dimensional confocal and light-sheet microscopy along with advanced image analysis. Velocity and strain maps revealed a complex morphogenetic kinematic, comprising alternate active and passive periods. Condensation, during the active phases, proceeds centripetally from both ends of the VNC and exhibits local oscillatory behavior. Further, spatial and temporal quantifications of material stiffness showed that the VNC displays a correlative, segmentally iterated, tensional landscape and stereotyped material stiffness inhomogeneities. We built a viscoelastic model and revealed that the periodic oscillations are consistent with the different viscous and elastic mechanical behaviors observed during tissue condensation. The combined experimental and theory results show that large-scale mechanical forces are essential for condensing and shaping the VNC. Its final shape depends on the concerted actions of neurons and glia through the dynamic modulation of their cytoskeleton. Overall, this work reveals that the nervous system behaves as a solid viscoelastic tissue and that its biomechanical properties are key, in concurrence with a complex series of coordinated cellular actions, for its morphogenesis.

RESULTS

VNC cytoarchitecture

The structural organization of the embryonic VNC has been described in detail (Landgraf et al., 2003; Sanchez-Soriano et al., 2007; Zlatic et al., 2009). The axonal scaffold links repeated neuromere units and displays iterated transversal commissures and longitudinal tracts. Almost every neuron has been mapped and their lineages identified. To characterize the mechanical properties of the VNC, we first monitor the allocation of cell bodies and the distribution of cytoskeletal components.

We performed a cross-correlation analysis, employing the pan-axonal marker acetylated tubulin, to define the 3D organization of the VNC axonal network. This study revealed a pattern of axonal assemblies segmentally iterated along the anterior posterior (AP) axis, which could serve as anchoring architectural nodes (**Figure S1A** and **Movie S1**). We also found that neuronal cell bodies, from early stages, arrange along the AP axis in a periodic, contralaterally symmetric, segmental pattern, with most cells accumulating at the VNC ventral side (**Figure S2A-C**). This segmental periodicity is lost as the 3D topology consolidates.

The stereotyped architecture of the VNC (cell density and axonal scaffold) associates to a discrete mesoscopic distribution of cytoskeletal components. Microtubules uniformly distribute along all axonal protrusions, while Non-Muscle Myosin (NMM - Myosin II) and Actin (Phalloidin) show distinct distributions (**Figure S1B-C** and **Movie S1**). Actin periodically accumulates at intracommissural areas (and at the nodes) in each segment and Myosin II builds up along contralateral single-cell domains at the dorsomedial edge of the neuropile. Both, also, decorate longitudinal components.

VNC condensation dynamics

To understand *Drosophila* VNC condensation mechanics, we characterized its progression *in vivo,* from the initiation of germ band retraction to larval hatching. Midway through embryogenesis, the VNC undergoes a dramatic compaction along the AP axis, shortening from over 700 to less than 250 µm (**Figure S2D** and **Movie S2**). This process depends on different cellular: the remodeling of the extracellular matrix (ECM) by

hemocytes; the cytoskeletal dynamics of glia and neurons; and regulated apoptosis (Evans et al., 2010; Olofsson and Page, 2005; Page and Olofsson, 2008). We live-imaged Fasciclin 2 (Fas2)-GFP embryos (Buszczak et al., 2007) by confocal microscopy and embryos expressing the nuclear marker Histone2A-mCherry by light-sheet microscopy (Krzic et al., 2012). Importantly, to reconstruct the VNC 3D morphology from stage 16 onwards we had to overcome the embryos movements and we developed an image processing pipeline that "detwitched" embryos digitally re-locating the VNC along the central midline at each time-point from *in toto* light-sheet images **(Figure 1A, Movie S2** and STAR methods**)**.

We generated length and velocity profiles for the VNC throughout condensation **(Figure 1B)** that revealed that it proceeds in five dynamic steps. First, the VNC pulls back, in parallel to the retraction of the germ band, until its posterior end positions near the tip of the embryo (compaction phase 1 – CP1). The condensation speed follows that of the germ band (Lynch et al., 2013). Second, the VNC reaches an almost stationary phase by the end of germ band retraction (end of stage 13). This phase lasts up to the end of dorsal closure and head involution by late stage 15 (pausing phase 1 – PP1). Third, the VNC, uncouples from the epidermis, and actively contracts (compaction phase 2 – CP2). Fourth, condensation rest again (pausing phase 2 – PP2). Last, the VNC undergoes a final slow progressive compaction, concurrent with peristaltic embryo movements, up to the end of stage 17 (compaction phase 3 – CP3). Variability in VNC length between embryos is very small (<10%) except during CP1 (see also (Tiwari et al., 2021)), which highlights the robustness of the condensation process, structured in active and passive phases **(Figure 1B)**.

VNC condensation is oscillatory

At its onset, VNC condensation passively follows the movements of the germ band. The successive phases of contraction are, on the other hand, active processes. We undertook an analysis of these late steps quantifying, using particle image velocimetry (PIV) (Vig et al., 2016), the local velocities along the whole length of the VNC (phases CP2, PP2 and CP3) **(Figure 1C**, **Movie S2)**. Remarkably, we found that, both during the CP2 and CP3 phases, condensation is oscillatory, with contractile periods of around 30 minutes. The frequency of the oscillations is quite regular, while their amplitude varies.

Oscillations with opposing directionalities are present at the anterior and, prominently, at the posterior of the VNC **(Figure 1C)**. They lead to the bidirectional convergence of the VNC towards a central stationary domain between the third thoracic and the first abdominal segments (**Figure 1D** and **Movie S2**).

We conclude that the active periods of VNC condensation are not monotonic. Tissue-scale oscillations suggest a complex spatiotemporal mechanical coordination across the whole tissue. We hypothesize that the dynamic combinatorial activities of the glia and neurons leads to this complex tissue-scale behavior.

Material properties of the VNC vary both spatially and temporally

The complexity of VNC condensation kinematics hints to potential spatiotemporal changes in its material properties. To evaluate tissue stiffness, we used Atomic Force Microscopy (AFM). The elastic Young' modulus (E), a stiffness proxy, was measured segment-to-segment at the midline and at the lateral neuropile of stage 14 and late stage 16 embryos (**Figure 2A-C** and STAR methods).

At stage 14 of embryogenesis (PP1), E was 0.08 ± 0.01 KPa (mean ± SD, n=15) in the midline, and 0.06 ± 0.01 KPa in the lateral regions (abdominal segments 1 to 5) **(Figure 2B)**. No statistical differences were found either between midline and lateral positions or along the AP axis ($p > 0.05$). At late stage 16 (CP2), the midline stiffness increased significantly when compared to the lateral cortex (0.17 ± 0.03 KPa vs 0.06 ± 0.02 KPa (n=15) ($p < 10^{-3}$). The central domain of the embryonic VNC, where most axons bundle, becomes more rigid than the lateral domains, where the somata are predominantly located. We also found that stiffness decreased towards the most posterior segments (**Figure 2C**; see also **Figure S3**).

In summary, the *Drosophila* embryonic VNC is extremely soft, consistent with previous measurements in neural tissues from different organisms (Spedden and Staii, 2013). Further, our results indicate that the neural tissue stiffens with time in an axially graded fashion, as also observed in neural crest morphogenesis (Barriga et al., 2018; Shellard and Mayor, 2021).

The tensional landscape of the VNC is temporally and spatially patterned

To infer large tissue scale forces, we first measure local strain rates. Strain rates measure how rapidly neighboring regions move relative to each other (Petridou and Heisenberg, 2019). They determine the resulting tissue stresses, which also depend on the viscoelastic tissue properties; its bulk viscosity and the local shear modulus (STAR Methods). The strain rate maps reveal that tissue deformations get restricted to specific subdomains of the VNC (**Figure 2D** and **Movie S2**). At the active CP2 and CP3 phases, the strain rates display alternating positive and negative values along the AP axis. The strain rate has a marked change in magnitude immediately after the second pause phase, slowly tending back towards zero. Distinct strain rate domains appear to correspond to iterative contractile regions repeated along the VNC. These regions map to the space between the posterior commissure of one neuromere and the anterior commissure of the next (intercommissural) **(Figure 2E)**.

Together, the strain rates and AFM data suggest that the VNC is mechanically organized in repeated units, and its mechanical properties temporally modulated. To evaluate these propositions, we studied the VNC response to mechanical perturbation. We utilized laser microsurgery to sever the VNC at specific times and positions (**Figure 3A**, **Figure S4A** and STAR methods). Cutting transversally to the AP axis in the intercommisural domains between the abdominal neuromeres of stage 11-14 embryos resulted in an isotropic recoil faster than in intracommissural regions (**Figure 3B**, **Figure S4B** and **Movie S3**). Thus, early (PP1), the intercomissural domains appear to be under significantly higher tension than the intracommissural. By contrast, tissue recoil during late condensation (CP2/PP2 - stages 16-17), was lost **(Figure 3C)**. Importantly, severing an individual intercommissural space does not affect the condensation of adjacent neuromeres that continue to condense (**Figure 3D** and **Movie S3**); they act as independent units.

Laser cuts also enable by analyzing the recoil rate (see (STAR Methods)) an approximate characterization of the viscoelastic properties of the VNC. By severing the intercommissural domains at different time points we found a strong reduction of contractility and viscosity as the VNC condenses **(Figure 3E)**. Though we cannot discount possible differences between the inter- and intracommissural domains, such variations in viscosity are likely negligible as their tissue composition is equivalent.

We next matched the iterated architectural organization of the VNC and its biophysical properties (stiffness and viscosity) and found they spatially correlate with the distribution of cytoskeletal components (actin and Myosin II) (**Figure S1**). This suggests that the

dynamics of the actomyosin cytoskeleton could be crucial in the modulation of the VNC viscoelastic properties and condensation progression.

Last, to evaluate stress patterns along the VNC, we constructed a three-dimensional Finite Element (FE) model. We mapped the measured velocity field onto this model and reconstructed the strain and stress fields (**Figure 3F**, **Figure S4C-D**, **Movie S4** and STAR Methods). The evolution of the stress profiles along the AP-axis and the superposition of the stress minima (compression) onto the phase contrast kymographs, confirmed that maximum compression occurs at the intracommissural domains. Further, the active stress increased over time in the intercommissures **(Figure S4E)** pointing to a potential scenario in which the distribution of tension reflects the spaced contractions of the tissue. Segments contract as units directing condensation progression.

Oscillations are an emergent property of a viscoelastic tissue

The contraction of the intercommissural domains in between neuromeres explains the condensation of the VNC, but how do they coordinate? Can this coordination explain the origin of the global oscillations? To tackle these problems, we developed a one-dimensional rheological model that incorporates the viscoelastic properties of the VNC along with a delayed active contractility. At a particular time, *t*, the VNC is taken to have a rest length, *L(t)*. This internal variable depends on time, as the system gradually condenses. We define $\Delta L(t)=l(t)-L(t)$ as the difference in VNC length at time *t* from its rest length. The change in the rest length as a function of time would be

$$\frac{dL(t)}{dt} = \gamma \Delta L(t - \delta t) \qquad \text{Eq. (1)}$$

where γ is the remodelling rate, which measures the rate at which the tissue adapts its rest length and δt represents the time delay between the current strain measure ΔL and the active remodeling of the VNC through its rest-length *L*.

The VNC is surrounded by the neural lamella (Meyer et al., 2014) and it is connected early to the underlying epithelia and late by intersegmental and segmental nerves to the developing muscles and peripheral sensory organs. We then incorporated potential effects of surrounding tissues adding a frictional term proportional to the apparent VNC length rate *dl/dt,*

$$-\eta \frac{dl(t)}{dt} = k_1 \cdot \Delta l(t) + k_2 \cdot \Delta L(t) \quad \text{Eq. (2)}$$

where η is the friction coefficient, k_1 is the purely elastic component of the VNC, $\Delta l(t) = l(t) - l_0(t)$, where l_0 is the characteristic elastic length scale, and k_2 represents the stiffness of the viscoelastic component of the VNC, with a dynamic rest-length $L(t)$. (**Figure 4A**). The combination of Eqs. (1) and (2) yields a viscoelastic model with a delayed viscous response, which has the ability to exhibit oscillatory behavior (Dawi and Munoz, 2021).

We utilized our above quantitative measurements to constrain our model parameters. The time delay was chosen such that similar frequencies to the experimental ones were obtained *in silico*, when considering the measured stiffness and viscosity. In fact, the period of the oscillation is proportional to the delay (Muñoz et al., 2018), which allows us to define the delay value corresponding to the observed oscillation period. Our chosen values of viscosity and stiffness matched the characteristic time of the tissue, between 5 and 15 s, which in our model is equivalent to the factor $\eta/k_2 \sim 8$ s (see **Figure 4A**).

Similar rest-length models have been used in the context of embryogenesis (Cavanaugh et al., 2020; Doubrovinski et al., 2017; Sumi et al., 2018), epithelia remodeling (Clement et al., 2017; Staddon et al., 2019) and stress relaxation of monolayers (Khalilgharibi et al., 2019). The stability of such models with the delay rheology in Eq. (1) considering environmental viscous effects has only recently been analyzed (Dawi and Munoz, 2021).

Eqs. (1)-(2) form a system of Delay Differential Equations that can be analyzed through their characteristic equation (Erneux, 2009; Stépán, 1989) or numerically. Depending on the parameters η, γ, δt, k_1 or k_2, the apparent length $l(t)$ can exhibit either a stable regime (with no oscillations or oscillations showing a diminishing amplitude) or unstable oscillations (with increasing amplitude). The phase diagram in **Figure 4B** shows that decreasing values of k_2 render the system unstable, while decreasing values of viscosity η and γ render more stable oscillations. These results are consistent with the stabilization of the VNC as its stiffness increases **(Figure 2B-C)** and its viscosity is progressively reduced **(Figure 3E)**. The kymograph, in **Figure 4C,** shows an example of the stable oscillatory regime (see **Figure S5A-D** for other scenarios). Overall, our reduced one-dimensional model can explain the emergence of periodic contractions as a consequence of time delays conveyed by the material properties of the VNC and the effective friction

between the neural cortex and the surface glia. As the VNC stiffens during development, these oscillations are stabilized, ensuring the condensation of the VNC.

As described, the results above are based, assuming some simplifications, on parameters values matching the experimental data. We then tested how robust our model was to parameter variation. To analyze the sensitivity of the VNC condensation to changes in the viscoelastic and regulatory parameters (γ, k_1, k_2, η and δt) and to define the range compatible with an oscillatory regime, we performed *in silico* simulations, jointly analyzing changes in amplitude and frequency of oscillations and the effective condensation (**Figure 4D (i-v)**). We found that viscous values under $15*k_2$ or a time delay below ~15 s prevent the appearance of oscillations, or, at least, strongly reduce their amplitude, in the final stages of condensation. The frequency of the oscillations is in general unaffected, except for the delay δt, where frequencies increase upon reduction of the time-lag.

In our simulations, we found that both condensation and tissue oscillation were sensitive to γ (which represents the effective rate of cell remodeling to contractile forces and is probably dependent on actomyosin dynamics). A reduction of γ by only 10% led both to condensation defects and alterations of oscillatory patterns (**Figure 4D (i)**). The sensitivity of the model to changes in γ is consistent with our observations above on the distribution of actomyosin cytoskeleton components. These results suggest that actomyosin activity plays a significant role on the mechanics of VNC condensation.

VNC condensation requires significant mechanical contribution from glia

Can differences in material properties and emergent oscillations be connected to cell behaviors? Considering the complex mechanics of VNC condensation, we next asked if neurons or glia - play a mechanically active part in modulating tissue scale behavior and, if they do, we aimed to determine their effects on the VNC material properties. We genetically ablated either neurons or glia by overexpressing the proapoptotic gene Grim (Chen et al., 1996) employing the pan-neural Elav-Gal4 and the glia Repo-Gal4 drivers **(Figure 5A-D)**.

Excessive neuronal cell death heavily distorted the organization of the axonal scaffold **(Figure 5A)**, and, to a lesser extent, VNC condensation **(Figure S6B)**. Yet, apoptosis is

a slow process and, possibly, late neurons, born halfway during condensation, could escape death. Neuron elimination was not fully penetrant and many ELAV positive cells were negative for Dcp1 (dying cells marker) (**Figure S6A**). No spurious apoptosis was detected, although glia mis-positioned, probably in response to steric constrains resulting from alterations in axonal morphology **(Figure 5B)**.

Expressing Grim in glia promoted slight alterations in the 3D axonal scaffold **(Figure 5C)**. Contrary to neurons, most of the glia was removed **(Figure 5D)**, which resulted in a strong failure of the condensation process **(Figure S6B)**. Loss of glia also altered the VNC shape (**Figure 5E** and **Movie S5**). We further studied glia depletion employing light sheet microscopy (**Figure S6C** and **Movie S5**) and found that in its absence, condensation arrests at the last contraction phase (CP3) **(Figure 5F)**. PIV analyses revealed a substantial reduction of VNC strain rates and the loss of contractile oscillations **(Figure S6D)**.

Last, we explored by AFM the impact of neurons or glia on the VNC material properties. AFM measurements were performed at the stationary PP1 (stage 14, **Figure 5G**) and active condensation CP2 phases (stage 16, **Figure 5H**). Before active contraction, VNC rigidity slightly decreased at the midline after neuron ablation but it was not affected by glia depletion. On the contrary, during the active CP2 phase, significant softening upon glia removal was observed, both at the midline and at lateral positions, while neuronal ablation had no effect (**Figure 5H**).

From the stability diagram of our rheological model (**Figure 4B),** we infer that, as the VNC condensation progresses, the viscoelastic parameters, rigidities k_1 and k_2 and viscosity η, proceed from a sector where oscillations tend to increase to a sector where oscillations tend to diminish. Further, the sensitivity diagrams (**Figure 4D (ii-iv)**) indicate that an increase in rigidity causes a resistance to condensation that results in larger final VNC relative length. This is in accord with the slowdown of shortening (**Figure 1B**) and increase of stiffening (**Figure 2B-C**) observed as condensation progresses.

The sensitivity analyses do not predict a shortening of the VNC relative length upon drastic reduction in rigidity (**Figure 4D (ii-iii)**), opposite what it is found after glia removal (**Figures 5G-H**). In this case, however, softening affects oscillations, as shown on the stability diagram (**Figure 4B**), and this will eventually disrupt condensation (**Figure 5F**). On the other hand, when rigidity is only mildly disturbed, as occurs after

neuronal ablation (**Figures 5G-H**), the rheological model predicts that the oscillatory response would remain largely unaffected as it happens.

In summary, both glia and neurons contribute to the active contraction of the VNC and modulate its material properties. However, while glia has a major contribution on both of these aspects, the impact of neurons appears to be more subtle; they mainly influence the structural organization of the neuropile and not VNC material properties.

Myosin-mediated contractility in neurons and glia is required for VNC condensation

VNC condensation is an active process demanding mechanical efforts. To evaluate the mechanical impact that the active cytoskeleton may have in condensation, we analyzed actomyosin contractility in both neurons and glia.

We found that Zipper (Zip) (Non-Muscle Myosin II heavy chain) knockdown led to distinct spatiotemporal alterations on VNC condensation dynamics (**Figure 6** and **Movie S6**). Abolishing neuronal contractility by pan-neural expression of a RNAi transgene (Elav-Gal4/UAS-Zip RNAi) resulted in major defects in the structural scaffold and in condensation failure from CP2 onwards **(Figure 6D)** without any increment in cell death (**Figure 6A** and **Figure S7A**). Interestingly, although no condensation progression was detected, segmentally iterated displacements and strain patterns still occurred.

Interference on Zip expression in glia (Repo-Gal4/UAS-Zip RNAi) resulted in cessation of condensation at the PP2 phase **(Figure S7A)**. Glia looked smaller and failed to migrate properly and the neuronal longitudinal axonal tracts were misplaced closer to the midline **(Figure 6B)**. On the contrary to neurons, depletion of Zip in glia resulted in a strong disruption of iterative strain profiles **(Figure 6E)**.

The aberrant strain patterns observed after Myosin II depletion in glia suggest that in this condition the oscillatory regime may also be affected. We evaluated the oscillatory patterns upon mild and strong interference in Zip expression in glia (Repo-Gal4/UAS-Zip RNAi embryos at the CP2 phase, taking advantage of the temperature sensitivity of the Gal4 transactivator (weak at 18ºC and strong at 29 ºC). Importantly, we found that the developing temperature affects oscillations. In representative control animals, both periodicity and amplitude were larger (~53 vs. ~48 minutes and ~7 vs. ~4 μm) when

developing at low, than at high temperature. Upon Zip RNAi overexpression, the periodicity of the oscillations with respect to control animals was not affected (~54 and ~45.5 minutes at 18 and 29ºC respectively) while the amplitude was strongly reduced (~4 and ~3 μm at 18 and 29ºC respectively). Thus, the oscillations' amplitude correlates with the degree of contractility inhibition.

Concerning the rheological model (**Figure 4A),** the description of the VNC condensation process in terms of viscoelastic and regulatory parameters does not distinguish, in principle, between neurons and glia. Yet, reducing γ in the phase space plots (**Figure S5F and H**) results in a severe condensation defect in which the strain pattern is sustained that mimics the observed when Myosin II is downregulated in neurons. On the contrary, upon blocking contractility in glia, no compression of intercommissural regions occurs and the strain rate pattern is lost (**Figure 7A** and **7B** and **Movie S7**). These observations suggest that within the model framework, neurons are primarily associated to condensation regulatory parameters (remodeling rate γ and delay δt), which control the oscillatory behavior. Conversely, glia is associated with the material properties (stiffness k_1, k_2 and viscosity η) that enable condensation. Specifically, η likely relates to the friction between neurons and the glia/ECM. These roles are, most probably, not completely uncoupled since ablation of neurons has also minor effects on rigidity (see **Figure 5G**), while Myosin II depletion on glia induces a relevant decrease of oscillatory amplitude.

Altogether, our data support a model in which neuronal contractile capability, at all stages, has a permissive regulatory role but it is not sufficient for tissue compaction. Likely, in the absence of Myosin II, neurons resist the compression forces generated by the surrounding glia. The glia otherwise exerts an external compressive force at the VNC surface, which is spatiotemporally regulated and exploits the segmentally iterated architectural organization of the neuronal network to accommodate the periodic tensional pattern of the VNC into oscillatory condensation events **(Figure 7C-E)**.

DISCUSSION

In discussing the spatial design of the nervous system, we have to consider some specific features, the organism symmetry, the spatial configuration of its locomotor and sensorial machinery and the need to create an integrated functional design (Bullmore and Sporns, 2012; Swanson, 2007). The condensation of the VNC, within the global CNS

developmental plan, must satisfy these traits. The VNC sustains iterated axonal connections to all segments' muscles and sensory organs.

Multiple cellular events play key roles in VNC condensation, in particular interactions between neurons and glia, and apoptosis (Meyer et al., 2014; Olofsson and Page, 2005; Page and Olofsson, 2008). Several intrinsic and extrinsic events are also ultimately linked: the deposition of the ECM; dorsal closure and head involution or midgut' closure. While some signaling pathways have been shown to participate in VNC condensation, we do not understand the events leading to its mechanical control. We don't know either the details of the cellular rearrangements occurring within the packed 3D structure of the VNC. The cell bodies of neurons are essentially round and do not change shape much during condensation, nor intercalate. The more planar glia decorating the surface of the VNC does not suffer axial compression; instead, it remodels its shape adapting to the VNC contour changes. The role of ECM remodeling, which has been long recognized as an important element during condensation (Matsubayashi et al., 2020; Meyer et al., 2014; Olofsson and Page, 2005; Pastor-Pareja and Xu, 2011) remains undefined.

The condensation of the VNC is oscillatory and serves specific purposes

Condensation is a common morphogenetic event (Hall and Miyake, 2000) affecting multiple tissues. It plays an important role at earliest stages of organogenesis (*e.g.* cartilage, bone, muscle and tendon) (DeLise et al., 2000) and in shaping neural ganglia, both in arthropods (Bullock and Horridge, 1965) and vertebrates (Stark et al., 1997). In most of these cases, cells get together by migratory accretion or intercalary growth (Christley et al., 2007; Frenz et al., 1989; Singh and Schwarzbauer, 2012). The *Drosophila* VNC condensation follows specific allometric constraints to reach full functional competence (Karkali et al., 2020). This is achieved through sequential active and passive stages and oscillatory behavior. This complexity has not been observed before in any equivalent process.

Oscillations may arise on epithelia with planarly-connected cells (Peyret et al., 2019) and they can also be anticipated in a tissue structurally segmented or with repeated alternations of stiffer/softer, viscous/less viscous domains. Yet, there are examples of segmentally repeated tissues that do not oscillate as they change shape (e.g. *Drosophila* germ band extension (Bertet et al., 2004), and examples of tissues not segmentally iterated

that do oscillate (e.g. *Drosophila* amnioserosa (Solon et al., 2009)). For the VNC, oscillations, *a priori*, were not expected.

Are VNC oscillations linked to (or an aftermath of) the stereotyped alternating organization of the VNC? The reproducibility and robustness of the oscillatory regime appears to suggest so. However, interfering in contractility in glia or neurons affects the strain rates pattern and condensation regimes in different ways (**Figure 6D**-**E**), without affecting the VNC alternating architectural organization. In conclusion, the oscillatory regime does not appear to be an unavoidable side-effect of the organization of the tissue and to be biologically relevant.

Oscillations are an emergent property of the viscoelastic character of the VNC

The condensation of a tissue is the result of the spatially-patterned dynamics of its components. The directionality and magnitude of such patterning are critical for changes in the tissue fine structure and properties (Li et al., 2017; Shyer et al., 2017).

During condensation the VNC tissue material properties and tensional mechanics undergo progressive changes (**Figures 2** and **3**). The embryonic VNC is very soft (as other neural tissues (Franze et al., 2013)) and its stiffness is neither constant, nor homogeneous. Additionally, an iterated tensional pattern rises and falls following segmental structural landmarks along the AP axis. Overtime, condensation leads to a rigid structural configuration in equilibrium, in which tensional differences are smoothed out.

Up to now, the lack of suitable biophysical models has limited the study of the mechanics during condensation. There are multiple models that can mimic oscillatory responses, either through combining reaction-convection terms (Notbohm et al., 2016) or oscillatory polarization and alignment (Petrolli et al., 2019; Peyret et al., 2019). Here, we developed a simple one-dimensional viscoelastic model FE model to infer strain and stress maps. This not just simulates the periodic oscillations of the VNC, but it predicts the different oscillatory regimes associated to the changes of viscous and elastic mechanical properties observed **(Figure 4)**. Fitting the rigidity values retrieved from AFM measurements, it reveals oscillations in the absence of any external inputs. Oscillations arise as a consequence of the delayed remodeling of the tissue with respect to the compressing forces. The oscillatory regime of VNC condensation thus depends on its material properties and effective frictional interactions with its surroundings. The fact that

increasing viscosity destabilizes oscillatory behavior can be interpreted by an additional delay induced by frictional forces.

From the modeling point of view, the addition of a time delay is expected to induce oscillations in a dynamical system. However, time delays are not sufficient and, in our model, no oscillations are obtained below threshold values of δt. We note that in the field of biological clocks there has been extensive analysis of oscillatory models (Le Novère, 2015; Negrete and Oates, 2021; Novák and Tyson, 2008), and including time delays does not trivially make a system oscillate (Muñoz et al., 2018).

In determining oscillatory robustness, our rheological model predicts that viscosity and time delay are key factors (**Figure S5E-H** and **Figure 4D**). Double sensitivity analyses of model parameters (**Figure S5E-H),** with respect to relative final length (condensation), also revealed the non-linearity of this length relative to elastic stiffness. Indeed, an increase in rigidity opposes the active contractility of the VNC, while the observed decrease of viscosity over time, in accord with the sensitivity analyses (**Figure 4D (iv)**), may be responsible of the progressive slowdown of condensation (**Figure 1B**).

VNC condensation requires the mechanical contribution of glia and neurons

VNC condensation bears mechanical similarities to the compaction of accordion bellows, in which each pleat corresponds to a neuromere unit. Though each “pleat” in the VNC can contract autonomously, they are temporally and directionally coordinated across a long-range by force continuity and balance. This results in oscillatory regimes extending throughout the VNC. We have found that this long-range continuity is created by a precise coordination of the contractile activities of neurons and glia.

In the VNC around 60 glial cells are identified per neuromere (Ito et al., 1995). Amongst them, the Subperineurial Glia (SPG) is responsible for establishing the Blood Brain Barrier (BBB) (Schwabe et al., 2017). Two evidences point for a major role for glia in VNC condensation: the ablation of hemocytes, which causes severe defects in SPG morphology (Martinek et al., 2008; Olofsson and Page, 2005), and the interference with Rac1 or Heartless signaling in the lateral glia (Olofsson and Page, 2005), both blocking condensation progression. The mechanical contribution of glia to VNC condensation may be linked to its participation in casting the BBB. Yet, our evaluation of the mechanical consequences of glia removal indicates that it does not just act as a barrier, but it also

operates as a “compression sock”, wrapping the VNC cortex and providing rigidity (**Figures 6** and **7**). In its absence, condensation is irregular, shows a substantial reduction in its strain rates, and lacks contractile oscillations. We found that interfering in the contractile capability of the SPGs was sufficient to phenocopy pan-glial myosin activity depletion, both in terms of condensation **(Figure S7A)** and axon network organization (**Figure S7B**). The capability of glia to compact is strongly dependent on actomyosin contractility, and it is mainly allocated to the SPGs.

Each abdominal hemisegment of the VNC comprises around 400 neurons, whose axons arrange into segmental and intersegmental nerves and longitudinal connectives that constitute a potential force-generating source. Consistently, in some metamorphic insects, the longitudinal connectives loop during condensation (Pipa, 1973). We also found that the domains in-between neuromeres subside as condensation progresses. These domains are under tensional stress at early stages, relaxing as condensation proceeds. Thus, the axonal network appears to resist rather than to promote AP compaction. Although, ablation of neurons only marginally affects condensation, when their contractile capability was abolished, VNC condensation failed without significantly altering the strain patterns. Thus, neurons are not playing a purely passive role (**Figures 6** and **7**).

Overall, this work reveals that the viscoelastic and biomechanical properties of the nervous system, in concurrence with a complex series of coordinated cellular actions, are important for its morphogenesis. The generation of force patterns, and the ultimate acquisition of the VNC final shape, can be assigned to the concerted actions of neurons and glia through the dynamic modulation of their cytoskeleton. The neuronal contractile capability is secondary to the glial compacting power, but necessary to direct VNC condensation along the AP axis **(Figure 7C-E)**.

Finally, if we assume that VNC condensation is a way to respond to evolutionary pressure for functional optimization, we speculate that the segregation and coordination of mechanical activities between emergent neurons and glia is a key factor for natural selection.

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AUTHOR CONTRIBUTIONS

Conceptualization, KK, TES, JJM, and EMB.; Methodology, DN; Investigation, KK, PT, AS, ST, I.J, TES, JJM. and EMB.; Writing – Original Draft, EMB; Writing – Review & Editing, KK, JJM, TES and EMB; Funding Acquisition, TES, JJM, DN and EMB; Resources, DN and TES; Supervision, TES, JJM and EMB.

COMPETING INTERESTS

The authors declare no competing interests.

FIGURE LEGENDS

Figure 1: Dynamics of VNC condensation

A) Snapshots at 2-minute intervals from **Movie S2 (**multi-view light-sheet imaging of a live Histone 2Av-mCherry embryo, ventral view, late stage 17). mCherry marks all nuclei; raw data is shown on the left and “detwitched” images (blue masked) on the right. In all images, unless stated otherwise, anterior is to the left and posterior to the right. Lines indicate the ventral midline. Scale bar 50 µm. **B)** Quantification of VNC length (**i**) and condensation speed (**ii**) as function of time. Condensation (CP1, CP2 and CP3) and

pause (PP1 and PP2) phases are masked in pale green and red respectively. As a convention, t=0 corresponds to the onset of PP1, at the end of germ band retraction. Means (solid) and SD (dashed) are represented by red lines. Gray lines represent individual embryos (n=11). **C)** Condensation velocity dynamics. (**i**) Snapshot of a live Histone 2Av-mCherry embryo monitored by light-sheet imaging at stage 16. Scale bar 50 µm. (**ii**) Velocity kymograph derived from PIV analysis (STAR Methods). Position=0 corresponds to the hinge between the brain lobes and the VNC. Time axis (top to bottom) as in **(B)**. Color-coded positive (posterior-ward - white/yellow) and negative (anterior-ward - black/blue) velocities (neutral - red). (**iii**) Representation of velocity profiles (CP2, PP2 and CP3) with 5-minute resolution, along the AP axis from the most anterior (darkest blue) to the most posterior (darkest red lines) VNC positions. **D)** Kymograph along the VNC from a live embryo expressing Fas2-GFP. (**i**) Ventral view from **Movie S4**, at stage 16. Scale bar 50 µm. (**ii**) Stage 16 embryonic VNC, re-sliced over the Z-axis. (**iii**) Fluorescence intensity peaks mark individual segments landmarks (color coded as in **(C)**). Time and AP axis positions are as in **(B)** and **(C)**. (**iv**) Kymograph of condensation, with arrows denoting condensation direction.

Figure 2: Characterization of VNC material properties

A) Representative images of flat dissected embryos in stages 14 and 16. VNC perimeter (white) and midline (yellow) are highlighted. Anterior is to the top. Scale bar 50 µm. **B)** Measured VNC stiffness (E) at early stages (13-14). Bars denote mean values (abdominal segments A1 - A7). Mean stiffness was measured at the midline (blue) and at lateral positions of the cortex (red). Dots and diamonds correspond to individual measurements. **C)** as **(B)** but for older, stage 16-17, embryos. **D)** (**i**) Kymograph of VNC strain rates, from **Figure 1C** (see STAR Methods). (**ii**) Representation of strain rates profiles (CP2, PP2 and CP3) with 5-minute resolution, from most anterior (darkest blue line) to most posterior (darkest red line) positions. (**iii**) Distribution of strains along the AP- axis for all time points (earliest light to latest dark lines) during CP2 (green), PP2 (gray) and CP3 (green). **E)** Average size (and SD) of intra- and inter-commissural domains from early (E) and late (L) stage 15 and 16 and early (E), middle (M) and late (L) stage 17 embryos. Data was collected from 7-10 measurements per time-point from two embryos.

Figure 3: Laser microsurgery during condensation and tissue tension

A) Representative images of stage 14 embryos, expressing alpha Tubulin-GFP, before (top) and after (bottom) laser ablation. The yellow dashed line highlights the position of the laser cut, while green (anterior) and red (posterior) arrows indicate tensile recoil directionality **(Movie S3)**. Scale bar 10 μm. **B)** Recoil velocity after ablation at intercommissural (dark) and intracommissural (pale) domains, on stage 14 embryos. Bars represent mean recoil velocity of anteriorly (green) and posteriorly (red) retracting tissue. Individual measurements are denoted by yellow dots (intercommissural) and diamonds (intracommissural). * $p < 0.05$. **C)** Recoil velocity of anteriorly (green) and posteriorly (red) retracting domains after VNC ablation at different stages of embryonic development (n=12 embryos). **D)** (**i**) Tiled image of a stage 16 embryo expressing alpha Tubulin-GFP after laser cutting the intercommissural domain between the segments A1 and A2. The white arrow marks the direction of condensation. The anterior and posterior limits of the VNC and the abdominal segments (A1 to A8) are indicated (yellow). (**ii**) Snapshots, immediately post-ablation (masked blue), and 2 hours later (masked red), from **Movie S3**. Scale bar 20 μm. (**iii**) Superimposed intensity profiles of both time points. Black arrows indicate the magnitude of the anterior-ward displacement of individual segmental landmark. **E)** Characteristic recoil time τ computed from the rate of recoil at the intercommissural domain. **F)** Kymograph of the VNC during condensation (Fas2-GFP). White curves correspond to fourth order polynomial fitting of the points of maximum compression as deduced from the viscoelastic FE model (STAR Methods). (See also **Figure S4D** and **Movie S3**).

Figure 4: Rheological model of VNC condensation

A) Scheme of one-dimensional rheological model including a viscoelastic term with variable rest-length l, stiffness k_2 and remodeling rate γ (Eq. (1)). In parallel, carries an elastic component with stiffness k_1 and considers viscous contacts to the external environment, denoted by η. **B)** Phase diagram in the parameter space k_2 - η, showing that reduction of η and increase of k_2 stabilizes the oscillatory behavior. St 14 and 17 characterize the transition from early to late condensation stages, with a stabilizing effect. **C)** Kymograph of numerical simulation showing the oscillatory behavior of strains as a function of time. **D)** Sensitivity of VNC shortening and oscillatory frequencies to main

model parameters on (**i**) Remodeling rate, γ. (**ii**) Stiffness, k_1. (**iii**) Stiffness, k_2. (**iv**) Viscosity, η. (**v**) Time delay, δt. Shortening is measured as the relative final length, l_{final} / l_0. The dotted blue line indicates the initial amplitude of the oscillations for the reference parameters (γ, k_1, k_2, η, δt) = (0.2, 0.01, 1.9, 15, 20), while the gray area represents the final amplitude for the analyzed values indicated on the horizontal axis. The green line indicates the oscillations frequency as a function of the parameter values. Frequency is measured in min^{-1} *10.

Figure 5: Neurons and glia contribute to the architectural organization of the VNC and its condensation

A) CNS Flat-preps of WT (top) and Elav-Gal4>UAS-Grim (bottom) embryos, at stage 16, immunostained for Fas2 (red) and Dcp1 (green). **B)** Embryos as in **(A)**, immunostained for Dcp1 (red) and Repo (green). **C)** CNS Flat-preps of WT (top) and Repo-Gal4:UAS-mCD8-GFP>UAS-Grim (bottom) embryos, at stage 16, immunostained for Fas2 (red) and GFP (green). **D)** Embryos as in **(C)**, immunostained for Dcp1 (red) and GFP (green). Yellow arrowheads point to the disrupted axonal network in **A** and **C.** Pink arrowhead points to misplaced or surviving glia in **B** and **D**. Scale bar 10 µm. **E)** Snapshots from time lapse recordings of WT (Top) and Repo-Gal4>UAS-Grim (bottom) embryos, in an alpha Tubulin-GFP background (ventral view –stage 17) **(Movie S8)**. Yellow arrowhead points to the VNC misshaped buckling. Scale bar 50 µm. AP axis orientation is indicated. **F)** Quantification of VNC length (*elav*:mCD8-GFP marker) as a function of developmental time in WT (red, n=11) and Repo-Gal4>UAS-Grim (blue, n=4) embryos. Solid and dashed lines show mean and SD values. **G)** VNC stiffness (E) measured by AFM at early stages 14, for WT, Elav-Gal4>UAS-Grim and Repo-Gal4>UAS-Grim embryos. Bars denote mean values at the ventral midline (blue) and at lateral cortex (red). *$p < 0.05$, **$p < 10^{-2}$ and ***$p < 10^{-3}$. **H)** As **(G)** but for late stage 16 embryos.

Figure 6: Active contractility in neurons and glia have distinct roles.

A) Ventral and Dorsal 3D views of stage 16, WT (top) and Elav-Gal4>UAS-Zip-RNAi (bottom) embryos, immunostained for Fas2 (red) and Dcp1 (green). **B)** Ventral and

Dorsal 3D views, as in **A**, of stage 16, WT (top) and Repo-Gal4:UAS-mCD8-GFP>UAS-Zip-RNAi (bottom) embryos, immunostained for Fas2 (red) and GFP (green). Yellow arrowheads point to the disrupted axonal network in **A-B**. Pink arrowheads point to misplaced glia. Scale bar 10 µm. **C-E)** Condensation dynamics in control **(C)**, Elav-Gal4>UAS-Zip-RNAi **(D)** and Repo-Gal4>UAS-Zip-RNAi **(E)** embryos **(Movie S7)**. (**i**) Snapshots of live embryos, expressing Fas2-GFP at stage 17. Yellow arrowheads point to the posterior tip of the VNC in **D** and to the VNC misshaped buckling in **E**. Scale bar 50 µm. (**ii**) Representation of velocity profiles along the AP axis, from the most anterior (darkest blue) to the most posterior (darkest red line) VNC positions (as in **Figure 1C**). (**iii**) Kymograph of strain rates along the VNC (as in **Figure 2D**). Cyan marks point to strain oscillations.

Figure 7: Neurons and Glia cooperatively contribute to the oscillatory behavior

A) Snapshot from the 3D representation **(Movie S7)** of the 2D strain pattern of the VNC in WT animals. The 3D meshwork (top) is aligned to the corresponding raw image (bottom). **B)** Displacements and strains along the VNC in WT and in embryos with pan-neural (Elav-Gal4>UAS-Zip-RNAi) or pan-glial (Repo-Gal4>UAS-Zip-RNAi) Myosin II knockdown, at equivalent times [14 hours after egg laying (AEL) at 29ºC] **(**Snapshots from **Movie S7)**. **C)** Cartoon describing the condensation oscillatory regime during the CP2 and CP3 stages (at the level of the segments A4 and A7, data from **Movie S2** – see **Figure 1C**), highlighting the opposing displacements of thoracic (red) and abdominal (green) segments towards the central stationary domain. **D)** Cartoon representing the segmentally iterated intercommissural and intracommissural domains of the axonal network before (top) and after (bottom) condensation. Their mechanical properties (rigid or tensile) are shown. This representation depicts the first three abdominal segments actively contracting (green arrows). **E)** Cartoon presenting in 3D the VNC segmental axonal network (as in **D**) surrounded by the glial shell (SPGs), displaying centripetal and longitudinal contractile capability (blue arrows).

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