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1 Anteroposterior elongation of the chicken anterior trunk neural tube is hindered by interaction

2 with its surrounding tissues

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11 Abstract

12 The neural tube is the precursor of the central nervous system. Its early formation and growth are 13 known to be extremely biased along the anteroposterior (AP) axis. Several mechanisms including addition 14 of cells from the tail bud, lateral pressure from surrounding tissues and oriented cell divisions have been 15 proposed to contribute to this biased growth. Here we show that, contrary to what has been found in 16 posterior regions encompassing the tail bud region, the growth of the anterior trunk neural tube is slower 17 along the AP direction than in the other axes. We found that this is due to anchorage of the neural tube to 18 the matrix which favors apicobasal elongation at the expense of AP growth. In addition, as the neural tube 19 develops, we found a moderate slowdown of cell proliferation that could account for the overall reduction 20 of the pace of 3D growth in the same time window. However, as we found no preferred orientation of cell 21 division, changes in cell cycle pace are unlikely to directly contribute to the observed AP-hindered growth 22 of neural tube. Overall, these data indicate that neural tube growth is not intrinsically positively biased 23 along the AP axis. Rather it switches from AP-favored to AP-hindered regimes between the most posterior 24 and anterior trunk neural tube regions.

25

26 Keywords: neural tube, chicken embryo, growth, tissue interaction, proliferation

27 Introduction

28 In vertebrates, from gastrulation, the growth of the embryo is biased along the anteroposterior 29 axis giving rise to an extensive elongation. The neural tube, which is the precursor of the central nervous 30 system, sits at the center of the developing embryo and is no exception to this biased growth. In the chick 31 embryo, between 2 days and 4 days of development, the posterior region of the neural tube (from somite 32 15 to the tip of the tail) grows by a factor 3.56 along the anteroposterior (AP) axis (Ferreira et al., 2019). 33 By contrast, the neural tube only grows by a factor 2.45 and 2.3 in the dorsoventral (DV) and apicobasal 34 (AB) axes, respectively (Ferreira et al., 2019). Several phenomena could contribute to this favored 35 anteroposterior growth. Caudally, there is the tail bud, a progenitor zone that adds cells to the growing 36 neural tube and mesoderm in a rostrocaudal manner (Sambasivan and Steventon, 2020). In addition, it 37 has been proposed that the nascent mesoderm and the underlying notochord would exert a local 38 pressure on the forming neural tube favoring its elongation along the anteroposterior axis (Xiong et al., 39 2020). Further, analyses of cell division at early stages of chicken neural tube development (up to 2 days, 40 HH12) suggested that neural cells preferentially divide along the anteroposterior axis (Sausedo et al., 1997). Furthermore, neural progenitors display a differential proliferation rate along the anteroposterior 41 42 axis. Indeed, as the neural tube develops, proliferation slows down and differentiation kicks in such that 43 anterior portions of the neural tube have proportionally fewer progenitor cells with an increased mean 44 duration of their cell cycle (Molina and Pituello, 2017).

Altogether, these observations suggest that the anteroposterior bias of the neural tube growth is primarily due to fast proliferation with oriented divisions and local pressure from mesoderm in the posterior region which would concur to promote anteroposterior elongation. While this model of growth is widely accepted, numerous questions remain open. Is AP-biased growth a feature of the neural tissue and thus also observed outside of the most posterior region? As mesoderm develops into somites, is the neural tube still under lateromedial pressure favoring AP elongation? Are AP oriented divisions restricted

to early stages or a feature of the neural plate/tube regardless of the stage? What is the actual amplitude
of the lengthening of the mean cell cycle duration in a given region over time and could it bias growth in a
given axis or account for changes in growth rates?

54 To address some of these points, we used a combination of classical embryology techniques 55 coupled with mathematical modelling of the proliferating epithelium. First, we found that growth of the 56 neural tube is still anisotropic outside of the tail bud region. However, by contrast with the posterior region, AP elongation is slower than growth in the other axes between 2 and 4 days of incubation. Second, 57 58 extracting a portion of the anterior trunk from the embryo or detaching the neural tube from its 59 surrounding tissues leads to an AP expansion of the neural tube. This indicates that AP growth of the 60 anterior neural tube is hindered by neighboring tissues and its adhesion to the matrix. Third, we found no 61 evidence of a directional bias of cell division along AP axis in the developing neural tube between 2 and 4 62 days of development. Fourth, we calculated the expected mean cell cycle duration to account for the 63 growth of the neural tube facing the forelimb region from 2 to 4 days of development. We used a 64 mathematical model to simulate different regimes of proliferation using cell cycle durations extracted 65 from our experimental data or from the literature. We found that the growth of the neural tube at 66 forelimb level between 2 and 4 days of development corresponds to a shift of mean cell cycle length from 67 8h50 to 13h30min in neural progenitor cells together with a proportionally decreasing progenitor pool. 68 This shifts is likely due to a global change of mean cell cycle length rather than to the emergence of 69 lengthy cycles that would skew the mean towards higher values. Interestingly, the scale of this change is 70 of the same order of magnitude as the observed change in tissue growth suggesting that the lengthening 71 of the cell cycle is one of the main driver of the slowdown of neural tube elongation between 2 and 4 72 days.

Altogether, our data indicate that neural tube growth goes from a favored AP elongation in
caudal-most regions, most likely due to addition of new cells from the activity of the tail bud and lateral

pressure from the mesoderm, to a hindered AP growth due interaction with the matrix. Concomitantly,overall growth slows down due to changes in cell cycle duration.

77

78 Results

1. Anterior trunk neural tube growth is slower along the anteroposterior axis than in the other axes

80 Does neural tube growth switch from being AP-biased in posterior regions to isotropic in anterior regions? To assess this, we monitored the growth of the neural tube anterior to the tail bud. To measure 81 82 AP growth, we injected the fluorescent tracer Dil at different locations along the neural tube (Fig. 1A) in 83 embryos at 48h (Fig. 1B) or 72h of incubation (Fig. 1C). From these, we measured the distance between 84 the different Dil points in ovo at t0 and 24h later and calculated the mean growth rate along the AP axis in 85 each time window (Fig. 1D-E). In parallel, we assessed neural tube growth in the DV and AB directions by 86 measuring these axes on transversal sections of embryos at identical region and stages (Fig. 1F-J). Interestingly, the mean AP growth rates of 1.44 between 48 to 72h and of 1.27 between 72 and 96h are 87 88 significantly lower than the observed growth rates for the DV and AB axes at both time points (Fig. 1K). 89 These results indicate that neural tube growth is not isotropic outside of the tail bud region. Instead, the 90 anterior neural tube displays a reversed anisotropic growth in which AP growth is slower than growth in 91 the other axes.

92

93 2. Anterior trunk neural tube anteroposterior elongation is hindered by interaction with surrounding tissues

In posterior regions, local pressure from surrounding tissues, including the nascent mesoderm and the underlying notochord, has been proposed to favor AP growth (Xiong et al., 2020). Thus, we asked whether interaction with the surrounding tissues might have a similar role in the neural tube facing somites or on the contrary might contribute to the observed slower AP growth. First, we measured the length of the neural tube of individual embryos along the 5 most posterior somites *in ovo* and after

dissection and fixation (PFA4%) of that same portion at 2 days of development (Fig. 2A). The mean AP
length of the neural tube was significantly higher *ex ovo* compared to *in ovo* with a 14% length increase
upon extraction (Fig. 2B). Noticeably, the mean somite length also significantly increased after dissection
but only by 6.9% in the same explants (Fig. 2C), which was significantly lower than the neural tube
expansion (see figure legends for statistics). Given that aldehyde fixatives lead to tissue shrinkage, these
values are likely to be slightly underestimated.

105 Next, we assessed the role of the interaction of the neural tube with the surrounding tissues. For 106 this, we again harvested portions of the trunk corresponding to the 5 most posterior somites at 2 days of 107 development that we either fixed immediately after dissection or after 2h in culture medium (DMEM) 108 with or without Dispase, which digests collagens and Fibronectin and thus cuts links between the neural 109 tube and underlying basal lamina (Fig. 2D-E). After 2h in culture, in explants in DMEM, the neural tube 110 kept its overall shape and length compared to explants fixed at t0 (Fig. 2D-E). By contrast, the Dispase 111 treatment led to drastic changes of the explants shape, visible to the naked eye, with the superficial 112 ectoderm detaching and rolling up. In these Dispase-treated samples, the neural tube buckled and its AP 113 length significantly increased by 19% compared to control DMEM explants (Fig. 2D-E). Therefore, 114 adhesion of the anterior neural tube to the extracellular matrix plays a role in restricting its AP growth. 115 Interfering with the cell-matrix interaction also prevents normal interaction with the surrounding 116 tissues. To assess whether the elongation observed in Dispase-treated samples was due to the loss of 117 physical interaction with the surrounding tissues and/or to the loss of adhesion to the matrix per se, we 118 performed a similar assay but this time, the surrounding tissues were removed by microdissection. 119 Importantly, the proximal parts of the somites were kept intact to ensure that the basal lamina of the 120 neural tube was not damaged by the procedure (Fig. 2F). In this case, the dissected neural tube elongated 121 by only 5% compared to explants with surrounding tissues, a difference that was not found to be

statistically significant, indicating that the neural tube AP extension obtained after Dispase treatment wasmostly due to the loss of interaction with the basal lamina (Fig. 2F-G).

Collectively, these data indicate two levels of physical constraints that hinder AP elongation in the anterior neural tube. One associated with being part of the whole embryo *in ovo* that is released upon extraction of a whole piece of trunk and a second related to the anchorage to the matrix that is released upon enzymatic degradation of collagens and fibronectin.

128

129 3. Anchorage of the anterior trunk neural tube to the extracellular matrix favors apicobasal elongation

130 Next, in order to assess the consequences of the loss of cell-matrix interactions on the other axes, 131 we monitored the size of the neural tube in DV and AB directions and its pseudostratification on 132 transversal sections in DMEM and Dispase-treated samples. For that, we stained DNA using DAPI and 133 Actin with Phalloidin (Fig. 3A-B). The DV height, AB width and number of nuclear pseudolayers were 134 similar in controls (t0) and DMEM incubated samples (Fig. 3C-E). By contrast, in Dispase-treated samples 135 the DV height increased by 13.6% (Fig. 3C) while the AB width (Fig. 3D) and pseudostratification (Fig. 3E) 136 decreased by 44 and 38% respectively. This shows that anchorage to the matrix is essential for normal AB 137 elongation of the cells and the associated pseudostratification. In addition, matrix degradation led 138 simultaneously to AP lengthening, DV expansion and AB shortening of the neural tube. Importantly, these 139 changes take place in a short time window (2h), during which one can reasonably assume that cell 140 volumes remain constant. Therefore, this result suggests that AB elongation and pseudostratification 141 occur at the expense of AP and DV growth of the neural tube, in line with our previous observations 142 (Ferreira et al., 2019).

143

4. Mitoses orientations in the neural tube are not biased along a specific direction between 2 and 4 days of
 development

146 Next, we wondered whether changes in cell proliferation could account for tissue growth 147 dynamics either due to a change of orientation of cell divisions or to a change of the pace of proliferation. 148 First, we assessed whether cell divisions displayed a preferred orientation in the neural tube as its AP 149 growth gets hindered. Previous analyses on fixed samples were performed using DNA labelling. It was 150 proposed that in the trunk of embryos up to stage HH12 (2 days of development), nearly 55% of all 151 divisions in the neural tube were oriented along the AP axis (Sausedo et al., 1997). To analyze the 152 orientation of cell division, we performed an immunostaining for γ -tubulin together with DNA labelling 153 using DAPI, mounted the samples in open book configuration (Fig. 4A) and measured the angles of 154 divisions according to the AP axis of the neural tube (Fig. 4B). As it is known that metaphasis plates rotate 155 until the last minute before division, we restricted our analysis to situations with a clear separation of the 156 genetic material to two sides of the cell (anaphase) to avoid the possible misleading interpretation of early 157 mitotic figures. We used the axis between the virtual line passing through the two γ -tubulin dots and the 158 AP axis of the neural tube to measure the angle of division (Fig 4C-D). With this strategy, we analyzed the 159 orientation of anaphases in the neural tube in 48h embryo at the level of the presomitic mesoderm (psm) 160 or the forelimb. In these two regions, and contrary to what had been previously proposed, the divisions 161 were evenly distributed along the AP, DV and intermediate/diagonal orientation (Fig. 4E-F; 48h and 48h 162 psm). Next, we analyzed the orientation of cell divisions at the level of the forelimb along further 163 development, at 72 and 96h, and found again evenly distributed orientations of cell division along AP and 164 DV axes at both stages (Fig. 4E-F; 72h and 96h). Therefore, these results indicate that the orientation of 165 cell divisions is unlikely to contribute to the switch from AP-biased to AP-hindered growth of the neural 166 tube from posterior to anterior regions or to changes in growth in the anterior neural tube over time. 167

5. Dynamics of the anterior trunk neural tube growth over two days at forelimb level corresponds to a
 moderate and homogenous lengthening of the mean cell cycle duration

170 In absence of oriented cell divisions, we wondered whether a change of the pace of proliferation 171 might account for the observed slowdown of growth between 2 to 4 days of development. Indeed, our 172 measurements indicated an important change of anterior neural tube 3D growth between 72h and 96h as 173 compared to 48-72h growth rate (Fig. 1K). Between 2 to 3 days the mean 3D growth rate is 4.2 (1.44 x 1.7 174 x 1.72) while between 3 to 4 days it is only $2.74 (1.27 \times 1.52 \times 1.42)$ which correspond to a 35% drop. This 175 raises the question of whether cell proliferation dynamics in anterior trunk neural tube could contribute 176 to tissue growth temporal variations. Mean cell cycle length has been reported to vary widely in time and 177 space during chicken embryo development and in particular in the neural tube (Fig. 5A). There is a general 178 consensus that the duration of the cell cycle increases as the neural tube develops but in the chicken 179 embryo, there has been no global study in a given region over time to confirm this or to estimate the 180 amplitude of the change (Molina and Pituello, 2017) or whether such changes match observed changes in 181 growth.

182 To estimate cell cycle durations in the anterior chick neural tube between 48h and 96h of 183 development, we quantified the progenitor and total cell populations. We used Sox2 immunostaining to 184 label progenitor cells and DAPI to label all cells at 48h, 72h and 96h of development at the forelimb level, 185 on transversal sections (Fig. 5B-C). Since we found no oriented cell divisions at these stages of 186 development, between two time points, observation of cell numbers on series of transversal sections is a 187 proxy of cell numbers in a 3D volume. Indeed, without a bias of cell division orientation, the proportion of 188 daughter cells that are excluded from a given transversal section is the same as the proportion of newly 189 incorporated daughter cells coming from divisions that took place in adjacent transversal sections. Thus, 190 the evolution of cell number in sections over time reflects the evolution of the whole population. 191 Transversal sections at all time points were generated with the same thickness (14 μ m). However, 192 because of tissue growth, a 14 µm-thick section of the neural tube at 48h of development will no longer 193 be 14 μ m-thick at 72 or 96h. According to the AP elongation rates that we calculated from our Dil

194 injections (Fig. 1B-E), a 14 μ m-thick section will develop into a 20.16 μ m section (14 x 1.44) at 72h and 195 into a 25.6 μ m-thick section at 96h (14 x 1.44 x 1.27). Therefore, the number of cells observed on 14 μ m 196 sections at 72 and 96h needs to be corrected by the observed AP growth rates, respectively x1.44 at 72h 197 and x1.83 (1.44 x 1.27) at 96h. It is also important to note that cell translocation can occur independently 198 of cell division, as previously reported in the cephalic neural tube in mice (McShane et al., 2015). 199 Therefore, extrapolating the dynamics of the whole population from transversal sections will only be 200 correct if such translocations events are very rare or evenly distributed between all axes. 201 We first calculated the number of progenitor cells and total cell number dynamics from 202 histological sections of the neural tube (Fig 5C). From these data, we calculated the expected doubling 203 time required for the progenitor region at a given time point to generate the whole population at the next 204 time point (see methods for the calculations) with the two aforementioned assumptions that there are no 205 oriented divisions (our data) and that there no significant neural cell translocations (the simplest 206 hypothesis). From this, we calculated a mean cell cycle duration of 8h50 for neural progenitors between 207 48 and 72h of development and of 13h30 between 72 and 96h of development (Fig. 5A, green bars). 208 To compare the relevance of these cell cycle values with the previously published values, we ran a 209 computational model that we formerly validated (Ferreira et al., 2019) to simulate the growth of the 210 neural tube cell population over two days (Fig. 5D-E). Importantly, and unless otherwise specified, all 211 mean cycle values used for simulations are allowed a narrow range of 1h above and below the mean. We 212 first assumed that all cells are proliferative and simulated growth regimes with the fastest (5h, Fig. 5D, 213 open circles) and the slowest cell cycle published values (28h, Fig. 5D, black dots) (Fujita, 1962; Wilcock et 214 al., 2007) and an intermediate regime in which neural progenitor cells can proliferate with a cell cycle of 215 any duration between 5 and 28h (Fig. 5D, grey dots). All data were normalized to t0 such that each 216 population virtually starts with one cell, allowing direct comparison of growth dynamics (Fig. 5D). This 217 shows that none of the proliferation regimes tested accounts for the actual growth of the population.

Cycles of 5h give rise to an excessive growth whereas cycles of 28h, previously observed *in vivo* using live
imaging (Wilcock et al., 2007), or an intermediate 5-28h cell cycle length do not allow production of
enough cells.

221 We then use our calculated values of cell cycle length to perform new simulations of neural cell 222 growth between 48 and 96h of development, with all cells proliferating, normalized all datasets to t0, and 223 compared them to the biological data (Fig. 5E). We modeled 3 conditions, constant cell cycle length of 224 8h50 (Fig. 5E, open circles), constant cell cycle length of 13h30 (Fig. 5E, black dots) or a mixed regime with 225 a cell cycle of 8h50 for the first 24h and then switching to 13h30 for the next 24h (Fig. 5E, grey dots). The 226 mixed regime was the closest to the biological data (Fig. 5E, green dots). However, the mixed regime still 227 produced too many cells compared to the observed cell number over 2 days (+21%), with a divergence 228 between the predicted growth rate and the observed cell numbers that is mostly observed in the last 24h 229 (Fig. 5E). This discrepancy could be due to an underestimation of the actual cell cycle length between 3 230 and 4 days of development or to the fact that we modelled a population with 100 % of progenitors while 231 the biological data indicate that the proportion of proliferative cells (Sox2+) progressively shrinks over 232 time (Fig. 5B-C).

233 Thus, we plotted the percentages of differentiated cells at 48, 72 and 96h of development and 234 used a LOWESS fit to extrapolate the proportion of differentiated cells at intermediate stages of 235 development (Fig. 6A). From this, we calculated the proportion of available progenitor cells over time (Fig. 236 6B). We then used this information to repeat simulations of the mixed 8h50-13h30 proliferation regime 237 with different proportions of proliferative cells (Fig. 6C): 100% throughout (Fig. 6C, light grey dots), 100% 238 for 24h and then 85% (Fig. 6C, dark grey dots), 100% for 24h, 85% for 12h and 70% for another 12h (Fig. 239 6C, black dots). The first two conditions give rise to an excess of 21% and 8.5% of cells compared to the 240 biological data after two days, respectively. The last condition is the closest to the biological data with a 241 difference in total cell number inferior to 3% with the biological data. These results show that a slight

lengthening of the cell cycle duration (+50%) coupled with a progressively declining proportion of
progenitor cells is sufficient to reproduce the dynamics of the neural tube cell population at forelimb level
between 2 and 4 days of development.

245 The long cell cycle value that we calculated between 3 and 4 days of development is significantly 246 shorter than some values that have been previously proposed in the literature (up to 28h). The mean 247 increase of cell cycle duration between 2 and 4 days of development could either occur because all cell 248 divisions take slightly longer or by an increase of the range of cell cycle durations. In the last case, some 249 cells would still cycle fast while others would take a very long time skewing the mean towards higher 250 values while accounting for observed lengthy cycles. We tested these two scenarios by repeating 251 simulations with the declining progenitor populations in which the slowdown of cell cycle takes place with 252 the aforementioned narrow range (+/- 1h) around the mean (Fig. 6D, black dots) or a wide range (+/- 5h) 253 around the mean (Fig. 6D, open circles). In this last scenario, cells can proliferate with any cell cycle 254 duration between 8h30 and 18h30. Despite the mean duration being the same at 13h30, the wide range 255 simulation generates 16% extra cells compared to the biological data. This indicates that the shift of cell 256 cycle duration is most likely driven by an overall lengthening of cell cycle in all proliferative progenitors 257 rather than a widening of the range of cell cycle values towards lengthy cycles.

258 Can these moderate changes in cell cycle pace account for the observed slowdown of the neural 259 tube growth over two days? We compared the change in growth rates in 3D, calculated from the growth 260 rate in each axis, with the change in growth rates in terms of cell number (which is driven by the 261 lengthening of the cell cycle) during the same period. We have a mean 3D growth rate of the neural tube 262 of 4.21 between 2 and 3 days of development and of 2.74 between 3 and 4 days (Fig. 1K). This gives a 263 ratio of 0.65 for 3D growth between these two periods, corresponding to a 35% drop. By comparison the 264 cell cycle goes from 8h50 (8.8h) between 2 and 3 days to 13h30min (13.5h) between 3 to 4 days, which 265 also gives a ratio of 0.65 of cell cycle variation. This indicates that the slowdown of the cell cycle and that

of the 3D growth are of similar amplitude. This strong correlation between cell cycle variation and tissue
growth suggests that the lengthening of the cell cycle is likely to be one of the main driver of the declining
rate of 3D growth in the anterior neural tube. However, one should keep in mind that other changes that
are taking place concomitantly could contribute to the slowdown of tissue growth such as the declining
number progenitors and the delamination and subsequent differentiation of neurons.

In conclusion, our data indicate that the switch from AP-favored to AP-hindered growth of the
neural tube primarily depends on interaction with the matrix whereas the subsequent slowdown of
growth observed over two days is likely to be driven by a moderate and homogenous lengthening of the
duration of the cell cycle.

275

276 Discussion

277 Anteroposterior growth is notoriously biased along the AP axis and this is mainly driven by the 278 progenitor zone which adds cells to the growing tissues. Surprisingly, we found that rather than switching 279 to isotropic growth outside of the tail bud region, the neural tube displays a reversed anisotropic growth 280 with a slower AP elongation relative to AB and DV axes. More precisely, AP growth is hindered by 281 anchorage to the matrix. Surprisingly, we found that simply extracting a portion of the trunk was sufficient 282 to promote an immediate AP elongation of the neural tube, suggesting that it is under slight AP 283 compression in ovo. In addition, impairing interactions with the matrix also led to an additional increase in 284 AP length and DV height. This occurred concomitantly to a reduction of the AB width of the cells. This 285 echoes our previous observation that neuroepithelial cells in the forelimb region see their volume shrink 286 between 2 and 4 days of development while their size along the AB axis more than doubles in the same 287 time frame (Ferreira et al., 2019). This dramatic change of cell shape while cells are getting smaller means 288 that most of the cell material is used along the AB axis. Thus, any mechanism that favors or impairs AB

elongation would impact growth in the other axes. One example is interkinetic nuclear movements (INM)(see below).

291 In posterior regions, it has been proposed that the nascent mesoderm and the underlying 292 notochord contribute to a caudally oriented force on the growing neural tube favoring AP elongation 293 (Xiong et al., 2020). Our dissection and Dispase experiments suggest that this relationship no longer 294 stands in anterior regions where the neural tube faces somites. One possibility could be that 295 somitogenesis itself contributes to this hindered AP elongation. Somites aggregate by epithelialization 296 from a loose mesenchyme called the presomitic mesoderm. One could think that the epithelialization and 297 the local reorganization of the matrix that occurs during the process (Martins et al., 2009) might lead to a 298 local compressive force along the AP axis when somite precursors cluster to epithelialize. Indeed, the 299 region of the anterior presomitic mesoderm that contains the next somite to be formed is approximately 300 90 μ m long whereas a newly epithelialized somite is 50-60 μ m long (Martins et al., 2009). This local 301 "shrinkage" or "condensation" of the paraxial mesoderm during somitogenesis might contribute to a local 302 compressive force aligned with the AP axis that could participate to the hindered AP elongation of the 303 neural tube at this level. Interestingly, we also found that somites elongate in AP direction after dissection, 304 but to a significantly lower extent than the neural tube. A possible explanation for this is that the neural 305 tube is a long epithelial structure with bilateral anchoring to the matrix, while paraxial mesoderm consists 306 of a succession of epithelial somites separated by matrix boundaries that may buffer compression forces. 307 Regarding the cell cycle, we found, as previously reported, that the mean cell cycle duration 308 increases over time. However, our calculations and simulations indicate that the actual amplitude of the 309 proliferation slowdown at the level of the forelimb is about 35% over 2 days. Interestingly, these results 310 are in better agreement with analyzes in mice where cell cycle length was monitored over time in one region (Kicheva et al., 2014) than with studies in chicken that compared different regions along the AP axis 311 312 (Olivera-Martinez et al., 2014). This suggests that there may be important regional differences in cell cycle

313 length. Of note, our estimated cell cycle values are line with what was measured using none invasive 314 methods such as EdU/BrdU incorporations but differ significantly from cell cycle values that were 315 measured by live imaging. While some interpreted this difference as a new level of complexity discovered 316 by live imaging, one could also argue that these long cell cycle values are actually induced by the protocol 317 of imaging itself. For live imaging, neural tubes have to be cooled down, sliced, cultured under a 318 microscope and intensively exposed to laser along z stacks over long periods of time. All these treatments 319 might impact on cell fitness and the pace of the cell cycle (e.g. changes in temperature, phototoxicity). 320 Some cells may not be affected but some may actually be struggling to divide normally leading to 321 artefactual lengthy cell cycle durations. Strikingly, we found that the reduction of cell proliferation 322 between 2 and 4 days of development, is of the same order of magnitude as the observed slowdown of 323 3D growth during the same time period indicating that the slowdown of proliferation is likely to be the 324 main driver of this change. Nevertheless, the relationship between cell proliferation and tissue growth is 325 complex. As we found no preferred orientation of cell division, the simplest assumption would be that 326 proliferation impacts growth of the tissue equally in all directions. However, while this might be true for a 327 ball of cells, it is less likely for a pseudostratified epithelium. Indeed, pseudostratified tissues are 328 monolayers and all new cells are added within the plane of the epithelium. Thus, proliferation should 329 solely impact apical expansion, which in the case of the neuroepithelium corresponds to growth along the 330 AP and DV axes. In addition, proliferation in neuroepithelial cells involves interkinetic nuclear movements 331 (INM) with cells sending their nuclei apically prior to mitosis. We previously showed that INM favor 332 expansion of the apical domain (Ferreira et al., 2019) which should further regulate AP and DV expansion. 333 However, this is mitigated by cell shape changes over time, neuroepithelial cells growing extensively along 334 their apicobasal axis while experiencing a reduction of their volume (Ferreira et al., 2019). Therefore, while new cells are added in the plane of the epithelium a significant part of the cell material is 335 336 redistributed along the AB axis, thus competing with INM-driven apical expansion.

337 We previously discussed that changes in the size of the apical domain, either by contractility or by 338 INM, could impact pseudostratification and AB elongation (Ferreira et al., 2019). The logic being that 339 enlarging the apical domain would allow cells to redistribute their nuclei within the 2D plane of the 340 epithelium while a reduction of the apical domain would force pilling of the nuclei along the AB axis. 341 However, this relationship is most likely to apply only in cells with a short AB axis as very elongated cells 342 will be less influenced by small variations of their apical domain. Therefore, in the case of the anterior 343 neural tube between 2 and 4 days of development, the increase of the mean cell cycle duration would 344 primarily contribute to the pace of AP and DV expansion rather than controlling the balance between 345 apical expansion and AB elongation. Changes in cell cycle dynamics have been linked to fate decisions in 346 the neuroepithelium (Molina and Pituello, 2017). Given the link between INM and cell and tissue shape, it 347 is also possible that changes in the dynamics of the cell cycle directly contribute to the morphogenesis of 348 the neural tube itself. This way, proliferation would coordinate identity and shape of neuroepithelial cells 349 ensuring the formation of a functional organ.

350

351 Materials and Methods

352 Dil injections

After 48h of incubation, eggs were windowed using curved scissors. A solution of CM-Dil (Molecular Probes C7000) diluted 1 : 10 from a 0.5% stock solution in Ethanol 100%, in 0.3M sucrose solution as previously described (Kulesa and Fraser, 2000) was then injected using a pulled glass needle. Two to three injections were performed along the AP axis. A picture was taken before closing the eggs and putting them back in the incubator for 24h. The center of each Dil spot was used for measurements of distance.

360 Whole-Mount Immunostaining

361	After fixation in 4% paraformaldehyde, embryos are washed in PBS1X / 0.1% Tween 20 and
362	incubated 60 minutes in PBS1X/1% Triton/5% Bovine Serum Albumin (BSA). Embryos are then incubated
363	with primary antibody in PBS1X/1%BSA/0.1% triton 24-48 hours at 4°C (anti-Sox2, Merck Millipore AB
364	5603; anti- γ -tubulin, Sigma GTU-88), washed in PBS1X/0.1% triton X100 and incubated with secondary
365	antibodies. All secondary antibodies were coupled with Alexa fluorophores and purchased from Invitrogen
366	Molecular probes (Life Technology) and incubated with DAPI or Phalloidin in PBS1X/0.1% triton X100
367	overnight at 4°C. Embryos are washed in PBS1X/0.1% triton X100 before mounting in mowiol and
368	observation or embedding for histology.
369	
370	Cryosections and immunostaining
371	After fixation in 4% paraformaldehyde (PFA 4%) or immunostaining, embryos are washed in
372	Phosphate Buffer (PB) and embedded in PB/15% sucrose/7.5% gelatin as previously described (Andrieu et
373	al., 2020). Individual blocks are cut under a dissecting microscope to position the embryo in the desired
374	orientation for sectioning. Blocks are frozen in isopentane (Sigma, 615838) at -70°C and stored at -70°C
375	until sectioning. Cryosections were performed using a cryostat Leica CM1950 as previously described
376	(Theveneau et al., 2007). Sections of 14 μ m were incubated in PBS1X at 42°C until gelatin was totally
377	removed, treated with PBS1X/1% triton X100/5% BSA for 1 hour for permeabilization and blocking.
378	Primary antibodies were diluted as mentioned above and disposed on sections overnight at 4°C under a
379	coverslip. Secondary antibodies were diluted as mentioned previously and applied on sections for 2 hours
380	at room temperature. All washes were done in PBS1X.
381	
382	Measurement of neural tube and somite length
383	Neural tube and somite lengths were measured using the line tools in FIJI. For all explants, images
384	were taken dorsally except for DMEM explants (Fig. 2D) which tend to slightly curl. Thus, dorsal views lead

to an underestimation of the actual anteroposterior length of the tissue. To circumvent this problem,

386 neural tubes from DMEM explants were separated and imaged laterally.

387

388 Quantification of cells

Mean area occupied by a DAPI stained nuclei was estimated in FIJI by manually segmenting at least 20 nuclei either in Sox2+ or in Sox2- areas, on multiple cryosections at 48, 72 and 96h of development. At each stage, Sox2+ and Sox2- areas were measured manually on each cryosection and estimation of the number of nuclei was obtained by dividing this surface by the corresponding average nucleus surface.

394

395 Estimation of mean cell cycle durations

396 At 48h, a 14 μ m-thick transversal section of the neural tube contains 318.6 cells on average whereas at 72h there are 1208 cells. Since we found no preferred orientation of cell division, daughter 397 398 cells that may be produced outside of the transversal section would be replaced by equivalent daughter 399 coming from divisions in adjacent sections. In addition, the relationship between the evolution of the 400 population in transversal sections and the whole tissue only stands if there are no spatially biased post-401 mitotic intercalation of cells. We postulated that such intercalation are negligible. Therefore, using these 402 two assumptions, we can extrapolate the actual number of cells at 72h by multiplying the number of cells 403 on a 14 μ m-thick section by the AP elongation factor between 2 and 3 days of development (1.44). This 404 gives a mean cell number at 72h of 1739. From these cell numbers, we can calculate the growth rate by 405 dividing the total number of cells at 72h (1739) by the total number of cells at 48h (318.6), in this case 406 5.46. Then, from this, the expected doubling time is : 24h (the interval between 48 and 72h of 407 development), times 2, divided by the actual growth rate between the two considered time points, here 408 $(24h \times 2) / 5.46 = 8.8$ hours of doubling time (8h50). We can apply the same calculation from the next

409 interval between 72 and 96h. Although this time, the total number of cells generated between 72 and 96h 410 corresponds to the total number of cells at 96h minus the number of differentiated cells (Sox2-negative 411 cells) already present at 72h corrected by the AP elongation factor between 3 and 4 days of development 412 (1.27). This gives a total of 3645.7 cells at 96h that were generated by the mean 1026 Sox2+ neural 413 progenitors at 72h. From this, we get a growth rate of 3.55 which gives a doubling time of $(24 \times 2) / 3.55 =$ 414 13.5 hours (13h30). 415 416 Mathematical Modelling 417 The complete description of the mathematical model is available in Ferreira et al., 2019. In the 418 present study, we only used the cell cycle clock to model cell population growth. Each simulation was 419 done 20 times and mean values are plotted. Each initial condition was modelled for 12h. The mean 420 growth rate was computed and applied to calculate the evolution of the cell population over the next 48h. 421 422 Image acquisition and analysis 423 Images were taken on a stereomicroscope Leica MZF10F equipped with a camera Leica DFC450C

and the LAS software, a Nikon Eclipse 80i equipped with a camera DXM1200C and the NIS-elements
software, a Zeiss Axio Imager 2 equipped with a Hamamatsu ORCA Flash 4 camera and the Zen2 software,
a confocal Zeiss 710 with Zen software and Leica SP8 with LasX software. Images were then processed and
analyzed with FIJI.

428

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439	
440	Figure Legends
441	Figure 1. Anterior trunk neural tube growth is slower along the anteroposterior axis than along the
442	dorsoventral and apicobasal axes between 48 and 96h.
443	A, Diagram of a chicken embryo at 48h of development indicating the position of the Dil injections. B-C,
444	merge of bright field and fluorescent pictures showing the evolution of the Dil spots between 48 and 72h
445	and 72 and 96h. The center of the bright spots in the neural tube is used as landmarks of the
446	measurements. D-E, anteroposterior length between Dil spots, normalized to t0; 48-72h, n = 11; 72-96h, n
447	= 8. Fold changes are indicated above each graph. F-H, representative transversal sections of the neural
448	tube at the forelimb level at 48h (F, $n_{embryos}$ = 4, $n_{sections}$ = 52 (at least 10 sections per embryo), 72h (G,
449	$n_{embryos}$ = 4, $n_{sections}$ = 38 (at least 8 sections per embryo) and 96h (H, $n_{embryos}$ = 4, $n_{sections}$ = 46 (at least 9
450	sections per embryo) of development counterstained with DAPI. I-J, Dorsoventral height (I) and apicobasal
451	width (J) at 48, 72 and 96h. Fold changes between stages are indicated on the graphs. Scale bars in B-C, 1
452	mm; in F-G, 50 μm , in H, 75 μm . K, growth rates along the three spatial axes between 48h and 72h and 72
453	and 96h, with paired statistical comparisons (t-tests) between growth rates of the AP axis and those of the
454	DV and AB axes for each developmental time window.

456 Figure 2. Anterior trunk neural tube anteroposterior length is restricted by interaction with the surrounding 457 tissues and anchorage to the extracellular matrix.

458 A, diagram of a chicken embryo at 48h of development indicating the position of 5 posterior somites and 459 pictures prior (in ovo) or after dissection (ex ovo). B, anteroposterior length of individual neural tubes in 460 ovo and after extraction (ex vivo), normalized to the mean length in ovo, n = 11, paired t-test, p < 0.0001461 (****). C, anteroposterior length of the middle somite within the 5-somite long portion in ovo and after 462 extraction (ex vivo), normalized to the mean in ovo, n = 11, paired t-test, p = 0.0106 (*). Note that 463 expansion of the NT (14%) post-dissection is significantly greater than that of the somites (6.9%), unpaired 464 t-test, p = 0.034 (*). D, Explants at t0 (n = 10), fixed immediately after dissection (left), or after 2h in 465 DMEM (middle, n = 16) or DMEM and Dispase at 1U/mL (right, n = 22). E, mean anteroposterior length of 466 the neural tube in each condition in D normalized to mean to length, ANOVA with multiple comparisons, 467 t0/DMEM, p = 0.2 (ns), DMEM/Dispase, p = 0.0066 (**). F, Explants at t0, fixed immediately after 468 extraction from the embryo (t0, n = 17, left), or after the paraxial mesoderm has been surgically removed 469 (dissection, n = 20, right), t-test, p = 0.4 (ns). G, mean anteroposterior length of the neural tube in each 470 condition in F normalized to mean to length. Scale bars, 100 µm. 471 472 Figure 3. Adhesion of neural tube cells to the extracellular matrix is required for apicobasal elongation and 473 pseudostratification.

A-B, transversal sections of neural tubes from the different experimental conditions shown in Figure 2 D-E,
counterstained with DAPI (DNA, green) and phalloidin (actin, magenta). Pictures in B display zooms shown
in panel A (indicated by the dotted lines). C-E, Dorsoventral height (C), apicobasal width (D) and
pseudostratification (E, number of nuclei along the AB axis) were assessed in each condition, n_{t0} = 8, n_{DMEM}
= 14, n_{Dispase} = 16. C, ANOVA with multiple comparisons, t0/DMEM p = 0.68 (ns); DMEM/Dispase, p =
0.0119 (*). D, ANOVA with multiple comparisons, t0/DMEM p = 0.35 (ns); DMEM/Dispase, p < 0.0001

480 (****). E, ANOVA with multiple comparisons, t0/DMEM p = 0.82 (ns); DMEM/Dispase, p < 0.0001 (****).
481 Scale bars in A 40 μm, in B 20 μm.

482

483 Figure 4. Mitoses are randomly oriented in the neural tube between 2 and 4 days of development.

484 A, diagram of a chicken embryo from 48 to 96h of development indicating the position of the regions 485 monitored, trunk portions are dissected, neural tubes open all along the dorsal midline and observed in 486 flat mount "open book" orientation (d, dorsal; v, ventral). B, categories of angles of division. Angles were 487 measured relative to the AP axis: 0-30° and 150-180° angles (white) were considered aligned with the AP 488 axis, 60-120° angles were considered perpendicular to the AP axis (black) and 30-60° and 120-150° angles 489 (grey) were considered to be in diagonal. C, example of the apical confocal plane of one half of a neural 490 tube at 48h of development in open book configuration; DNA in magenta (DAPI), γ-tubulin in green. D, 491 examples of divisions for each category color-coded as aforementioned. E, angles of all cell divisions 492 analyzed per region (n_{48-psm} = 144 divisions from 3 embryos, $n_{48-forelimb}$ = 136 divisions from 3 embryos, n_{72-} 493 forelimb = 568 divisions from 5 embryos, n_{96-forelimb} = 426 divisions from 6 embryos). F, angles of divisions per 494 category per region analyzed. Scale bar in C, 50 μm; in D, 5 μm.

495

Figure 5. Mean cell cycle length passes from 8h50 to 13h30 between 48h and 96h of development in the
neural tube at forelimb level.

A, Summary of previously published cell cycle durations measured during the first 5 days of development.
All obtained in chicken embryo, except for Kicheva et al., 2014 measured in the mouse neural tube. All in
the trunk region except for Fujita 1962 at 24h (anterior neural plate) and 5 days (144h, ventral brain). Data
points that are linked by a line indicate that authors have analyzed the cell cycle within this range of
developmental stages. Lack of line indicates that authors analyzed cell cycle time at specific stages but not
in between. Green bars correspond to our own estimation from biological data (see main text for details).

504	B, transversal sections of the chicken neural tube at the level of the forelimb region at 48, 72 and 96h of
505	development stained with DAPI (DNA, green) and Sox2 (neural progenitor cells, magenta). C, mean
506	number of total neural tube cells (black line) and Sox2-positive progenitors (grey line) per time point, per
507	transversal section (n_{48h} = 3 embryos-28 sections, n_{72h} = 3 embryos-28 sections, n_{96h} = 4 embryos-40
508	sections), values for 72 and 96h were corrected by the corresponding AP growth rates (see methods). D,
509	simulation of neural tube population growth versus biological data (green crosses) for 48h normalized to
510	t0 using fastest (5h, open circles) and slowest (28h, black dots) published durations of cell cycle in the
511	trunk neural tube and an intermediate regime (grey circles). E, simulation of neural tube population
512	growth versus biological data (green crosses) for 48h normalized to t0 using mean cell cycle durations
513	estimated from our biological data, i.e. constant 8h50 (open circles), constant 13h30 (black dots) and a
514	mixed regime of 8h50 for the first day and 13h30 the second day (grey dots), considering that all cells
515	proliferate throughout the simulation. Scale bars in B, at 48h, 50 μm ; at 72 and 96h, 80 μm . References in
516	panel A: (Fujita, 1962; Kicheva et al., 2014; Langman et al., 1966; Le Dreau et al., 2014; Olivera-Martinez et
517	al., 2014; Peco et al., 2012; Saade et al., 2013; Smith and Schoenwolf, 1987; Wilcock et al., 2007).
518	
519	Figure 6. Dynamics of the anterior neural tube growth fits with a homogenous lengthening of the cell cycle
520	duration together with a proportionally declining progenitor population
521	A-B, LOWESS curve fitting from our biological data to predict the evolution of the progenitor cell
522	proportion over time. C, Simulations with a mixed proliferation regime (cell cycle of 8h50 for the first day
523	and 13h30 the second day) with declining proportions of proliferating cells over time. Black curve and
524	dots: 100% for the first 24h of simulation, 85% between 24 and 36h and 70% between 36 and 48h. Dark
525	grey dots and curve: 100% for the first 24h of simulation, 85% between 24 and 48h. Light grey curve and
526	dots are the same as the mixed regime with 100% proliferating cells displayed in Figure 5 panel E,
527	provided for comparison. D, Simulations with a mixed proliferation regime and the declining proportions

- 528 of proliferating cells over time (100% for the first 24h of simulation, 85% between 24 and 36h and 70%
- 529 between 36 and 48h) with either a narrow range around the long cell cycle values (+/-1h, black dots and
- 530 curve) or a wide range around long cell cycle values (+/-5h, open circle and curve). All simulations were
- 531 done 20 times, mean is plotted.
- 532
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