

PERSPECTIVE

In preprints: releasing the brakes on neuronal maturation

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In mammals, the length of gestation and the timing of developmental milestones are fixed, with only minor variation between individuals of the same species. However, across species, developmental timing is fundamentally different and little is known about the mechanisms that determine species-specific tempo. Three recent preprints, two from Lorenz Studer's lab (Ciceri et al., 2022 preprint; Hergenreder et al., 2022 preprint) and one from Pierre Vanderhaeghen's lab (Iwata et al., 2021 preprint), describe different mechanisms that determine the timing of cortical neuron maturation in the developing central nervous system (CNS). The CNS is particularly interesting for the study of developmental timing because there is a high degree of conservation in molecular programmes that control formation, expansion and differentiation of the neural tube, but remarkable species-specific features in their timing and complexity. In humans, several mechanisms have been identified that contribute to increased cerebral cortex size (Espinós et al., 2022), but the protracted duration of neuronal maturation (neoteny) in comparison with other species remains far less understood.

Evidence from xenotransplantation of developing mammalian neurons into the brains of different species indicates that the timing of neural differentiation and maturation is primarily determined by cell-intrinsic programmes (Espuny-Camacho et al., 2013; Linaro et al., 2019). This species-specific pace is also maintained during pluripotent stem cell (PSC) differentiation and *in vitro* neuronal maturation (van den Amelee et al., 2014), which are the main model systems used by the selected preprints.

Hergenreder et al. developed a high-content imaging platform of homogeneous cultures of human PSC-derived deep-layer (TBR1-positive) cortical neurons, synchronised at cell cycle exit through Notch pathway inhibition. This allowed screening of 2688 molecules for the capacity to enhance morphological and functional parameters in these neurons, including nuclear size and circularity, total neurite length and branching, and the percentage of FOS- and EGR1-positive cells. From the screen, they selected 32 candidates that accelerated maturation across all features and ten that scored highly on a single parameter. This curated list of 42 molecules included inhibitors involved in epigenetic regulation, calcium signalling, cell cycle control, mitophagy and lipid and glucose metabolism, suggesting a function for all these pathways in neuronal maturation. Interestingly, the presence of compounds that selectively accelerate only one parameter may suggest that different aspects of neuronal maturation can occur independently of each other. After validation and manual curation, they chose four compounds that inhibit epigenetic regulators [the H3K4/9 demethylase LSD1 (KDM1A) and H3K79 methyltransferase

DOT1L] or activate L-type calcium channels and NMDA (N-methyl-D-aspartate) glutamate receptors. Treatment with a cocktail of these four compounds (aptly named 'GENtoniK') further enhanced maturation across all parameters. RNA sequencing and chromatin profiling showed a transcriptional upregulation and H3K79me2 enrichment, respectively, of genes with gene ontology terms or expression patterns suggestive of neuronal maturation.

In the second preprint, also from Lorenz Studer's lab (Ciceri et al., 2022 preprint), the authors took a different but equally ambitious approach, aimed at broadly characterising pathways involved in intrinsic maturation. They again used human PSC-derived TBR1-positive cortical neurons, but this time providing an in-depth description of morphological, electrophysiological, transcriptional and chromatin accessibility changes that accompany maturation over 100 days *in vitro*. In line with the findings from the compound screen (Hergenreder et al., 2022 preprint), chromatin regulators were among the most strongly downregulated genes during neuronal maturation. They propose that prolonged maintenance of an epigenetic signature in neurons may form a barrier for full neuronal maturation. Knocking out a selected list of chromatin regulators and transcription factors identified a set of genes required for slow maturation of human neurons. As the epigenetic regulators are already expressed in neural progenitor cells (NPCs), the authors also found that transient inhibition of the PRC2 regulator EZH2 or the histone lysine methyltransferases EHMT1/2 or DOT1L specifically in NPCs accelerates subsequent neuronal maturation. Whether these treatments affect the NPCs themselves remains to be determined. Temporal progression in NPCs, including through PRC2-mediated epigenetic modification (Hirabayashi and Gotoh, 2010; Telley et al., 2019), is known to determine neuronal subtype specification and the neuro-to-astrogenic transition. However, the authors do not describe obvious changes in neuronal identity upon NPC treatment. The lack of changes in the timing of spontaneous NPC differentiation could be masked because the protocol is optimised for the synchronisation of cell cycle exit to direct differentiation into TBR1⁺ neurons. Alternatively, these results may indicate that developmental time is measured differently in NPCs than in neurons.

In the third preprint (Iwata et al., 2021 preprint), authors from Pierre Vanderhaeghen's lab compared mouse and human PSC-derived neurons to identify species-specific features related to human neoteny. They used a comparable approach to the Studer lab, relying on a combination of Notch inhibition and NeuroD1 promoter-dependent genetic labelling to obtain homogeneous cultures of accurately birth-dated neurons, and observed a striking delay in mitochondrial growth in human neurons compared with mouse. Mitochondrial oxygen consumption gradually increases upon maturation in both species, but along a protracted time course in human neurons. In addition, metabolomic analyses revealed that human neurons display lower levels of glucose metabolism than mouse neurons at similar ages. Building on these observations, they showed that various aspects of human neuronal maturation can be accelerated by promoting mitochondrial pyruvate metabolism and

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oxidative phosphorylation. Although differentiation and maturation are clearly accompanied by profound rewiring of cellular metabolism, as also illustrated by the RNA sequencing and chromatin profiling results from Ciceri et al., functional experiments in the preprints from the Studer lab did not address a causal role for metabolism in controlling timing. The role of cellular metabolism on developmental rate is an active topic of research because it was recently shown that reduced metabolic rate in the human segmentation clock (Diaz-Cuadros et al., 2021 preprint) or in *Drosophila* NPCs (van den Ameele and Brand, 2019) and sensory organs (Cassidy et al., 2019) drives slower differentiation.

Whereas Iwata et al. compare maturation in cortical neurons from human and mouse, the preprints from the Studer lab investigate the time of maturation only in human. It would be interesting to determine whether the perturbations have a similar effect on mouse cortical maturation. Measuring morphological development of mitochondria could be a useful way to assess the species-specific pace of cortical differentiation in mouse and human. Hergenreder et al. also treated PSC-derived spinal motor neurons, melanocytes and pancreatic β cells with GENtoniK and saw an analogous effect – quicker maturation of these other differentiated cell types. Studies on the rate of development in spinal motor neuron differentiation and the segmentation clock of mouse and human have identified an association with the speed of biochemical reactions, whereby slower development in human coincides with higher protein stability in NPCs (Matsuda et al., 2020; Rayon et al., 2020). It would be interesting to investigate the epistatic relationship between GENtoniK and the speed of biochemical reactions. It is possible that epigenetic rewiring, calcium signalling and protein turnover act in parallel because transient inhibition of chromatin modulators in NPCs in the preprint by Ciceri et al. does not cause an obvious temporal change in progenitor timing. Similarly, it will be interesting to see whether the changes in mitochondrial metabolism identified by Iwata et al. act upstream, downstream or in parallel to the epigenetic programmes identified by the Studer lab, because cellular metabolism can modify the epigenome (Dai et al., 2020). Identifying the specific epigenetic mechanisms and genetic loci involved will be crucial, not only because differentiation and maturation are generally thought to be accompanied by chromatin compaction and repression to prevent aberrant de- and trans-differentiation (Arabaci et al., 2021), but also because DOT1L and H3K79me were previously shown to promote epigenetic memory and resistance to reprogramming (Onder et al., 2012). This seemingly contrasts with the notion put forward by Ciceri et al. that these epigenetic brakes need to be released to allow maturation, and opens interesting questions for future studies.

Although developmental time can be slowed or accelerated in most non-mammalian organisms, for example by changing temperature, humidity or nutrient availability (Shimizu et al., 2014), modulation of these factors is clearly more challenging in mammals. Intrauterine development provides a relatively constant and protected environment in which developmental time is highly stereotyped, but we know very little about how tempo regulation is encoded in the genome. These three preprints provide an intriguing glimpse into the cell-intrinsic accelerators or brakes that evolution has put in place to safeguard developmental time. It will be interesting to see whether releasing them comes at a cost, or whether there are limits for speeding up or reducing the tempo both in culture and in the womb.

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Competing interests

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