



Programa de Doctorado en Neurociencias

Diversification of neural progenitor cells in the radiation of the amniote cerebral cortex

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Dr. Víctor Borrell Franco, director, and Dr. Adrián Cárdenas Castelló, co-director of the doctoral thesis entitled “Diversification of neural progenitor cells in the radiation of the amniote cerebral cortex”

inform:

that Mr. Eduardo Fernández Ortuño has carried out under our supervision the above referred thesis, in accordance with the terms and conditions defined in its Research Plan and in accordance with the Code of Good Practices of the Miguel Hernández University of Elche, satisfactorily fulfilling the objectives set for its public defense as a doctoral thesis.

Which we sign for the appropriate purposes, in Sant Joan d’Alacant, on 23rd December 2025.

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informs:



That Mr. Eduardo Fernández Ortuño has carried out under the supervision of our Doctoral Program the work entitled “Diversification of neural progenitor cells in the radiation of the amniote cerebral cortex” in accordance with the terms and conditions defined in its Research Plan and in accordance with the Code of Good Practices of the Miguel Hernández University of Elche, satisfactorily fulfilling the objectives set for its public defense as a doctoral thesis.

Which I sign for the appropriate purposes, in Sant Joan d’Alacant, on 23rd December 2025.

Dra. Maria Cruz Morenilla Palao

Coordinator of the Doctoral Program in Neuroscience





This Doctoral Thesis, entitled “Diversification of neural progenitor cells in the radiation of the amniote cerebral cortex”, has been carried out at the Institute de Neurosciences, a joint center participated by the Universidad Miguel Hernández de Elche (UMH) and the Spanish Council for Scientific Research (CSIC), and with the distinction Excelencia Severo Ochoa.

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Eppur si muove. Y, sin embargo, se mueve. Aunque probablemente Galileo no pronunciara la famosa frase tras ser enjuiciado y condenado por la Iglesia, en cualquier caso, creo que estas tres breves palabras concentran poderosamente la esencia ética de cualquier científico genuino y de la ciencia en sí. Más allá de todo enmascaramiento de la verdad, la verdad es. En términos metafísicos, no importa cómo de poderosa sea la voz que niega la realidad. Esta sigue siendo, y sigue teniendo un efecto. El trabajo científico tan solo trata de expresar la verdad de la forma más cercana a la realidad. Este empeño, nacido y alimentado por la curiosidad, tiene profundas implicaciones. Porque, en términos epistemológicos, éticos y sociales, la forma en que el humano interpreta el mundo sí tiene una enorme importancia. Por ello, al defender Galileo una tesis heliocéntrica contraria al dogma eclesiástico, estaba haciendo mucho más que acercar nuestra verdad a la realidad. Estaba acercando, trayendo justicia a la vida humana. Que la Iglesia estuviera equivocada implicaba que no poseía la verdad, como había detentado durante miles de años y, por tanto, que no tenía la autoridad ni legitimidad que se autoconfería. En consecuencia, el poder terrenal de reyes absolutistas, basado en el derecho divino, perdía también su legitimidad. Y así la observación del cielo nocturno añadió otra pieza más para la creación de sistemas parlamentarios y democráticos, más justos, contruidos, supuestamente, sobre principios de igualdad, libertad y fraternidad. La actividad científica es, pues, revolucionaria para las sociedades humanas, a niveles que van mucho más allá que la curiosidad y la tecnología.

La historia de Galileo es tan solo un ejemplo. Otro también muy famoso es el de la teoría de la evolución. Hay muchos otros. Las compañías petrolíferas negaban que el plomo de la gasolina fuese dañino. Fumar no generaba cáncer. El hombre blanco era superior al resto. Las vacunas producen autismo. Siempre ha hecho este asfixiante calor en verano. Y, sin embargo, la realidad se mueve. Habrá quien niegue el cambio climático, pero este seguirá aumentando el riesgo de DANAs. Habrá quien niegue la nocividad del aire contaminado de las ciudades. Las muertes prematuras por enfermedades respiratorias seguirán ocurriendo.

¿Qué tiene que ver esto con la evolución del desarrollo cerebral en amniotas? Bueno, todo ello forma parte del sistema científico. Cuando crecía, disfrutaba mucho aprendiendo y descubriendo cuántas cosas había. Creo que tengo mucha suerte de disfrutar tanto aprendiendo. Nunca me aburro. Fue tomando forma la idea de ser científico. De formar parte de todo aquello. De yo poder contribuir a la expansión del conocimiento humano. Aunque mi curiosidad tiene un enorme peso en mi actividad, al final, creo que fue este compromiso con la verdad y la nobleza del trabajo científico lo que hizo que me decantara por una carrera científica, frente a otras opciones que también me apasionan, el arte y la historia. Así que creí oportuno, en este momento, cuando estoy adquiriendo a través de un ritual humano (Defensa de la tesis), una categoría oficial como científico, incluir una reflexión sobre qué significa para mí serlo. Independientemente de nuestro tema de estudio, ya sean terremotos, la colonización de la Luna, los progenitores neuronales o cualquier otra cosa, como científicos debemos mantener un compromiso férreo con la verdad. Debemos proteger la voluntad de que la actividad científica mejore la vida. Ignorar, negar la realidad, tiene fatales consecuencias. De modo que, como miembro de la comunidad científica, agradezco a todas aquellas personas cuya actividad y compromiso científicos han mejorado nuestra forma de vida, consolidado la justicia social, y nos han acercado más a las maravillas de este mundo.

Pero sí, teniendo en cuenta esto, lo que me mueve en el día a día es la curiosidad, las ganas de aprender. He tenido la suerte de encontrar un camino científico muy divertido. Me fascina la belleza de la biodiversidad y la complejidad de los sistemas biológicos. También, no sé por qué, cómo el pasado ha generado el presente me despierta un gran interés. Agradezco aquí a todos los excelentes profesores que tanto me han inspirado mis ganas de aprender. Así, dedicarme a entender el origen de las formas biológicas a través de la evolución y el desarrollo es una actividad donde encuentro un gran sentido. Agradezco pues, al doctor Víctor Borrell que pudiese quizás ver esta motivación cuando le contacté como un año y medio antes de acabar el máster, para incorporarme a su grupo de investigación.

Durante estos años, de lo que más he disfrutado han sido las reuniones sobre el proyecto con mis directores, Víctor Borrell y Adrián Cárdenas. En esta institución hay muchos casos donde las reuniones con los directores de tesis suponen estrés y miedo. No ha sido esta mi situación. Para mí, suponían la interpretación de resultados, la discusión de ideas y, aunque a veces no lo pareciera quizás por la forma que tengo de escuchar, el aprender de científicos consolidados. En general, por la mayor parte del tiempo, he sentido que había regularidad, tiempo para discutir sobre el proyecto y dirección atenta. También me he sentido intelectualmente valorado. Víctor y Adrián me han escuchado muchas largas horas. Además, me he sentido totalmente seguro a la hora de comunicar mis fallos, mis dudas, despistes, y demás dificultades. Ante esto, he tenido una escucha activa y proactiva para resolver los problemas inherentes al trabajo de investigación. Simplemente, he sentido que la generación de errores se entiende como una parte inevitable, integral, natural del trabajo científico. Creo que esto es de suma importancia para el trabajo cotidiano en el laboratorio. Habría sido mucho peor, y fatal para mi formación científica y bienestar, encontrarme en una situación donde solo poder comunicar buenos resultados. He participado en cinco conferencias internacionales, he podido presentar mis resultados todavía en un estado temprano (no todos los IPs permiten esto), y he podido colaborar en otros proyectos y publicaciones. Finalmente, tanto Víctor como Adrián me han enseñado técnicas para obtener resultados y cómo dar forma a esa información para convertirla en conocimiento. Todo esto ha formado parte de la buena dirección de la tesis y, por ello, expreso mi sincero agradecimiento.

Además, quisiera añadir otro especial reconocimiento a Adrián. Su intervención experimental ha sido fundamental. Aparte de enseñarme muchas de las técnicas y protocolos que he utilizado, él ha llevado a cabo una parte considerable de las electroporaciones, y la práctica totalidad de los experimentos de microscopía, cuyo análisis de imagen fue un esfuerzo conjunto. El alcance de esta tesis y sus implicaciones habrían sido mucho menores sin su trabajo, y se lo agradezco enormemente.

Otros miembros del laboratorio, del instituto, y amigos también han apoyado mi trabajo. Gracias a Esther por su asistencia. A Álex y Anna por su supervisión con las cirugías en ratón. Gracias también a Álex por compartir parte de su histología para el análisis de PH3 a lo largo de la neurogénesis. Gracias a Anna por el apoyo con los primeros experimentos de single cell. Aquello supuso una adaptación de las vacaciones de verano y de su propio plan experimental, debido al momento en que las serpientes iban a poner huevos, en otro acto de la gran profesionalidad que caracteriza a Anna. Creo que hicimos un buen trabajo. Le agradezco personalmente esta profesionalidad y todas aquellas otras cosas innumerables del laboratorio de las que se ha encargado. Enrico ha sido una fuente casi constante de ideas y de entusiasmo científico. Cuando compartimos mesa, casi discutíamos todas las tardes

de algún tema denso. En concreto para este proyecto, agradezco sus consejos para mejorar el análisis de imagen y su feedback, así como las tantas discusiones sobre paletas de colores. Agradezco a Rafa y Lucía sus consejos para el análisis de los datos de single cell. Y, en general, agradezco todos los demás gestos de ayuda cotidiana que he recibido, por parte de miembros del grupo y de otros miembros del Instituto de Neurociencias. Agradezco también a Antonio Caler su disponibilidad y compromiso con sacar adelante estos complicados experimentos. Gracias al personal del animalario y a todo el personal que profesa un profundo respeto por la vida animal al tratar con los modelos. Ah, y por supuesto, agradezco a mis compañeros de laboratorio haber sabido apreciar a través de mis silbidos a Mahler, Rachmaninov, Wagner et al.

Todos estos elementos han apoyado el trabajo de esta tesis y han permitido su realización. Aprovecho para agradecer aquí a la Fundació La Caixa. Nos ha cuidado. Además, escuchó nuestra petición de extender la duración de la beca a 4 años, de modo que gracias. Sin embargo, otros elementos lo han hecho todo mucho más difícil. *Es el sistema*, se oye decir. Para intentar cambiar algo, un grupo de doctorandos decidimos trabajar por mejorar el programa, desde aspectos administrativos y vida social, hasta aspectos de salud mental y laboral. Más allá de la odiosa respuesta frente a los problemas de *es el sistema*, queríamos tratar de hacer algo, aunque fuera a nivel interno, por cambiar, mejorar en lo que fuese, la situación de los doctorandos y cambiar la cultura laboral hacia una más sana. Pienso que el trabajo científico requiere pasión, compromiso personal, paciencia, capacidad de soportar la frustración, curiosidad y motivación. Pero es un mito que tenga que estar precarizado. Por la parte que nos toca a mí y a mis compañeros, es un mito que haya que sufrir en el doctorado. Puede haber la tensión, la frustración, el miedo y la emoción por la incertidumbre del trabajo experimental. Es esta emoción lo que hace que también nos encante. Pero no puede ser que dos de cada tres personas contestaran que necesitaban atención psicológica debido al doctorado en el informe que realizamos. Y que sirva este dato como la parte por el todo. Los resultados que obtuvimos se suman a los de tantos otros estudios (basta un poco de Google para encontrarlos). Y he aquí una verdad inefable, como la del plomo en la gasolina: aunque la ciencia sea maravillosa, el sistema académico actual muele el bienestar psicológico de sus miembros. Gracias a todos los científicos y científicas que, a pesar de ello, hacen ciencia.

Mi mayor agradecimiento es para todas aquellas personas que, frente al reaccionarismo y al inmovilismo, creían que podían contribuir a mejorar las cosas. Al final, creo que conseguimos avances importantes. Mejoramos el apoyo administrativo a los estudiantes extranjeros. Conseguimos que se nos diera membresía y voz en la junta directiva. Conseguimos que la comisión académica de este programa de doctorado se comprometiera a mejorar el sistema de evaluación y seguimiento. Propusimos y organizamos actividades sociales con el personal del IN. Conseguimos que hubiese una cultura en el IN donde lo que era un secreto a voces fuese una situación que resolver con urgencia. Quedan cosas por mejorar. Agradezco la escucha de la anterior dirección del IN bajo Ángel Barco y la actual bajo Juana Gallar. Agradezco sinceramente el esfuerzo de la CAPD por mejorar el programa y nuestra situación, en concreto, a Elvira de la Peña y a Cruz Morenilla. Por su compromiso, valentía, voluntad de cambio, tiempo, y por lo conseguido, agradezco profundamente y doy la enhorabuena a todos los doctorandos que formaron parte de este empeño, en especial, a Manuela, con quien compartí por dos años el honor y deber de representación de los doctorandos. El mundo no es así. Nosotros hacemos así el mundo.

Pero bueno. Como decía arriba, yo he disfrutado del trabajo científico, y lo agradezco. Además, durante estos años, tanto yo como los otros a quienes ahora me refiero, hemos podido disfrutar de tener un grupo con mucha energía social. Ha habido planes de todo tipo, y muy buenos recuerdos que quedan asociados a esta etapa. Junto a las dificultades de la tesis, hemos tenido amistad en abundancia. Agradezco pues que haya habido esta dinámica tan buena, que nos ha dado tanta diversión, y a las personas que la han hecho posible. Quizás debería incluir una tabla suplementaria con todas ellas o, ¿un aplauso, no? Entre ellas, como en todos los sitios por los que he pasado, he encontrado personas a quienes llamar amigo con la completa profundidad de la palabra. Y puedo decir que he sido bastante rico en buenos amigos.

Francamente, en estos años de tesis, esta ha sido la menor de mis preocupaciones. Personalmente he tenido experiencias muy duras. Estas personas han constituido un apoyo esencial para mí. En relación a la tesis, este apoyo me permitió seguir trabajando en ella durante meses muy difíciles. Merecen mi más sincero agradecimiento y saben que soy afortunado de tenerlas. Gracias a Sofía, Bea, Alicia, Francesco, Mirjam, Bárbara, Isa, Marina, Marina y a Manu por haberme ayudado en estos años. Con ellas, y con unos cuantos más, también ha habido muchas risas, intereses compartidos, geniales conversaciones e intercambios de ideas y, simplemente, el disfrute del otro y de su amistad. Gracias también a Esteban por haberme ayudado a aprender de otra forma y otro tipo de conocimiento distinto al científico.

Aunque no hayan estado directamente relacionadas con la tesis, otras amistades anteriores y que van envejeciendo también me han dado mucho en esta etapa. Agradezco su ayuda y apoyo, a Kia, Elena, Julián, Ana, Cris, Lola, Marina, Constan, Adri y Mauro. Se entiende la amistad como una relación de expectativas recíprocas. Aristóteles distingue tres tipos de amistad: por utilidad, por placer, y por carácter. La tercera, la más profunda, requiere conocer a fondo a la persona, pasar mucho tiempo juntos y descubrir ese carácter. Precisamente por conocer el carácter de estas personas a lo largo de años que se van acumulando, así como el de las que he descubierto en esta etapa, tengo grandes expectativas sobre mi amistad con ellas y tendré más motivos para agradecer tenerlas en el futuro.

Los padres nos dan la vida y nos abren el mundo. Se olvida esto. Probablemente ni siquiera sea consciente de cuánto de mí es ellos. Pero sé que me han transmitido gran parte de quién soy. Si la defensa de la tesis se entiende como uno de los hitos en la vida a los que llegar, agradezco a mis padres haber fraguado sólidamente mi desarrollo personal. Los valores que se cocinaron en casa me han permitido tener éxito académico, forjar sólidas amistades, exigir sin vergüenza aquello a lo que tengo derecho y expresar mi cariño a través de la dedicación en crear algo que compartir. Y todo lo demás. Gracias pues por permitirme ir llegando a todos los sitios por un buen camino.

La brújula, el descanso, el sol de mi camino es mi hermana, Caro. Gracias por ello.





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Abbreviations

Abbreviation	Meaning
aIP	apical intermediate progenitor
ANR	anterior neural ridge
AP	apical progenitor
APall	arcopallium
aRGC	apical radial glia cell
bIP	basal intermediate progenitor
BP	basal progenitor
bRGC	basal radial glia cell
CCA	canonical correlation analysis
Cl	claustrum
CoRC	core regulatory complex
CP	cortical plate
DEA	differential expression analysis
DEG	differentially expressed gene
dpo	day post-ovoposition
DVR	dorsoventricular ridge
ent. ortex	entorhinal cortex
EPall	entopallium
GO	gene ontology
GW	gestation week
HA	hyperpallium apicale
Hc	hippocampus
HD/HI	densocellular hyperpallium/intercalated hyperpallium
Hpall	hyperpallium
HPC	homeostatic property cluster
IHA	interstitial hyperpallium
IHC	immunohistochemistry
INM	interkinetic nuclear migration
iSVZ	inner subventricular zone
IZ	intermediate zone
K-Pg	Cretaceous Paleogene
MPall	mesopallium
NA	numerical aperture
NEC	neuroepithelial cell
NL	neuronal layer
NPall	nidopallium
NPC	neural progenitor cell
oSVZ	outer subventricular zone
PA	pallial amygdala
pir. ortex	piriform cortex
PSBP	pallial subpallial boundary
RGFs	radial glia fibre
RPCA	reciprocal principal component analysis

sAPs	subapical progenitor
sNPs	short neural precursor
SVZ	subventricular zone
TF	transcription factor
VS	ventricular surface
VZ	ventricular zone



Note to the reader

I have tried to keep a relaxed writing style, while keeping the academic rigor. Whenever specialized terms appear for the first time in the text, I have tried to clarify their meaning, just to be safe independently on the background of the reader.

The first part of the introduction is devoted to a broad evolutionary and developmental context for cortical development. I could have skipped this, or addressed it in a much briefer manner, going straight to the neural progenitor cells in the cortex, which is the topic of this thesis. But, to enrich my view and that of the reader, and to better contextualize the evolution of cortical development within the evolution of the whole brain, I took the chance to extend myself a bit more here and get to talk about dinosaurs. Besides, many of the discussions held for neuronal evolution can apply to neural progenitors, and it is good to have an idea of the neural landscape that neural progenitors generate when studying these.

Finally, I have divided the introduction in a series of sections whose content sometimes might seem redundant, but, for the sake of clarity, I have preferred to present the different aspects I considered opportune in a compartmentalized manner.





Abstract

A defining feature of **mammalian evolution**, compared to the much smaller homologous brain region in sauropsids (reptiles and birds), is the **massive expansion of the cerebral cortex**, forming the neocortex. The evolutionary mechanisms giving rise to the mammalian neocortex from its ancestors remain largely unknown. We hypothesize that changes in the genetic program of cortical development led to the **emergence of new types of cortical progenitor cells in the mammalian lineage** higher neurogenic capacity, thus expanding the cerebral cortex. In this project we carried out a multifactorial analysis of cortical progenitor cells comparing a gyrencephalic (ferret) and a lissencephalic mammal (mouse) with a bird (chicken) and a reptile (African house snake). This analysis comprises histological, molecular, morphological, proliferative and neurogenic traits, including modes of division and transcriptomic diversity at single cell resolution. Our results show **conserved principles of corticogenesis** across amniotes, such as limited expansion of the primary germinal layer, coexpression of Pax6 and Tbr2 in mitotic basal progenitors and presence of mitotic basal radial glia in birds. But they also evidence their **divergence**, in the progressive increase in basal progenitor number and proliferative capacity, the emergence of a basal radial glia pool and a dedicated SVZ in mammals and the distinct apical-contacting bRGC morphology retained only in birds. Altogether, we continue uncovering how neural progenitors evolved in mammals differently from sauropsids, changing the developmental plan that originated the neocortex and caused its massive expansion.

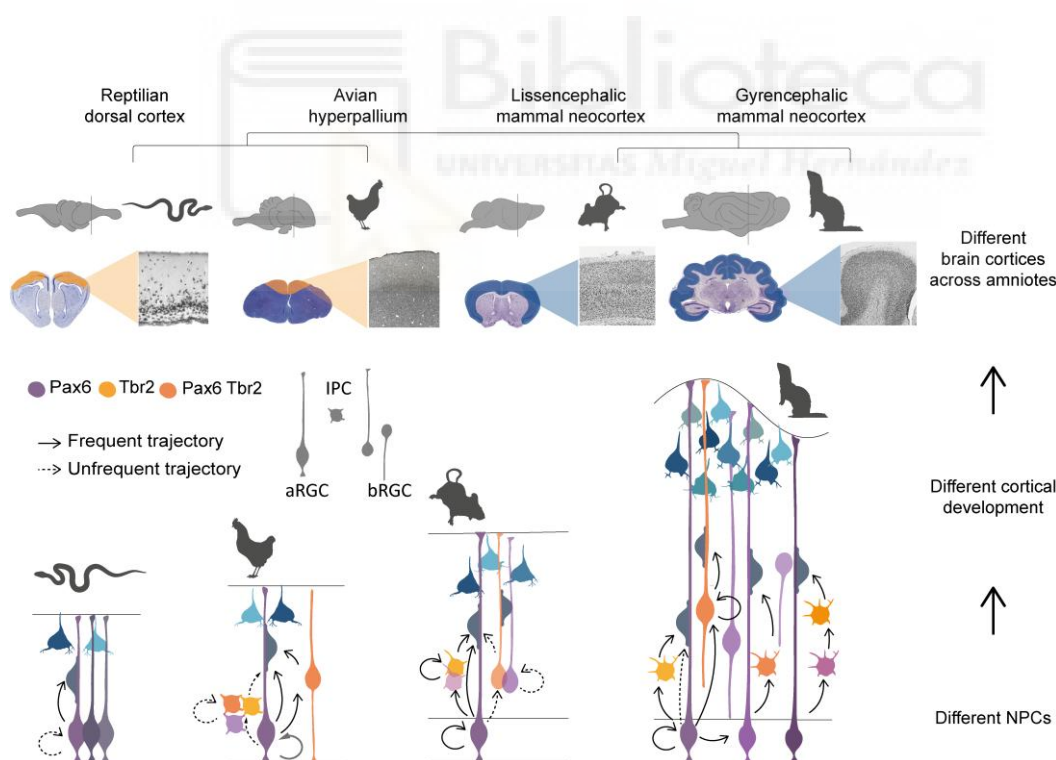
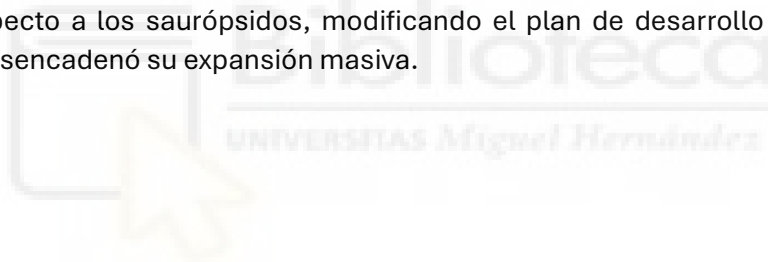


Figure 1. Graphical summary.



Resumen

Una característica definitoria de la **evolución de los mamíferos**, frente a la región homóloga mucho más pequeña que presentan los saurópsidos (reptiles y aves), es **la gran expansión de la corteza cerebral** que da origen al neocórtex. Los mecanismos evolutivos que generaron la neocorteza a partir de sus ancestros siguen siendo en gran medida desconocidos. Planteamos que cambios en el programa genético del desarrollo cortical propiciaron la **aparición de nuevos tipos de células progenitoras**, con mayor capacidad neurogénica, a lo largo de la línea mamífera, ampliando así la corteza cerebral. En este proyecto realizamos un análisis multifactorial de las células progenitoras corticales comparando un mamífero girencefálico (hurón) y uno lisencefálico (ratón) con un ave (pollo) y un reptil (serpiente doméstica africana). El estudio abarca rasgos histológicos, moleculares, morfológicos, proliferativos y neurogénicos, incluidos los modos de división y la diversidad transcriptómica a resolución de célula única. Nuestros resultados revelan **principios conservados de corticogénesis** entre los amniotas—como la limitada expansión de la capa germinal primaria, la coexpresión de Pax6 y Tbr2 en progenitores basales mitóticos y la presencia de glía radial basal mitótica en aves. Pero también ponen de manifiesto **divergencias clave**: el incremento progresivo en número y capacidad proliferativa de los progenitores basales, la aparición de un reservorio de glía radial basal y de una zona subventricular especializada en mamíferos, y la morfología distintiva de las bRGC con contacto apical, conservada únicamente en aves. En conjunto, continuamos revelando cómo los progenitores neurales evolucionaron de forma diferenciada en mamíferos respecto a los saurópsidos, modificando el plan de desarrollo que originó la neocorteza y desencadenó su expansión masiva.





1. - Introduction

1.1. - A developmental approach to understand the evolution of the brain cortex

The tree of life is composed of the most diverse and wonderful creatures, a diversity shaped by the two great creative processes of biology: **evolution** and **development** (Carroll, 2005). Indeed, from a developmental perspective, evolution can be seen as the modification of the preexisting forms and their associated functions, which then are tested for natural selection (Arthur, 2021; Breuker et al., 2006; Raff, 1996). These modifications can apply to the overall fundamental design of the body plan (e.g., a bilaterian body with a head, a torso and four limbs), to certain themes within the body plan (e.g. the formation of wings in bats or in birds from preexisting forelimbs), or to specific variations of such themes (e.g., the different shapes of the wings that different bat and bird groups can have). Although the appearance of wings in mammals and birds can be quite a fascinating topic to study, the variation that this work focuses on has to do with a specific part of the central nervous system: the dorsal pallium (**Figure 2**).

The **pallium** corresponds to the **dorsal telencephalon** and may be broadly defined as the roof of the cerebrum. This part of the brain underwent extensive variation after the Cretaceous-Paleogene (K-Pg) extinction in the amniote group (Briscoe & Ragsdale, 2019). **Amniotes** are a group of vertebrates comprising reptiles, birds and mammals that develop in their embryonic stage of life an envelope called amnion. In the case of reptiles and birds, collectively referred to as **sauropsids**, the modification of the pallium involved the expansion of its ventral region into the **dorsoventricular ridge** (DVR). After this, reptiles may have maintained an anatomy similar to the ancestral state (Briscoe & Ragsdale, 2018; Tosches & Laurent, 2019), whereas further changes occurred in birds, with a rearrangement of the neuronal populations and an increase in their numbers (Balanoff et al., 2013; Ksepka et al., 2020; Torres et al., 2021). In the case of mammals, the modification of the pallium entailed a massive expansion of the dorsal domain (referred to as **dorsal pallium** and, for the rest of the text, considered synonym to **dorsal cortex**). This expansion occurred radially, together with a 6-layered arrangement of neuron types, and tangentially, which resulted in the dorsal cortex covering the whole surface of the cerebrum and becoming its main part. As a result of these drastic innovations, a specific term for the mammalian dorsal cortex was coined: the **neocortex** (Rakic, 2009; Smaers et al., 2021). The neocortex, also termed isocortex, is actually one of the major innovations of mammalian evolution, one that has had definitory and transcendental implications (Sousa et al., 2017; Zilles et al., 2013). This is because, even if neocortex size does not necessarily imply a higher cognitive capacity, the enlargement of the neocortex might have untethered the development of complex behaviour and higher cognitive capabilities by allowing a better accommodation of a vast number of neurons into functional circuitries (Buckner & Krienen, 2013). At this point, several questions may be raised, but specifically for this work, we ask by which mechanisms the dorsal pallium of mammals expanded so much more compared to reptiles and birds.

To understand the evolutionary origin or modification of a form, in our case the dorsal pallium, one must know its provenance. In the case of hard structures, we can make use of the fossil record (if available). However, for the case of the brain, only a trace of its external appearance remains imprinted inside the skull (Balanoff & Bever, 2020). Nevertheless, as mentioned above, evolution works through changes in the developmental program of *preexisting forms*. Differently put, the developmental program found in each species is the

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result of the accumulated evolution in the past and is the starting point for the evolution of its lineage in the future.

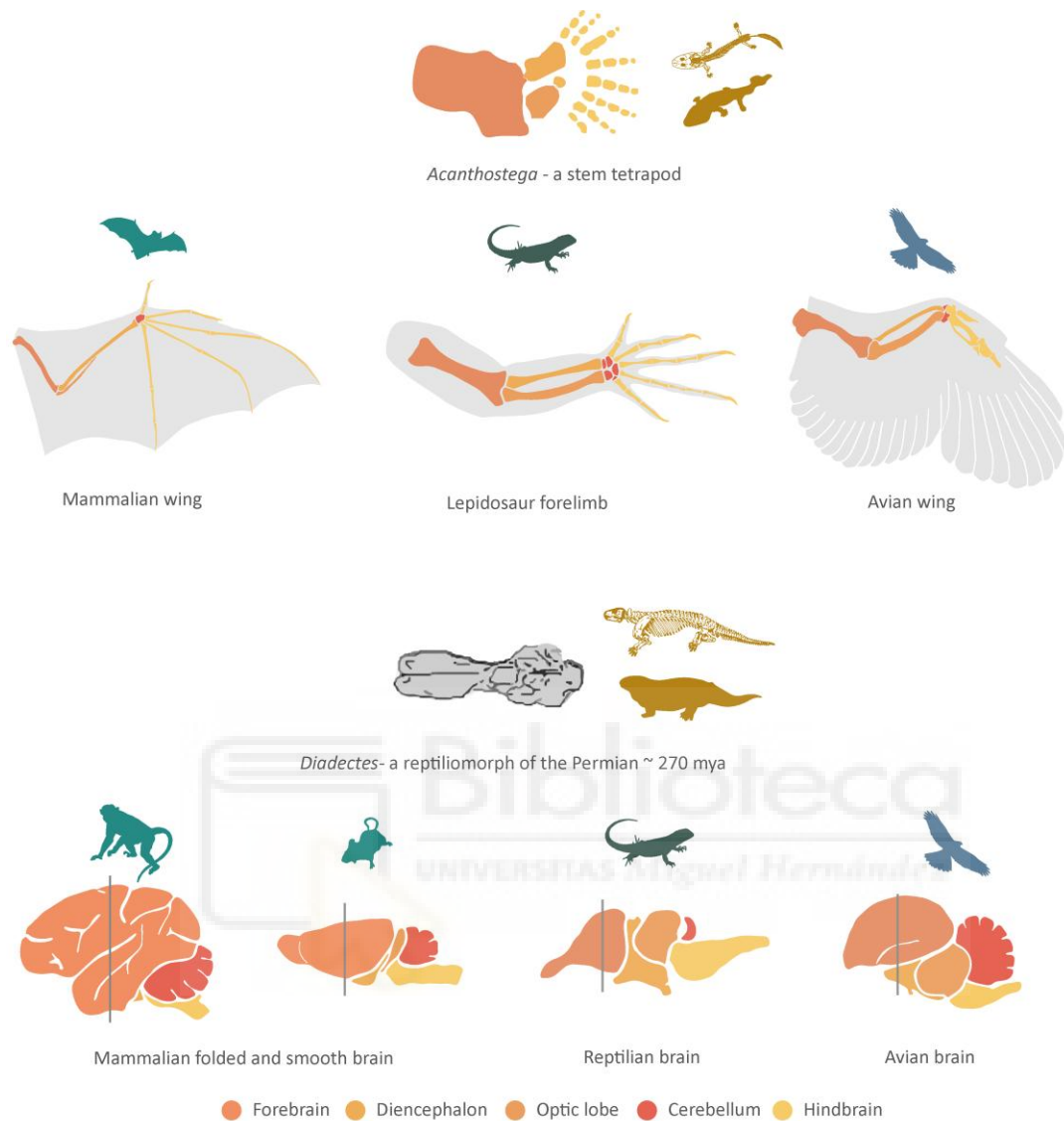


Figure 2. Evolution operates by the modification of preexisting forms through developmental repatterning.

Changes in the developmental program led to the modification of equivalent, i.e., homologous structures along the evolution of independent lineages, resulting into the modification of their shape and functions.

Linked to the notion of **developmental trace in evolution** is the notion of the **hierarchical nature of development**, in an ontogenic (i.e., developmental) and a phylogenetic sense, both related. Ontogenic hierarchy was already anticipated in the two first laws of embryology proposed by Karl Ernst von Baer, which remain very relevant still today (Abzhanov, 2013; Baer, 1828): (1) the more general characters of a concerned taxon appear earlier in their embryos than the more special characters; (2) the less general characters develop from the more general ones, and so forth, until the most specialized ones are formed. This means that, when evolutionary change occurs in a developmental trajectory, the modification at a lower level leaves the higher one in place. Because of this, and connecting with the phylogenetic sense, more general features define big phylogenetic groups, whereas the more specialized

traits define more specific, crownward and evolutionarily more recent phylogenetic groups. The relationship between the ontogenic and phylogenetic hierarchy was very nicely formulated by Keith Thomson in his book, *Morphogenesis and Evolution* (Thomson, 1988):

In principle we should be able to reconstruct for any species or any higher group a sequence of levels of morphological characteristics that define all the higher groups to which the taxon belongs, and to match these up with particular points in the hierarchy of morphogenesis.

This hierarchical nature of development offers us a way to understand the specific question of the origin of the neocortex. If we compare the developmental trajectories of the dorsal pallium in mammals with the one of sauropsids, this should reveal an early pattern of similarity, derived from the common ancestor, and the later divergence, exposing the specificities of the mammalian development of the dorsal pallium that led to its massive expansion. But what should we compare?

Development is an extremely complex biological process that operates at the molecular, cellular and tissue level, and in interaction with the environment. Developmental genes (and the effector genes they activate) determine the properties of stem and progenitor cells, as well as the progression of their differentiation lineages. These originate the differentiated adult cells in a very specific way in time and space, which position themselves in the tissue as this grows and acquires its final shape (morphogenesis). The whole process can be termed as **developmental patterning**. Thus, for novelty to follow after **mutations** (the cover term for change at the genetic level), this must be realized during development via an alteration at any of these levels, be it genetic/molecular, cellular or tissue level. Such adjustment of the developmental process is termed **developmental repatterning** (the cover term for developmental change) (Arthur, 2011). This concept and the one of hierarchy in development will be fundamental later when I address the problem of establishing a correspondence in comparative developmental studies across non phylogenetically close species.

Developmental repatterning occurs through four main types of change: **heterotopy** (change in place), **heterochrony** (change in time), **heterotypy** (change of types) and **heterometry** (change of rate/amount) (Arthur, 2021). In the specific case of the brain, this could be transcribed into changes in the location of neurons, in their pace and order of generation, in the types generated and their numbers (total and relative). This would apply to the resulting adult brain of the developmental process. Studying these changes in the neuronal populations between the brains of different amniote groups is a way of exploring their divergence, which has been previously studied and that is enriching our understanding of brain evolution across vertebrates (Hain et al., 2022; Hecker et al., 2025; Lamanna et al., 2023; Lust et al., 2022; Rueda-Ala  a et al., 2025; Tosches et al., 2018; Woych et al., 2022; Zaremba et al., 2025). I will address this in a later section of the introduction.

In this PhD Thesis project, we decided to focus on the developmental process itself. Accordingly, the previous question translates into what to compare when trying to understand the developmental repatterning of the expansion of the dorsal pallium that originated the neocortex in mammals differently to sauropsids. A possible answer could be the amount, the location, the types and the time of generation of the cells that are responsible for brain cortex development: **neural progenitor cells (NPCs)**.

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NPCs are the progenitor cells that generate the neurons and provide the scaffold for their migration to their final location in the cortex. The different properties of NPCs determine the number of neurons generated and therefore have a direct influence on the size of the brain and, in particular, of the dorsal cortex. It is our working hypothesis that an increase in the proliferative capacities of NPCs, in their numbers and in their diversity provided the substrate for the massive expansion of the mammalian dorsal cortex into the neocortex. By contrast, the neurogenic potential of NPCs would have remained more restricted in this particular area of the developing brain in reptiles and birds.

Specifically, we have decided to focus on the cellular level of developmental repatterning, undertaking a thorough characterization of NPCs during an early moment of cortical development in their histological context, and also exploring the genetic level through their active transcriptomes. This characterization has been performed in four animal models that span the amniote phylogeny and the diversity of brain cortices: **African house snake** (*Boaedon fuliginosus*), a reptile; **chicken** (*Gallus gallus*), a bird; **mouse** (*Mus musculus*), a mammal species with a smooth cortex; and **ferret** (*Mustela putorius*), a mammal species with a more expanded and folded cortex. The comparative characterization of NPCs in these four species should add another piece to the comprehension of the principles at the cellular level that took part in the developmental repatterning of the dorsal pallium towards the appearance of the mammalian neocortex.

In the following sections of the introduction of this thesis, I will first take the space to offer a brief background on the fundamental differences of the adult brains of reptiles, birds and mammals and their evolution. After all, even if to understand the divergence of adult structures we need to understand the divergence of their developmental programs, we still need to know how the adult structures themselves are. Following this first part of the introduction, I will move to the specific background of the matter here studied: cortical development across amniotes. Here, I will address the overall cortical developmental programs, the properties of NPCs and what we know about them in species of sauropsids and mammals. Finally, I will devote the last section of the introduction to the technical and theoretical aspects of how to study NPCs, and which strategy in particular we have chosen to do so.

1.2. - General view on pallial evolution in reptiles, birds and mammals

In this section, I present a broad vision on the objects of study, that is, the brains of reptiles, birds and mammals. I expose, first, the overall shared organization of the amniote brain and, second, the general external morphological and allometric changes that the brains in each of these groups underwent since the K-Pg mass extinction, focusing on the forebrain.

1.2.1. - General plan of the amniote brain

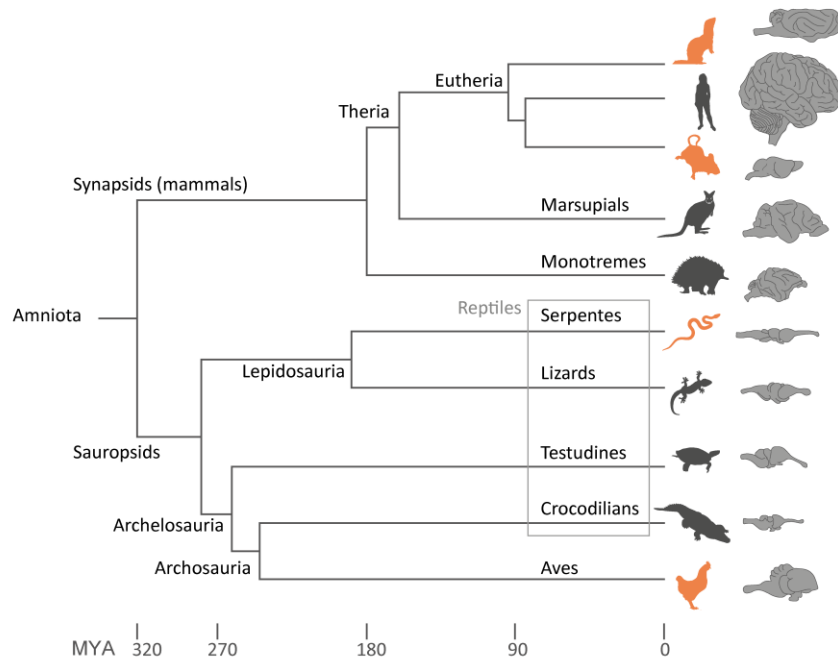
Amniotes are a group of vertebrates that are defined by their embryos developing inside a series of protective extra-embryonic membranes (Zug, 2008). This adaptation was key for the occupation of terrestrial ecosystems at the end of the Carboniferous period (ca. 350 Ma), and since then the group diversified into what we know as reptiles, birds and mammals (Irisarri et al., 2017). In a more phylogenetically accurate manner, amniotes are divided in two main clades: synapsids and sauropsids. Synapsids include **mammals** and their extinct relatives. Sauropsids include the extinct relative groups and the modern extant groups lepidosauria, or lizards and snakes; testudines, or turtles; and archosauria, which includes crocodilia and dinosauria, whose only survivors are **modern birds**. Therefore, by the common name “**reptiles**” we refer to a paraphyletic group that includes all sauropsids except birds (**Figure 3a**).

All amniotes share a common basic brain organization (Wullimann, 2016). Following the ontologically based prosomeric model nomenclature, the brain is divided into the **forebrain**, **midbrain** and **hindbrain** (Puelles et al., 2013) (**Figure 3b**). The telencephalon and diencephalon develop from the forebrain, the mesencephalon, from the midbrain, and the rhombencephalon, from the hindbrain. This basic plan is shared by other vertebrate groups, but not by more ancient chordate stem groups (Albuixech-Crespo et al., 2017; Murakami et al., 2005; Striedter, 2005). Within the forebrain, the telencephalon and the hypothalamus constitute the secondary prosencephalon. The diencephalon develops into three major domains, the pretectum, the thalamus and the prethalamus, plus into the corresponding segmental regions. For the hindbrain, this model recognizes 12 segments, which develop into the isthmus, cerebellum, pons and medulla oblongata.

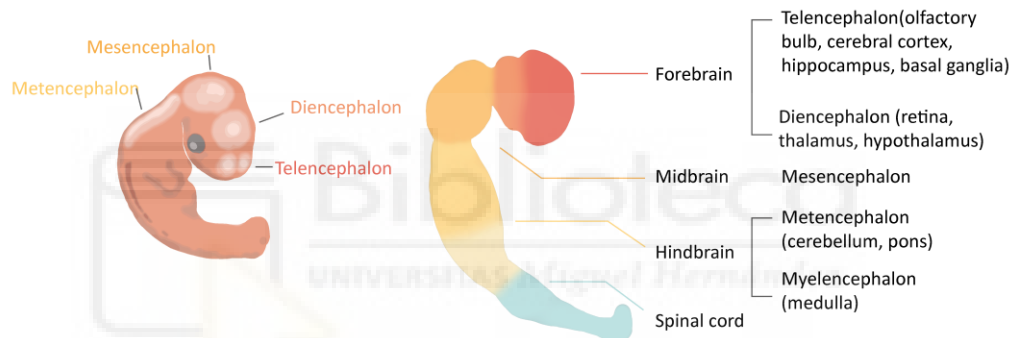
Of major interest for this text, the telencephalon is partitioned into the pallium and the subpallium. These two subpartitions are commonly defined very vaguely: the **pallium** is “the roof of the telencephalon”, or dorsal telencephalon, and the **subpallium**, or ventral telencephalon, lies “under it” (Montiel & Aboitiz, 2015; Pessoa et al., 2019; Striedter & Beydler, 1997; Tosches & Laurent, 2019; Yamamoto et al., 2024). In any case, this rough subdivision has a molecular and developmental foundation based on a complex pattern of expression of transcription factors (TFs) and morphogen gradients (Leung et al., 2022; Montiel & Aboitiz, 2015). For now, what we need to know is that the pallium is divided in a medial, dorsal, lateral and ventral domains, and that it originates the neocortex, the olfactory bulb, claustrum and pallial amygdala in mammals, and their equivalents in reptiles and birds. The subpallium originates the basal ganglia, the striatum, the pallidum, the diagonal-basal area and the preoptic area (Medina, 2009; Puelles et al., 2013). In a later section I will address in more detail the developmental organization and progression of the pallium across amniotes (**1.3.2, 1.3.3, 1.3.4**).

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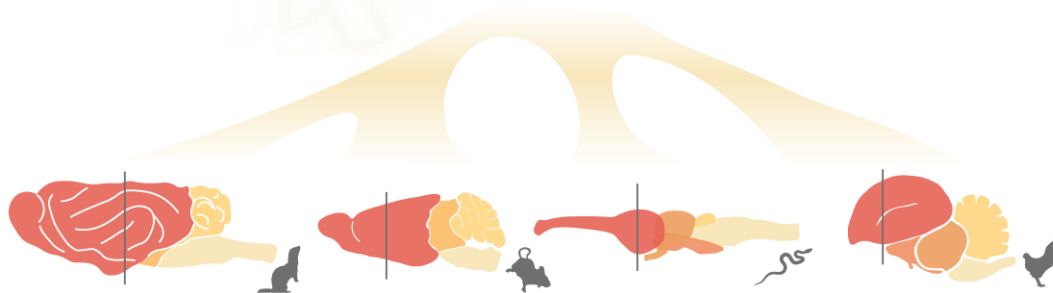
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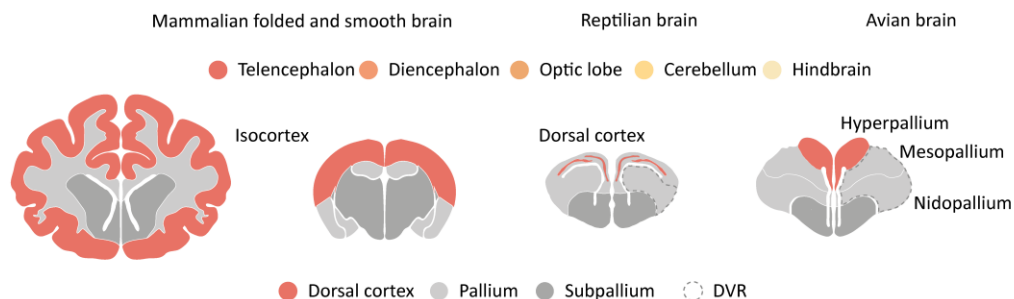


Figure 3. Amniote brain evolutionary diversification and developmental repatterning.

Amniote brains have largely diverged during their evolutionary history (a). From a common starting embryonic brain bauplan (b), the developmental program diverges into brains whose homologous components acquire markedly different shapes. In both mammals and birds, the forebrain has largely expanded (c). In particular within the forebrain, the dorsal cortex has experienced a drastic enlargement in the mammalian lineage (d). Animal silhouettes are from <http://phylopic.org/>.

Although all amniotes share this common broad bauplan of their brains, these have greatly diversified in the different groups of reptiles, birds and mammals, and within each group (**Figure 3c**). Each of them is characterized by a different degree of elaboration of the regions and subregions of their brains (Medina, 2009). In the case of the forebrain, all amniote groups underwent an expansion of the telencephalon compared to their stem groups (Briscoe & Ragsdale, 2019). Within the telencephalon, whereas the subpallium maintained a more conserved organization, the pallium greatly diverged in its structure and functions (González et al., 2014; Pessoa et al., 2019). Here, there are two points to make. (1) On the one hand, in sauropsids the pallium expanded more in the ventrolateral domains, originating the dorsoventricular ridge (DVR). On the other and, in mammals it was the dorsal domain of the pallium that expanded, originating the neocortex (**Figure 3d**). (2) The overall expansion of the pallium remained more moderate in the different reptile clades, whereas in both birds and mammals it was massive, with a huge increase in its size relative to the rest of the brain, in neuron numbers and in neuron density (Kverková et al., 2022; Olkowicz et al., 2016). In both groups, this was accompanied by the development of complex behaviours, including elaborate vocalization and communication, higher degree of cognition, learning abilities, social interactions and communities, prolonged parental care, nesting, etc. (Baciadonna et al., 2021). However, as just stated, developmentally and anatomically, the expansion of the pallium occurred very differently in birds and mammals, affecting different regions in a different manner. Additionally, and worth mentioning, birds and mammals also independently evolved huge cerebellums, in contrast to reptiles. Let us examine now in greater detail the different evolutionary routes that the brain of reptiles, birds and mammals took. For this, the improvement of imaging technologies has allowed a deeper exploration of fossilized skulls, revealing their internal surface or **cranial endocast**, which can provide a remarkable level of detail on the external morphology of the contained brain (Balanoff & Bever, 2020b; Early et al., 2020; Fabbri et al., 2017).

1.2.2. - Amniote brains diversification after the Cretaceous-Paleogene mass extinction

The evolution of reptilian brains

Although reptiles constitute a paraphyletic group, and there are differences in the pallial organization between lepidosaurs, testudines and crocodylians (Naumann & Laurent, 2020), these clades still share extensive anatomical and functional features, which makes reasonable talking of a “reptilian brain” (Naumann et al., 2015). Compared to the more “globular” form in birds and mammals, modern reptilian brains have kept a “tubular” organization, as the different regions of the forebrain, midbrain and hindbrain are linearly arranged in the dorsocaudal axis (Paulina-Carabajal, Jiménez-Huidobro, et al., 2023). While their telencephalon constitutes a fraction of the brain higher than in amphibians’, the sister group of amniotes (Faltine-Gonzalez & Kebschull, 2022), it has nowhere near acquired such a preponderant space as in birds and mammals (Tsuboi et al., 2018) (**Figure 3**). It has a broad elongated shape, and usually the olfactory bulbs extend conspicuously rostrally, conforming a relatively large proportion of the forebrain. The reptilian brain is also characterized by having a very low neuronal density, which is on average 20 times lower than in birds and mammals of similar body sizes (Kverková et al., 2022). It is possible that the fact that reptiles did not develop endothermy posed a metabolic constraint to the generation of an energy-demanding abundant neuron population (Kverková et al., 2022; Osvath et al., 2024).

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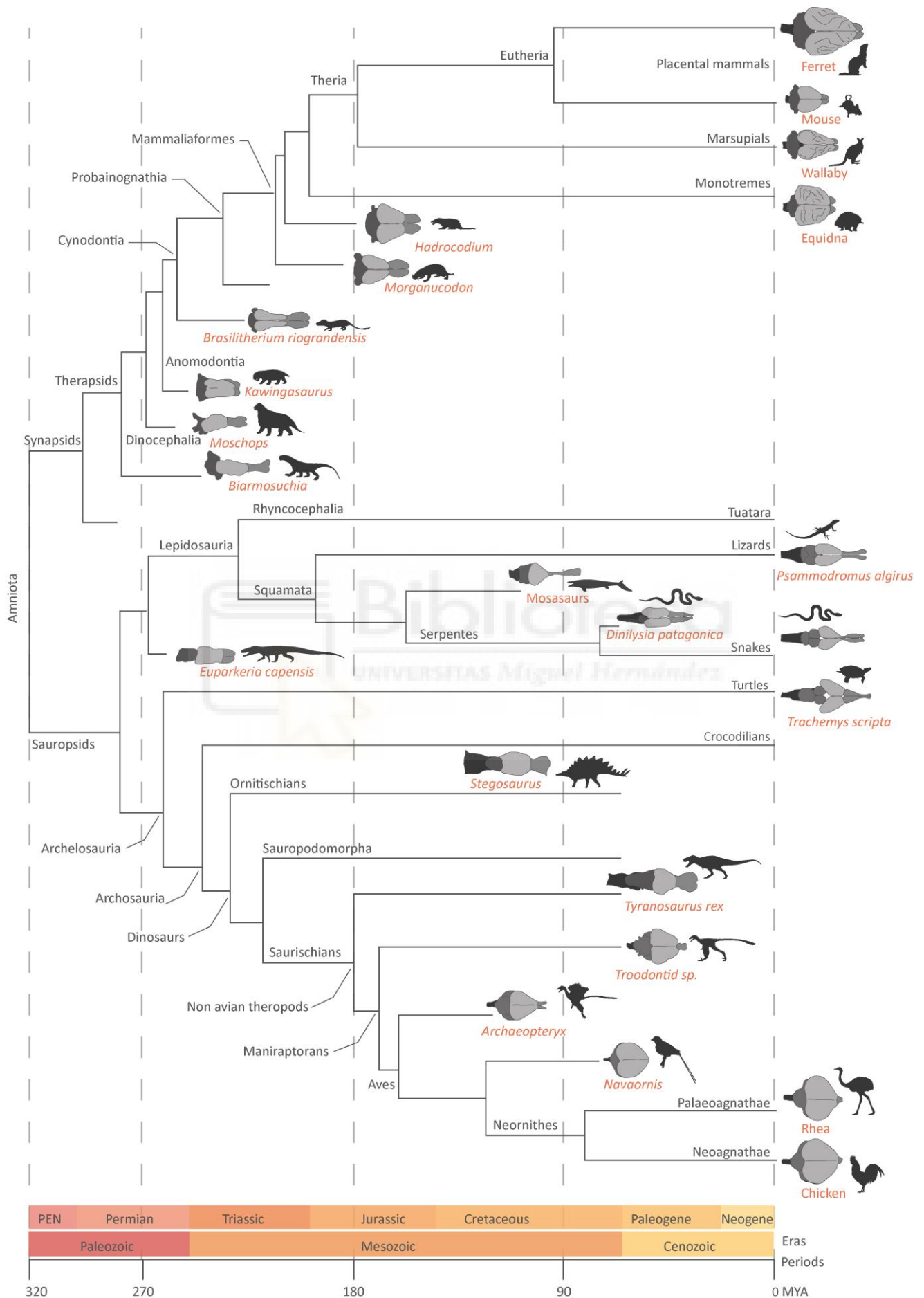


Figure 4. Endocasts illuminate the evolutionary changes of the brain in the amniote lineages leading to mammals, reptile groups and birds.

Early synapsids and diapsids presented elongated and tubular brains, with a modest telencephalon. This general anatomic arrangement is similar to the one of today's reptiles. On the other hand, both lineages leading to mammals and modern birds underwent an expansion, globularization and topological rearrangement of the telencephalon. Brains and animal size are not scaled. Animal silhouettes are from <http://phylopic.org/>.

Tracking the overall external morphological changes of reptilian brains along their evolution has revealed that the plesiomorphic condition was very similar to today's, with an elongated morphology and the several regions linearly arranged (Fabbri et al., 2017). This assembly was conserved in all basal forms of dinosaurs, including ornithischians, sauropodomorphs and early theropods (Balanoff & Bever, 2020; Witmer & Ridgely, 2009). It is not until the appearance of dinosaur groups phylogenetically closer to birds (maniraptora) that the fossil record shows an endocast more reminiscent of the modern shaped avian brain (Balanoff et al., 2013b; Balanoff & Bever, 2020b; King et al., 2024) (**Figure 4**).

Altogether, the facts that the different orders of extant and extinct reptiles show a remarkably similar overall architecture and their low neuron numbers make plausible to state that, details apart, the reptilian brain did not undergo much evolutionary change since the K-Pg extinction. This makes reptiles good models to get a closer proxy to the brain organization of the common ancestors of mammals and sauropsids (Naumann et al., 2015; Nomura, 2013), allowing more accurate inference of the mammalian specific novelties of pallial development.

The evolution of avian brains

The evolution of the avian brain is characterized by (1) a huge expansion of the forebrain, the cerebellum and the optic lobes (Jarvis et al., 2005; Reiner et al., 2005); (2) by a huge increase in neuron numbers and density (Ksepka et al., 2020; Kverková et al., 2022; Olkowicz et al., 2016); (3) by a modification of the linear arrangement and tubular shape into a sinusoidal arrangement and globular shape (Balanoff et al., 2013), such that the brain of many extant birds is flexed in an S-shaped manner along the rostro-caudal axis, connecting ventrally with the spinal cord, differently to the more linear arrangement of stem avian and non-avian theropods (Widrig et al., 2024); and (4) by an internal reorganisation of the functional domains of the pallium (Jarvis et al., 2005, 2013; Rueda-Alaña et al., 2025; Zaremba et al., 2025). In particular for the forebrain, as in reptiles, the structures that arise from the ventral and lateral domains of the pallium are the ones that have more substantially expanded. However, the increase in their size has been much more evident, both absolute and relative to body size. In addition, the dorsal pallium also exhibits a more elaborate and neuron-dense structure than in reptiles (Jarvis et al., 2005). As a result of all these changes, birds are the only group of vertebrates whose brains match the mammalian ones in terms of size, relative size, neuron numbers, density and cognitive capacities.

It was thought that the expansion of the avian pallium and cerebellum was linked to the acquisition of flight (Domínguez Alonzo et al., 2004). However, the study of the fossil record is allowing to reconstruct how such changes occurred in the dinosaur lineage that led to modern birds, showing that non-avian theropods already experienced reshaping of their cranium and brain close to the avian condition. Maniraptorans experienced an expansion of the cranial vault, of the ocular orbits and a shortening of the facial region (Balanoff & Bever, 2020; Lee et al., 2014). For instance, oviraptorosaurs and troodontids endocasts exhibit a trend in brain-to-body ratio (Balanoff et al., 2013), a degree of brain flexion into sinusoidal

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arrangement, and an expansion, albeit modest, of the telencephalon and cerebellum close to extant birds (Balanoff et al., 2013; Chiappe et al., 2024; Torres et al., 2021). Therefore, evolutionary changes leading to the avian brain started to occur during the Cretaceous before the appearance of modern birds. The size of the avian brain would not have been a primary adaptation for flight, but an exaptation (an adaptation that once consolidated allows for the development of other features) (Balanoff et al., 2016; Beyrand et al., 2019; Knoll & Kawabe, 2020). In the more crownward avian lineage, these changes were followed by a significant increase of the telencephalon (Watanabe et al., 2021), whose hyperinflation was actually the main driver of the high degree of encephalization measured in birds (Amy M. Balanoff et al., 2016) (**Figure 4**).

Later, the diversification and encephalization of the avian brain seem to have experienced a burst in the aftermath of the K-Pg extinction, according to measurements in brain-to-body size, as the disappearance of many competing groups opened the occupancy of a wide variety of ecological niches by birds (Ksepka et al., 2020). These measurements show that many avian groups reached a higher brain-to-body size because there was a decoupling between the body miniaturization and a corresponding decrease in brain size. That is, as there was an overall decrease in body size, a relatively big brain was maintained. This pattern is especially prominent in parrots, one of the bird groups with highest cognitive capacities. On the contrary, the other of such groups, corvids, experienced an opposite tendency. They followed the same pattern as humans: as they increased in body size, their brains grew much faster (Ksepka et al., 2020).

Finally, numbers-wise, bird brains have acquired a much higher and denser neuronal population than reptiles (17-fold increase in telencephalic neurons, 45-fold increase in cerebellar neurons) and, depending on the clade, can be higher than some mammalian groups, even primates (Kverková et al., 2022; Olkowicz et al., 2016). Specifically, the increase in neuron numbers in birds is proportionally much higher in the telencephalon than in other brain regions. This could be one of the variables enabling the development by birds of complex behaviours as we observe in mammals (Baciadonna et al., 2021), very likely related to the wiring of complex neuronal circuits (Nieder et al., 2020; Stacho et al., 2020). The independent evolution of complex behaviour and cognition by birds and mammals and whether, despite the homoplasy (similarity of a character that is not the result of inheritance from a common ancestor), there are common basic neuronal principles after it (Calabrese & Woolley, 2015; Stacho et al., 2020a) is an extremely interesting research topic, (Güntürkün et al., 2021). All in all, the fact that the avian lineage experienced rapid pallial evolution, but keeping a more restricted dorsal pallium compared to other regions and to the mammalian neocortex, positions birds as yet another model to compare when highlighting the properties of the NPCs of the dorsal pallium in mammals.

The evolution of mammalian brains

Mammalian brain evolution is markedly characterized by (1), as in birds, an overall change in shape, from tubular and linear to globular and sinusoidal, and arrangement, sitting ventrally to the spinal cord, and (2) a great expansion of the telencephalon, that includes portions of the diencephalon and the cerebellum, but which affected massively and disproportionately the dorsal pallium. This region expanded radially, but mostly tangentially, increasing its surface, covering the whole exterior of the telencephalon, and folding into gyri and sulci in the majority of mammalian species (Briscoe & Ragsdale, 2019; Kaas, 2009; Llinares-Benadero & Borrell, 2019). As definitory of the mammalian lineage, the dorsal cortex receives

a specific name, the **neocortex** (also known as **isocortex**). The expansion of the isocortex allowed the development of another mammalian brain trait: allocation of specific sensory, motor and association functions to specific regions throughout the cortex. This functional compartmentalization goes from 15-20 regions in mammals with smaller brains up to the 150 regions that we distinguish in humans (Cadwell et al., 2019). The increase in brain size was accompanied by a huge increase in neuronal numbers, density (Kverková et al., 2022) and types (Lodato & Arlotta, 2015). This neuronal diversification was functionally reorganized in six layers across the radial axis that span most of the cortical surface, while other parts of the brain (hippocampus, olfactory bulb and olfactory cortex) retained the three-layered structure that reptiles also have. Apart from this, mammalian brain evolution is also characterized by an increase in the size and neuron numbers of the cerebellum (Kebschul et al., 2020).

Synapsids diverged from diapsids (the stem group of sauropsids) about 300 mya in the Carboniferous period. The reconstruction of the brain morphology of these animals (therapsids) is difficult due to the partial ossification of their skulls (Benoit et al., 2017), but therapsid brains seem to be all narrow and small, similar to the reptilian ones. A few fossils of some very early therapsids with a fully ossified endocast have allowed to reconstruct some of the earliest variations in the synapsid lineage. At this point (Permian period), the brain of these animals (dinocephalia and biarmosuchia) is still rather tubular, but is strongly flexed at the midbrain, creating an angle between the forebrain and the hindbrain. Their brains are also primitive in other aspects, as there is no enlargement of the telencephalon, nor the cerebellum, there is probably no separation of the cerebral hemispheres and the olfactory bulb was likely very small (Benoit et al., 2017) (**Figure 4**).

Another fossil of the Permian period that belongs to a more derived, although still primitive, group, anomodontia, shows some first evidence of traits that are attributable to the neocortex (**Figure 4**). The endocast of the *Kawingasaurus* exhibits inflated cerebral hemispheres, these are demarcated by the medial sulcus and a possible rhinal fissure is detected. This fissure is used in modern mammals to delineate the ventral piriform cortex from the dorsal neocortex. *Kawingasaurus* also shows a degree of encephalization larger than the one of other non-mammalian therapsids. Additionally, although this brain is still narrow, it is dorsally inflated. All in all, Laaß & Kaestner (2017) interpret these data as the common ancestor of anomodonts and mammals having a primitive thin layer of neocortex dorsally positioned and limited in surface.

Moving closer to crown mammals, in cynodonts, a stem clade to mammaliaforms, the medial sulcus is also observed, as well as an enlarged cerebellum, but the cerebrum maintains the primitive tubular shape (Kielan-Jaworowska et al., 2004; Quiroga, 1980, 1984). These animals are thought to have maintained poor sensory capacities and motor coordination, given the small size of their olfactory bulbs and the lack of ossified nasal turbinates (Rowe et al., 2011). The cynodonts *Brasilitherium riograndensis* and *Trucidocynodon riograndensis* show nevertheless other more mammalian-like traits, with a larger olfactory bulb, presence of hemispheres and encephalization degree higher than other cynodonts (Rodrigues et al., 2014) (**Figure 4**).

Within the proper crown lineage that led to mammals, mammaliaforms, deeper reshaping of the brain into the mammalian condition occurred. The endocasts of *Morganucodon* and *Hadrocodium* from the early Jurassic (~ 175 mya) suggest that the evolution from the primitive therapsid brain into the mammalian brain occurred in three steps (**Figure 4**). In the first step

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of encephalization, as inferred from the *Morganucodon* endocast, the mammalian brain would have undergone a profuse enlargement of the olfactory bulb and cortex, together with an increase of the cerebral hemispheres and cerebellum. The cortex is no longer narrow, but enlarged laterally, covers the midbrain and is wider than the cerebellum. The endocast of *Hadrocodium*, the fossil closest to extant mammals, shows a second stage, where there was a further increase in brain size to the range of modern mammals. Again, this increase affected mostly the olfactory bulbs and the hemispheres. Lastly, in the third stage, there was still a further increase of the olfactory sensitivity, with an expansion of the olfactory epithelium and new nasal bones. Therefore, olfaction seems to have been crucial for these early base stem mammals, according to their presumed nocturnal and burrowing habits (Northcutt, 2011; Rowe et al., 2011).

Finally, the fossil record shows that much of the repatterning of the mammalian brain occurred during the last 65 Mya, after the K-Pg mass extinction. During mammalian evolution, the enlargement of the brain affected the neocortex much more than other of its parts, and so it became its biggest portion. This is referred to as **neocorticalization** and has occurred in all the evolutionary lines within mammals, monotremes, marsupials and placental mammals (Lewitus and Kalinka, 2013; Lewitus et al., 2013; Striedter, 2005) (**Figure 4**). Here it is worth mentioning that monotremes split from therian lineages very early ~200 mya. Therefore, a six layered neocortex was already present in the ancestral forms of modern extant mammals (Krubitzer et al., 1995) and must have originated between the emergence of stem-mammals from cynodonts (~250 mya) and the appearance of monotremes. In the tree lineages there are species with smooth and folded brains, and then it seems likely that gyrification occurred several times through mammalian evolution, as the independent loss of a character in different lineages is less likely to have occurred. Additionally, no present-day mammal species of the size equivalent to that of the postulated common ancestor presents folds. The phenomenon, which appears linked to cortical size exceptions apart (Lewitus et al., 2013; Reillo & Borrell, 2011), might simply, in combination with an early genetic patterning, come along with the increase in proliferation, neuronal migration and differential mechanics of the outer and inner layer of the neocortex (Franchini, 2021; Llinares-Benadero & Borrell, 2019), and probably the conditions for folding were present in the mammalian ancestor.

As in birds, in mammals there was decoupling between the rate of change of body size and brain size (Smaers et al., 2021; Tsuboi et al., 2018). Most early shifts occurred soon after the K-Pg extinction, suggesting a rapid diversification of mammals. Later changes occurred predominately in cetaceans, large-brained carnivores and primates. Regarding the numbers and density of neurons, as already stated, there was a dramatic surge in neuron numbers, with an average 9-fold increase compared to reptiles in the telencephalon, and a 69-fold increase in the cerebellum. Interestingly, differently to birds, a higher brain size in mammals is not predictive of a higher neuronal density, a pattern only excepted by primates (Kverková et al., 2022).

Altogether, the synapsid lineage that separated from sauropsids maintained a primitive, tubular and small brain shape for much of its evolutionary history, and it was not until the emergence of mammaliaforms and specially after 66 mya, that profound reshaping of the brain and, especially, telencephalon occurred. Putting this in perspective, although we do not have access to the internal brain organization of therapsids nor of stem sauropsids, at least the external tubular and linear arrangement of the brain was maintained in both

lineages for hundreds of millions of years. This poses the question of how similar the brains of base stem mammals were to the ones of the reptile models used today and whether the mammalian brain evolved from so to speak a reptilian one, or from the dorsal pallium of an amphibian-like ancestor. (Aboitiz et al., 2002; Kemp, 2009; Woych et al., 2022).

1.3. - Homology of embryonic pallial structures and analogy of the derived adult circuitry in amniotes

In the previous section we have seen how the fossil record has informed us of the major changes of brain repatterning that occurred across amniotes and that extensively affected the pallium. Now, to understand how these changes occurred, we need to look into the divergence of the developmental program (**section 1.1**). The use of this word implies that there is a common point of origin. A key issue in evo-devo, when trying to understand the origin or reshaping of structures through comparative analysis between different species is being aware of until which extent the processes are comparable, how developmental time can be translated between species, and whether the final output is a result of “mere” tinkering of the developmental program or whether it involves novelty. In this section, I will try to offer an overview on the parallel progressive divergent pallial development in amniotes. We will see how, early in development, the developmental program is very similar across amniotes but, later on, the growing structures begin to be different. At this later point, I will shift the focus to the dorsal cortex, where it will remain for the rest of the manuscript.

1.3.1. - The problem of establishing developmental correspondences between phylogenetically distant species

When trying to understand the appearance of an evolutionary novelty, this is a matter of identifying the incorporation of changes via developmental repatterning. But *changes* with respect to what? With respect to the **plesiomorphic state**. The fossil record offers a way to know to a certain extent these ancestral states and to track the changes in a given phylogenetic lineage, provided there is fossil record available. However, this is not valid to understand the developmental aspect of the question of evolutionary novelty, given that, simply we cannot know the developmental program from extinct animals. To solve this issue, a cladistic approach is used (de Pinna, 1991). The basis of this approach is that the most likely evolutionary pathway is the most parsimonious one, that is, the one that involves the least changes. Following this premise, if two compared species share a same feature, the most parsimonious explanation of this is that such feature was present in the common ancestor. In our case, divergence of the dorsal pallium development, the comparison of their nature across amniotes should highlight the specificities leading to the mammalian neocortex.

A key point in such comparisons is what we consider to be *the same* in different species. On this regard, in biology, similarity does not imply equivalence of identity (Wake, 2007). This is because of **homoplasy**, which is a phenomenon that goes in the opposite direction to the principle of maximum parsimony: the similarity of characters in two or more taxa that is not the result of inheritance from a common ancestor. For instance, vertebrates and invertebrates have eyes, but they are not the *same* thing (despite showing syngeny, sharing toolkit genes in their development). Rather, by *equivalent* features we refer to **homologous features**. There are different definitions of homology. The most spread one is that homologous features are those that are present and similar in different taxa because they derive from a common ancestor where the feature was present (E. O. Wiley, 1982). This is called **historical homology**. I prefer though the definition proposed by Roth (Roth, 1984):

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“the basis of homology in the broad sense is the sharing of pathways of development, which are controlled by genealogically related genes”. In other words, a structure is homologous in two taxa if they have a common embryonic origin, even if their development in each taxon might diverge in later stages. This definition can be named as **biological homology** (A. B. Butler, 2009). I prefer it because it allows us to recognize different degrees of homology, which is something convenient for this work. For instance, in the case of the wings of birds and bats, we can recognize them as homologous if considered forelimbs (they share the forelimb developmental program), but not so much if considered as wings (their developmental programs have diverged resulting in a different wing structure).

Accordingly, to understand the appearance of the mammalian neocortex within amniotes we will compare the developmental program of the homologous regions in birds and reptiles, i.e., the dorsal pallium, to infer which properties of neural progenitor cells were ancestral and which ones diverged. Specifically, we will try to understand which properties allowed in the mammalian lineage their generation of a higher and more diverse number of neurons. However, there is a difficulty, which is how to keep track of homology along the developmental program of different species. Let me develop this.

Development is a dynamic process during which the properties of the tissue, as well as those of stem cells, progenitor cells, and newly differentiated postmitotic cells, progressively change (Telley et al., 2019). If we compare the developmental trajectory of an organ in two species that are phylogenetically very close, we can precisely track the equivalence between the developmental stages due to their similarity. But if we compare it between two species that are phylogenetically distant, as development progresses the trajectory in each species will diverge and become more species-specific, even with profound morphogenetic variation, losing structural reference in the comparison. In addition, there is the problem of heterochrony. That is, a developmental process in different species does not take the same time, does not follow the same pace, does not scale linearly, or might even not follow the same sequence of events, the more so as larger is the phylogenetic distance (Fenlon, 2022; McNamara, 2012). Therefore, the stage of cortical neurogenesis in mouse at, for example, 50% of the whole neurogenic period may well not correspond to the stage of neurogenesis at 50% of the whole neurogenic period in chicken. Because of this, if we are trying to infer the specificities of evolutionary novelty in developmental repatterning in different phyla but we do not know the developmental correspondence, there will be confounding between the phylogenetic and the developmental variability. Following the previous example, in this project we will not be able to discern whether the specific properties of NPCs at 50% of neurogenesis in mouse and chicken are due to their belonging to different species or to developmental mismatch.

There are two solutions to this issue. Either we establish the developmental correspondence using transferable ontogenic landmarks, e.g., closing of the neural tube, beginning of neurogenesis, generation of neurons of layer 5; or we compare the whole developmental processes of the species considered. In this case, the second option was not available due to practical limitations, and therefore, as it will be explained later, in this project we chose a specific point of neurogenesis in snake, chicken, mouse and ferret based on a number of criteria that will be defined in due time.

Nevertheless, it should be as well noted that the mammalian neocortex is an evolutionary novelty. Although there was an obvious homologue in the ancestor, it has become radically different in the derived species (Günter P. Wagner, 2014). Therefore, there is no structure in

sauropsids strictly homologous of the mammalian neocortex (Briscoe & Ragsdale, 2018). This means that, although the early pallial developmental plan across amniotes is homologous, there will be a point, when neurogenesis starts, where developmental repatterning will be profuse, resulting in divergence and partial conservation of the spatiotemporal generation and placing of neurons, of neuronal populations and functions, and of neuron types and subtypes (glutamatergic specially), activity and connectivity. Consequently, a certain level of ambiguity between homology and parallelism will have to be withstood (Roth, 1984). In addition, evolutionary change can act at several levels (genes, gene expression regulation, cell types, embryological patterning, neural circuits, etc.) and homology at one level is independent of the others. Nonhomologous structures can share homologous features (Briscoe & Ragsdale, 2018). Evolutionary changes in the brain and in its development can occur at these different levels of organization, which makes it inherently complex to track the homology or the homoplasy of novelty in the brain (Günter P. Wagner, 2014; Rueda-Alaña et al., 2025; Tosches, 2021; Zaremba et al., 2025).

Altogether, although in this work we will be comparing the development of a structure that is homologous — arising from the same embryonic brain field— across amniotes, we will be doing so at a point where the biological homology between mammals and sauropsids starts to be diffuse, as different pallial structures will be being generated. Therefore, although we aimed at developmental correspondence between the stages studied in each species, we just acknowledge the fact that such correspondence can only be so broadly allocated. The homology at this point will lie in the equivalence of NPC types, in the set of toolkit genes these cells will be using, and the types of neurons generated, topics addressed later in more detail (sections 1.3.6, 1.5.1).

1.3.2. - An early common developmental program of amniote and vertebrate pallium diverges at the onset of neurogenesis

Brain development in vertebrates can be understood in three stages. The early stage is **neurulation**, when the neural tube is formed after embryonic gastrulation. The intermediate one is **brain patterning and segmentation**, when the neuromeres (brain embryonic fields) are established. And the late stage is **neurogenesis and maturation**, when neurons are generated, migrate to their final location and acquire their connectivity and functionality (Luo, 2020). In this text I will leave out the first stage.

Across vertebrates, and more so within amniotes, after the initial stage of gastrulation, where more variability in the early organization of the embryo is observed, probably due to the inheritance of maternal geometries (Abzhanov, 2013; Steventon et al., 2021); their embryos converge into a very stable and structurally similar form during brain patterning and segmentation, the **phylotypic stage**. This dynamic reflects that there is a common **bauplan** (invariant set of morphogenetic and structural features which are shared among evolutionarily related animal forms) for the main parts of the brain, and pallium specifically. Then, once the brain has been regionalized and the primary germinal zones are established, neurogenesis starts. It is at this point that each vertebrate group initiates a specific part of the developmental program, and the divergence in the growing structures between amniote groups begins to be realized, with the pallium showing the most flagrant variation in size, organization, neuron number and composition (Senovilla-Ganzo & García-Moreno, 2024).

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1.3.3. - Patterning and regionalization of the forebrain and pallium

Specifically in the telencephalon, its early patterning is controlled by the expression of a complex topological combination of transcription factors (TFs) based on the gradients of morphogens secreted at three main signalling centres (Montiel & Aboitiz, 2015) (**Figure 5a**). At the dorsomedial location, the **cortical hem** secretes Wnt and BMP molecules, delimiting dorsal pallium development. In the lateral domains, the **antihem** secretes FGFs and Frizzled related proteins, establishing the lateral and ventral pallial identity. These proteins neutralize the action of the dorsally derived signals, establishing the boundary with the subpallium. In the anterior forebrain, the **rostral patterning centre**, or **anterior neural ridge**, secretes FGF8 and FGF17, shaping the frontal cortex. Shh and downstream FGF effectors determine the ventral telencephalon or subpallial domain. Then, the expression of different sets of TFs delineates the boundaries between pallial regions and between the pallium and subpallium, conferring **positional identity** to the progenitors (Hébert & Fishell, 2008; Leung et al., 2022). This patterning morphogens and TFs are conserved across tetrapods (amniotes plus amphibians) (Brox et al., 2003, 2004; Desfilis et al., 2018; Puelles, 2018; Puelles et al., 2000; Smith Fernandez et al., 1998; Striedter, 1997) and derive from a complexification of the program of stem vertebrates where many of these agents were used (Albuixech-Crespo et al., 2017; Lamanna et al., 2023; Murakami et al., 2005; Sugahara et al., 2016, 2017).

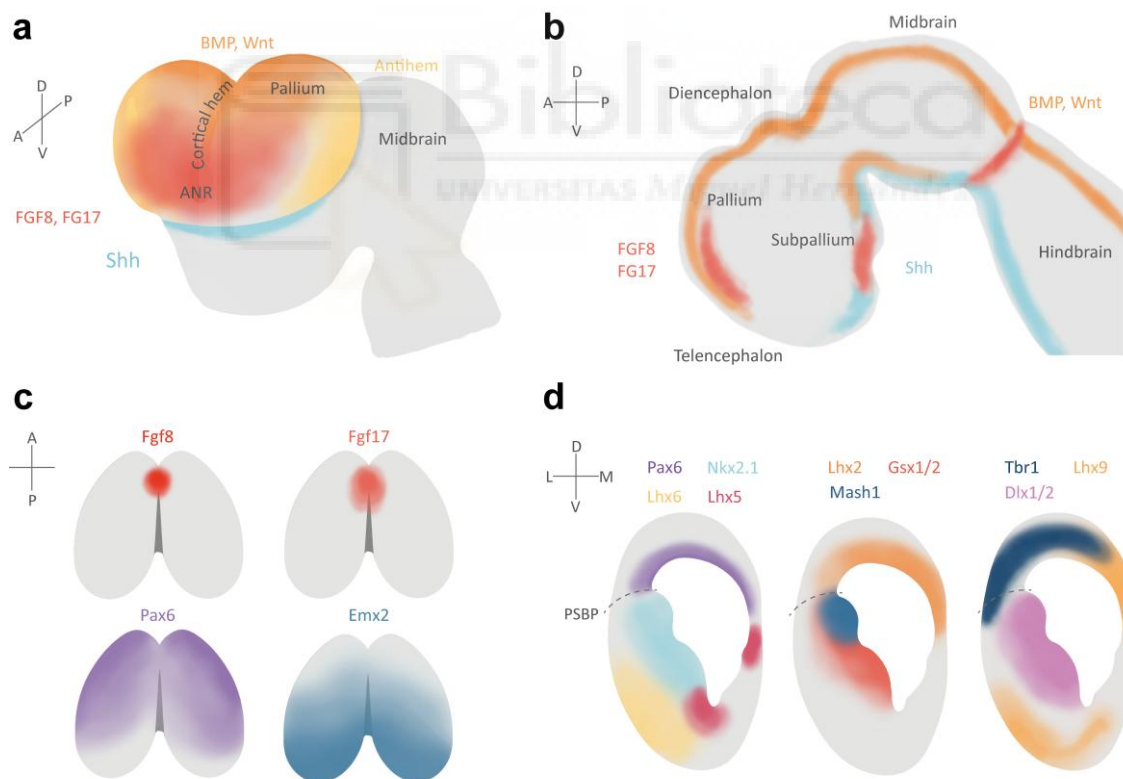


Figure 5. Brain and pallial patterning.

The different territories of the brain are set by a combination of morphogen gradients, secreted from three main signaling centers (**a**, **b**). A combinatorial and topologically distributed expression of TFs determines the fate of the pallial regions (only some are depicted) (**c**, **d**). ANR, anterior neural ridge, PSBP; pallial-subpallial boundary.

The regionalization of the pallium is determined by at least the following TFs. *Lhx2* maintains the pallial identity, preventing its ventralization. *Emx1* and *Emx2* are highly expressed dorsocaudally, influencing hippocampal and occipital development, whereas *Pax6* is highly expressed dorsorostrally, promoting progenitor proliferation and patterning of the cortex. The pallium also expresses *Tbr1*, a key transcription factor in the specification of excitatory glutamatergic neurons. In the subpallium, the identity is maintained by the expression of other TFs, such as *Nkx2.1*, *Gsx1/2* and *Dlx* TFs, which regulate the production of interneurons in the ganglionic eminences. The generation of interneurons in the ventral pallium is a process conserved across amniotes. The boundary between the expression of *Pax6* and *Nkx2.1* delineates the pallium from the subpallium (pallial/subpallial boundary, **PSPB**). *Pax6* induces the expression of *Ngn1/2*, which inhibits *Mash1* (*Cash1* in chicken), the inductor of a cascade of expression of ventral domain TFs, such as the mentioned *Dlx* genes. Later, the *Pax6* and *Nkx2.1* expression domains become further defined by the expression of *Gsh2* at the PSPB (Hébert & Fishell, 2008; Leung et al., 2022; Montiel & Aboitiz, 2015) (**Figure 5b**).

Based on the specific combination of these and other TFs, as well as on the anatomical progression through development, several models of pallial progenitor fields have been proposed, recognizing from 3 to 6 different domains, or rejecting the idea of sharp boundaries between domains (Yamamoto et al., 2024). In any case, the models claim that the pallial domains are conserved across tetrapods, from amphibians to mammals, and constitute the embryological fields to state homology for the derived regions. According to the tetrapartite model of Luis Puelles (Puelles, 2017) the pallium is divided into a medial, dorsal, lateral and ventral domain. The dorsomedial domain shows high expression of *Lhx2* and *Emx1/2* and lower expression of *Pax6*. The lateroventral domain shows stronger expression of *Pax6* and *Lhx9* and low expression of *Emx1*. In particular, the ventral pallium is characterized by an absent expression of *Emx1* in the germinal zone and by strong expression of *Pax6* and *Lhx9* in the mantle (Medina, 2009).

Each of these domains constitutes an **embryological field** that develops into different adult brain structures. The medial pallium generates the hippocampus and the entorhinal cortex. The dorsal pallium, the dorsal cortex in reptiles and birds (for the latter the name hyperpallium or Wulst are more commonly used) and the neocortex in mammals. The lateral pallium, the insular cortex, the claustrum, the endopiriform cortex and the amygdala. Finally, the ventral pallium generates the olfactory bulbs and claustrum and amygdaloid nuclei and, in reptiles and birds, a big structure that bulges into the ventricle, called the dorsoventricular ridge (DVR). Although all amniotes share the same homologous embryological pallial domains, these develop differently, giving rise to some pallial structures with a higher degree of conservation, but also to others whose developmental program has undergone substantial repatterning, as it is the case of the neocortex and the DVR (Medina, 2009; Puelles, 2018; Puelles et al., 2013).

I would like to add here that the tetrapartite model of pallial regionalization is not devoid of critic, interpretation and alternatives. It is not clear yet how many true pallial sectors exist and how different they are, their fate mapping is not completely resolved, in particular in the ventral and lateral pallium; and because of this it is not clear either with precision how the pallial domains translate into one-to-one homologues across vertebrate groups and within amniotes (Desfilis et al., 2018; Moreau et al., 2021; Tosches, 2021; Wullimann, 2017; Yamamoto et al., 2024). But, as Yamamoto and colleagues state, I will maintain the dorsal pallium nomenclature out of convenience. In any case, the region studied in this work,

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independently on the model to explain field homologies, was confined to that originating the dorsal cortex; we studied the properties of NPCs in charge of generating the cortex, what makes the NPCs of this specific embryonic region more proliferative in mammals compared to the corresponding topological/anatomical/molecular/ontogenic region in sauropsids, independently on how certain the boundaries between different developmental fields of the pallium are.

1.3.4. - A later divergence of the developmental program results in a different arrangement of the pallial structures in reptiles, birds and mammals

After the pallial regions and the positional identity of the neural progenitors have been established, these begin the process of neurogenesis. From now on, the neurons that will form the adult brain are generated, the developmental program triggers differential gene expression and modes of neurogenesis (Cárdenas et al., 2018; Nomura et al., 2013), neuronal migration (García-Moreno & Molnár, 2020), lamination (García-Moreno et al., 2018), connectivity (Karten, 2013) and cytoarchitecture (Stacho et al., 2020; Y. Wang et al., 2010), which determines the disparate variations of pallial morphogenesis in reptiles, birds and mammals (Senovilla-Ganzo & García-Moreno, 2024). The main difference in terms of pallial growth between mammals and sauropsids lies in the massive growth of the dorsal pallium in mammals, forming the neocortex, and the greater growth of the lateral and ventral pallium, forming the DVR in sauropsids (García-Moreno & Molnár, 2020). In birds, the territory of the DVR is also much more enlarged, so that the lateral ventricle is extremely narrow, and is divided into the mesopallium and nidopallium (Jarvis et al., 2005). Also in birds, the dorsal cortex has a major growth, and a higher neuronal population compared to reptiles, and acquires a different topological organization, forming the hyperpallium (**Figure 6**).

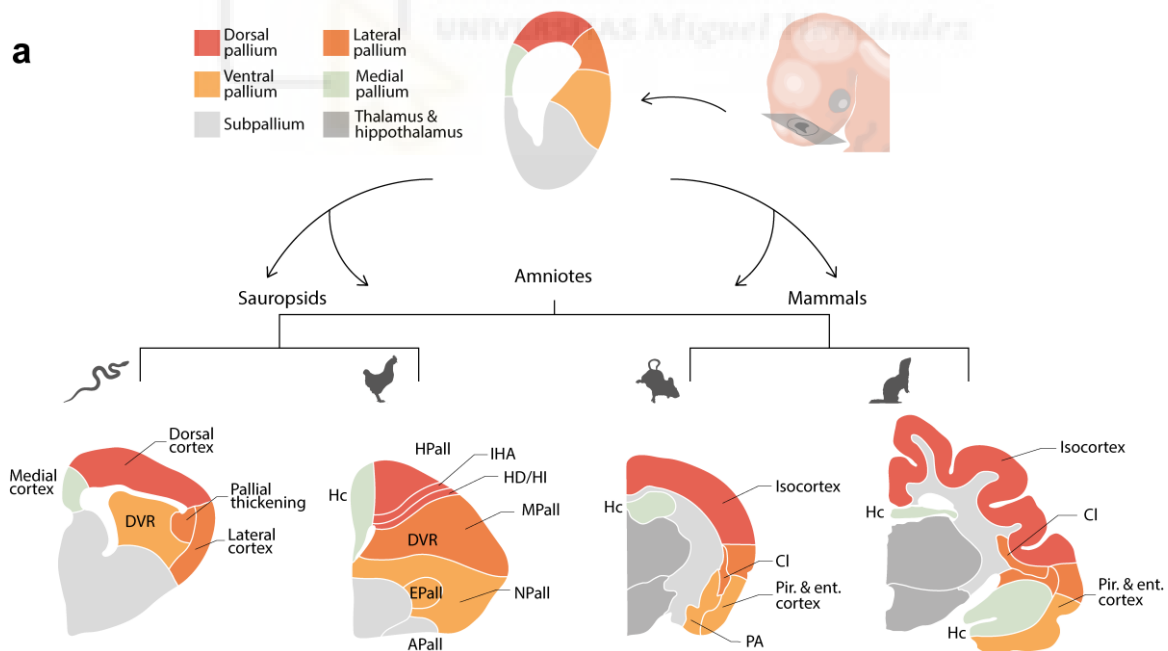


Figure 6. Pallium developmental divergence.

Common early pallial embryonic fields develop into different telencephalic regions in reptiles, birds and mammals. APall, arcopallium; CI, claustrum; DVR, dorsoventricular ridge; ent., entorhinal cortex; EPall, entopallium; HA, hyperpallium apicale; Hc, hippocampus; HD/Hi, densocellular/intercalated hyperpallium; HPall, hyperpallium; IHA, interstitial hyperpallium; MPall, mesopallium; NPall, nidopallium; PA, pallial amygdala; Pir, piriform cortex. The EPall and APall would not be seen in the same coronal view as they are not located at the same anterior-posterior level but are depicted together for clarity.

There is the hypothesis that a differential strength in a mediolateral pattern of signalling pathways involving Wnt signalling, Pax6 and its associated genetic network could provide a molecular basis for this differential pattern of growth based on the different use of common developmental toolkit genes (Montiel & Aboitiz, 2015). In mammals, the stronger upregulation of dorsal and anterior signals would lead to the expansion of the isocortex. These authors venture that in mammals, the boundary between the dorsal and the lateral/ventral domains of the pallium would have been shifted ventrally due to a stronger dorsalizing signalling centre and, consequently, the territory previously patterned to originate an alleged DVR acquired a cortical identity. Recent single cell data, however, support a non-homologous origin of the neocortex and the DVR (**section 1.3.6**). The initial pool of progenitors could be also due to the different activity of species-specific enhancers that regulate the expression of transcription factors key for the identity/phenotype of neural progenitor types, like *Pax6* and *Dbx1* (García-Moreno et al., 2018; Yamashita et al., 2018).

1.3.5. - Cortical arrangement in reptiles, birds and mammals

As a result of the diverging developmental program, the adult pallium of reptiles, birds and mammals show a disparate organization, which nevertheless holds conservation of important functions and neuronal circuits. The functions of the mammalian neocortex though are allocated in reptiles and birds into both their dorsal cortices and the DVR territory, something that has drawn much curiosity on their homologous or convergent evolution (Karten, 2013; Rueda-Alaño et al., 2025). I will explain now the generalities of the cortical and pallial organization. I will invert the order, explaining first mammals as reference, to then position the equivalent functionalities of the mammalian neocortex into the corresponding territories in reptiles and birds (**Figure 7**).

Mammalian cortical organization

In mammals, the dorsal pallium has massively expanded tangentially, increasing further its surface through folding in most species, covering most of the telencephalon and becoming its main part (Briscoe & Ragsdale, 2019). It has also expanded radially, holding a very dense and abundant neuron population that is cytoarchitectonically and functionally arranged in six layers (Brodmann, 1909; Rakic, 2009). Neurons of different layers participate in different aspects of a basic trisynaptic circuit of thalamic input, cortical integration and output (**Figure 7a**). Thalamic recipient neurons are found in layer 4. Intratelencephalic neurons are present in all layers. Neurons in layers 2 and 3 establish cortico-cortical projections, either columnar, ipsilateral within the same hemisphere, or interhemispheric. Finally, corticofugal neurons, extending projections outside of the telencephalon, belong to the layer 5 (subcerebellar projections) and to layer 6 (thalamic projections) (Molyneaux et al., 2007).

Other regions of the pallium have a thinner 3-layered structure, such as the olfactory bulbs, the piriform (olfactory) cortex and the hippocampus, so one would claim that the developmental repatterning has occurred much more profusely in the dorsal cortex, and that these other regions have remained more conserved (Cárdenas et al., 2018). Comparison of single cell data between these regions of mammals, reptiles, birds and amphibians go in this line (**section 1.3.6**).

During cortical development, the six layers are generated in and inside-out pattern, so the first-born neurons will conform the deeper layers, and the later born neurons will migrate beyond them until positioning themselves in the outer layers. Accordingly, output neurons are generated first, then input neurons and finally the integrator neurons (Rueda-Alaño et al., 2025).

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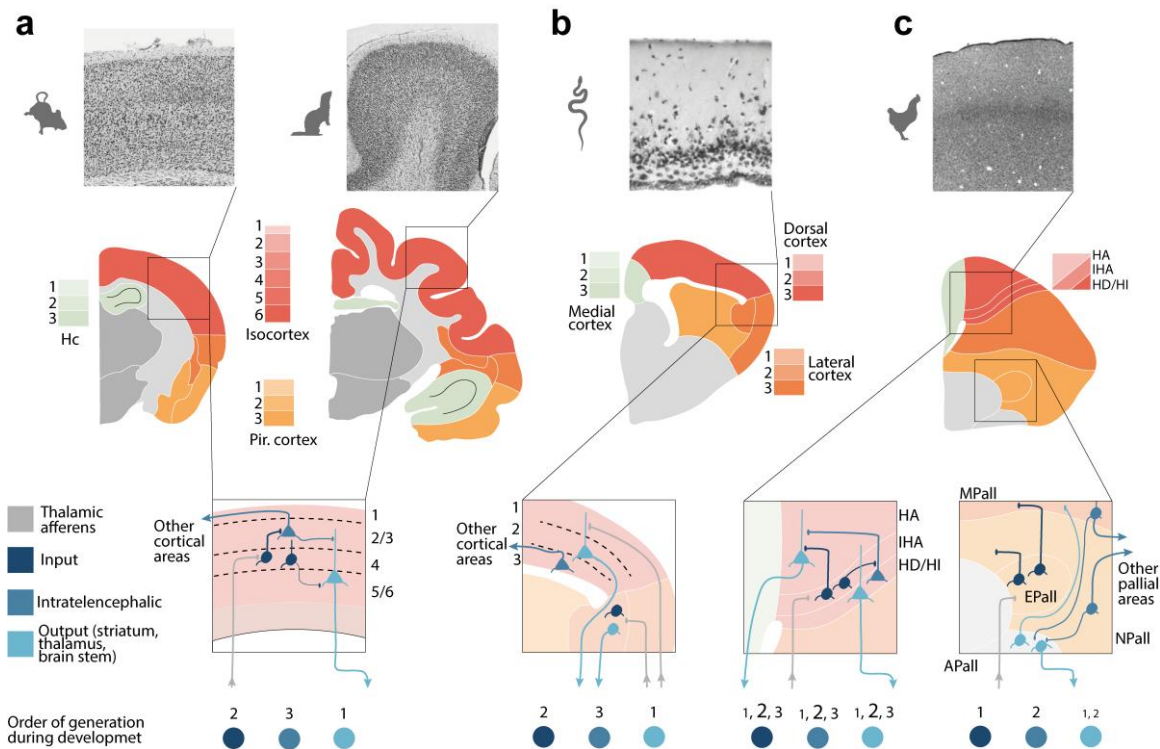


Figure 7. Correspondence of the trisynaptic circuit in the dorsal cortex of reptiles, birds and mammals.

The functionally analogous circuits of mammals (**a**), reptiles (**b**) and birds (**c**) present a very different cytoarchitecture. Only the mammalian isocortex presents a six-layered arrangement. The reptilian cortex shares a three-layered arrangement with the hippocampus and piriform cortex of mammals, whereas the functional regions of the avian pallium are distributed in centers. The order of generation of input, inter, and output neurons is equivalent in the dorsal cortex of mammals and reptiles, but is distinct in birds (bottom row). APall, arcopallium; HA, hyperpallium apicale; HD/HI, densocellular/intercalated hyperpallium; IHA, interstitial hyperpallium; Hc, hippocampus; MPall, mesopallium; NPall, nidopallium.

Reptilian pallial organization of the analogous neocortical circuits

In reptiles, the dorsal pallium expands much less than in mammals into the dorsal cortex. It occupies a much more restricted territory within the telencephalon and it is much thinner. It maintains a layered organization, but with only three layers and a much sparser neuronal population (Naumann & Laurent, 2020) (**Figure 7b**). The outer layer 1 is the plexiform or molecular layer; it contains scattered interneurons. The intermediate layer 2 is the cellular layer, and it contains most neurons, the majority of which are excitatory. The innermost layer 3 is the deep plexiform or subcellular layer and contains again loosely packed interneurons (Güntürkün et al., 2016). This simpler organization of the dorsal cortex is more similar to the three-layered mammalian hippocampus and olfactory cortex. Besides, these layers are seeded in an inwards pattern, contrarily to the mammalian neocortex. Differently, the lateral and ventral pallium grows much more during development, generating the DVR, a globular structure that bulges into the ventricle. Interestingly, neurons in the reptilian DVR seem to be generated also in an inward sequence, as revealed by birth dating in gecko (Rueda-Alaña et al., 2025).

In reptiles, the functions that are observed in the mammalian neocortex are allocated in two telencephalic regions, the dorsal cortex (Bruce & Butler, 1984; Hoogland & Vermeulen-Vanderzee, 1989; Martiánez-García et al., 1990) and the DVR (Balaban & Ulinski, 1981;

Bruce & Butler, 1984). For instance, the latter contains areas for auditory, somatosensory and visual processing (Naumann & Laurent, 2020). In both regions, a trisynaptic circuit with thalamic recipient neurons, integrator neurons and output neurons is found. In the dorsal cortex of gecko (*Paroedura picta*), thalamic recipient neurons are found in layer 3. Integrator neurons seem to be found also in layer 3. Long-range output neurons are found in layer 2. In the DVR, thalamic recipient neurons are in the outermost region of the DVR, and integrator and output neurons seem to locate in inner regions of the DVR. These circuits are inferred from the expression of TFs known to mark the putative neuronal populations in mammals and that are predicted markers of functionally analogous neurons in reptiles, as well as from tracing experiments (Rueda-Alaña et al., 2025), in agreement with results found in other lepidosaurs (Martínez-García et al., 1990)(Hoogland & Vermeulen-Vanderzee, 1989) (Bruce & Butler, 1984).

In reptiles, there are two patterns of order of birth, one in the dorsal cortex and another in the DVR. In the dorsal cortex, early born neurons are output neurons, intermediate-born neurons contain thalamic recipient neurons and late-born neurons settle close to the VZ (the authors do not comment on their connectivity). Therefore, the sequence of birth of functional groups of neurons is the same as in mammals, but are positioned in a topologically inverse order. In the DVR, there is also an outside-in pattern of neuron positioning, but the role of these neurons in the trisynaptic circuit does not correspond. As such, early born neurons in the DVR locate in the outermost part and are thalamic recipient. Integrator neurons locate in the central areas of the DVR and late-born neurons, close to the ventricle (Rueda-Alaña et al., 2025).

Avian pallial organization of the analogous neocortical circuits

In the avian lineage, the layered organization and the sequential order of neuronal birth is not present. Instead, their neurons are arranged in what is frequently named as “nuclei”, although I would prefer another term with a lower feeling of compaction or topological definition, such “centres”, “areas”, or “hubs” (Jarvis et al., 2005; Montiel et al., 2016). The dorsal cortex grows into the **hyperpallium (HPall)**, where three functional areas are distinguished: the **apical hyperpallium (HA)**, the **interstitial apical hyperpallium (IHA)** and the **densocellular/intercalated hyperpallium (HD/HI)**. The lateral and ventral pallium develops into, from more dorsal to more ventral, the **mesopallium (MPall)**, the **nidopallium (NPall)** and the **arcopallium (APall)**. Within the nidopallium there is another functional centre named as the **entopallium (EPall)** (Figure 7c).

In birds, similarly to reptiles, components of the trisynaptic circuit and functions, that in the mammalian are allocated in the neocortex, are found in the homologous hyperpallium and in territories of the DVR that include the mesopallium, nidopallium, entopallium and arcopallium. In the HPall, the connectivity of its columns reminds of the functional organization of the neocortex. The IHA contains the thalamic recipient neurons, the HI/HD contains the integrator neurons, and the HA contains the long-range output neurons (Dugasford et al., 2012a; Reiner & Karten, 1983; Shimizu et al., 1995). At the anterior DVR lie the visual and somatosensory circuits, whereas the posterior NPall holds the auditory circuit. Visual sensory input from the thalamus is received at the EPall (Krütfeldt & Wild, 2004), so neurons here would be functionally analogous to L4 of mammals. Neurons in the dorsal NPall act as integrators (Stacho et al., 2020), being functionally analogous to neurons in the L2/3 of mammals. Long-range output neurons locate in the APall, being functionally analogous to neurons in L5/6 of mammals.

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In the avian DVR, the order of birth is like in reptiles. Input neurons are first generated during early stages of neurogenesis. Integrator neurons are generated mostly at intermediate stages. Output neurons are generated during early and intermediate stages of neurogenesis. In the HPall, differently, input, integrator and output neurons are born simultaneously during intermediate, mostly, and late stages of neurogenesis. (Rueda-Alaña et al., 2025). Therefore, the neurogenic order in the DVR seems to be conserved in sauropsids, and is different to mammals, given that the initial neurons to be generated are thalamic recipient neurons.

1.3.6. - Homology and analogy of neuronal populations and circuits in the adult brains of reptiles, birds and mammals

The fact that common functions appear in sauropsids in brain regions that do not derive from the same pallial homologous field has puzzled researchers in the field. How can neurons that are born in different developmental regions and times end up participating in an equivalent circuitry? In this regard, it seems that the core circuits of the amniote pallium have been conserved, but the pallial architectures and the temporal and spatial regulation of neuron generation and specification have been not (Briscoe & Ragsdale, 2018). A few studies in the recent years have started to answer a big part of the question and I think it is worth summarising their main conclusions.

But before I address those studies, three different models had tried to approach the correspondences between neuronal populations across amniotes. The first is the **equivalent circuit hypothesis**, based on the work of Harvey Karten's in the physiology of axonal projections in the bird brain. This hypothesis states that the different types of neurons in the mammalian neocortex are homologous to neurons in reptiles and birds that are spatially segregated between the dorsal cortex and the DVR (Briscoe & Ragsdale, 2018; Dugas-ford et al., 2012b; Karten, 2013). These authors have claimed to establish homologies between neuronal types of specific mammalian layers with sauropsid types. Accordingly, glutamatergic types of the reptilian dorsal cortex would be homologous to the mammalian cortical layers 5 and 6, whereas the late born mammalian neurons of layers 2-4 would be mammalian specific (Reiner, 1991). In contrasting view, another hypothesis proposed that the neocortex would have originated only from intra-telencephalic type neurons in the amniote ancestor, and corticofugal neurons in layers L5-6 would have appeared only in mammals (Shepherd & Rowe, 2017). However, there is the observation that some aspects of both mammalian upper and deep layers (gene expression and connectivity) can be found in the dorsal pallium of sauropsids. This has led to another line of thought regarding layer homology that suggests that there are one-to-one homologues of mammalian L2/3, L4 and L5b in reptiles and birds (Briscoe & Ragsdale, 2018; Dugas-ford et al., 2012; Karten, 1969, 2013; Suzuki & Hirata, 2014). In my opinion, even though similarity can be an indication for homology, it is not the same as homology (**section 1.3.1**), and therefore if we used this model, we would fall short in explaining the correspondences from an evolutionary perspective (**section 1.1**). Nevertheless, it holds true for the functional analogy of neuronal circuits across amniotes.

The second view is the **pallial domain hypothesis**, based on ontology, and I have been talking about this in previous sections. The tetrapartite and other models (Puelles & Ferran, 2012; Yamamoto et al., 2024) understand that there are homologies between the embryonic fields of the pallium across all jawed vertebrates, and therefore the neuronal populations derived from them can be considered homologous types. However, as explained in the previous sections of this chapter, these homologous fields experience profuse divergence in

the neurogenic stage of brain development. A third view, the **continuum domain hypothesis**, is also based on ontology, but it has a nuance compared to the previous one. This model understands that there is actually not a distinct border between pallial domains, but a continuous ground of progenitor cells along the ventricle (Chen et al., 2013; Gedman et al., 2021; Jarvis et al., 2013; Medina et al., 2022). This model though seems to be more specific to the avian case, given that it is based on the similarity of expression profiles of TFs between the dorsal and the ventral domain of the pallium in avian models.

Lately, a new way of generating data through single cell transcriptomics has permitted to tackle the problem at much more depth. With this approach, transcriptomic equivalences and specificities between neuronal populations of different species are tried to be established, and then, based on correspondence, on cell type specification and evolution due to expression of defining sets of TFs (**section 1.5.1**), on overlapping sets of effector genes and invoking maximum parsimony, claims are made on homology or convergence (Colquitt et al., 2021; Hain et al., 2022; Hecker et al., 2025; Lust et al., 2022; Rueda-Ala  a et al., 2025; Tosches et al., 2018; Wei et al., 2022; Woych et al., 2022; Zaremba et al., 2025). Detailed though may such data be, few of these studies have incorporated the developmental aspect (Rueda-Ala  a et al., 2025; Zaremba et al., 2025). In these studies, many specific comparisons between very specific clusters and subpopulations of neurons are established. A detailed summary of these results would extend too much beyond the scope of this text, but I want to provide the general tendencies that recurrently appear in these articles.

1. GABAergic neuronal types show a high degree of conservation.
2. Glutamatergic types have diverged into specific types.
3. Usually, correspondence can be established when the data is organized in broad categories, there not being one-to-one homologs at more minute levels of partition.
4. Closer glutamatergic types in cross species comparisons are found in regions of the brain considered to be more “primitive”, such as the hippocampus, the olfactory cortex, the amygdala, the claustrum or the cornu ammonis.

Of notice, these tendencies also appear when comparisons are established with amphibians (Faltine-Gonzalez & Kebschull, 2022; Woych et al., 2022a). In particular, Woych and colleagues (2022) found that the dorsal pallium of a salamander species (*Pleurodeles waltl*) lacks gene expression signatures of the mammalian neocortex, bear similarities with neurons of the entorhinal complex, the subiculum and hippocampus; and that the ventral pallium shares similarities with the anterior DVR of reptiles but not with DVR neurons related to processing thalamic input. These findings suggest that the mammalian neocortex appeared as a novelty from the common ancestor, whereas the DVR derives from neurons present in a common ancestor of tetrapods. This implies that both structures are indeed not homologous, despite the similarities in functions.

Therefore, mammalian and sauropsid brains evolved via an independent expansion of different pallial territories (dorsal pallium into the neocortex in mammals, lateral and ventral pallium into the DVR in sauropsids), with a diversification of specific neuronal types, mostly glutamatergic, especially in mammals (Colquitt et al., 2021; Hain et al., 2022; Tosches et al., 2018). This diversification of neuronal types must come from different programs of neuronal maturation, mostly due to the rewiring and compartmentalization of regulatory networks through changes in *cis*-regulatory elements and divergence of transcriptional programs (Espin  s et al., 2022; Hain et al., 2022; Senovilla-Ganzo & Garc  a-Moreno, 2024). As an

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example, Maria Tosches and colleagues (Tosches et al., 2018) found that the same sets of toolkit TFs are expressed collectively in the reptilian dorsal cortex and in the mammalian neocortex, but these appear in different combinations at the cell-type level. For instance, in both turtle and lizard, the neurons of layer 2 have distinct mixtures of markers found in the upper and deeper layers of mammalian neocortex neurons, in agreement with a previous work that found coexpression of *Ctip2* and *Satb2* in gecko and chicken, two TFs that in mammals are mutually exclusive (Nomura et al., 2018). Besides, in gecko and chicken, neurons that express only *Ctip2* or *Satb2* are not segregated radially, as in mammalian neocortical layers, but tangentially, which points towards a different dynamic of neuronal specification in cortical development of sauropsids. As another example of compartmentalization of genetic networks, TFs that in mammals are expressed in different interneurons (*Lamp5 Ndnf* and *Lamp5 Lhx6* interneurons) are coexpressed in the salamander (Woych et al., 2022).

Altogether, the basic components of the circuit and forebrain must have been present in the common ancestor of amniotes, but there was divergence in the developmental routes of constructing and complexifying this circuit, especially in mammals and birds. This process of developmental repatterning involved the demarcation of different embryonic fields giving rise to a different allocation of neuronal circuits (heterotopy), shuffling in the order of generation of neurons (heterochrony), specification of neuronal types (heterotypy) and increase in neuron numbers and density (heterometry). Such reorganization of the developmental program entailed a differential combinatorial use of TFs, with a parallel progressive divergence of cell fate determination that resulted in the different arrays of neuronal types existing across amniotes, and their further diversification in birds and, especially, mammals. As of the specific enlargement of the dorsal pallium into the neocortex in the mammalian lineage, this came defined by three developmental mechanisms: diversification of neuronal types, temporal sequential generation of such types, and massive increase in the proliferative capacities of NPCs, both in the number of neurons derived from each clonal unit, and in the sustained neurogenic developmental time. Probably, the three processes are linked.

1.3.7. - The missing link between neural progenitors and neurons

Although we are learning a lot with these recent studies in terms of correspondences and specificities of adult neuronal populations and starting to guess their convergent developmental origins, we still do not know specifically how these varied neuronal populations are generated specifically during development, neither whether, at the level of the neural progenitor cells, we can already appreciate divergence in the sense of different types or homologous types with variations.

The common and different neuron populations detected across amniotes, are they paralleled at the progenitor level, or are their identities acquired post mitotically (something that in any case would require some sort of priming at the genetic and or epigenetic level in the NPCs)? Are the more conserved neuron types *equally* generated? That is, are they generated in the same order and by *same* NPCs, or by the *same* NPCs in a different order? In other words, whichever the way a neuron type is generated, is there a common set of features a NPC must have to generate it, independently of the species, or can a given neuronal fate be acquired through a different developmental trajectory? In the case of the neuron types specific of each lineage, do they derive from a diversification of ancestral conserved types? If so, how was this achieved at the level of both NPC priming and post-mitotic modifications

in the newborn neurons? How much is the appearance of new types due to shuffling of a common set of developmental toolkit genes and pathways and how much is it due to the incorporation of new genetic and molecular players?

If, as it seems, there has been a change in the developmental plan of neuronal specification, how can differently generated neurons arrange into equivalent circuits and functions? Is this dependent on the expression of a series of conserved proteins involved in the guidance towards and connectivity with the proper targets? Is there really *convergence* of the common neural circuitry, or is it rather *simply* a rearrangement (heterotopy) of the topological organization of neurons already present in the common ancestor and programmed to wire in a certain manner, with the incorporation of new classes in each lineage? Is it maybe the case then that novelty at the neuronal level has been built on top of a conserved basic structure, especially in birds and mammals? If more specific types of neurons are found in mammals (provided this is not due to a sampling bias, given the much deeper study of mammalian models and genomes) compared to sauropsids, how is this finer specification achieved during cortical development in mammals compared to sauropsids? A reordering of the genetic networks and specification of TF combinatorial expression, their targets, and adjustment of signalling mechanisms, as already shown in a few case studies, must be involved. These and others are questions that are still far from being fully answered and makes the developmental repatterning of the brain a fascinating field of study if you like evolution and development. A daunting endeavour that would comprise large-scale projects aimed at characterizing across species the chromatin landscape, the epigenetic regulators, the regulatory regions, the cascades of TFs and their effector genes of their NPCs along neurogenesis, as well as at tracking the neuronal populations born by NPCs in each given state and their postmitotic modifications until maturation.

As far as this project is concerned, a question I have left out is, how does NPC diversity link to the increased expansion of the dorsal cortex and what is the basis for the increased proliferative potential and increase neuronal output of mammalian NPCs in the dorsal pallium domain? It is but one of the pieces needed to comprehend the evolution of cortical development and the appearance of the mammalian neocortex. It is time now to really zoom into the topic of the thesis.

1.4. - Differences in cortex size stem from the different types and properties of neural progenitor cells

So far, I wanted to contextualize pallium evolution and emphasize its developmental repatterning, slightly addressing as well the puzzling issue of neuron evolution. Now, in this part of the introduction, I will specifically address the developmental basis of the massive expansion of the dorsal pallium into the neocortex in mammals and the modest growth into the dorsal cortex of sauropsids. I will explain first the basic principles of cortical development as we know it occurs in the mouse model. Next, having this as a reference, I will specify which particularities of cortical development other mammalian species with a folded and expanded neocortex have, and which ones reptiles and birds have. Next, I will address the cellular aspects, relating what we know about the different types and properties of NPCs across amniotes, and how this affects cortical size. In this last part, I will restrict the focus to the cellular type aspects, leaving out genetic and molecular mechanisms, for which there are many reviews addressing transcriptional, genetic and epigenetic regulation, signalling pathways, organelle and cytoskeletal dynamics and extracellular matrix interplay with NPCs

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(Amin & Borrell, 2020; Andrews et al., 2022; Espinós et al., 2022a; Namba et al., 2020; Stoufflet et al., 2023; Taverna et al., 2014; Vaid & Huttner, 2020; Wimmer & Baffet, 2023).

1.4.1. - An overview of cortical development using the mouse as a model

Cortical development begins when the cells of the neuroectoderm that line the neural tube grow and form an early pseudostratified epithelium composed of **neuroepithelial cells (NECs)**. As they progress through the cell cycle, NECs move their nucleus through the cytoplasm in the apico-basal direction. When the nucleus locates at the most apical side, in contact with the ventricular surface, cell division takes place. This process is called interkinetic nuclear migration (INM) and because of it, at any given time, the nuclei of NECs can be found at any position within the thickness of the neuroepithelium, giving it its pseudostratified appearance (F. C. Sauer, 1935; M. E. Sauer & Walker, 1959; Taverna et al., 2014). If mitosis can only occur at the apical surface, INM allows for accommodation of a higher number of NECs throughout the ventricular surface.

NECs first increase their population through self-proliferating divisions. Later soon, they further commit to the neural lineage, giving rise to a population of NPCs that delineates the ventricular cavities of the developing brain and with which neurogenesis begins (Hartfuss et al., 2001; Malatesta et al., 2000; Noctor SC, Flint AC, Weissman TA, Dammerman RS, 2001). These are the **apical progenitors (APs)**. APs keep a neuroepithelial character, as they maintain contact with the apical surface and also undergo INM. As their cell bodies are confined to the most apical side of the growing cortex, they constitute a germinal zone with densely packed and pseudostratified nuclei known as the **ventricular zone (VZ)**. By far and large, the main type of APs is **apical radial glia cells (aRGCs)** (**Figure 8**). These not only keep contact with the apical surface, but they also extend radially a long basal process that contacts the basal lamina and that is maintained as the developing cortex grows (Rakic, 1972). Other types of APs, much less abundant, include cells that keep the apical contact, divide at the apical surface, and extend a very short or no basally directed process, known as **apical intermediate progenitors (aIPs)**, or as originally named, short neural precursors (sNPs) (Gal, 2006; Tyler & Haydar, 2013). Within the VZ, a third progenitor is distinguished, that undergo mitosis within the VZ, but not at the apical surface. Hence named **subapical progenitors (SAPs)** (Kowalczyk et al., 2009), these typically have a bipolar morphology, with short basal process and an apical basal process that keeps contact with the apical surface. This can be integrated into the apical junctional belt and so exposed to the ventricular lumen or can form an apical adherens junction without exposition to the ventricular lumen.

Upon cell division, APs can give rise to neurons through **direct neurogenesis**. However, this mode of neuron production, which leads to an early exhaustion of the progenitor pool and a limited neurogenic output, is very infrequent in mammals (Cárdenas et al., 2018). Instead, APs mostly commit to the production of basal progenitors, as specified below. Within AP types, direct neurogenesis is characteristic of aIPs, whereas aRGC cells maintain a proliferative state, generating NPCs and/or neurons. Some of these aRGC-generated NPCs remain as APs (aRGC or aIPs) in the VZ (Tyler & Haydar, 2013), but others delaminate from the apical surface, migrate radially and position themselves basally to the VZ, where they constitute a second proliferative niche called the **subventricular zone (SVZ)**.

NPCs that divide in the SVZ are called basal progenitors (**BPs**). The fact that aRGCs generate BPs prior to neurons exponentially increases the neurogenic output of each clone, as there is an increase in the number of NPCs. This process is known as **indirect neurogenesis** and is fundamental in the larger growth of the dorsal cortex characteristic of mammals (Thor,

2024). As BPs are delaminated from the adherens junctional belt, they lack a proper apical membrane (Betizeau et al., 2013; Fietz et al., 2010; Hansen et al., 2010; Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Reillo et al., 2011). Within BPs, we further distinguish between **basal intermediate progenitors (bIPs)**, which present a multipolar morphology with short processes (Haubensak et al., 2004; Miyata et al., 2004), and **basal radial glia cells (bRGCs)**, which present long processes that extend basally towards the pial surface, apically towards the VZ, or both, but that do not establish contact with the apical surface (Shitamukai et al., 2011; X. Wang et al., 2011). In mammalian species in which the dorsal cortex expansion has not been so drastic, such as mouse, BPs account for a minority of the total pool of progenitors. Most of them are bIPs and have a very low proliferative capacity, undergoing one or two rounds of cell divisions before committing to neurogenesis. bRGCs, although can present a higher proliferative capacity, are limited to ~5% of total progenitors, (Wang et al., 2011). This low proliferative capacity of BPs in mice leads to a faster exhaustion of the pool of NPCs and a smaller final neuron population, compared to other mammals with more expanded cortices.

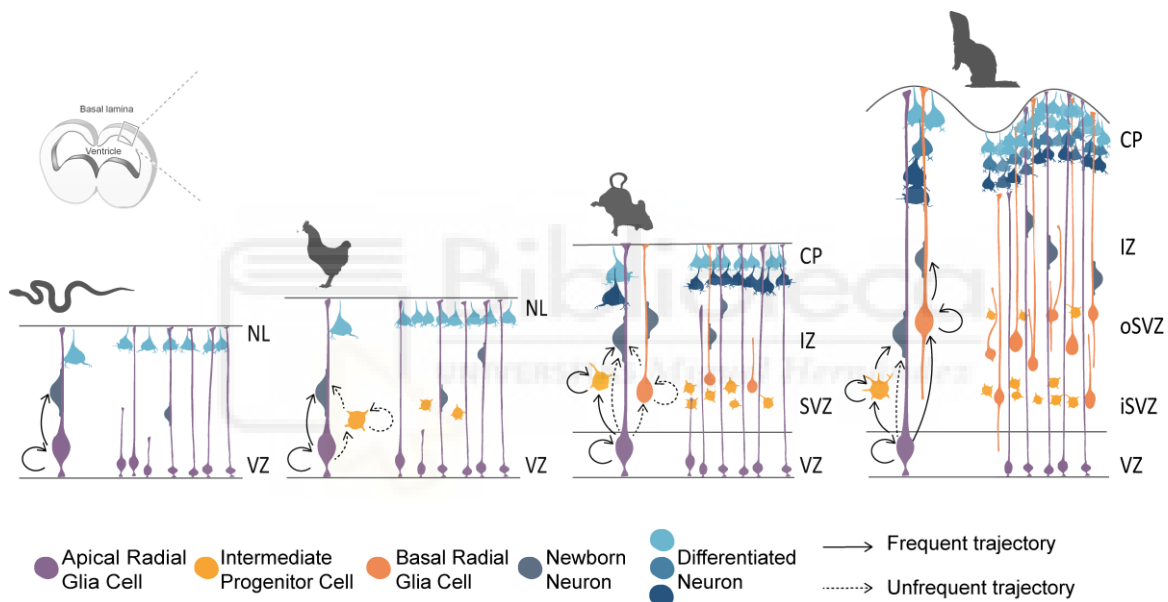


Figure 8. Cortical development in amniotes.

A different combination of NPCs, with varying properties and clonal trajectories towards neurogenic divisions, determine the neuron output and size of the cortex in each lineage. VZ, ventricular zone ; oSVZ, outer subventricular zone ; iSVZ, inner subventricular zone ; IZ, intermediate zone ; CP, cortical plate ; NL, Neuronal Layer.

1.4.2. - An expanded pool of progenitors enlarges the cortex in gyrencephalic mammals

In the case of species with an expanded and folded neocortex, as it is the case of the ferret, or more evidently, the case of primates, BPs acquire a much higher presence, becoming the main pool of progenitors, thanks to their capacity to maintain a prolonged proliferative state in the SVZ (Betizeau et al., 2013; Hansen et al., 2010). First, APs commit at a mid-stage of neurogenesis into cell divisions that seed a founder population of BPs that migrates basally and locate at the SVZ (Martínez-Martínez et al., 2016). Then, BPs undergo prolonged self-renewing and self-amplifying divisions, which leads to their accumulation in the SVZ, which is not subject to the space limitations of the VZ. Consequently, the SVZ expands and splits into the **inner SVZ (iSVZ)** and **outer subventricular zone (oSVZ)**. Eventually, neurogenic divisions of the enlarged pool of BPs in the iSVZ and mostly in the oSVZ result in a much higher

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population of mature neurons in the cortical plate, with a marked expansion of the most superficial layers of the cortex (DeFelipe et al., 2002; Kriegstein et al., 2006; Reillo et al., 2011; Smart et al., 2002) (**Figure 8**).

It is accepted that bRGCs play a crucial role in the expansion of the neocortex and its folding (Llinares-Benadero & Borrell, 2019; Namba & Huttner, 2017). This is for three reasons. First, they have been found to undergo several rounds of proliferative divisions (Betizeau et al., 2013; Hansen et al., 2010; X. Wang et al., 2011). Second, the accumulation of BPs is higher in the developing areas of the cortex prospective of gyri, and lower in the prospective areas of sulci (Reillo et al., 2011). In relation to this, variations in embryonic neurogenesis across regions partly manifest as differences in immature-neuron density within the cortical plate before it begins to fold. However, at an adult stage, the density of neurons is homogeneous across the cortex. This is because the higher number of neurons in the gyri extend more tangentially throughout the fold, which evens out their density with the sulci (Llinares-Benadero & Borrell, 2019). Third, the extra radial glia fibres (RGFs) provide a fanning scaffold for the radial and tangential migration of newborn neurons towards the cortical plate and into the cortical folds (Gertz & Kriegstein, 2015; Martínez-Martínez et al. 2019, Reillo et al., 2011). As such, whereas in mouse, bRGCs account for only ~ 5% of the total pool of progenitors (Wang et al., 2011), in ferret, a species with a moderately folded cortex, they make up for ~20 % of the total pool of NPCs (Fietz et al., 2010; Kalebic et al., 2019; Pilz et al., 2013; Reillo et al., 2011), and in humans, SVZ progenitors make up for up to 80% of total mitosis at peak neurogenesis (Fietz et al., 2010, Hansen et al., 2010). In fact, in human, at mid stages of neurogenesis, the apical radial scaffold is truncated, and then bRGCs take over in the generation and guidance of neurons, although this has been shown only once (Nowakowski et al., 2016). These percentages are estimates and might vary depending on the technique used to measure (usually phospho-vimentin staining) and on the developmental time, but what seems clear is that bRGCs become extremely much more abundant in gyrencephalic mammals. Nevertheless, they must not be the only cause of gyrencephaly, given that in marmosets, a small lissencephalic primate, bRGCs also account for 30-40% of BPs (Kelava et al., 2012).

1.4.3. - An apparently less complex cortical developmental plan in sauropsids

In the case of reptiles, the small and thin dorsal cortex (**section 1.3.5**) arises from a simpler and faster neurodevelopmental program. Indeed, reptiles show a major absence of BPs or abventricular mitoses in the developing dorsal pallium (**Figure 8**). This observation has been made in several reptile groups, including snakes (Cárdenas et al., 2018), lizards (Martínez-Cerdeño et al., 2016; Nomura et al., 2013), turtles (Clinton et al., 2014; Martínez-Cerdeño et al., 2016; Nomura et al., 2016) and crocodilians (Charvet et al., 2009). Therefore, the vast majority of the neuron population is rapidly generated via direct neurogenesis from aRGCs, massively limiting the neuronal output. The low proliferative capacity of aRGCs in reptiles is further limited by the long cell cycle length, which limits the number of cell divisions they undergo during cortical development (Nomura et al., 2013). Although already addressed in section 1.3.5, the other two important aspects of cortical development in reptiles with respect to mammals are the different arrangement of neurons in both a radial and tangential direction. In the radial direction, the limited pool of neurons does not experience as complex patterns of neuronal migration as in mammals but instead position themselves in their final location by somal translocation (García-Moreno & Molnár, 2020). As a result, neurons in the reptilian cortex are not generated in an inside-out pattern, but in an outside-in pattern. In the tangential direction, neurons that express markers that in mammals are radially segregated

in different layers, in reptiles are tangentially segregated (Nomura et al., 2018). Therefore, at the genetic level, there must be fundamental changes in the capacity to generate large numbers of neurons and in the dynamics of neuronal specification along cortical development.

In the case of birds, their dorsal cortex does not present a layered organization, but a nuclear or semi-layered one, with a higher neuronal population than reptiles do (Montiel et al., 2016; Nomura & Izawa, 2017). Notwithstanding, cortical development follows the same starting scheme, with a germinal layer of aRGCs lining the ventricular surface. The majority of neurons are generated directly from aRGCs. However, differently to reptiles, abventricular mitoses have been described in chicken, dove, quail and parakeet, and some authors interpret this as the existence of a rudimentary SVZ (**Figure 8**) (Cárdenas et al., 2018; Charvet & Striedter, 2008; Verónica Martínez-Cerdeño et al., 2016). Nevertheless, the abventricular mitoses detected in the dorsal pallium are much scarcer than in the lateral and ventral domains (Charvet et al., 2009), reflecting the much more modest expansion of the avian dorsal pallium compared to the DVR. Considering the morphology of BPs, they can be multipolar, as bIPs (Nomura et al., 2016), or they can retain an apical, basal or both processes, similar to bRGCs, but their proliferative/neurogenic potential has not been described (Nomura et al., 2016). The fact that different types of neurons are generated quickly during mid and late stages of neurogenesis (Rueda-Alaña et al., 2025), as well as the tangential segregation of neurons characterized by markers that in mammals are radially segregated (Nomura et al., 2018) implies that, as in reptiles, the dynamics of proliferation vs differentiation and of neuron specification in birds must be fundamentally different to mammals (at least as far as the few markers used to establish such different types of neurons are concerned).

1.4.4. - Overall developmental variables governing cortical size

Having studied different cortical developmental programs across different groups, a series of general variables that interplay and affect cortical size can be drawn. If we were engineers trying to generate a given cortical structure, these are the variables whose values we would be playing with to achieve the outcome. At the risk of redundancy and for the sake of clarity, I explain them now, and how they vary across amniotes.

Neuroepithelium surface area at the onset of neurogenesis

The neuroepithelium that spans the lateral ventricles is the ground for the growth of aRGC. An increase of its surface means a higher number of “founder” cortical progenitors (Rakic, 1995). The neuroepithelium is grown prior to the onset of neurogenesis. Hence, the extension of this pre-neurogenic period of neuroepithelial growth is a conserved evolutionary mechanism for the expansion of the cortical surface area. In mammals, species with a gyrencephalic cortex, such as ferret, macaque or human, have a neuroepithelial surface much larger than mouse or rat (Florio & Huttner, 2014; Reillo et al., 2011; Smart et al., 2002). This happens also in birds. Songbirds and parrots, with larger brains, have a developmentally delayed onset of neurogenesis compared to chicken (Charvet & Striedter, 2008). As for reptiles, measurements in a turtle species showed not much increase of the VZ surface during neurogenesis, reflecting a limited amplification of aRGCs before neurogenesis (Martínez-Cerdeño et al., 2006).

Neurogenic period length and cell cycle length

Once the population of aRGCs is defined, the next key determinant is the total number of proliferative and neurogenic divisions. This can depend on **the length of the**

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proliferative/neurogenic period and on the **cell cycle length**. For a given pool of aRGC and other NPCs, the longer they can maintain a proliferative and neurogenic state, the more neurons and glia they will be able to generate. In reptiles and birds, the period of neurogenesis is much shorter and finishes much earlier during embryogenesis (Nomura et al., 2013; Tsai et al., 1981), whereas in mammals, it extends for a major part of embryogenesis and even after birth (Dehay & Kennedy, 2007; Florio & Huttner, 2014a; Gilardi & Kalebic, 2021; Martínez-Cerdeño et al., 2006).

As for the cell cycle, given a certain neurogenic length, a higher number of cell proliferative divisions would result into the generation of more neurons, therefore, a shorter cell cycle would relate to a greater cortical size. Within mammals, however, this is not the tendency, as primates (macaques) (Betizeau et al., 2013; Kornack & Rakic, 1998) show a much longer cell cycle than mouse (Caviness & Takahashi, 1995). However, all things considered, the neurogenic period in primates is much longer (months) than in mouse (a few days). Curiously, in birds something contrary occurs. Chickens have larger brains than quails. Well, in quails, prior to neurogenesis, the cell cycle length doubles the length of the cell cycle in chicken, resulting in a much lower neurogenic output of the former (Charvet & Striedter, 2010). Also, parakeets, which have one of the highest neuronal densities among birds, also have a shorter cell cycle compared to quails (Charvet & Striedter, 2008). Considering reptiles, geckos have a very extended cell cycle length already at the onset of neurogenesis (~ 50 h), which together with the very short neurogenic period, explains their small neuron population (Nomura et al., 2013).

The length of the cell cycle is not constant through the neurogenic period. In mouse, it extends as neurogenesis progresses, from 8h to 18h at the end (Caviness & Takahashi, 1995). The change in cell cycle length is associated with a change in the mode of division, from proliferative to neurogenic. The experimental modification of cell cycle length affects the fate of the daughter cells and so the neurogenic output. Christian Lange and colleagues found in mouse that, if the G1 phase is shortened through cdk4/cyclinD1 overexpression, this delays the switch from proliferative to neurogenic divisions and enhances the generation of basal progenitors, increasing the thickness of the SVZ and the cortical surface area (Lange et al., 2009). In macaque, the cell cycle length extends from 23 to 54h by mid-gestation during layer IV formation. Then it shortens to 27h, coinciding with an increase in proliferative divisions prior to layer II/III formation, when the OSVZ is formed (Betizeau et al., 2013; Kornack & Rakic, 1998). In ferret there is also cell cycle shortening at mid neurogenesis, coinciding with the seeding of supragranular layers (Reillo & Borrell, 2011).

Mode of cell division, cell cycle re-entry and proliferation versus differentiation

The final number of neurons is determined by how many neurogenic cell divisions occur during the neurogenic period. This means that, the larger the pool of NPCs that are available to undergo neurogenic divisions, the larger the neuron output. It follows that, for an increase in neurons, there must be a prior increase of NPCs. Because of this, in species with enlarged cortices, NPCs do not promptly commit to neurogenesis but instead spend some time multiplying themselves through proliferative divisions; that is, they have a higher **proliferative capacity**. As such, given a certain neurogenic period duration, and a certain number of cell divisions provided a certain cell cycle length, there will be a number of proliferative divisions prior to neurogenic divisions that gives a maximum neurogenic output.

Other variables though come into play. The clonal lineage from aRGC to neuron does not only involve aRGC and neurons, but other intermediate progenitor stages, and that cell divisions

are not simply either multiplicative, generating two progenitors, or consumptive neurogenic, generating two neurons, but there are other modes (Florio & Huttner, 2014). NPCs can generate other progenitors, can divide and generate a progenitor and a neuron, the progenitor generated can be of the same type or further committed towards neurogenesis, an aRGC can consume itself into two basal progenitors, etc. These and other possibilities of cell divisions (**section 1.5.2**) complicate the model, and much research has been carried out trying to disentangle the lineages from aRGC to neurons (Gao et al., 2014; Hippenmeyer, 2023; Ma et al., 2018; Mihalas & Hevner, 2018). In any case, the higher the proliferative capacity of the clonal lineage, the higher the neurogenic output.

Abundance and types of progenitors

Given a neuroepithelium area and that APs almost exclusively divide at the apical surface, the ventricular zone poses a spatial limitation to the abundance of aRGCs. In species with enlarged folded cortices, an increase in the pool of NPCs able to multiply and generate neurons has occurred through their detachment and placing basal to the VZ, in the SVZ. That is, through the generation of basal progenitors. Among these, bIPs show a lower proliferative capacity, whereas bRGCs show a higher one. Summing up, an increase in the diversity of NPCs comprising BPs and, in particular, bRGC, is associated to a higher neurogenic output (Kalebic & Huttner, 2020; Lin et al., 2021).

Direct vs. indirect neurogenesis

Finally, as yet another possible variable, and related to the types of progenitors and mode of cell division, is the dichotomy between direct neurogenesis and indirect neurogenesis (defined in **section 1.4.1**). This is a frequently addressed topic in the field of cortical development, referring to the presence of BPs and their relation to a bigger cortical size, and key to understand the lineage trajectory of NPC towards neurons and their ability to generate a lower or bigger neuron population (Borrell and Reillo, 2012; Thor, 2024).

In reptiles, dorsal cortex neurogenesis is virtually only direct, as nearly no basal progenitors have been detected in snakes, lizards and turtles (Cárdenas et al., 2018; Nomura et al., 2013; Suzuki & Hirata, 2014). Nevertheless, this might not be true for all reptiles, as proliferative BPs have been detected in a turtle species and a crocodilian species, although located laterally and ventrally, close or at the DVR (Charvet et al., 2009; Clinton et al., 2014). In birds, direct neurogenesis also predominates in the dorsal pallium, while the lateral and ventral pallium can have a significant population of BPs (Cárdenas et al., 2018; Charvet et al., 2009; Charvet & Striedter, 2008; Cheung et al., 2007a; Martínez-Cerdeño et al., 2016). In mammals, although direct neurogenesis occurs, it is believed that the vast majority of neurons are generated through indirect neurogenesis, both in lissencephalic rodents via bIPs (Kowalczyk et al., 2009; Miyata et al., 2004; Noctor et al., 2004; Noctor SC, Flint AC, Weissman TA, Dammerman RS, 2001), and in gyrencephalic species via both IPCs and bRGCs, especially in primates (Betizeau et al., 2013; Coquand et al., 2024; Hansen et al., 2010; Kelava et al., 2012; Martinez-Martinez et al., 2016, Nonaka-Kinoshita et al., 2013, Pilz et al., 2013; Reillo et al., 2011, Reillo and Borrell, 2012).

A signalling pathway that has been shown to be very related to these modes of neurogenesis is Notch signaling. Notch signaling is a highly conserved pathway that plays a crucial role in cell fate determination, including the regulation of neurogenesis (Nian & Hou, 2022). In the developing nervous system, Notch receptors (Notch1-4) interact with ligands such as Delta-like 1 (Dll1), Dll4, Dll3, and Jagged1 (Jag1) to mediate lateral inhibition, a process where neural progenitor cells (NPCs) prevent neighboring cells from adopting the same fate

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(Kageyama et al., 2008; Yoon & Gaiano, 2005). Activation of Notch by Dll1 or Dll4 typically maintains NPCs in a proliferative state, promoting indirect neurogenesis—where progenitors undergo multiple rounds of division before differentiating into neurons (Mizutani & Saito, 2005; Lui et al., 2011). In contrast, the absence or inhibition of Notch signaling allows NPCs to exit the cell cycle and differentiate directly, leading to direct neurogenesis (Borrell et al., 2012). Interestingly, different ligands exert distinct effects on neurogenic outcomes. While Dll1 and Dll4 are potent activators of Notch signaling and promote progenitor maintenance (Nelson et al., 2013), Dll3 acts as an inhibitory ligand, sequestering other Delta proteins and reducing Notch activation, thereby favoring neuronal differentiation (Ladi et al., 2005). Jagged1, on the other hand, can have context-dependent roles, sometimes sustaining progenitor proliferation and other times promoting gliogenesis (Parras & Berdón, 2022). Thus, the balance between these ligands fine-tunes the choice between direct and indirect neurogenesis, ensuring proper brain development and cellular diversity.

1.4.5. - On the evolutionary origin of a subventricular germinal zone in the developing cortex and its role in cortical expansion

In reptiles, observations in several species that include snakes (Cárdenas et al., 2018), lizards (Martínez-Cerdeño et al., 2016; Nomura et al., 2013), turtles (Cheung et al., 2007; Clinton et al., 2014; Martínez-Cerdeño et al., 2006, 2016; Nomura, Ohtaka-maruyama, et al., 2016; Suzuki & Hirata, 2014) and crocodilians (Charvet et al., 2009) have not found abventricular mitoses in the developing dorsal cortex. However, mitotic cells do appear in a more ventral position, in the pallial region that develops into the DVR in alligator (Charvet et al., 2009), and Clinton and colleagues found PCNA cells at the basal end of the VZ in a turtle species (*Trachemys scripta*), although with no counterstaining for mitosis (Clinton et al., 2014), which prevents against making any statement about a rudimentary SVZ.

In birds, although there are basal mitoses in the developing dorsal cortex, they are much more abundant in the lateral and ventral pallium (data obtained in chicken, parakeet and quail) (Cárdenas et al., 2018b; Charvet et al., 2009; Charvet & Striedter, 2008; Cheung et al., 2007; Nomura et al., 2016; Suzuki & Hirata, 2014). Specifically, parakeets, which have a higher neuronal density, have a higher density of PH3+ cells in both the dorsal and lateroventral regions of the pallium compared to the quail, stating that only parakeets, but not quails, have enough PH3 numbers to speak of a possible SVZ (Charvet & Striedter, 2008).

Therefore, in both reptiles and birds, the pallial regions that show a greater expansion (dorsal cortex of birds compared to reptiles', and DVR in both reptiles' and birds') have BPs distributed in what some authors claim to be a putative SVZ.

Within mammals, monotremes show mitoses basal to the VZ (Ashwell & Hardman, 2012). The authors claim that there is a putative SVZ in these species but point out that it is not in the dorsal cortex, but laterally to the lateral ganglionic eminence. Therefore, this study, which was made on very few old samples available, is not enough to state the presence of a cortical SVZ. Of note, whereas the platypus is lissencephalic, the equidna is gyrencephalic, which poses the question on how folding occurs in this species if, apparently, there are very few BPs (Ashwell & Hardman, 2012).

In marsupials, we find also both lissencephalic and gyrencephalic species. In the small mouse-sized lissencephalic dunnart, no BPs have been observed (Paolino et al., 2023). Opossum and tammar wallaby have a demarcated SVZ with mitotic cells in the developing cortex. However, despite the long neurogenic period that occurs in marsupials, the number

of both upper and deeper neurons is much lower than in placental mammals (Cheung et al., 2010). This could be due to the very delayed emergence of the SVZ during brain development in these species. However, a conflicting study found opossum to have Tbr2+ cells localized between the VZ and IZ but that were already post-mitotic, thus not forming a population of IPCs and so without a proper SVZ (Puzzolo & Mallamaci, 2010). The contradiction might be due to a different position along the rostro-caudal axis of the tissue analysed. A third study in the tammar wallaby found mitotically active Tbr2+ bIPs as well as Pax6+ bRGCs in a SVZ that thickens at later stages of neurogenesis (Sauerland et al., 2018). Of interest, opossums have smooth cortices, whereas wallabies have folded ones, so these differences in the SVZ and dynamics of neural progenitor cells may explain such anatomical difference and neocortex surface differences. This observation supports the idea that the appearance of bRGC is key to the development of gyrencephaly.

Finally, in placental mammals, I explained (**section 1.4.3**) how lissencephalic species present a limited SVZ, with BPs that quickly commit to neurogenic divisions, and how in gyrencephalic species, especially primates, the SVZ expands into an inner and an outer SVZ, the latter further growing and being the seat of a large population of BPs with high proliferative capacities and hence a higher neurogenic potential.

Having considered all these cases (**Table 1**), despite some exceptions (e.g., echidna), the detachment of NPCs from the apical surface and their cell division at a basal location appears to be a conserved developmental solution in amniotes enabling the generation of more neurons in those regions of the pallium with a major expansion. In the case of mammals, this phenomenon reaches its maximum expression in those species with a higher neuronal density and gyrification indexes, like primates or, more moderately, in our model, the ferret.

In particular, it is the extended proliferation of BPs, specially of bRGCs, that is thought to be critical for the expansion of the neocortex (Dehay et al., 2015; Fernández et al., 2016; Fietz and Huttner, 2011; Lui et al., 2011; Nonaka-Kinoshita et al., 2013; Penisson et al., 2019, Reillo et al., 2011; Reillo et al., 2012). Newborn neurons generated in the germinal zones migrate basally and accumulate below the basal lamina in the prospective **cortical plate (CP)**, where they will end up forming the six layers of the mature cortex. In relation to this, apart from generating neurons, bRGCs play another crucial role for cortex expansion, since their long basal processes that extend towards the pia and are called **radial glial fibres (RGFs)** provide a scaffold for the radial migration and tangential dispersion of newborn neurons as they move towards the cortical plate. This tangential dispersion occurs most at regions where the cortical plate later grows into folds and is a contributing phenomenon to the local expansion of the cortical surface in these areas (Borrell and Götz, 2014; Johnson et al., 2018; Reillo et al., 2011).

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Table 1. Tbr2 distribution and BPs in studied vertebrate species. Based on references from Cárdenas and Borrell (2020) and Hevner (2023).

Clade	Species	Scientific name	Tbr2 in VZ	Tbr2 in SVZ	Basal divisions (in dorsal pallium)	bIPs (Tbr2+ basal mitoses)	bRGCs (Pax6+ basal mitoses)
Cartilaginous fish	Small spotted catshark	<i>Scyliorhinus canicula</i>	+	+	+	~ (Tbr2 ISH, PH3 IHC)	unknown
Bony fish	Zebrafish	<i>Danio rerio</i>	+	+	+	-	unknown
Amphibians	African clawed frog	<i>Xenopus laevis</i>	+	+	+	-	unknown
	Axolotl	<i>Ambystoma mexicanum</i>	unknown	unknown	+	unknown	unknown
Crocodylians	American alligator	<i>Alligator mississippiensis</i>	unknown	unknown	-	-	-
Testudines	Red-eared slider turtle	<i>Trachemys scripta elegans</i>	+	+	+	~	unknown
	European pond turtle	<i>Emys orbicularis</i>	unknown	unknown	+	unknown	unknown
	Chinese softshell turtle	<i>Pelodiscus sinensis</i>	unknown	unknown	-	-	-
Lepidosaurs	Madagascar gecko	<i>Paroedura pictus</i>	+	-	-	-	-
	Green anole lizard	<i>Anolis carolinensis</i>	+	-	-	-	-
	African house snake	<i>Boaedon fuliginosus</i>	unknown	unknown	-	-	-
Birds	Mourning dove	<i>Zenaida macroura</i>	+	+	unknown	unknown	unknown
	Chicken	<i>Gallus gallus</i>	+	+	+	debated	unknown
Monotremes	Short-beaked echidna	<i>Tachyglossus aculeatus</i>	unknown	unknown	+	unknown	unknown
	Platypus	<i>Ornithorhynchus anatinus</i>	unknown	unknown	+	unknown	unknown
Marsupials	Dunnart	<i>Sminthopsis leucopus</i>	+	+	+	-	unknown (Sox2+)
	Opossum	<i>Monodelphis domestica</i>	+	+	+	~	+
	Tammar wallaby	<i>Macropus eugenii</i>	+	+	+	+	+
Placental mammals	Mouse	<i>Mus musculus</i>	+	+	+	+	+
	Rat	<i>Rattus norvegicus</i>	+	+	+	+	+
	Ferret	<i>Mustela furo</i>	+	+	+	+	+
	Marmoset	<i>Callithrix jacchus</i>	+	+	+	+	+
	Macaque	<i>Macaca mulata</i>	+	+	+	+	+
	Human	<i>Homo sapiens</i>	+	+	+	+	+

Looking from a broad perspective at the different cortical developmental programs, it appears that the VZ germinative layer has a developmental limitation to generate a thick cortex. The imposition of mitosis of APs in contact with apical surface, the limited number of APs that fit throughout the ventricular surface and the limited time they dispose of to proliferate and generate neurons might explain it. In species with an enlarged cortex, the way of circumventing this has been through the generation of a secondary germinal layer, the SVZ, where BPs do not have the space limitation to expand, enabling the generation of a higher pool of NPCs available for the generation of a higher neuronal output.

1.4.6. - Diversification of NPC types and modification of their properties determine the differences observed in cortical size across amniotes

Altogether, as the dorsal cortex has become a bigger structure, with a bigger size, a more varied and denser neuronal population, additional cohorts of the NPCs that build it have been added. In the mammalian lineage, bIPs appeared as an abundant secondary population of NPCs, demarcating a clear secondary germinal layer, the SVZ. Additionally, in those species with yet bigger cortices, the appearance of a more proliferative population of NPCs, the bRGCs, enabled a further generation of neurons and their guidance into the folding cortex. In birds, with a bigger cortex than reptiles, BPs also appeared, but probably with properties different to the ones of mammals. Such BPs can have a bRGC-like morphology, but their proliferative potential has not been characterized.

Apart from a diversification of types, a modification of their properties has occurred. At the level of APs, it is likely that aRGCs in birds show properties different to those in reptiles, that enable them to generate a more abundant neuronal population, and different to those in mammals. For instance, Pax6 (Paired Box 6) appears to act differently in the aRGCs of birds and mammals. Whereas in chicken Pax6 overexpression promotes differentiation independently of the developmental stage, in mouse it can promote either proliferation or neuronal differentiation depending on the developmental stage (Nomura et al., 2018). In relation to this, in mammals, a key question is how aRGCs produce large amounts of BPs, and how BPs in gyrencephalic species are more proliferative than in lissencephalic ones. Another candidate gene that is associated to different genetic networks is *Tbr2* (T-Box Brain Protein 2, also known as *Eomes*, Eomesodermin). In mammals, Tbr2 is required for the generation of bIPs from aRGCs, as its loss leads to aRGCs committing to direct neurogenesis (Sessa et al., 2008). Then, Tbr2 expressing bIPs undergo some rounds of cell divisions before generating neurons. Actually, Tbr2 must be involved in the balance between proliferative and neurogenic divisions in BPs, since its downregulation in ferret reduces the abundance of bIPs and bRGCs, with the consequent reduction in upper layer neurons and folding (Toda et al., 2016a). Very differently, in reptiles, birds and even non-placental mammals, Tbr2 appears associated to post-mitotic cells (Nomura et al., 2013; Nomura, Ohtaka-Maruyama, et al., 2016; Paolino et al., 2023). Nevertheless, author studies claim that Tbr2+ BPs do have mitotic behaviour (Cárdenas et al., 2018; Clinton et al., 2014).

It is expected that much of the differences in properties between different types of NPCs across amniotes must be due to their differential use of common genetic and molecular mechanisms, despite the possibility of genetic innovation (Espinós et al., 2022). As an example, genetic manipulation of Robo and Delta signalling in snake and chicken increases the clonal neuron production, and this comes accompanied by a substantial increase in basal mitosis both in snake, where they are otherwise absent, and in chicken (Cárdenas et

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al., 2018). Therefore, NPCs have an intrinsic responsiveness to a higher proliferative behaviour to locate at a subventricular location.

Finally, with a much more extended period of neurogenesis, the diversification of NPCs in mammals has not only occurred in the spatial dimension, but also in the temporal one. Indeed, the pattern of generation of neurons in mammals is markedly different than that of sauropsids. As NPCs in mammals generate different types of neurons in an ordered and paced sequential manner as neurogenesis progresses, their properties, in terms of their ability to generate different types of neurons, must progress also during development (Ludovic Telley, 2016; Telley et al., 2019).

A thorough comparative study between the types and properties of the varied NPC across amniote phylogeny has not been carried out in a standardized manner, and there is still a lack of a level of description of NPCs in sauropsids as detailed as in mammals. Because of this, in the pursuit to understand the mechanisms of developmental repatterning of the dorsal pallium variations across amniotes, from the thin reptilian cortex to the massive neocortex of gyrencephalic mammals, we set up to carry out a standardized comparative study of the diversity and properties of NPCs in snake, chicken, mouse and ferret. For this, we aimed at a series of cellular and histological characteristics that I explain in the next section, and we deemed best appropriate. Furthermore, the NPCs that histologically are categorized within the same type, might show a variety of behaviours and be implicated in different modes of neuronal generation (Del-Valle-Anton et al., 2024a). In this regard, further comparative analysis using single cell omics may allow us to better disentangle the different properties, at the genetic expression level, of the common and varied types of NPCs found across amniotes. In the next and last part of the introduction, I will go through the relevance and adequacy of the properties studied, and, again, reflect on the issues of homology for NPCs that single cell data might have.

1.5. - Experimental approaches to establish and characterize neural progenitor cells

1.5.1. - The epistemological problem of defining cell types in developmental contexts and between species

As the whole research project of this doctoral thesis has to do with the comparison between NPC types across species not so phylogenetically close, I deem appropriate to give some space to the issue of what a cell type is, a question that is being hotly revisited with the incorporation of single cell information (Arendt et al., 2016; Dance, 2024; Jonas Simon Fleck et al., 2023; Huang et al., 2022; Is et al., 2017; Shainer et al., 2025; Zeng, 2022).

What does it make two cells belong to different types? Are cell types real? That is, do cell types correspond to natural kinds or are they a product of nominalism? If yes, what is the biological natural basis for the determination of cell type identity? How does this identity arise during development? How is it maintained or modified and how do new cell types appear during evolution?

Some authors have proposed most living entities resist the classical essence-based taxonomy of the physical sciences. Boyd's introduced the concept of **homeostatic property cluster** (HPC), which addresses a biological kind as a constellation of highly correlated—but not universally shared—properties maintained by underlying causal, homeostatic mechanisms (Boyd, 1991; Assis & Brigandt, 2009). Cells could be understood using such concept: morphology, physiology, marker genes and epigenomic state tend to co-vary because a regulatory circuitry stabilises them, even if no single feature is absolutely definitory on its own. The size and coherence of the cluster depend on how finely our methods can define it (Bird & Tobin, 2024). As single-cell omics expand the list of candidate properties, correspondence among modalities (e.g., transcriptome versus electrophysiology) might become blurry, raising the open question of how far transcriptomic “cell types” uniquely correspond to other attributes (Huang et al., 2022; Marioni & Arendt, 2017; Shainer et al., 2025).

Whichever properties different cell types have, these are a result of an evolutionary history and developmental context. Because of this, Detlev Arendt and others have proposed a definition of **evolutionary cell type** (Arendt, 2008; Arendt et al., 2016): a cell type comprehends a group of cells in an organism that change together in evolution, in a partially independent manner to other cells, and are evolutionarily more related to each other than to other cells. They state that cell types are characterized by the use of some genomic information not used by other cell types, which provides them with **genetic individuation**, and is achieved by the cooperation of a unique combination of regulatory transcription factors, which conform the **core regulatory complex (CoRC)**. These transcription factor set is determined hierarchically through differentiation, and is composed of the **terminal selector** TFs, that control the expression of the specific **effector genes** that provide the cell with its functions and its responsiveness to the environment (Deneris & Hobert, 2014; Hobert, 2008). In each cell type, the CoRC allows the evolution of independent gene expression, and thereby the appearance of new sister cell types. Importantly, this definition sets a boundary between the cell type identity and its phenotype and allows the recognition of homologous cell types across organisms based on the conservation of the regulatory genetic network, which, additionally gives the cell a **stable cell identity**. A set of transcription factors that maintains the cell identity through self-regulation and inhibition of

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alternative TFs constitutes a good biological substrate for the homeostatic property cluster that would define a cell type, since it still holds room for limited variation.

NPCs, however, are not static. In mammalian cortical development, there is gradual transition of transcriptional states of NPCs (Telley et al., 2019), with a progressive restriction of their potential, and a sequential generation of the neuronal populations that occupy the different layers of the cortex (McConnell, 1988; Molyneaux et al., 2007; Oberst et al., 2019), but with the possibility of such populations being generated by different types of progenitors in parallel trajectories (Del-Valle-Anton et al., 2024). Within a single clone, an apical radial glial cell (aRGC) may divide to produce an intermediate progenitor (IP), then a basal radial glial cell (bRGC), then neurons. As the global transcriptional programs shift, so may cell identities, e.g., an early aRGC does not share the full identity of a late aRGC (Fischer & Morin, 2021).

If not complex enough, another issue appears when we try to keep track of cell types across different species, the more so as the phylogenetic distance is bigger. It was addressed earlier in the text (**sections 1.3.1, 1.3.6**) the relevance of homology to maintain comparability, with more emphasis on the topological acquisition of identity during embryo/brain patterning to keep track of homology. I stated as well that, when the temporal dimension starts to unfold, the topological homology can get diffuse, as developmental programs diverge. Here, at the cell identity level, the same issues apply.

To be able to build some knowledge, some degree of ambiguity or flexibility must be withstood, and we can work with different levels. How well does the meaning of a variable used to define a cell type in a species translates into another species? For instance, does the expression of *Tbr2*, classically used to define BPs, in mammals represent a same set of NPC properties as in sauropsids? These issues have to do with the very nature of evolutionary change through modification of the regulatory landscape onto which developmental toolkit genes can act, which determines the independent evolution of cell types in different lineages (Carroll, 2008). Then, how much change across species can be withstood within a same cell type? Let's use again the aRGC as an example. We can observe a same morphology in the chicken and mouse. But does it translate to a same cell type? Which properties do they share, which ones make them different? Are there subtypes of aRGCs in each species, some common and others species-specific? Does their similarity depend on developmental time? Is the diversity of mammalian neurons paralleled at the level of primed NPCs? (Li et al., 2020). The extent of overlap of their transcriptome might provide some clues at this respect. The detailed study of NPCs in their histological context, with a special relevance given to their proliferative/neurogenic potential, and the exploration of their functional properties through single cell omics (other technologies apart) should provide a way to answer these questions, and this is precisely what this project is aimed at.

1.5.2. - Establishment of main neural progenitor classes using histological approaches

Despite the philosophical considerations about NPC types, aimed at epistemological caution, in this project I have used a set of variables to demarcate NPC types, based on their relevance in the field of cortical development. However, I considered convenient a reflection on the limitations of categories, and on the variability of the implications that the values of such variables can have, depending on the developmental and phylogenetic context. In these sections, I explain which variables have been used to define NPCs histologically, which ones we have chosen based on their relevance, and how single cell omics, in particular

transcriptomics, is greatly affecting the understanding of NPCs, which nevertheless are named based on classical markers.

For this part, I will mainly use the comprehensive review written by Elena Taverna, Magdalena Götz and Wieland Huttner (Taverna et al., 2014), on top of which I will add some specificities.

Mitosis location

Depending on their location, mitotic events are defined as apical or basal. **Apical progenitors** (APs) undergo mitosis at or very near to the luminal surface of the VZ. **Subapical progenitors** (SAPs) undergo mitosis within the SVZ, but not at the apical surface. **Basal progenitors** (BPs) undergo mitosis at an abventricular location, beyond the VZ, at the SVZ (Betizeau et al., 2013; Fietz et al., 2010; Hansen et al., 2010; Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Reillo et al., 2011). Location of mitosis is a parameter with a deep relevance. As discussed earlier, although APs can have different properties depending on the phylogenetic context, the VZ has a developmental constraint in its neurogenic capacity, and it is the extended proliferation of BPs, specially of bRGCs, that is thought to be critical for the expansion of the neocortex (Borrell and Götz, 2014; Dehay et al., 2015; Fernández et al., 2016; Fietz and Huttner, 2011; Lui et al., 2011; Nonaka-Kinoshita et al., 2013; Penisson et al., 2019, Reillo et al., 2011; Reillo et al., 2012). In support of this, even if experiments of genetic manipulations, with an effect in an increase of the NPC pool and/or neuron output, can increment the AP population, an increment of BPs is more frequently observed (Villalba, Götz and Borrell, 2021; Tynianskaia and Heide, 2024). Besides, as NPCs can substantially change their position and morphology during cell cycle, as I address now, their position at mitosis presents a reference.

Morphological characterization and extent of polarity

NPC morphology is related to their polarity, and polarity is determined in two ways:

1. The presence or absence of apical-basal cell polarity, reflected by the **presence or absence at mitosis of constituents of the plasma membrane both apical-specific** (e.g., prominin-1) **and basolateral-specific** (e.g., N-cadherin).
2. The **presence or absence of polarized, apically directed and/or basally directed processes at mitosis**, independently on whether the apically directed process exhibits apical plasma membrane identity.

In these criteria, mitosis is chosen as the defining stage of the cell. This is for two reasons. First, because this is when distinct cell constituents are distributed to the daughter cells (equally or differentially). Second, NPCs can change their morphology during the cell cycle (references in **Table 2**). Therefore, confining the analysis of cell polarity and morphological features to the mitotic stage avoids ambiguities in progenitor classification. Applied to the apical and basal progenitors:

- **APs:** all three principal types of apical progenitors (NECs, aRGCs and aIPs) exhibit apical-basal cell polarity and extend apical and basal processes. In the case of NECs and aRGCs, the basal process extends across the entire cortical wall until the basal lamina. In the case of **aIPs**, the basal process tends to be confined within the VZ and fully retracted at mitosis. SAPs can extend either both an apical and basal process, only an apical process, or no processes at mitosis (Pilz et al., 2013)
- **BPs:** BPs delaminate from the ventricular surface and lack an apical membrane. Therefore, BPs do not have apical-basal cell polarity. However, bRGCs do present

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polarization. They can be either bipolar, with a basal and an apical process, or monopolar, with a basal or an apical process. The types and absolute and relative abundance depend on the species and developmental stage. BPs undergo drastic morphological changes during their cell cycle. After being born, they can remain initially attached with a bipolar morphology at the apical surface, then migrate to the SVZ keeping a bipolar morphology. In the case of bIPs, these acquire a multipolar shape with multiple short processes which at the time of mitosis are typically retracted. As for bRGCs, they typically retain the basal process of their mother aRGC, maintaining the contact with the basal lamina. Then, along their cell cycle, they can grow an apically directed process, retract their process, or regrow a basally directed process that does not reach the basal lamina (Betizeau et al., 2013; Florio & Huttner, 2014). Proliferative capacity and cell division mode

By proliferative capacity we understand the number of cell division rounds that a neural stem or progenitor cell go through giving rise to other NPCs before committing to a neurogenic division. NPCs can undergo multiple rounds of cell division before neurogenesis or they can just divide once, generating two neurons in a self-consuming division. For each different NPC type, its proliferative capacity depends on the species. For instance, in the case of mouse, aIPs and bIPs generally divide only once, in a self-consuming division, generating two neurons. All other types of NPCs are capable of undergoing multiple rounds of cell division in which they either proliferate or self-renew before undergoing their final self-consuming neurogenic division.

Related to their proliferative capacity is the **mode of cell division**. Cortical stem and progenitor cells can divide either symmetrically or asymmetrically, depending on the identity of the daughter cells when compared with each other. A cell division is considered **symmetric** when it leads to two identical daughters, and **asymmetric** when the two daughters are different from each other.

When we compare the identity of the daughter cells with that of the mother, depending on whether an NPC gives rise to two, one or no self-renewed daughters, its pool can be amplified, maintained or depleted, respectively. Accordingly, we define cell division as **self-amplifying** (i.e. proliferative), **self-renewing** or **self-consuming**. Also, comparing the identity of the daughter cells compared with that of the mother cell, we can use an additional terminology: we can refer to cell divisions as **proliferative** when both daughters adopt the same identity as the mother progenitor, and **differentiative** when one or both daughters differ from the mother (**bi-differentiative** when the two daughters also differ from each other). **Neurogenic** divisions are differentiative divisions in which at least one daughter withdraws from the cell cycle as a neuron. Thus, there are four principal modes of cell division:

- (a) symmetric self-amplifying (i.e., symmetric proliferative)
- (b) symmetric consumptive (differentiative)
- (c) asymmetric self-renewing (differentiative)
- (d) asymmetric consumptive division (differentiative).

In principle, each NPC type can undergo each of the different cell division modes. However, again it is found that the frequency of cell division mode varies depending on the NPC type and on the species (references in **Table 2**). Therefore, in each case, the cell division mode

has to be corroborated by observations. How to characterize experimentally the proliferative and cell division mode? With live microscopy and the use of known cell markers.

Videomicroscopy characterization

An almost direct way to assess the behaviour, changes in morphology, location and division outcome of NPCs is using live-imaging experiments. In this setting, a fluorescent labelling is applied to NPCs, live slices are obtained, submerged in a maintaining culture medium and then imaged alive with periodic frames for a duration of time that hopefully is enough to capture at least one round of division. Additionally, after the last frame, the tissue can be fixed and immunolabelled to molecularly characterize the progeny of the observed divisions. An important issue however is the fact that NPCs are observed *ex vivo*, out of their natural context but, currently, there is no other way to visualize them alive within the embryo with a high enough resolution. This technique allowed the discovery and characterization of the main classes of NPCs in seminal works in the field of cortical development, initially only through capture of movement, cell division and morphology of the daughter cells, and later with the incorporation of the immunolabelling (**Table 2**). Nowadays, it is still one of the most complete techniques for the study of NPCs.



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Table 2. Selection of research articles where time-lapse has been used for the characterization of NPCs.

Article	Species	Age	NPCs characterized	Time lapse max. duration	N cell divisions
(Noctor SC, Flint AC, Weissman TA, Dammerman RS, 2001)	rat	E16-E19	aRGCs	60h	14 aRGC divisions
(Noctor et al., 2004)	rat	E17-E22	aRGCs, bIPs	120h	51 clones, 146 daughter cells
(Haubensak et al., 2004)	mouse	E12	aRGCs, bIPs	10h	Not explicated
(Miyata et al., 2004)	mouse	E13-16	aRGCs, bIPs	30h	E13: 15; E14:4; E13 + E14 long culture: 70; E15: 6
(Gal, 2006)	mouse	E13-16	sNPs	24h	Not explicated. 3 sNPs mentioned
(Kowalczyk et al., 2009)	mouse	E10, E12	bIPs, sAPs	15h	111 cell divisions (57 basal, 31 apical, 23 subapical)
(Hansen et al., 2010)	human	GW15	bRGCs	60h	"hundreds of oRG cell divisions observed", not specified
(Shitamukai et al., 2011)	mouse	E13-E16	bRGCs	45h	30-570 cells, depends on experiment
(Wang et al., 2011)	mouse	E12-13	bRGCs	60h	17 bRGC divisions, 52 aRGC divisions
(Lamonica et al., 2013)	human	GW16-18	aRGCs, bRGCs	6 days	70 apical divisions
(Pilz et al., 2013)	Mouse (LGE, not cortex)	E14	saPs, SNPs	56h	52 clones
(Betizeau et al., 2013)	macaque	E65, E78	BPs	250h	487 divisions
(Tyler & Haydar, 2013)	mouse	E14-E15	sNPs	48h	Not explicated
(Gertz et al., 2014)	Human	GW17-18	bRGC	5 days (?)	128 divisions
(Gertz et al., 2014)	ferret	E39, P3	bRGC	5 days (?)	512 divisions
(Martínez-Martínez et al., 2016)	ferret	P6, P18	aRGC, bRGC	142h	Not explicated
(Coquand et al., 2024)	Human (cortex)	GW14-18	BPs	48h	335 bRGC divisions
(Coquand et al., 2024)	Human (organoid)	7-9,13-15 weeks	BPs	48h	1101 bRGC divisions

Immuno-labelling characterization

The study of NPCs has relied heavily on the use of immunolabelling, a classical technique that allows the visualization and characterization of the different types of NPCs based on the expression of protein markers. These markers are used to distinguish between proliferating progenitors, the different types (APs, BPs, aRGC, bRGC, bIPs, etc.), to mark newborn neurons, neurons of upper and deeper layers, etc. When combined with TL and clonal analysis, they can provide insight into the lineage commitment of the NPC. The utility of using markers is that they are representative of a series of other properties. However, the use of immunolabelling to identify NPC types is very limited in the capacity to distinguish subpopulations that might differ in the expression of a vast number of other genes relevant for their properties. A few main markers used are summarised in **Table 3**.

Of special interest in the field have been two markers: Pax6 and Tbr2. Pax6 is a canonical marker of APs and/or RGCs, and Tbr2 is a canonical marker of IPs. However, bRGCs can express Tbr2, and IPs, both aIPs and bIPs can express Pax6 (Florio & Huttner, 2014). But this has been described not to happen equally in different species. Whereas in rodents, the majority of bIPs are neurogenic, express Tbr2 and do not express Pax6 (Englund et al., 2005a), in primates, bIPs are proliferative and show sustained Pax6 expression (Betizeau et al., 2013; Fietz et al., 2010).

Frequently, reviews articles state that, in mouse (or rodents), BPs do not express or downregulate Pax6 and express only Tbr2, or they obviate the expression of Pax6 by mouse bIPs (Amin & Borrell, 2020; Fernández et al., 2016; Florio & Huttner, 2014; Franco & Müller, 2013; Manuel et al., 2015; Martínez-Cerdeño & Noctor, 2018; Namba & Huttner, 2017; Vaid & Huttner, 2020; Villalba et al., 2021). This has led to the perception, at least to me, that coexpression of Pax6 and Tbr2 in BPs is restricted to mammalian gyrencephalic species. However, evidence does not support that view. Even in the cited original paper (Englund et al., 2005) they state that ~10% of NPCs were Pax6+Tbr2+, although interpreting them as bIPs just born from aRGCs. Importantly, these percentage is obtained from Pax6 and Tbr2 labelling, without colabelling with any proliferation marker. Indeed, in another study, Sylvie Poluch and Sharon Juliano showed how mitotic (PH3+) BPs in mouse do coexpress Pax6 and Tbr2, with varying frequencies along development (~20% of basal PH3+ cells at E12, ~50% at E14, ~90% at E16). As such, although it is true that mouse mitotic BPs may have a lower frequency of Pax6 Tbr2 coexpression compared to gyrencephalic mammals (as ferret in the same article) (Poluch & Juliano, 2015), a very high proportion of them do coexpress these markers.

Specifically, for bRGCs, there are also conflicting results. In mouse, one of the seminal articles describing bRGCs showed that bRGCs, identified by Sox2 and pVim labelling the soma and a basal process, were all Tbr2- (Wang et al., 2011). Differently, a later research found bRGCs, identified by pVim labelling the soma and a basal process, to have abundant (close to 100%) expression of Tbr2 (Florio et al., 2015), which would be in agreement with their lower proliferative potential (Wang et al., 2011). In gyrencephalic species, there are also contradicting findings. In human and ferret, mitotic bRGCs detected with pVim labelling the soma and a basal process were found to express Tbr2 in a very scarce proportion (Fietz et al., 2010; Florio et al., 2015). But in macaque, a third of mitotic bRGCs detected with EGFP and Ki67 were found to coexpress Tbr2 and Pax6 (Betizeau et al., 2013). Therefore, the simplification of Tbr2 labelling bIPs and Pax6 labelling bRGCs is a poor representation of a reality that is not definitively described.

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Jumping into sauropsids, whether Tbr2 is expressed by BPs is not clear either. A series of articles have associated Tbr2 to a postmitotic state (Nomura et al., 2013; Nomura, Ohtaka-Maruyama, et al., 2016), a later research showed its expression in mitotic BPs (Cárdenas et al., 2018). Finally, there might be a different genetic associated network to Pax6 and Tbr2 transcription factors in mammals and sauropsids, something I explained in **section 1.4.6**.

Table 3. Relevant markers used in cortical development research. Between brackets, NPC type if it can express the marker but not in a defining manner.

Marker	Cell types	Function
BrdU/Edu	Cycling progenitors	Incorporated into DNA during S-phase, used to track proliferation
PH3 (Phospho-Histone H3)	Dividing NPCs	Marks cells in M-phase (mitosis)
PCNA (Proliferating Cell Nuclear Antigen)	All proliferating NPCs	DNA replication factor, found in S-phase cells
Ki67	All proliferating NPCs	Expressed in all actively cycling cells
BLBP (Brain Lipid-Binding Protein, FABP7)	aRGCs	Important for RG self-renewal and gliogenesis
GLAST (Slc1a3)	aRGCs	Astroglial marker highly expressed in aRGs
Hes1, Hes5	aRGCs	Maintains self-renewal and prevents premature differentiation
Nestin	RGCs	intermediate filament initially characterized in neural stem cells
Pax6	APs/ RGCs (IPs)	Master regulator of radial glial identity; maintains progenitor status
Sox2	RGCs (IPs)	Essential for stem cell maintenance and neuroepithelial identity
Vimentin (Vim)	RGCs	Cytoskeletal protein in RGs, involved in cell structure and migration
GFAP (Glial Fibrillary Acidic Protein)	RGCs	Astroglial marker, highly expressed in aRGCs, also in bRGCs, but not in IPs
HOPX	bRGCs (not all)	Highly enriched in bRGs, maintains their self-renewal
Filamin A (FLNA)	bRGCs	Actin-binding protein, essential for bRG expansion in the oSVZ
Tbr2 (Eomesodermin)	IPs, (bRGCs)	Promotes transition from apical radial glia (aRGs) to intermediate progenitors (IPs) and promotes neurogenic divisions
NeuroD1	IPs, newborn neurons	Drives differentiation of IPs into neurons
Cux2	IPs	Expressed in upper-layer neuron-producing IPs
Tbr1	Postmitotic neurons	Specifies deep-layer cortical neurons
DCX (Doublecortin)	newborn neurons	Microtubule-associated protein, marker of migrating neurons
βIII-Tubulin (Tuj1, Tubb3)	newborn neurons	Structural cytoskeletal marker of immature neurons
Map2 (Microtubule-associated protein 2)	Mature neuron	Dendritic marker, mature neuron identity
NeuN (Rbfox3)	Mature neuron	RNA-binding protein, found in fully mature neurons

1.5.3. - Omics technologies expand our understanding of the properties of NPCs

The advent of single-cell transcriptomics (scRNAseq) and other single cell omics technologies (single nuclei, single ATAC, single spatial transcriptomics) has introduced dramatic change in the paradigm of studying NPCs during cortical development by providing unprecedented resolution of their molecular diversity, lineage trajectories, and dynamic gene expression changes over time. Traditionally, studies of cortical development had relied on the above-mentioned histological techniques and on bulk RNA sequencing, which averaged gene expression across populations of cells, masking heterogeneity within neural progenitor subtypes. With scRNAseq, researchers isolate and analyse individual NPCs, revealing a diverse spectrum of progenitor states beyond the classical divisions of aRGCs, IPs, and bRGCs. The main outbreaks in the field in recent years have been:

- Identification of previously unknown neural progenitor subtypes with distinct molecular signatures, particularly in the outer subventricular zone (oSVZ) of gyrencephalic species. This has come together with the identification of new markers (Del-Valle-Anton et al., 2024; Pebworth et al., 2021; Pollen et al., 2015; Zhong et al., 2018).
- Unravelling the temporal dynamics of NPC differentiation, showing how transcription factors regulate fate transitions from stem-like progenitors to neurogenic IPs and how neuronal states are primed in NPC prior to mitosis (Di Bella et al., 2021; Fan et al., 2020; Li et al., 2020).
- Modelling cortical development using brain organoids, where scRNAseq has been crucial in validating their fidelity to in vivo corticogenesis (Camp et al., 2015; Fleck et al., 2021; Kanton et al., 2019; Uzquiano et al., 2022).
- Combination with barcoding strategies has allowed tracking the transcriptomic progression of cells along their clonal lineage and at different developmental times, enabling the reconstruction of differentiation trajectories based on cells belonging to true lineages, rather than using only computational inference methods (Bandler et al., 2022; Chan et al., 2019; McKenna & Gagnon, 2019; Ratz et al., 2022; Zhisong et al., 2021).
- Finally, and of higher interest for this study, single cell omics have introduced a new platform to address evolutionary comparative studies, introducing a high-dimensional approach that can capture the correspondence between cell types across species in a much more comprehensive manner than through the comparison of a few morphological, molecular and functional characteristics (**section 1.3.6** and (Butler et al., 2018; Feregrino & Tschopp, 2022; Shafer, 2019). Although, some caution must be taken before jumping to conclude homology, given that phenotypic transcriptomic similarity can be reached through different developmental origins and trajectories (Zaremba et al., 2025), these technologies can also provide access to the similarity or convergence of TF codes used by cell types in different species (Hecker et al., 2025).

Overall, single-cell transcriptomics has provided a molecular roadmap of NPC identity and differentiation, deepening our knowledge of how neural progenitors generate the intricate

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architecture of the cerebral cortex. Nevertheless, they have come with the problematic of how the transcriptomic clusters correspond to the cell types that were defined by histology and physiology. Some articles go along the line that cellular taxonomies based on transcriptomics are congruent with morphology, connectivity and electrophysiology, and are therefore accurate and complete datasets (Huang et al., 2022; Initiative & Census, 2021; Peng et al., 2021; Yuste et al., 2020). Other researchers claim that cells are much more than their transcriptome, and their identity depends on other intrinsic features and their relationship with their environment (Dance, 2024; Pebworth et al., 2021; Shainer et al., 2025). For instance, apical and basal NPCs separately dissected did not cluster separately, but we know that they have different roles in cortical morphogenesis and neuronal generation (Del-Valle-Anton et al., 2024).

1.5.4. - What is needed to define an NPC type?

All in all, a series of variables can be applied to define boundaries between NPC types. Some of these boundaries are not firmly demarcated, and the type of a NPC may vary depending on which variables are studied, e.g., different marker combination; the moment of cell cycle when they are defined or the meaning of the variable in different species. Because of this, there could be minor inconsistencies in the naming of NPCs observed in different research articles. In the research part of this manuscript, NPC between amniote species have been studied in a comparative manner, using the following criteria, based on the above background: histologically, NPC are defined at the moment of mitosis, main NPC types are distinguished based on the expression of Pax6 and Tbr2, their morphology is added *in vivo* and live characterized along the cell cycle *ex vivo*, the cell division mode of NPC types defined by morphology and mitosis location is analysed, and single cell transcriptomics is used to explore the possibility of species or class-specific types, as well as the different genetic basis for the different properties that equivalent or similar NPC show across amniotes. Other interesting layers of analysis that could enrich the understanding of the transcriptomic variety of cells in tissue (spatial transcriptomics)(Moses & Pachter, 2022) and the divergence in genetic regulation (single cell ATAC, chromatin 3D interactions)(Acemel & Lupiáñez, 2023; Baysoy et al., 2023) have not been included in this Thesis.



1. - Introduction



2. - Objectives

An essential evolutionary innovation of the mammalian lineage is the enlargement of the dorsal cortex and its histological organization, achieved during development. The main objective of this thesis is to characterize the diversification of neural progenitor cells in the radiation of the amniote cerebral cortex, thus contributing to the understanding of the developmental origin of this mammalian evolutionary novelty. To this aim, we performed a thorough comparative characterization of NPCs in the developing cortices of a reptile (*Boaedon fuliginosus*), bird (*Gallus gallus*), lissencephalic (*Mus musculus*) and gyrencephalic (*Mustela putorius*) mammal, to identify which NPC types and properties may have enabled the appearance of cortices that reach a higher size and neuronal diversity during development. We addressed this via the following specific aims:

1. To establish a pseudotime correspondence along the period of dorsal pallial neurogenesis among our four animal models.
2. To classify and compare the main types of NPC populations in the dorsal pallium based on histological features, including location, cell morphology and marker gene expression.
3. To characterize and compare the proliferative dynamics and lineage of NPCs in the dorsal pallium of our model species.
4. To identify transcriptomic features that define NPC diversity in reptiles, birds and mammals, as well as the genetic regulation of their lineage dynamics, and determine the conservation or divergence of these features across phylogeny.

2. - Objectives



3. - Materials and methods

Animals

Animal subjects in this project were not used in prior procedures. All subjects were test and drug naïve. Animal procedures were approved by an ethics committee of the Generalitat Valenciana under authorization number 2022 VSC PEA 0074, and animals were treated according to the Spanish law (RD 53/2013) and European regulations (Directive 2010/63/EU).

Snakes (*Boaedon fuliginosus*) were obtained from our colony kept at the Animal Facilities of the Universidad Miguel Hernández. Snakes were crossed, fertilized eggs were obtained and incubated on wet vermiculite at 28 °C and 95% humidity using an incubator specific for reptiles (ReptileMax b0). The day eggs were laid was considered as the day 0 post-ovoposition (dpo). Embryos were obtained at day 6 dpo and processed the same day either for histological characterization of mitotic NPCs (PH3+) and of Pax6+ &/or Tbr2+ mitotic NPCs (PH3+) or for single cell transcriptomics.

Fertilized chicken eggs were obtained externally (Granja Santa Isabel, Córdoba) and incubated at 38 °C with intermittent rotation of the eggs recipient. The day eggs were laid was considered as the day 0 post-ovoposition (dpo). For histological characterization of mitotic NPCs (PH3+) during the neurogenic period, embryos were obtained at consecutive days from dpo 4 to dpo 8. For histological characterization of Pax6+ &/or Tbr2+ mitotic NPCs (PH3+), embryos were obtained at 6 dpo. For histological morphological characterization of the latter, embryos were electroporated with Cre-recombinase-dependent membrane GFP (1 µg µL⁻¹) plasmid and a Cre recombinase plasmid (10 ng µL⁻¹) at 4 dpo and processed at 6 dpo. For live-imaging of cultured forebrain slices, embryos were electroporated with the mentioned plasmids at 4 dpo and processed at 5 and 6 dpo. For single cell transcriptomics, embryos were processed at 4 dpo and 6 dpo.

Adult ICR (Institute of Cancer Research) wild type background mice were bred and kept at the animal facility of the Institute of Neurosciences of Alicante. Detection of a vaginal plug was considered as embryonic day 0.5 (E0.5). Mouse embryos were obtained by caesarean section from mothers sacrificed by neck dislocation. For histological characterization of mitotic NPCs (PH3+) during the neurogenic period, embryos were obtained at consecutive days from E12 to E16. For histological characterization of Pax6+ &/or Tbr2+ mitotic NPCs (PH3+), embryos were obtained at E14. For histological morphological characterization of the latter, embryos were electroporated with Cre recombinase dependent membrane GFP (1 µg µL⁻¹) plasmid and a Cre recombinase plasmid (10 µg µL⁻¹) at E12 and processed at E14. For live imaging of cultured forebrain slices, embryos were electroporated with the mentioned plasmids at E12 and processed at E13 and E14. For single cell transcriptomics, embryos were processed at E12.

Adult wild type domestic European ferrets (*Mustela putorius furo*) were acquired from Euroferrets (Denmark) and bred and kept at the animal facility of the Universidad Miguel Hernández. Detection of a vaginal plug was considered as E0.5. Ferret embryos were obtained by caesarean section under deep anaesthesia with sodium pentobarbital (250 mg/Kg, Dolethal). Ferret mothers were sacrificed using an intraperitoneal overdose of sodium pentobarbital (Dolethal), after being sedated with an intramuscular dose of ketamine (0.15 mL/Kg), xylazine (0.1 mL/Kg) and buprenorphine (0.06 mL/Kg). Ferret cubs were obtained by natural labour. For histological characterization of mitotic NPCs (PH3+) during

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the neurogenic period, embryos were obtained every other day from E34 to E40, and cubs were sacrificed and processed at P0 and P2. For histological characterization of Pax6+ &/or Tbr2+ mitotic NPCs (PH3+), embryos were processed at E36. For histological morphological characterization of the latter, embryos were electroporated with Cre recombinase dependent membrane GFP (1 µg/µL) plasmid and a Cre recombinase plasmid (10 ng/µL) at E34 and processed at E36. For live imaging of cultured forebrain slices, embryos were electroporated with the mentioned plasmids at E34 and processed at E36. For single cell transcriptomics, embryos were processed at E34 and P1 cubs were sacrificed and processed.

DNA Constructs and *in utero* and *in ovo* electroporations

To show the morphology of progenitors and for live-imaging experiments, a mix of pCAG-Floxp-EGFP-farnesylated plasmid at 0.5 µg /µL and pCAG-Cre plasmid at 10 ng /µL together with Fast Green (2.5 µg/µL; Sigma) in PBS was delivered via electroporation (*in ovo* for chicken, *in utero* for mouse and ferret) into the progenitors lining the apical surface of the forebrain lateral ventricles. The low concentration of Cre plasmid was aimed at sparsely labelling individual progenitor cells upon recombination of the floxed stop cassette to ease distinction between electroporated cells and their progeny.

For mice, embryos were *in utero* electroporated at E12 as described (Saito, 2006). Briefly, mothers were deeply anesthetized with isoflurane (5 % flux for induction, 1-2 % for maintenance) and administered analgesic (buprenorphine 0.03 mg/Kg). The uterine horns were exposed on the abdomen and kept wet using saline solution. Embryos were held using ring forceps and the DNA solution was injected through the uterine wall into the forebrain ventricles using pulled glass capillaries. Five unidirectional electric pulses (35 V, 50 ms duration with 950 ms intervals) were delivered using 5mm rounded platinum electrodes (Cuy650P5, Nepa Gene) with an electric stimulator (Cuy21EDIT Bex C., LTD). The uterine horns were reintroduced into the abdominal cavity, the abdominal wall and skin were sutured, the mother was recovered and monitored with administered analgesia until sacrifice.

For ferret, embryos were *in utero* electroporated at E34 as described (Kawasaki et al., 2013). Briefly, ferret mothers were deeply anesthetized with isoflurane and administered analgesic (0.2 mg/Kg Meloxicam, buprenorphine 0.03 mg/Kg) and antibiotics (amoxicillin 15 mg/Kg). The uterine horns were exposed on the abdomen and kept wet using saline solution. Embryos were held with the hands, and the DNA solution was injected through the uterine wall into the forebrain lateral ventricles using pulled glass capillaries. Five unidirectional electric pulses (75 V, 50 ms duration with 950 ms intervals) were delivered using 7mm rounded platinum electrodes (Cuy650P7) with an electric stimulator (Cuy21EDIT Bex C., LTD). The uterine horns were reintroduced into the abdominal cavity, the abdominal wall and skin were sutured, the mother was recovered and monitored until sacrifice.

For chicken, embryos were *in ovo* electroporated as described (Cárdenas & Borrell, 2021). Fertilized eggs were incubated as described above. The day before electroporation, a small amount of yolk was extracted through a whole on the eggshell using a syringe, the hole being sealed afterwards, to ease manipulation. At 4 dpo, a small window was open on the eggshell and the DNA solution was injected in the forebrain lateral ventricles using pulled glass capillaries. Five unidirectional electric pulses (30 V, 50 ms duration with 950 ms intervals) were delivered using 3 mm rounded platinum electrodes (Cuy650P3, Nepa Gene) with an

electric stimulator (TSS20 Ovodyne Electroporator, MCI). The window was sealed, and the embryos were kept in incubation at 38 °C until 5 or 6 dpo.

Tissue processing for histology

Tissue fixation: all samples were fixed using 4% paraformaldehyde in phosphate buffer (PFA) at 4°C. For snake samples, whole heads by immersion for 20 min. For chicken samples, whole heads were fixed by immersion for 30 mins; forebrains were dissected out of the head in cold PBS and immediately after were further fixed by immersion for another 30 mins. For mouse samples, forebrains were dissected out of the head in cold PBS and fixed by immersion for 90 min. For ferret samples, embryos were fixed by trans cardiac perfusion; forebrains were dissected out of the head in cold PBS and further fixed by immersion for 2h.

For cryostat sectioning, forebrains were washed with cold PBS and cryoprotected immersing them overnight in 30% sucrose in PBS at 4 °C. Forebrains were embedded in Cryo-medium Neg-50 (Thermo Scientific), frozen and sectioned with a cryotome at 20-30 µm.

For vibratome slicing, forebrains were washed with cold PBS and sectioned with a vibratome (Leica VT1000S) at 150-250 µm. For histological characterization, forebrains were sectioned in cold PBS. Vibratome slices were cryoprotected using a solution containing 30% glycerol, 30% etilenglycol and frozen until being used. For live imaging slices, details are provided below.

Immunohistochemistry

Tissue sections/slices were permeabilized using 0.25% Triton X-100 (Sigma) in PBS and blocked in 10% Horse Serum (Gibco) and 2% BSA (Sigma) for two hours. Tissue sections/slices were incubated in primary antibodies in blocking solution overnight at 4 °C, followed by appropriate fluorescently conjugated or biotinylated secondary antibodies and fluorescently conjugated streptavidin, in case of using a biotinylated secondary antibody, in blocking solution for 2-4 hours at room temperature. Primary antibodies used were: chicken polyclonal anti-GFP (1:750, Aves Lab), mouse monoclonal anti-Pax6 (1:400, DSHB), rat monoclonal anti-Pax6 (1:400, BioLegend), mouse monoclonal anti-phosphohistone H3 (1:3000, Millipore), goat polyclonal anti-NeuroD1 (1:400, Invitrogen), rabbit polyclonal anti-Tbr2 (1:400, abcam). Secondary antibodies used were: donkey alexa488 anti-chicken IgY (Abcam), donkey alexa647 anti-mouse Fab IgG (Jackson ImmunoResearch), donkey Alexa555 anti-rabbit IgG (Thermo Fisher Scientific), goat alexa647 anti-rabbit IgG (Jackson ImmunoResearch), donkey Cy3 anti-rat Fab IgG (Jackson ImmunoResearch), goat biotinylated anti-mouse IgG (Vector Laboratories), DyLight Alexa405 streptavidin (Jackson ImmunoResearch) (**table 4**); all used at 1:200 dilution.

Samples stained only with anti PH3 antibody were counterstained with DAPI 1:1000 for 3 min.

Samples stained for four different markers (GFP, Pax6, PH3, Tbr2) were immunostained in two rounds as it follows: first, they were stained with primary antibodies for GFP, Pax6 and Tbr2 followed by appropriate secondary fluorescently conjugated antibodies, as indicated above; these primary and secondary antibodies were lightly fixed to their antigens immersing the tissue sections/slices in 4% PFA in PBS for 10 min at room temperature; tissue sections/slices were washed using PBS and blocked again with Lys 0.2 M and Gly 0.2 M in the same blocking solution indicated above for two hours; sections were then stained as indicated above against PH3 followed by appropriate secondary biotinylated antibody and

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fluorescently labelled streptavidin. The purpose of this sequential staining was to circumvent the observed cross-affinity of the secondary antibody used for Pax6 staining for the primary antibody used for phosphohistone H3 staining. To make sure that this observed crossing was appropriately solved, the ganglionic eminences (GEs) of the stained brain slices were checked: in this brain region, there are cells undergoing mitosis (PH3+) but these do not express Pax6 (Pax6-). If we observed that PH3+ cells were negative for the secondary antibody used against Pax6 in the GEs, the immunostaining was successful, and samples were used. If we observed that PH3+ cells were positive for the secondary antibody used against Pax6 in the GEs, the sample was discarded.

Table 4. Commercial antibodies used for IHC.

Antibody	Source or reference	Identifier
Chicken polyclonal anti-GFP	Aves Lab	Cat# GFP-1020, RRID:AB_10000240
Mouse monoclonal anti-Pax6	DSHB	Cat# NA, RRID:AB_528427
Rat monoclonal anti-Pax6	BioLegend	Cat# 939802, RRID:AB_2888904
Mouse monoclonal anti-phosphohistone H3	Millipore	Cat# 5-806, RRID:AB_310016
Goat polyclonal anti-NeuroD1	Invitrogen	Cat# PA5-47381, RRID: AB_2607915
Rabbit polyclonal anti-Tbr2	Abcam	Cat# ab23345, RRID:AB_778267
Donkey Alexa488 anti-chicken IgY	Jackson ImmunoResearch	Cat# 703-545-155, RRID:AB_2340375
Donkey Alexa647 anti-mouse Fab IgG	Jackson ImmunoResearch	Cat# 715-607-003, RRID:AB_2340867
Donkey Alexa555 anti-rabbit IgG	Thermo Fisher Scientific	Cat# A-31572, RRID:AB_162543
Goat Alexa647 anti-rabbit IgG	Jackson ImmunoResearch	Cat# 111-605-144, RRID:AB_2338078
Donkey Cy3 anti-rat Fab IgG	Jackson ImmunoResearch	Cat# 712-167-003, RRID:AB_2340670
Goat biotinylated anti-mouse IgG	Vector Laboratories	Cat# BA-9200, RRID:AB_2336171
DyLight Alexa405 streptavidin	Jackson ImmunoResearch	Cat# 016-470-084, RRID:AB_2337248

Image acquisition of fixed tissue samples

For histological characterization of PH3+ cells during neurogenic period, z-stack images comprising the full thickness of the tissue slices were acquired using a fluorescence microscope (Zeiss Axio Imager Z2, Carl Zeiss) with Apotome.2 (Carl Zeiss) coupled to two digital cameras (AxioCam MRm and AxioCam ICc) with the following objectives: 2.5x, 10x and oil immersion 20x (Zeiss EC-PLAN NEOFLUAR 20X/0.5 NA) and 40x (Zeiss EC-PLAN NEOFLUAR 40X/1.3 oil) objectives. The distance between z planes was set at 1 μ m with the 20x objective and 0.5 μ m with the 40x objective. Same image parameters (light power, gain, wavelength) were kept for images from the same slide and same used objective. For each imaged sample, a mosaic of tiles with the region of interest was acquired and then automatically stitched using ZEN blue software (Carl Zeiss). For coronal sections (all snake, chicken and mouse samples, E36, E38 and E40 ferret samples), the whole span of the dorsal cortex between the hippocampal region and the contact with the DVR (snake and chicken) or GEs (mouse and ferret) in equivalent rostro-caudal regions was imaged. For ferret sagittal sections (E34, P0 and P2 samples), equivalent rostro-caudal and latero-medial regions were imaged. To establish the correspondence to the coronal sections, the GEs were used as reference. The use of both coronal and sagittal sections in the case of the ferret was not due to a design error, but to take advantage of samples used in previous projects and reduce the number of animals used.

For histological characterization of Pax6+ &/or Tbr2+ mitotic NPCs (PH3+) and their morphology, z-stack images comprising the full thickness of the tissue slices or of the cell of

interest were acquired using a fluorescence confocal microscope (Leica SPEII, Leica Microsystems) with the following objectives: oil immersion 20x (ACS APO 20x/0.60 IMM CORR), 40x (ACS APO 40x/1.15 OIL CS) and 63x (HCX PL APO 63x/1.40 OIL CS) objectives with a 1024 px resolution. The distance between z planes was set at 1-4 μm with the 20x objective, at 0.44 μm with the 40x objective and at 0.33 μm with the 63x objective. Same image parameters (laser power, gain, pinhole aperture, wavelength) were kept for images from the same slide and same used objective. For each imaged sample, a mosaic of tiles with the region of interest was acquired and then automatically stitched using Leica Application Suite X software (Leica Microsystems). The imaged regions were contained within the dorsal cortex between the hippocampal region and the DVR (snake and chicken) or the GEs (mouse and ferret).

Slice-culture and time-lapse imaging

For live imaging of electroporated progenitors and their progeny, embryos were electroporated as indicated above at the following ages: chicken, 4 dpo; mouse, E12; ferret, E34. Forebrains were dissected out in ice-cold dissection medium (DMEM-F12 (Sigma) supplemented with glucose (Sigma) NaHCO_3 (Sigma) and PenStrep (100U/ mL) (Sigma) at the following ages: chicken, 5 and 6 dpo; mouse, E13 and E14; ferret, E36. Forebrains were sliced at 250-300 μm thickness using a vibratome (Leica VT1000S) in ice-cold dissection medium fizzed with carbogen (5% CO_2 and 95 % O_2). Slices were embedded in a collagen matrix (Nitta gelatine) on a filter membrane (Millipore) and cultured in DMEM-F12 (Sigma) supplemented with foetal bovine serum (5%), horse serum (5%), N2 (1:100, Invitrogen), B27 (1:50, Invitrogen), PenStrep (100 U/mL), buffered with HEPES (Pilz et al., 2013) at 37.5 °C for at least 3 hours to let the sample and matrix stabilize. Imaging was performed with a Andor Dragonfly spinning disk inverted confocal microscope with a 25x objective (HC Fluotar L 25x/0.95 W) in a 5% CO_2 atmosphere 37 °C. For each imaged region, a z-stack of 220-260um images with a separation of 4 μm was acquired every 20 min during 24-48 hours. Immediately after the last time point was imaged, slices were fixed in 4% PFA for 10 minutes, washed with PBS and later processed for IHC.

Image analysis

PH3 counting. Cell counts and distances to the apical surface were obtained semiautomatically using Fiji software (Schindelin et al., 2012). Briefly, each z-stack image was reduced to a z-stack spanning a maximum of 2 nuclei-thickness. The z-stack was projected onto a single plane using maximum intensity; the background intensity was subtracted from the image; a gaussian blurring filter ($\sigma = 1$) was applied to smooth the edges of the nuclei; contrast was enhanced and the image was automatically classified (background and object) using the default threshold plugin of Fiji; a watershed was applied to separate individual nuclei; individual ROIs for each nuclei were obtained using “analyze particles” and visually inspected to discard artifacts; the length of the ventricular surface of the image was measured, the number of nuclei was quantified and their distances to the apical surface was measured using a macro (https://microscopynotes.com/imagej/shortest_distance_to_line/index.html). For each developmental stage of each species, quantifications were obtained from at least three different brain slices from each embryo and at least three different embryos.

Marker analysis. Cell counts, intensity values and distances to the apical surface were obtained semiautomatically using Fiji software (Schindelin et al., 2012). A mask of each PH3+

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nuclei along the z-stack was obtained in the PH3 corresponding channel as it follows: the background intensity was subtracted from the image; a gaussian blurring filter ($\sigma = 1$) was applied to smooth the edges of the nuclei; contrast was enhanced and the image was automatically classified (background and object) and binarized using the Trainable Weka segmentation tool (Arganda-Carreras et al., 2017); the binary image was then segmented into individual masks for each nuclei using the Morpholibj plugin (background value = 0) (Legland et al., 2016); the masked image was loaded into 3D manager, the volume of the masked objects was inferred and these were filtered with a lower and upper threshold; these masked objects were used in the Pax6 and Tbr2 channels as ROIs to measure the mean intensity across the z-stack using 3D manager in each channel individually (Ollion et al., 2013). To automatically measure the distance to the apical surface: a binary mask delineating the ventricle and the tissue was generated; this image was used to generate a Geodesic Distance map in 3D using the Morpholibj plugin (the intensity values in this type of image correspond to the distance to a reference, i.e., the ventricle); the ROIs generated for each cell were loaded onto the Geodesic Distance map to record their intensities, i.e., the distances to the apical surface. For each species, quantifications were obtained from at least three different brain slices from each embryo and at least three different embryos.

Morphological determination and time lapse clone tracking. Images were analysed using Imaris software (Bitplane). Images were contrast-enhanced and visually inspected in 3D and through the z-stack to determine the morphology of cells positive for PH3 and GFP. To show representative examples, surface masks were obtained to isolate the GFP signal of the cell of interest from the rest of the image. Long processes of these cells were isolated to their end or until they could be mixed with the signal of other cells. To analyse the dynamic behaviour of progenitors and their progeny, mitoses were identified at the GFP channel in the time-lapse experiments. The mother cell was then tracked back to the beginning of the time-lapse (or until it was no longer visible) and the progeny was tracked forward until the end of the time-lapse (or until it was no longer visible). The morphology and location of the daughter cells was used to establish their identity. This was further specified with immunolabelling for NeuroD1, Pax6 and Tbr2, as indicated above. The correspondence between tracked cells in the live-imaging files and the cells in the fixed tissue imaging files was visually achieved using the position and morphology of the cells.

Histological cell type definition

Apical, subapical and basal categorization. To assign cells to a domain in the radial axis of the growing cortex, the distance of its nucleus at mitosis (PH3+) was measured and three categories were established: apical, subapical and basal. A cell was considered to be apical if the distance of its nucleus to the apical surface was less than 25 μm ; to be basal if the distance of its nucleus to the apical surface was higher than 40 μm for snake and chicken or higher than 60 μm for mouse and ferret; to be subapical if the distance of the nucleus to the apical surface had a value between these thresholds. Each of these thresholds had its rationale: the apical threshold was set at 25 μm to account for the beveling of the tissue slice edges that can occur when slicing it; the basal threshold was set at 40 and 60 μm to consider as basal cells those undergoing mitosis in the transition area between the VZ with tightly and radially arranged Pax6+ nuclei and the basal area, with no such trait.

Lateral, medial and central (in-between the other two) categorization. To assign cells to a domain in the latero-medial axis, the dorsal cortex was divided in three sectors: a lateral one,

adjacent to the DVR (snake and chicken) or the GEs (mouse and snake), a medial one, adjacent to the hippocampal domain, and a central one, lying between the other two.

To determine whether cells were positive for Pax6 or Tbr2, the mean intensity of each channel for each marker was quantified as explained above (Image analysis). Then, to account for the intrinsic variability of intensity measurements due to species, antibody batch, experimental batch and tissue fixation, these values were normalized making a ratio over the background intensity for each channel. To further reduce the error of counting false positives, in case of doubt, the highest value of known true negatives (visual inspection) was used as threshold. For each nucleus, if the normalized intensity value was higher than 1, it was considered to be positive for the putative marker; otherwise, it was considered to be negative.

Morphological categorization. To show the morphology of the cell, membrane GFP was electroporated into apical progenitors as indicated above (DNA Constructs and *in utero* and *in ovo* electroporations) and they and their progeny were allowed to develop for one or two days. A cell was called nonpolar if it showed a rounded shape with no distinguishable processes, either long or short; multipolar if it showed a rounded shape with several short processes; monopolar with an apical process if it showed a long process directed to the apical surface; monopolar with a basal process if it showed a long process directed to the pia; bipolar if it showed both a long apical process directed to the apical surface and a long basal process directed towards the pia.

Data processing and visualization

Processing of raw data, quantifications and data visualization were performed using R software version 4.3.2 (R Core Team, 2020).

Tissue dissection for single cell experiments

Ferret data was obtained from a previous project (Del-Valle-Anton et al., 2024). For snake, chicken and mouse, tissue dissection took place in ice-cold Leibovitz's (1×) L15 medium with l- glutamine and without phenol red (Gibco). Forebrains were dissected from the heads and meninges were removed. Then, tissue straps from the dorsal cortex were dissected and transferred to a 1.5 mL LowBind tube (Eppendorf) with ribonuclease (RNase)-free filter pipette tips (JETbiofil). For chicken, tissue straps from the most medial and most lateral domains of the dorsal cortex were dissected and transferred separately. Each replicate was a pool of the dissected tissue of two embryos in the case of the snake and three embryos in the case of chicken and mouse. Mouse embryos were from a single litter. Number of replicates per species and developmental stage: snake 6 dpo, 2; chicken 6 dpo medial domain of the dorsal cortex, 2; chicken 6 dpo lateral domain of the dorsal cortex, 2; mouse E12, 2.

Single cell suspensions preparation

For cell dissociation, the extra L15 medium was aspirated from the tube containing the tissue pieces. A preheated mix of 500 µL of HBSS-phenol red without Ca^{2+} Mg^{2+} (Gibco) together with 40 µL of trypsin (Sigma) was added to the tubes for enzymatic digestion. Samples were incubated in a 37 °C water bath for 8 minutes, gently tapping the tubes every two minutes. Enzymatic digestion was stopped adding 200 µL of FBS (Equilabo Scientific). Enzymatic digestion was followed by mechanical dissociation of the tissue pipetting gently 12 times with a P1000 set at 1000 µL. Dissociated cells were filtered through a 40 µm PluriStrainer Mini

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(PluriSelect) prewet with Hank's balanced salt solution (1×) without $\text{CaCl}_2/\text{MgCl}_2/\text{pH}$ indicator (Gibco) into a 2 mL LowBind tube (Eppendorf) precoated with 1% BSA in PBS (Gibco). Individual cell dissociation and viability was checked with trypan blue twice in a Neubauer chamber. A minimum of 90% cell viability was considered as threshold to consider the sample valid to load into the Chromium chip (10X Genomics).

Single cell isolation and cDNA library preparation

Immediately after, the cell suspensions were FACS-sorted to remove cell debris, dead cells and doublets or multiplets (BD FACSAria III). Sorted cells were collected in a 2mL LowBind tube (Eppendorf) precoated with 1% BSA in PBS. For the preparation of single cell transcriptomics libraries, Chromium Next GEM Single Cell 3' Reagent Kit v3.1 was used (PN-1000269 and PN-1000127) and its protocol followed (document ID CG000315). To obtain an estimated cell recovery of 10000 cells per sample, ~17500 cells per sample were loaded onto 10X Genomics Chromium platform for GEMs (Gel bead-in-emulsions) generation to assign per-cell-specific barcodes with unique molecular identifiers (UMIs) to RNA transcripts. GEMs were lysed, RNAs were retrotranscribed into cDNAs. Silane magnetic beads were used to purify cDNAs from the GEMs lysate. cDNA was amplified via PCR, and it was quantified and its quality assessed with Agilent Bioanalyzer. cDNA was stored at -20 °C. The day after, 3' gene expression libraries were constructed following the manufacturer's protocol, quantified and quality-checked with Agilent Bioanalyzer and stored at -20 °C.

Library sequencing

Sequencing was configured to obtain a rough estimate of at least 30000 reads per cell, vastly surpassing the 20000 reads per cell recommendation by 10X Genomics. Libraries were sequenced at the Centre of Genomic Regulation (Barcelona, Spain) with Illumina NovaSeq 6000 S2 and NovaSeq6000 S1 systems. One sample per lane was loaded, with paired-end sequencing, according to the sequencing format for 10x libraries: 28 bp for TruSeq Read 1 (barcode and UMI), 90 bp for TruSeq Read2 (cDNA), and 10 bp for sample indexes i5 and i7 each.

Genome alignment

Read alignment and demultiplexing were performed with Cell Ranger v7.1.0. Reference genomes used were HLLamFul1 (*Boaedon fuliginosus*), GRCg7b (*Gallus gallus*), and GRCm39 (*Mus musculus*).

Data quality assessment and filtering

Single cell expression matrices and metadata were loaded in R (v4.2.0) and Seurat (v5) was used for further analysis. Individual sample libraries were examined for quality parameters (**Figure S3**). All cells had at least 250 features and 500 UMIs. Cells with a mitochondrial percentage > 5%, cells classified as empty droplets or damaged cells (DropletQC)(Muskovic & Powell, 2021) or as doublets (DoubletFinder)(McGinnis et al., 2019) were filtered out. Due to the high fraction of damaged cells/empty droplets, one of the chicken samples was removed from further analysis.

Normalization, cell cycle regression and excitatory lineage subsetting

For each species, samples were merged and examined for batch effects (technical/biological replicate) (**Figure S4a, b**). Only in the case of the ferret were samples

integrated regressing out the batch effects due to different versions of 10x that had been used in the generation of the six libraries taken from (Del-Valle-Anton et al., 2024). In the other species, provided the extensive overlap between samples and to avoid overcorrection, there was no integration of the cells from different samples. Cells were then evaluated for their cell cycle phase according to their expression of genes characteristic of G2/M and S phases, using the default list of cell cycle genes provided by Seurat and Seurat's *CellCycleScoring* function. Next, species datasets were normalized using SCT (Hafemeister & Satija, 2019) without regressing out the cell cycle, regressing out the cell cycle (distinguishing between G1, G2/M and S phases), and partially regressing out the cell cycle (distinguishing between G1 and G2/M/S phases). PCA on all variable genes and on cell cycle genes were performed to corroborate the corresponding regression (**Figure S4c**). For further processing, we proceeded with datasets with partial cell cycle regression. PCA was applied to the normalized and scaled dataset and nearest neighbours were found using the top 30 scoring PCs). Expression of microglial, endothelial cells, pericytes, erythrocytes and inhibitory neurons was checked to identify clusters nonbelonging to the excitatory lineage and these were removed out from the Seurat objects for further processing (**Figure S5**). After subsetting, each species' dataset was normalized again (SCT with partial cell cycle regression) and nearest neighbours and clustering recomputed. Subset dataset structures were visually inspected in 2d-UMAP representations.

Clustering, resolution

Clustering of the datasets was performed with varying resolutions (0.5, 0.7, 0.8, 1.0, 1.3, 1.5, 2). Clusters were visualized with UMAP representations and Clustree was used to assess cluster stability across resolutions (Zappia & Oshlack, 2018) (**Figure S6a**). Clustering resolution was established as a balance between clustering stabilization (no shuffling or partition of one cluster into others from one resolution to the next) and over clustering (partitions of clusters that have kept stable in a series of preceding resolutions). After this preprocessing, cluster composition by sample origin in each species dataset was checked to assess uniform distribution of clusters by sample origin and cell cycle phase (**Figure S6b, c**).

Cell type identification

To identify main cell types in each of the datasets, three approaches were followed. First, the scores of gene modules of main cell types of developing cortices (**table 5**) were explored across the datasets. These markers were extracted from studies in mouse, human and ferret (Del-Valle-Anton et al., 2024; Di Bella et al., 2021; Li et al., 2020; Loo et al., 2019). Second, in each species dataset, the top 20 significantly differentially expressed genes of each cluster, by average log fold change with respect to the other clusters' expression, were inspected for their function (obtained with *FindAllmarkers*, applied to the SCT assay, with Wilcoxon rank sum test, reporting only genes with a positive fold change, present in a minimum of 25 % of cells in each cluster and with a minimum 0.25 log fold change). This function segregates cells by condition (cluster) and carries out differential expression analysis for each cluster against the all the others. Third, the individual expression of canonical markers was checked through clusters to corroborate their annotation. Main categories were RGC, IPC, IPC-newborn, immature neuron, immature excitatory neuron, neuron. RGC and IPC clusters were further annotated using the main cell cycle phase composition of their cells. Other categories were further annotated with the expression of conspicuous characterized genes, such as *LHX1*, *RELN*, *DLL1* and *NOTCH1*.

Table 5. Canonical markers used in the module scores of main cell types of early excitatory lineage.

Main cell type	Canonical markers
Proliferating cells	<i>MKI67, TOP2A, APOE, SLC1A3, CST3, PCNA, SPC24, BIRC5, UHRF1, WDR76</i>
RGCs	<i>HES1, HES5, CYR61, PAX6, GAS1, ARHGEF39, ASPM, NDE1, SOX2, NES, GLI3, NOTCH1, NOTCH2, FGFR, TOP2A, DBI, CCND1, SLC1A3, FABP7, VIM, PON2, ATP1A2</i>
IPCs	<i>EOMES, UNC5D, MFAP4, SSTR2, NEUROG, BTG2, CHD7, ELAVL2, CELSR1, NEUROD1, SLC17A6, NEUROG1, MFNG, NEUROD2, NEUROD6, TUBB3, SFRP1, ELAVL4, NELL2, ILKAP, SCOC</i>
bRGCs	<i>HOPX, PTPRZ1, PTN, CST3, FAM107A, MOXD1, PEA15, SEPT7, TMEM132B, TNC, ITGB5, FBN2, MFGE8, LPL, SPARCL1, HTRA1, HTR2A, PLOD1, GPLD1, F3, DDR1, PRKCA, GPM6B, LIFR, CTNND2, IL6ST, SDC3, HS6ST1, LGALS3BP, BMP7, GFAP, GLI2, HEPACAM, VEPH1, CD38, SLC15A2, HERC6, KAL1, ATP10B, CD82, AQP4</i>
Immature neurons	<i>NEUROD2, TUBB3, NEUROD6, DCX, NEUROD1, NRP1, TBR1, STMN1, NCAM1</i>
Migrating neurons	<i>TIAM2, NEUROD2, TUBB3, NEUROD6, NRP1, NEUROD1</i>
Deep layer neurons	<i>BCL11B, NDRG1, SATB2, SLA, PLXNA4, SOX5, FEZF2, PTN, LMO4, LDB2, PANTR1, TBR1, BCL11B, TLE4, SOX5, LDB2, PCP4, NR4A2, FEZF2, POU3F1</i>
Layers II-III neurons	<i>CUX1, CUX2, PLXNA4, POU3F2</i>
Projection neurons	<i>FEZF2, TLE4, TBR1, BCL11B, NFE2L3, HIVEP2, NFIA, SOX5, CUX1, POU3F1, SATB2, PTPRK, FAM19A2, THY1, LDB2, PEX5L, FAM19A1</i>
Excitatory neurons	<i>NEUROD2, NEUROD6, BHLHE22, NRP2, SOX5, TLE4, DAB1, FEZF2, ALCAM, ID2, SYT4, MEF2C, CBLN2, TBR1, CADM1, ARPP21, DCX, TUBB3, SLC17A6, CUX2, NSG2</i>

Pseudotime analysis

Pseudotime was assigned to cells using Monocle3 (v.1.3.7) (Cao et al., 2019; Qiu et al., 2017; Trapnell et al., 2014). Briefly: Seurat objects were converted into cell data set objects (package Seuratwrappers, v.0.4.0), using the SCT assay data with partial cell cycle regression. Clustering and partition metadata entries were created (1 partition, for one differentiation trajectory, and provided there were no isolated clusters in the datasets). UMAP coordinates and metadata were transferred from the annotated Seurat objects to the cell data set objects. Trajectories were established using the Monocle3 function *learn_graph* and pseudotime calculated on the obtained trajectory (*order_cells*). Cells of origin for each trajectory were identified as the centre of RGC clusters with the highest G2-M score in a graphical user interface. Module scores and gene expression of main cell types were visualized along pseudotime to infer different dynamics of expression with respect to the proliferation score in each species.

Ferret samples integration

Due to a non-uniform composition by sample origin of the ferret clusters depending on the version of 10x chemistry (v2 and v3) used in the preparation of the scRNAseq libraries (Del-Valle-Anton et al., 2024) (**Figure S4a**), the merged ferret dataset was integrated after excitatory lineage subsetting, cell cycle scoring, merging and SCT normalization and scaling with partial cell cycle regression. Samples were integrating using *IntegrateLayers* on the SCT normalized and scaled data by CCA integration. Cluster uniform distribution by sample origin

was checked by visual inspection, confirming the integration of the different samples in every cluster (**Figure S6b, c**).

Cross-species integration

Prior to integration, one-to-one ortholog genes across *Boaedon fuliginosus*, *Gallus gallus*, *Mus musculus* and *Mustela putorius* were obtained, as follows. High confidence orthologs to *Mus musculus* were downloaded for *Gallus gallus* and *Mustela putorius* using the biomaRt package (v.2.58.2) (Durinck et al., 2005, 2009) from the ensemble database and filtered to common entries in the genes found in our single cell objects for each species. Since *Boaedon fuliginosus* was not included in this database, orthologs to mouse for the other two snake species included (*Notechis scutatus*, *Pseudonaja textilis*) were downloaded, combined and subset to the common genes found in our single cell data of *Boaedon fuliginosus*. This resulted in 10891 orthologs to *Mus musculus* found in our *Boaedon fuliginosus* dataset, 12518 in our *Gallus gallus* dataset and 14470 in our *Mustela putorius* dataset. Altogether, the four species shared 8600 orthologs, 7087 of which were commonly expressed in our datasets. This list of genes was used later for the integration, as now related.

For cross species integration, we used the workflow introduced in Seurat v3 (<https://satijalab.org/seurat/archive/v3.1/integration.html>). Single cell datasets of each species were randomly downsampled to match the snake dataset cell number (10946 cells) using the subset function from base to have an equal contribution of each species dataset to the integrated dataset. In the case of the chicken, we had retrieved a higher proportion than in the other species of neurons vs. NPCs. Because of this, chicken dataset downsampling was adapted to keep a contribution of NPCs clusters to the total population of cells of 50%, in the range of the datasets of the other species. Next, read counts from the RNA assay and metadata were extracted from each species dataset. Gene names of the mouse extracted RNA count matrix were adapted to the format of the other species to have a common nomenclature. New Seurat objects were generated for each species with the extracted RNA counts and metadata for each species. The objects were merged into a single Seurat object. Before integrating, the merged Seurat object was split into a list to match the variables to integrate by: species, plus medial and lateral regions in the case of the chicken, and plus 10x chemistry version used in the case of the ferret (snake, mouse, chicken medial, chicken lateral, ferret v2, ferret v3). The split object was normalized and scaled with partial cell cycle regression, increasing the number of variable features to 5000 from the default 2000 (SCT, method glmGamPoi set for a less memory-heavy performance, 5000 variable features). Integration features were selected from the SCT assays of the Seurat objects list (*SelectIntegrationFeatures*, 5000 minimum features) and reduced to the features present in the four species datasets and in the common orthologs. PCA was calculated on the SCT assay values of these features in the Seurat objects list. Finally, integration anchors among the common ortholog genes found in the integration features were computed, using RPCA and CCA methods (*FindIntegrationAnchors* function, normalization.method = "SCT", reduction = "cca" or "rpca"), and the Seurat objects list was integrated (*IntegrateData* function, normalization.method = "SCT"). Following integration (either RPCA or CCA integration), the integrated object counts of the integrated assay were scaled, its principal components computed, and nearest neighbours obtained for UMAP visualization. RGCs and IPCs were subset from the integrated object to respective independent objects to carry out further analysis.

Pseudobulk-aggregated analysis by species in the integrated dataset

As a first step in finding genes with greatest difference in expression between species, we compared the pseudobulk aggregated expression data by species in the RGC and IPC integrated objects. For this, in each object, the aggregated expression of the RNA assay was obtained using *AggregateExpression*, and plotted for visualization, comparing two species at a time (**Figure S12b-g, Figure S13b-g**). Top 50 deviant genes by difference in log2 average expression were obtained from each two-species comparison.

DEA analysis by species in the integrated dataset

Differentially expressed genes (DEGs) between species were computed for RGCs and IPCs. For this, significantly DEGs between species were calculated on the normalized data matrix of the RNA assay using *FindAllmarkers*, with Wilcoxon rank sum test, reporting only genes with a positive fold change, present in a minimum of 25 % of cells in each cluster and with a minimum 0.25 log fold change. The obtained DEG lists were filtered by an adjusted p-value < 0.01 and an average log2 fold change ≥ 0.59 (about 1.5 times more expressed in one of the species compared to the others). Histone genes and genes not annotated were also filtered out of the output. Top100 DEGs by average log fold change were inspected to find functionally relevant genes expressed specifically in each species, in sauropsids and in mammals. Mean expression per cluster of the top 50 DEGs (**Figure 20d & Figure 21d**) and of the top 50 ortholog DEGs (**Figure S12a & figure S13a**) in each species was visualized in a heatmap, ordered by a cluster similarity dendrogram. The variable for selecting top genes was average log2 fold change.

GO term enrichment analysis

Biological gene ontology analysis of the species-specific RGCs and IPCs was carried out using the EnrichR web (<https://maayanlab.cloud/Enrichr/>). Differentially expressed genes for each species in the RGC and IPC integrated objects were uploaded to the EnrichR web and results for *BiologicalProcess* 2025 were downloaded. Terms were filtered by adjusted p-value < 0.5 (p-value in case of ferret) and top 20 terms by adjusted p-value (p-value in case of ferret) were plotted for visualization (**Figure20f-i & Figure 21f-i**).



3. - Materials and methods



4. - Results

4.1. - Location and abundance of NPC mitoses during the neurogenic period

To infer an equivalence of dorsal pallial development across our model species, we determined the abundance of NPCs undergoing mitosis, by immunolabelling for phosphohistone 3 (PH3). Several developmental stages within the neurogenic period were analysed (except for snake, where we could only obtain tissue from a single developmental stage) (**Figure 9a, b**). The reason why we chose to study the PH3+ population, instead of other markers of proliferative cells, is that one of the main histological features of NPCs is the location along the apico-basal axis where they undergo mitosis (**section 1.5.2**). Accordingly, the distance of PH3+ nuclei to the apical surface was measured and normalized to the thickness of the VZ (**Figure 9c**). In the case of snake, at 6 dpo, virtually all mitoses were located at the apical surface, basal mitoses being largely anecdotic (5 basal divisions out of 560 mitoses analysed). In the case of chicken, the vast majority of mitoses were found at the apical surface. Basal mitoses were scarce and located at the basal border of the VZ at E5 and E6. This provides evidence for the existence of a secondary germinal layer as in the case of mammals, i.e., the SVZ. In the case of mouse, a similar pattern was observed: a majority of NPCs undergoing mitosis at the apical surface, with a minor population of NPCs dividing at basal locations. Finally, in the case of ferret, the distribution of mitoses along the apicobasal axis was very different, for two reasons. (1) There was a vast abundance of abventricular mitoses at all ages analysed, (2) although at E34 and E36 abventricular mitoses were more abundant around the basal end of the VZ, their location extended basally several times the thickness of the VZ. This corresponds to the expansion of the SVZ into an iSVZ and a large oSVZ, typical of gyrencephalic mammals (DeFelipe et al., 2002; Kriegstein et al., 2006; Reillo et al., 2011; Reillo & Borrell, 2011; Smart et al., 2002).

In spite of the different amplification capacity of RGCs between synapsids and sauropsids, it was very interesting to determine that VZ thickness is similar (**Figure 9d**). The fact that VZ thickness is similar between species in which the full cortical thickness differs dramatically already during development (~1450 μm in P2 ferret vs. ~140 μm in E5 chicken) suggests that there is a conservation in the structure of this primary germinal layer, or a limitation in the thickness of the VZ independent of final cortex size and developmental trajectory.

Next, we categorized NPCs into two main populations depending on their location: apical or basal. APs were considered as located on the apical surface of the VZ (distance of its nucleus to the apical surface was less than 25 μm). BPs were considered as located in the basal end of the VZ and beyond (distance of its nucleus to the apical surface was higher than 40 μm for snake and chicken or higher than 60 μm for mouse and ferret). SAPs were not considered for this part of the analysis. The VZ was defined using DAPI staining as previously described: a layer with tightly packed and radially arranged nuclei, differently to the looser organization of cells with rounded nuclei in a more basal location (Angevine et al., 1969; Bentivoglio & Mazzarello, 1999; Reillo & Borrell, 2011). For simplicity and due to their low abundance, subapical NPCs (undergoing mitosis in the middle area of the VZ) were counted together with apical NPCs at this stage of the analysis.

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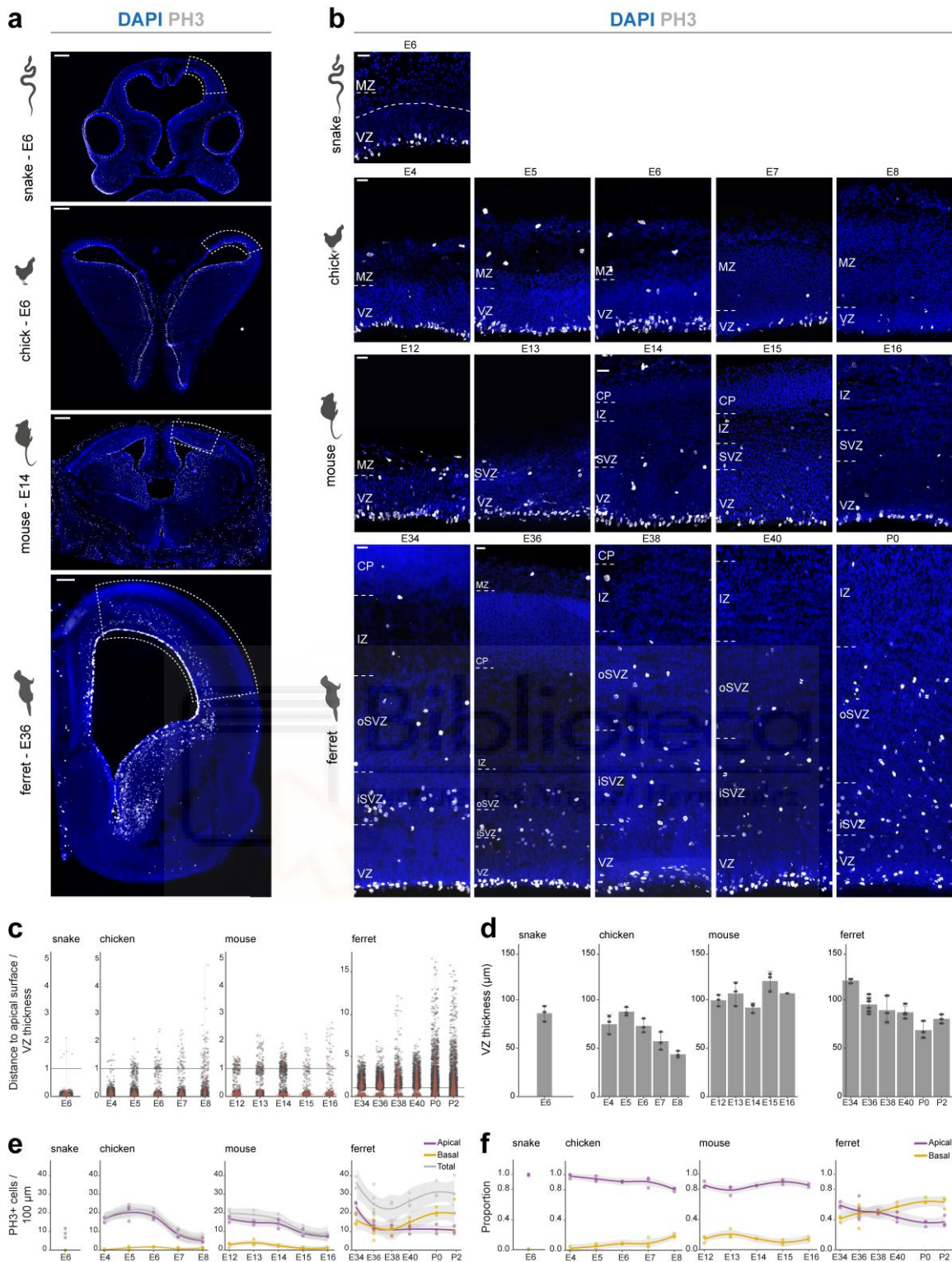


Figure 9. PH3+ cells distribution along neurogenic period.

a) Coronal sections of developing cortices of snake (E6), chicken (E7), mouse (E14) and ferret (E36) immunostained for PH3 and DAPI, with the region of analysis demarcated with a dashed line. Scale bars, 250 μm . **b)** Detail images as in (A) at the developmental stages indicated. Dashed lines indicate layer borders. Scale bars, 20 μm . **c)** Distribution of the distance of PH3+ nuclei to the apical surface relative to the VZ thickness. **d)** Average VZ thickness along neurogenic stages. **e)** Progression of apical and basal mitosis density along neurogenic stages. Lines indicate average. Shadowed areas indicate 95% confidence interval. **f)** Average proportion of apical and basal mitoses to the total pool of mitoses along neurogenic stages. Average data per embryo are shown. Shadowed area indicates the 95%

confidence interval. Data structure: 3 embryos per stage and species, 3 tissue slices per embryo. N PH3+ cells: snake, 560; chicken, 4082; mouse, 3932; ferret, 11682. . Error bars, SD. CP, cortical plate; IZ, intermediate zone; iSVZ, inner subventricular zone; MZ, marginal zone; oSVZ, outer subventricular zone; SVZ, subventricular zone; VZ, ventricular zone.

When we consider APs in chick, mouse and ferret, there was a point during neurogenesis in all the three species when they started to diminish. However, they did so with a different dynamic (**Figure 9e, f**). In the case of the chick, AP mitosis density reached a maximum at E5 (~ 20 mitosis/100µm in 20 µm thick slices), and from E6 on its value abruptly diminished to ~ 5 mitosis/100µm. In the case of mouse, between E12 and E14, AP mitosis density behaved as a slowly decreasing plateau around 15 mitosis/100µm and, from E14 on, its value diminished more smoothly than in chicken to ~ 7.5 mitosis/100µm. In ferret, between E34 and E36 there was an abrupt decrease in AP mitosis density into a plateau that maintained a stable value slightly higher than 10 mitosis/100µm for the rest of the period studied. Comparing quantitatively between species, AP mitosis density during mid neurogenesis in chicken reached a value fairly higher than in mouse (~ 33% higher), and similar to the maximum values observed in ferret at E34. However, at later stages, AP mitosis density in chicken fell to values ~ 33% lower than in mouse and more than two times lower than in ferret.

As for BPs, in the chick they maintained a very low value of mitosis density, reaching at E6 a maximum contribution to the total pool of NPCs undergoing mitosis (~ 10 % of total mitoses) (**Figure 9f**). In the mouse, BP mitotic density also maintained a low value, reaching a maximum contribution to the total pool of NPCs of at E13 (~20% of total mitosis). In the case of the ferret, BP mitotic density values and dynamic were drastically different. Not only did it reach much higher values in the middle of neurogenesis, but, at later postnatal stages, BPs continued proliferating, being their cell divisions up to twice as abundant as those of APs.

Altogether, this analysis showed (1) a modest apical mitotic density in the snake, in a range of values similar to those observed at late stages of neurogenesis in mouse; (2) a similar topology of the chicken and mouse proliferation curves, with a drop in apical mitosis density at mid neurogenesis and with a slightly higher apical density in the former and basal density in the latter; and (3) a marked extension of the mitotic pool in the ferret, in which not only are apical mitoses maintained stable after a drop in their abundance, but the basal ones take over and keep increasing in numbers.

Based on these results, we performed a further histological characterization of cortical progenitors to determine the diversity of NPCs according to molecular canonical markers (Pax6 and Tbr2), their morphology and cell division mode (**section 1.5.2**), which is developed in the following sections. This thorough characterization was performed at a single developmental stage (snake, 6 dpo; chicken, 6 dpo; mouse, E14; ferret, E36). The choice of this developmental point was made after several considerations: (1) we chose an stage in the middle of the neurogenic period in all species, (2) to establish the correspondence between sauropsids and mammals, we chose an stage where, in both chicken and mouse, the density of mitosis starts to diminish (**Figure 9e**); (3) to establish the correspondence between mouse and ferret, we chose a developmental stage where in both species layers IV/V are being seeded (Jackson et al., 1989)(Polleux et al., 1997); (4) additionally, the age chosen in ferret corresponds to a developmental time when the oSVZ has been seeded (Martínez-Martínez et al., 2016). This allows to further emphasize the particular aspects of

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NPCs and cortical development of gyrencephalic mammals, that is, the enlargement of the SVZ into the iSVZ and oSVZ.

4.2. - Cortical progenitor types across amniotes identified by expression of Pax6 / Tbr2 and mitosis location

Within cortical development, two markers have been used to broadly distinguish between two main types of progenitors: Pax6 for apical aRGC, Tbr2 for IPCs. In addition, bRGC frequently coexpress Pax6 and Tbr2 (Englund et al., 2005; Hevner et al., 2006; Kowalczyk et al., 2009; Poluch & Juliano, 2015). Generally speaking, Pax6 expression is related to a less differentiated and more proliferative state, whereas Tbr2 expression is related to a more neurogenic state (Englund et al., 2005). However, depending on the species, NPCs that express these markers may have different properties within this proliferation-to-neurogenesis axis (Betizeau et al., 2013; Fietz et al., 2010; Florio & Huttner, 2014). There has been some ambiguity in the field when comparing lissencephalic vs. gyrencephalic species, and when mammals vs sauropsids (**section 1.5.2: Immunolabelling characterization**). In addition, most times the analysis of the expression of these canonical markers has been done without taking into account the time of cell division. As stated above, the location of mitosis is determinant for the definition of NPC types and their contribution to neuron generation.

Here, to characterize NPCs according to these two main markers at the specific moment of mitosis, we performed immunolabelling for PH3, Pax6 and Tbr2 in the dorsal cortices of E6 snake, E6 chicken, E14 mouse and E36 ferret (**Figure 10a, b**). As shown above (**Figure 9**), PH3+ cells were confined to the apical surface in snake, very scarce at a basal location in chicken and mouse, and abundant at a basal location only in ferret. As for the overall expression patterns of Pax6 and Tbr2, in the four species Pax6 showed a homogenous expression in the VZ, with a high density of positive cells, tightly packed and with radially orientated spindle-shaped nuclei. Basal to the VZ, there were scattered Pax6+ cells, more evident and much more abundant in ferret. Tbr2 showed a more species-specific pattern of expression (in snake we were not able to successfully perform IHC for this marker). In chick, Tbr2 was mainly expressed in a thin stretch of cells with rounded nuclei positioned basally and adjacent to the VZ, with minimal overlap with the Pax6+ tissue region. In the VZ, there were very few scattered Tbr2+ cells, still distant to the apical surface. In mouse, Tbr2 was expressed extensively in the VZ except for cells lining the apical surface, with abundant overlap with Pax6+ cells; and in the basal border of the VZ, where Tbr2+ cells were tightly packed. Basal to this band, there were few isolated Tbr2+ cells. In ferret, there was a similar pattern of Tbr2 expression as in the mouse, but with two differences: (1) Tbr2+ cells in the VZ start to be abundant a bit further from the apical surface than in mouse, (2) there is a more abundant and extended presence of scattered Tbr2+ cells in the SVZ and, especially, in the OSVZ. At this point, there is an important remark to make: the pattern of expression of Tbr2 in chick is noticeably different to that in mammalian species. Whereas in mammals there is an extensive overlap in the VZ between Pax6 and Tbr2, in chick there is a stark demarcation between a Pax6+ VZ and a basal thin band of Tbr2+ cells.

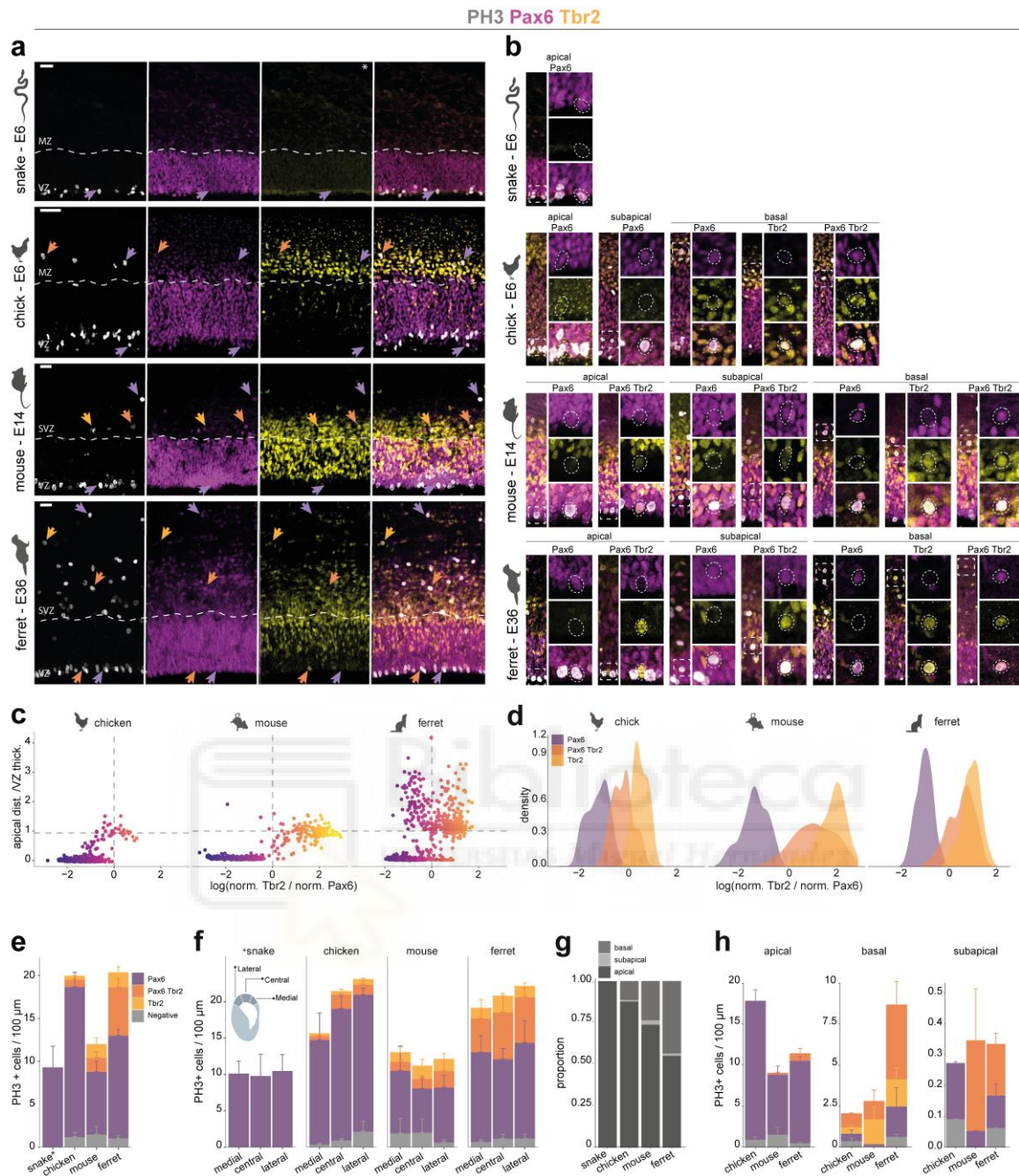


Figure 10. Expression of Pax6 and Tbr2 in mitotic NPCs.

a) Triple immunofluorescence for PH3 Pax6 and Tbr2 in cortical sections of snake (E6), chicken (E6), mouse (E14) and ferret (E36). Dashed lines indicate the basal end of the VZ. Arrowheads indicate PH3+ cells positive for only Pax6 (purple), only Tbr2 (yellow) or for both (orange). Scale bars, 20 μ m. **b)** Close-ups of representative examples of combinatorial staining of Pax6 and Tbr2 in PH3+ cells in different locations. For each case, a column showing the location of the high magnification area is shown. PH3+ cell showcases are indicated with dashed lines. **c)** Distribution of PH3+ cells by relative normalized expression of Tbr2 and Pax6 and distance to the apical surface relative to the VZ thickness. Colour code indicates the log ratio of normalized Tbr2 expression (more yellow) over normalized Pax6 expression (more purple). **d)** Density plots of the relative normalized expression of Tbr2 and Pax6 of categorized PH3+ cells according to their measured integrated signal intensity. **e)** Quantification of Pax+Tbr2-, Pax-Tbr2+, Pax6+Tbr2- and Pax6-Tbr2- cells in PH3+ cells over 100 μ m of apical surface. **f)** Quantification of Pax+Tbr2-, Pax-Tbr2+, Pax6+Tbr2- and Pax6-Tbr2- cells in PH3+ cells over 100 μ m of apical surface in medial, lateral and in-between (central) domains. **g)** Proportion of PH3+ cells in an apical, subapical and basal locations. **h)** Quantification of Pax+Tbr2-, Pax-Tbr2+, Pax6+Tbr2- and Pax6-Tbr2- cells in PH3+ cells over 100 μ m of apical surface in apical, subapical and basal locations.

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Pax6-Tbr2- cells in PH3+ cells in an apical, subapical and basal location. Data structure: 3 embryos analysed per species. N cells: snake, 560, chicken, 791, mouse, 629, ferret, 973. Error bars, SD. MZ, mantle zone ; SVZ, subventricular zone ; VZ, ventricular zone.

To define common and specific populations in our model species, we measured the intensity signal for Pax6 and Tbr2 (normalized by background signal) in each PH3+ nucleus, and plotted their log ratio against the distance to the apical surface (normalized by the VZ thickness; **Figure 10c**). This allowed to define, based on the combination of Pax6 and Tbr2 expression and the location of mitosis, common and specific populations in our model species. In all three, there was a group of PH3+Pax6+ cells lining the ventricle (presumably aRGCs). At a subapical level, PH3+ cells were very scarce in all three species, but showed species-specific trends of expression: in chick, subapical PH3+ cells were shifted towards higher Pax6 expression; in mouse, shifted towards Tbr2 expression; and in ferret, both situations were present. Finally, at the basal level differences between species were most evident: in chick, basal PH3+ cells seemed to be mostly coexpressing both markers; in mouse they seemed to have a low Pax6 and high Tbr2 expression; and in ferret, PH3+ cells were very abundant, extended much further basally and showed all combinations of high and low Pax6 and Tbr2 expression.

The signal intensity measurement of Pax6 and Tbr2 was used to automatically categorize PH3+ cells into four populations: Pax6+Tbr2-, Pax6-Tbr2+, Pax6+Tbr2+ and Pax6-Tbr2- (**Figure 10d**). Notwithstanding, we acknowledge that signal intensity from IHC cannot be considered as a direct measurement of protein expression and is subject to high variability. This categorization allowed to measure the abundance of PH3+ cells for each category (**Figure 10e**). Strikingly, the total density of PH3+ cells in the chick was equivalent to the amount observed in ferret, and almost two-fold greater than in mouse. The lowest abundance was observed in snake. The contribution of each NPC type per marker expression to the total was species specific (**Figure 10e**). In the case of snake, in the absence of Tbr2 staining, it can only be stated that all PH3+ cells were Pax6+. In the case of chick, Pax6+Tbr2- made up the vast majority of PH3+ cells, very different from ferret where this group accounted for ~60% of all PH3+ cells. Pax6+Tbr2+ were most abundant in ferret (~25%), in mouse made up ~12% of all cells, and about 5% in chick. Finally, Pax6-Tbr2+ were proportionately most abundant in mouse (~12.5 %), slightly less in ferret and scarce in chick. In chick, mouse and ferret, PH3+ cells negative for both Pax6 and Tbr2 were detected, possibly corresponding to endothelial cells or glial lineage progenitors, as found in other studies (Reillo & Borrell, 2011b), or false negatives. When we considered specifically the lateral, medial and intermediate domains of the dorsal cortex, the abundance of PH3+ cells greatly increased in a medial to lateral pattern in chick (**Figure 10f**), as previously reported (Cárdenas et al., 2018). This pattern was also observed in a subtler manner in the ferret but was absent in snake and mouse.

We wanted to assess also the abundance of PH3+ cell types per combination of Pax6 and Tbr2 expression in each species in the apical, subapical and basal region of the developing cortex (**Figure 10g, h**). In this analysis, snake was excluded due to the absence of Tbr2 staining and of basal mitoses. Considering first only the location of PH3+ cells, basal PH3+ accounted for 10% of the total in chick, 25% in mouse, and almost half in ferret; the rest of cells being mostly apical in the three species, as subapical cells were very scarce (**Figure 10g**). Combined with marker expression analysis, most apical mitoses were Pax6+Tbr2- in the three species (**Figure 10h**). However, in mammals there were also a few Pax6+Tbr2+ cells

undergoing mitosis at the apical surface. At the subapical level, again Pax6+ cells undergoing mitosis were detected in the three species, and only mammals presented also mitotic Pax6+Tbr2+ cells. Finally, at the basal level we found Pax6+ only, Tbr2+ only and Pax6+Tbr2+ cells undergoing mitosis in the three species, but in different proportions and amounts. In chick, the majority of the very scarce basal mitoses were Pax6+Tbr2+ (about half of total mitoses), Pax6+ only and Tbr2+ only cells, contributing each equally to the total pool. In the case of mouse, the majority of the slightly more abundant basal mitoses were Tbr2+ only (about more than half of total mitoses); about less than half of basal mitoses were Pax6+Tbr2+ only, and Pax6+ only basal mitoses were barely present. Finally, in the case of the very abundant basal mitoses in ferret, about half of them were made up by Pax6+Tbr2+ cells, Pax6+ only and Tbr2+ only cells contributing each equally to the total pool.

These data show that amniotes with different cortical organization display during development a different composition of NPC types, in both absolute and proportional terms, defined by the combinatorial expression of Pax6 and Tbr2 during mitosis and the location of mitosis. Several claims are drawn: (1) In sauropsids species, the vast majority of mitoses are Pax6+ and occur at an apical location. Tbr2 expression is markedly restricted to a thin band of cells basal to the VZ. Accordingly, mitotic NPCs express Tbr2 only at a basal location, either alone or in coexpression with Pax6. (2) In mammals, most Pax6+ NPCs undergo mitosis at an apical location. Although Tbr2 starts being expressed in the VZ, only a few of the NPCs undergoing mitosis at the apical surface express it. Basal mitotic NPCs are more abundant, particularly in the case of the gyrencephalic model (the ferret), in which they contribute to almost half of total mitoses. (3) Between mammals, differences between lissencephalic and gyrencephalic species are evident at a basal location. Here, in the lissencephalic species, basal mitoses account for 25 % of total mitoses and express Tbr2, either alone or together with Pax6. In the gyrencephalic species, basal mitoses are three-fold more abundant in absolute terms than in the mouse, the majority of Tbr2 mitoses coexpress Pax6, and a third population of Pax6+ only mitotic NPCs appears.

4.3. - Species with an enlarged cortical structure show a higher morphological heterogeneity of basal Pax6^{+/+}/Tbr2^{+/+} NPCs at mitosis

One of the central definitory aspects of the different types of NPCs is their morphology. In addition, the morphology of NPCs has been linked to their molecular identity in terms of Pax6 and Tbr2 expression: Pax6 is definitory of RGCs, Tbr2 is definitory of IPs. However, depending on the species, especially at a basal location and in gyrencephalic mammals, the identity between morphology and marker expression is not unequivocal: bRGCs can also express Tbr2, and IPs can also express Pax6 (**section 1.5.2: Immuno-labelling characterization**). At this point, we find that the link between the morphology and the molecular identity of NPCs has been done at times without considering the location of mitosis. As previously stated, this is the main reference system to classify NPCs, especially considering that not only the location but also the morphology of the cell can greatly vary along the cell cycle.

Here, to precisely characterize the NPC types that exist across amniotes with cortices of different size and histological structure, we revisited the morphological types that different populations of Pax6 and/or Tbr2 expressing cells can present at mitosis (PH3+). To do so, we electroporated APs lining the apical surface with a floxed mGFP plasmid and a low concentration of a Cre plasmid to sparsely label them. Then, these were allowed to proliferate for two days before fixing the tissue (chicken, E4 to E6; mouse, E12 to E14; ferret, E34 to E36) (**Figure 11a**). The mGFP signal was used to reconstruct the 3D morphology of

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NPCs in thick vibratome slices (150-250 μm). Two technical limitations were encountered: (1) we were not able to obtain enough embryos from our snake colony to do this manipulation, (2) the colocalization of GFP and PH3 was extremely low, limiting the number of observations despite the high number of embryos processed.

At an apical domain, we observed in the three species two possible morphotypes, which expressed Pax6 but no Tbr2 (**Figure 11b, c**, & **Figure S1a, b**). These included cells with a rounded soma undergoing division at the apical surface and bearing a long basally directed process that reached the pia, i.e., aRGCs (**Figure 11** & **Figure S1a**), and nonpolar cells that presented no observable process or very short ones, i.e., aIPs (**Figure 11c** & **Figure S1b**).

At a basal domain, we captured different combinations of radial glia morphotypes and Pax6 Tbr2 expression in each species. In the chicken, two different types of radial glia were observed: a cell with a rounded soma and a monopolar process basally directed expressing both markers (monopolar-bp Pax6+Tbr2+) (**Figure 11d**) and a cell with a rounded soma, one apically directed, and one basally directed process expressing only Pax6 (bipolar-abp Pax6+Tbr2-) (**Figure S1d**). In the mouse, a single morphotype was observed, rounded soma and basally directed process (monopolar-bp), expressing Pax6 only, or both Pax6 and Tbr2 (**Figure 11d**). In the ferret, more variation of radial glia morphologies was observed, although all of them expressed Pax6 and Tbr2, but one, which expressed Pax6 only. We found bRGCs with basal or apical short processes, bRGCs with long basal processes and bRGC with a process growing parallel or horizontal to the ventricular surface (**Figure 11d**). We also distinguished bipolar-abp bRGCs, expressing either Pax6 only or both Pax6 and Tbr2 (**Figure S1d**). Of notice, the bipolar-abp Pax6+ aRGC presented a bifurcation in the basal process.

For basal cells with an IP morphology, we detected that cells could show either short processes around the soma at mitosis (multipolar), or no processes (nonpolar), in the three species (**Figure 11e**). In the chicken, we were only able to capture bIPs expressing either only Pax6 or both Pax6 and Tbr2. In the mouse we captured a higher variability, with both multipolar and nonpolar cells expressing Pax6 only, Tbr2 only, both or none. Finally, ferret mitotic NPCs with an IP morphology were found to be Pax6+Tbr2-, Pax6-Tbr2+ or Pax6+Tbr2+.

At a subapical domain, we were not able to observe colocalizing GFP+PH3+ cells in the chicken. In mouse and ferret, we observed a shared morphotype: bipolar cells with an apically and basally directed processes, expressing both Pax6 and Tbr2. In the mouse, we also observed a nonpolar cell expressing Pax6 but not Tbr2 (**Figure 11f**).

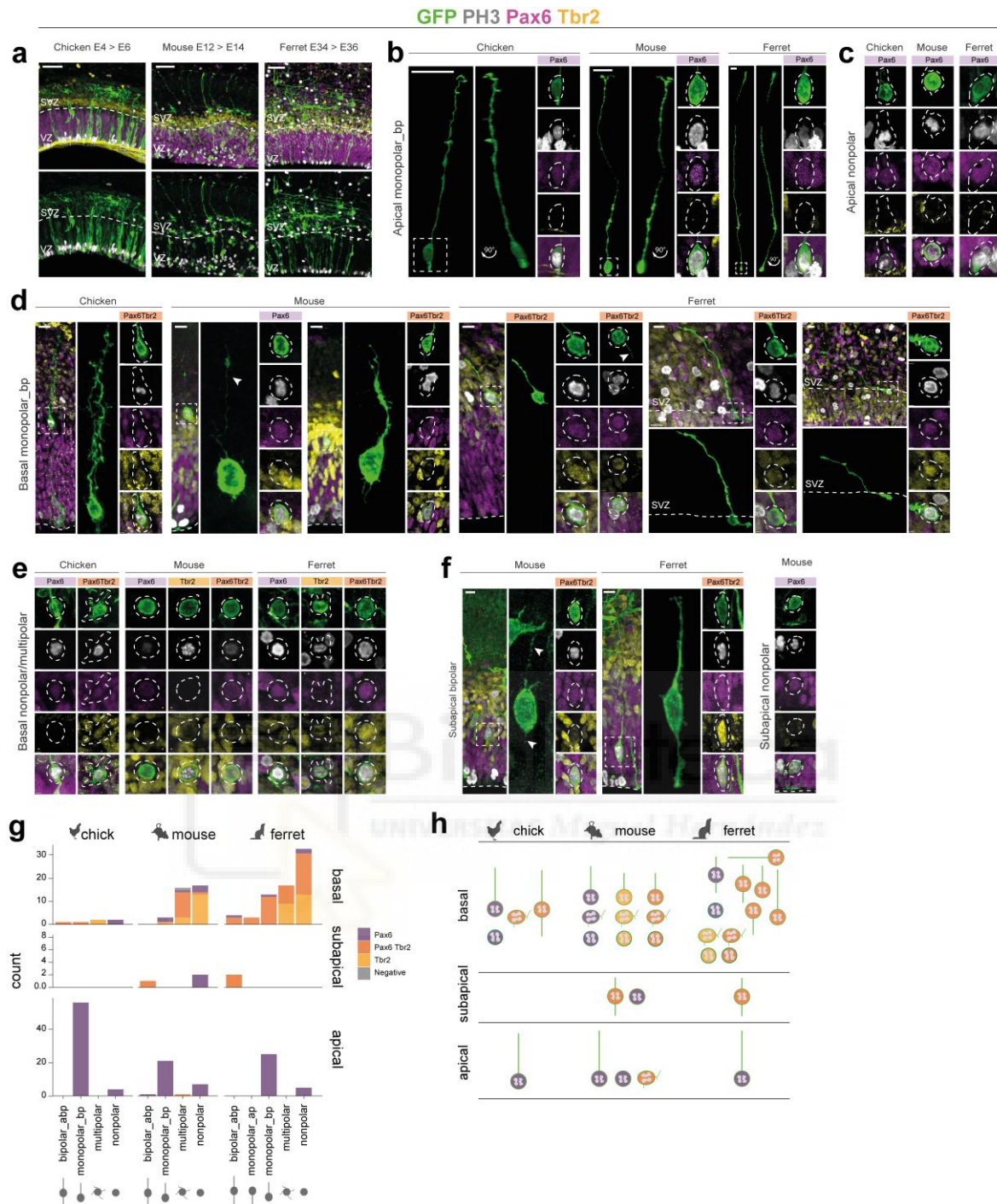


Figure 11. Morphological diversity of Pax6 and/or Tbr2 mitotic NPCs.

a) Images of thick sections across the dorsal cortex of GFP-electroporated embryos, immunostained for GFP (green), PH3 (white), Pax6 (magenta) and Tbr2 (yellow). Scale bars, 50 μ m (B), 20 μ m (D, F). Time of electroporation and time of analysis are indicated for each species. Dashed lines indicate the border between VZ and SVZ. **b-f)** Detail examples of NPCs with different morphologies, with high magnifications of the soma for cells with long processes (dashed squares). Each NPC type is indicated for each example. Arrowheads point to processes. Horizontal dashed lines indicate the apical surface or the border between VZ and SVZ. **g-h)** Absolute frequencies and summary representation of the types of NPCs observed according to mitosis location, Pax6 and Tbr2 expression, and morphology. abp, apically and a basally directed processes; ap, apically directed process; bp, basally directed process; SVZ, subventricular zone; VZ, ventricular zone.

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The number of observations of each combination of a morphological type with Pax6 and Tbr2 expression, and their mitosis location for each species, was low in some cases (**Figure 11g**). A cause for this was the very infrequent colocalization of GFP and PH3 obtained. Another reason is the natural rare occurrence of some NPC types depending on the species. According to this, subapical GFP+PH3+ cells were very scarce in all species; in the chicken, we obtained very scarce basal GFP+PH3+ cells; in the mouse we were able to obtain a broader heterogeneity of Pax6 and Tbr2 expression in cells with a classical IP morphology, but very scarce observations of cells with a radial glia morphology; and in the ferret we also obtained a low number of observations for some bRGC morphotypes (monopolar-ap, bipolar-abp). This sample size prevents us from making statements at a population level, and we do not preclude the possibility of there being other combinations of morphologies and Pax6 Tbr2 expression for apical, subapical and basal NPCs. Notwithstanding, these data constitute proof of existence of a more than expected and previously described combinatorial complexity of morphologies and Pax6/Tbr2 expression for mitotic NPCs (**Figure 11h**). In particular, we show (1) that BPs with a non-radial glia morphology can express Pax6 but not Tbr2 during mitosis in chicken, mouse and ferret, which challenges the unequivocal correspondence between this molecular profile and a RGC morphology; and (2) species with a bigger cortical structure show an increase in the variety of NPC types by morphology and Pax6 / Tbr2 expression only at a basal location. The long-standing question of whether there is unequivocal correspondence between morphological and molecular identity appears. The data here presented challenges this view in the three species analysed, as cells of a given morphology show different combinatorial expression of Pax6 and Tbr2 TFs.

4.4. - NPC proliferative and differentiative potential

One of the most important attributes of a progenitor cell is its proliferative or differentiative state (**section 1.5.2: videomicroscopy characterization**). To capture this aspect, we performed *ex vivo* time-lapse microscopy of sparsely mGFP-labelled APs and their progeny in chicken (E6), mouse (E14) and ferret (E36), tracking the position and morphology of individual progenitor cells and the daughters they generated. We were able to film the main types of classical NPC types in the three species, distinguishing aRGCs, sAPs, bIPs and bRGCs (**Figure S2a**).

APs

Considering aRGCs, we distinguished three types of daughter cells according to their morphology (**Figure 12a-c**): (1) bipolar cells that remained anchored to the apical surface through an apical process and that also had a basal process. This basal process might be inherited from the mother cell and span the whole thickness of the developing cortex or might be a new process that was growing. We interpreted this type as an aRGC (**aRGC**). (2) Cells that detached from the apical surface, were migrating basally and exhibited a bipolar spindle-like morphology (**BP**). (3) Cells that detached from the apical surface and acquired a multipolar morphology (**MP**). In the three species, aRGCs showed a capacity not only to self-sustain their pool, but also to increase it through amplifying divisions (**Figure 12b**). Interestingly, the composition of daughter cells by morphotypes was rather similar in chick and ferret, with a higher generation of detached cells compared to mouse (**Figure 12b**).

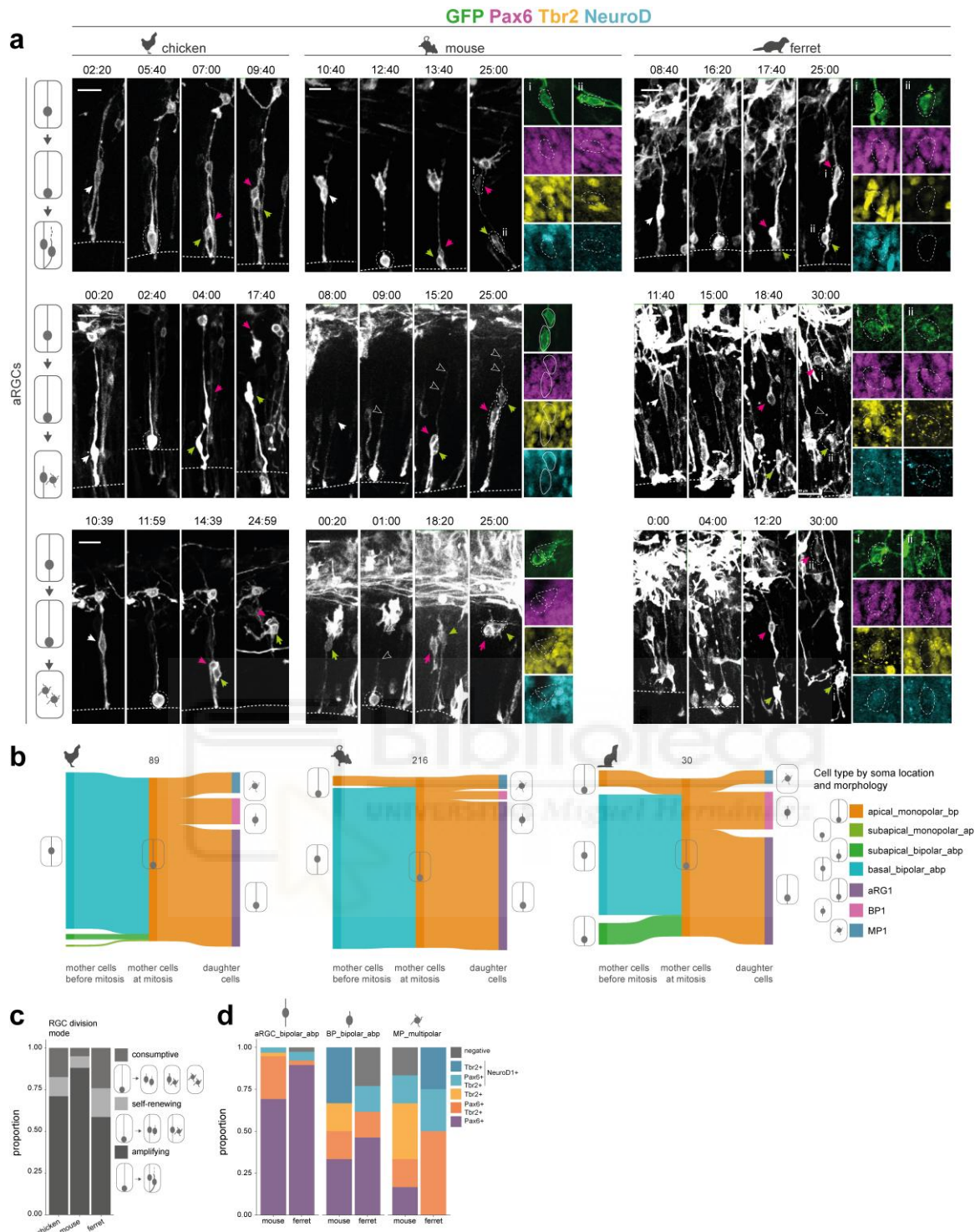


Figure 12. Apical progenitor divisions.

a) Time-lapse imaging frames of aRGs dividing, generating two aRGs daughters (top row), one aRG (middle row) and no aRG (bottom row), with high magnification of the daughter cells soma, immunolabelled for GFP, Pax6, Tbr2 and NeuroD1. Horizontal dashed lines indicate the ventricular surface. Beveling sectioning might cause the GFP signal of other z-planes to project below the dashed line. Each daughter cell is indicated by a differently coloured arrowhead and by dashed lines in the high magnifications. b) Morphological and location transitions of mother cells from the beginning of the film to the time of mitosis, and types of NPCs they give rise to according to their morphology. c) Quantification of aRG mitosis type, according to the morphology of both daughter cells. d) Quantification of daughter cell types, according to their morphology and immunolabelling. Sankey plots and c) barplot number of aRG clones: chicken,

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89, mouse, 216, ferret, 30. aRGC, apical radial glia cell; BP, bipolar; MP, multipolar; abp, apically and a basally directed processes; bp, basally directed process. Immunolabelling of chicken samples was unsuccessful.

Sankey plots representations consider the whole population behaviour, but do not allow to discriminate the type of cell division according to the couple of daughter cells generated: amplifying, self-renewing or consumptive divisions (**section 1.5.2: Proliferative capacity and cell division mode**). We therefore also assessed the mode of cell division of aRGCs, attending to the morphology of the two daughter cells (**Figure 12c**). In the three species, according to the behaviour plotted in figure 4B, the majority of aRGCs were undergoing amplificative divisions. The proportion of amplificative divisions was higher in mouse, as both chicken and ferret aRGCs generated more BP and MP daughter cells in self-renewing and consumptive divisions. However, as I next interpret, the fate of these cells in each species was likely different.

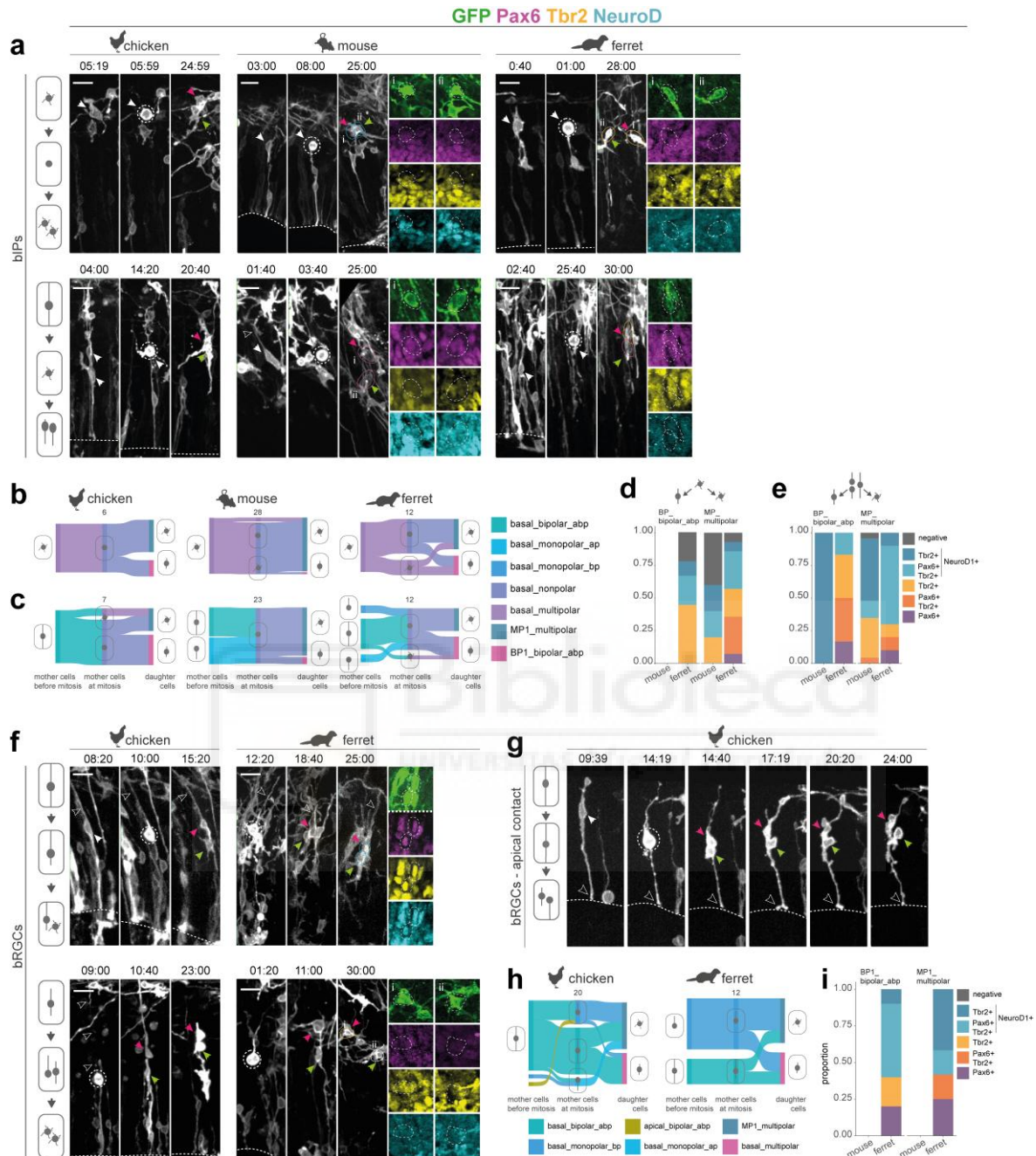
To further confirm the fate of daughter cells, we combined the morphology with immunolabelling for Pax6, Tbr2 and NeuroD1 (a newborn neuron marker) (although we were not able to carry out successful IHC in chicken samples) (**Figure 12d**). Attending to their morphology alone, aRGC daughters (with apical attachment and inherited or growing basal process) could be very likely considered to maintain a mitotic state. In the mouse case, about a quarter of them expressed Tbr2, whereas in the ferret, the vast majority expressed only Pax6. This indicated that, even if mouse aRGCs had undergone a higher fraction of self-renewing divisions (**Figure 12c**), they generated a more differentiated aRGC progeny than ferret aRGCs (**Figure 12d**). As for BP and MP daughter cells, these could be newly generated basal progenitors or newborn neurons in each species (MP cells not having acquired yet the bipolar elongated morphology typical of migrating neurons). In the chicken case, these detached cells were likely newborn neurons, according to the predominant direct neurogenesis observed in this species (Cárdenas et al., 2018). In the mouse, there was a mix of basal progenitors and neurons, as indicated immunolabelling (**Figure 12d**). In the ferret, there was also a mix, with a tendency towards a higher proportion of progenitors, as suggested by the more abundant presence of Pax6. Indeed, at this point along neurogenesis aRGCs in the ferret are generating the progenitors that will seed the oSVZ (Martínez-Martínez et al., 2016). Of note, the numbers of BP and MP daughters analysed with IHC were scarce, and therefore the distributions of marker combinatorial expression might largely vary when more data are added. Altogether, aRGC maintain an extended self-amplifying state in the three species, but with a likely higher commitment to direct neurogenesis in chicken and a higher generation of basal progenitors in the ferret.

We were not able to find rounded nonpolar cells bearing no processes dividing at the apical surface (i.e., aIP), as we had found in the fixed tissue (**Figure 11c**). This might be either due to sample size or to the fact that such a type of cell does not appear in *ex vivo* cortical slices.

BPs

Considering bIPs, we distinguished the cases where these were born from multipolar cells (**Figure 13a, top row, b**), and those born from RGCs (**Figure 13a, bottom row, c**). With caution towards the limited sample size, chicken and ferret showed similarity in the morphotype composition of the daughters generated, (**Figure 13b, c**). In both species, bIPs divided into multipolar and bipolar cells. Differently, in the mouse bIPs almost only generated multipolar cells. Regarding the fate of daughter cells, again we observed a more

sustained expression of Pax6 in ferret compared to mouse, either alone or in coexpression with Tbr2 and NeuroD1, indicating their IPC and neuron identity, respectively (**Figure 13d, e**).



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record and immunolabel a single mouse bRGC). Horizontal dashed lines indicate the ventricular surface. Beveling sectioning might cause the GFP signal of other z-planes to project below the dashed line. Sankey plots number of clones: b, chicken, 6, mouse, 28, ferret, 12; c, chicken, 7, mouse, 23, ferret, 12; h, chicken, 20, ferret, 12. Immunolabelling of chicken samples was unsuccessful.

In the case of bRGCs, we had a single observation of this kind of progenitor in the mouse (**Figure S2a**). Once more, the progeny of this NPC type had a similar morphotype distribution in chick and ferret, with the generation of multipolar and bipolar cells (**Figure 13f, h**). Interestingly, we observed that a fraction of chicken cells with radial glia morphology dividing at a basal location kept the apical contact (**Figure 13g, Figure S2b**), as previously reported (Nomura, Ohtaka-Maruyama, et al., 2016), and differently to mammals, where bRGCs have been described to lose contact with the apical surface (Florio & Huttner, 2014). The cell fate of the ferret MP daughter cells seemed to be associated to a more differentiated state than BP daughter cells (**Figure 13i**).

Finally, we also observed subapical divisions in the three species but, due to their much lower numbers, an equivalent cell fate analysis was not performed (**Figure S2a**).

4.5. - Cell division transition map of NPCs at mid neurogenesis

As we were very precise with the definition of NPC types precisely at the moment of mitosis, we next wanted to generate a map of the morphological and location transitions that all NPCs can go through until mitosis. A Sankey plot visualization of NPCs classified by location and morphology before mitosis, at the time of mitosis and of the daughter cells classified by morphology revealed a restricted set of transitions for basal progenitors and a diverse but biased variety of transitions for RGCs, with particularities in each species (**Figure 14a**).

Basal multipolar cells showed a common morphological transition to M-phase, either retracting the short processes or keeping them, and stayed at a basal location in the three species. Of notice, in the ferret we recorded one of such cells having grown a basal process by the time of mitosis (**Figure 14a**).

Differently, NPCs that at the beginning of the time lapse presented their soma in the VZ and extended long apical and basal processes (RGC-apicobasal-P) showed different sets of topological and morphological identities at mitosis in the chicken, mouse and ferret cortices. In the three species, most of such cells underwent INM to divide at the apical surface as aRGCs. Very few of them divided subapically and a large fraction of them, greatest in ferret, fed different types of progenitors undergoing mitosis with the soma at a basal location. Here, we observed two main transitions. (1) In the first one, RGC-apicobasal-P detached from the apical surface, migrated to a basal location and fully retracted the apical and basal processes into a nonpolar morphology at mitosis. This mode was observed in the three species. In other words, in the three species we observed generation of bIPs from RGCs. (2) (**Figure 14a, b**). In the second one, RGC-apicobasal-P maintained the apical and basal process and underwent mitosis at a basal location. This mode was observed in the ferret and the chicken, but not in the mouse, in agreement with the notion of bRGCs being very scarce in the latter species (X. Wang et al., 2011) (**Figure 14a, b**). Altogether, RGCs-apicobasal-P in the chicken and mouse kept the contact with the apical surface in a similar proportion, whereas in the ferret, a much larger fraction of them detached and migrated to a basal location to undergo mitosis, according to the seeding of the oSVZ NPCs at this developmental stage (**Figure 14b**)(Martínez-Martínez et al., 2016).

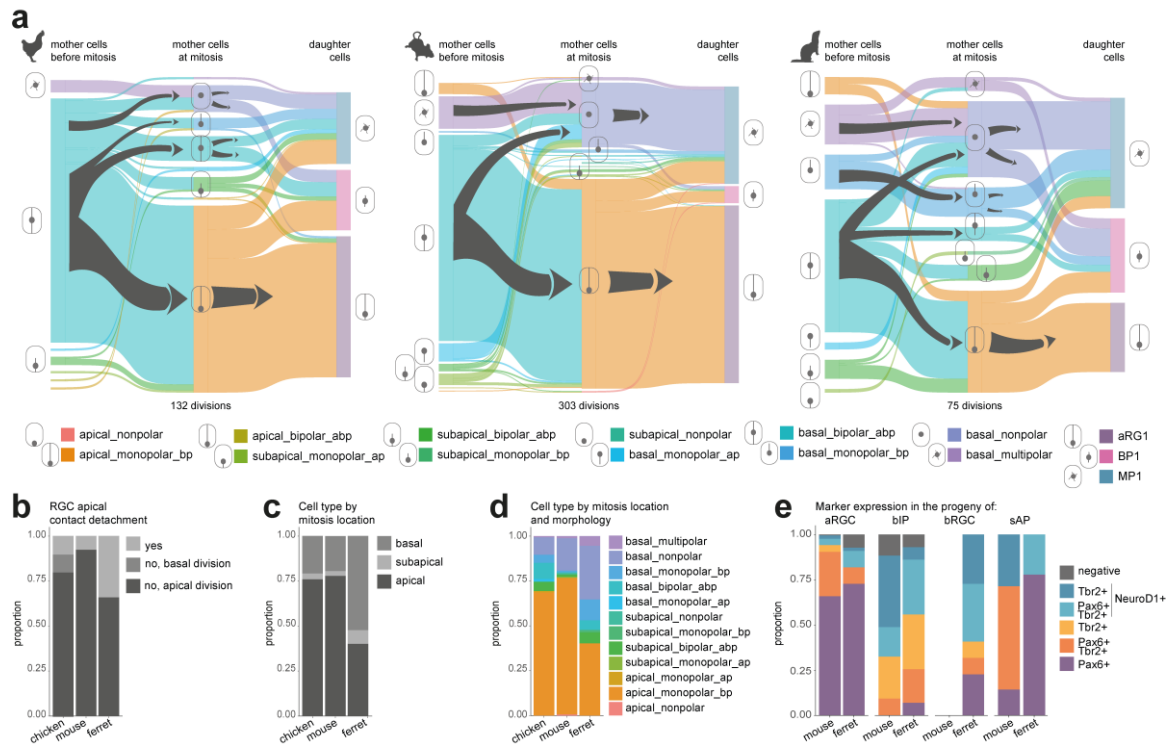


Figure 14. Morphological transition map of NPCs during division.

a) Summary of all morphological and location transitions of NPCs, from the beginning of the film to the time of mitosis, and types of NPCs they give rise to according to their morphology. Main transitions are highlighted in the bottom row. b-d) Quantifications of aRGCs that detach from the apical surface by the time of mitosis (b), of NPC mitosis location (c) and of NPC location and morphology at the time of mitosis (d). e) Quantification of marker expression in the progeny of all aRGCs, bIPs, bRGCs and sAPs in mouse and ferret. Total number of clones (in a, c and d): chicken, 132, mouse, 303, ferret, 75. Number of daughter cells in q: mouse: aRGC, 208; bIP, 45; sAP, 7; ferret: aRGC, 55; bIP, 47; bRGC, 22; sAP, 9. Mouse bRGC are not shown because only 2 could be included in the IHC analysis.

According to the criterium of defining NPCs by their mitosis location, and considering their changes in morphology and location during the cell cycle, keeping track of the morphology and location of NPCs has allowed us to determine the possible errors in NPC definition when observed out of mitosis:

- Basal multipolar cells can be assumed to be bIPs (as long as they are stained for a marker of proliferation to preclude the possibility of it being a postmitotic newborn neuron).
- Complementarily, basal nonpolar or multipolar cells at the time of mitosis derive from a variety of morphotypes.
- RGCs extending long apical and basal processes, however, cannot be assumed to be aRGCs. Although the majority of them will divide apically, an important fraction of them turn into different types of BPs (especially in ferret), and a minor fraction of them divide subapically.
- In ferret, bRGC-basal-P will most likely keep such morphology at mitosis.

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Importantly for the coherence of our multifactorial NPC characterization, we obtained a distribution of NPC types by location of mitosis (**Figure 14c**) very similar to the one obtained in the fixed tissue analysis, especially in the mouse dataset (the most numerous) (**Figure 10g**). Additionally, this dataset constituted another source of morphological characterization of NPC types at mitosis, which is relevant given the very few GFP+PH3+ cases of BPs obtained *in vivo*. In particular, here we provide further evidence of basal radial glia-like cells and multipolar bIPs in chicken with proliferative behaviour, of the virtual absence of the former at this stage in mouse and describe an abundant population of BPs in ferret at an earlier stage than previously studied (**Figure 14d**) (Gertz et al., 2014; Martínez-Martínez et al., 2016). These results show, at the morphological level, a parallelism between the three main types of NPCs between the chicken and the ferret, whereas in the mouse there seems to have been a restriction of the morphological diversity of NPC types.

When considering the marker distribution of each classical NPC type generated, overall, the progeny of each type presented a less differentiated composition in ferret compared to mouse (**Figure 14e**). aRGCs in the three species showed extensive self-renewing and proliferative divisions, with a lower proportion of Tbr2 expressing cells in ferret compared to mouse. bIPs generated in both mouse and ferret a more committed population compared to that of aRGCs, based on the expression of Tbr2 and NeuroD1. But specifically in ferret, the bIPs progeny maintained a more extensive expression of Pax6, either alone or in combination with Tbr2, reflecting the higher proliferative capacity of BPs in this species. This fraction was still higher in ferret bRGC daughters, according to the greater proliferative character of bRGCs vs bIPs. Finally, the few observations we reported of subapical divisions followed by immunolabelling (mouse, 4, ferret, 6) also provided a more proliferative character for their progeny in the ferret compared to mouse.

4.6. - scRNAseq datasets of developing dorsal cortices of snake, chicken, mouse and ferret

To investigate the genetic activity behind the properties of the main histological NPC types identified in our 4 model species, we generated single cell transcriptomic libraries of the developing parietal cortices and analysed it using mainly Seurat-5 and monocle3. For the ferret, we used a previously generated data (Del-Valle-Anton et al., 2024). This dataset was composed by cells derived from the VZ and OSVZ microdissected from the splenial gyrus and lateral sulcus of E34 (only VZ) and P0 ferrets. For our present analysis, only the E34 samples were used, from both the gyrus (E34VZSG1-3) and sulcus (E34VZLS1-3). For each species (snake, chicken and mouse), two replicates composed of a pool of several embryos (2 for the snake, 3 for the others) were made. Datasets of each species were filtered through stringent quality control parameters (**Figure S3**) to proceed only with high quality cells. Briefly, we discarded cells with a low number of features detected, high percentage of mitochondrial RNA molecules, cells with a high fraction of nuclear mRNAs, predicted using DropletQC (Muskovic & Powell, 2021) and doublets, as predicted by DoubletFinder (McGinnis et al., 2019).

After constructing the Seurat objects, the dataset of each species was processed individually (**Figures S4-6**). Briefly, for each species, datasets from each replicate were merged into a single Seurat object (**Figure S4a, b**). Cluster compositional analysis showed overall homogeneous composition of clusters per sample origin in the snake, chicken and mouse, but not for ferret. In this latter species, clustering was affected by the 10x version used to generate the libraries, as cells from samples processed with v2 (E34VZLS2,

E34VZSG3) and processed with v3 (E34VZLS1, E34VZLS3, E34VZSG1, E34VZSG2) clustered together, respectively. Cells were scored for cell cycle and their gene expression normalized and scaled (SCT transformation) (Hafemeister & Satija, 2019). After assessment of the effect of applying cell cycle regression on cell clustering, we chose to proceed with partial cell cycle regression, making the distinction between cells in G1 and cells in G2/M/S. Despite regression, progenitors still separated in the reductional dimension between G2M and S phases, especially in the chicken and ferret datasets (**Figure S4c**). After cell cycle regression, only in the case of the ferret were samples integrated, due to the separation of cells coming from different samples in a number of clusters (**Figure S4b**). As the clusters of the other species presented overall homogeneous composition by replicate, we decided not to batch-correct them, in order to preserve the biological information.

For further processing, cells not belonging to the excitatory lineage were filtered out (mainly inhibitory neurons and very few endothelial cells, microglia and erythrocytes) (**Figure S5**). These populations were identified by exploration of expression of a set of canonical markers of the excitatory and inhibitory lineages (**Figure S5b**), and by exploration of the top 20 genetic differentially expressed genes of each cluster (**Figure S5c**). Ventral progenitors and inhibitory neurons were detected by expression of genes such as *DLX1*, *DLX2*, *DLX5*, *DLX6*, *GAD1*, *GAD2*, *NXPH1*, *LHX6*, etc. Haemoglobin genes (*HBB-BH1*, *HBBA*, *HBAD*, *HBA1*, etc.) were the main identifiers of erythrocytes in the chicken and ferret datasets. *FOXC1* and *FOXC2*, expressed in neural crest mesenchymal cells, together with collagen related genes (*COL1A1*, *COL6A3*, *COL3A1*), identified mesenchymal/endothelial cells in the snake. Endothelial/pericyte populations (*ACTA2*, *APOA1*, *VTN*, *RGS5*, *PECAM1*, *CDH5*, etc.) and microglia (*C1QB*, *C1QC*, *CX3CR*, *TREM2*, etc.) were identified also in the ferret. Finally, in the mouse, a population identified as epithelial cells from the preoptic area or choroid plexus was also removed (*Lhx1*, ciliogenesis genes, *Mcidas*, *Gmnc*, *Ccno*, *Trp73*, desmosomal component genes, *Dsp*, *Perp*).

After removal of these clusters, we set cluster resolution of the excitatory lineage datasets as a compromise between cluster stability and over clustering (resolution = 1.3) (**Figure S6a**). At this point the single cell objects were ready for annotation (**Figure S6b, c**). This preprocessing resulted into: snake dataset, 10946 cells and 16851 features, chicken dataset, 15971 cells and 19302 features, mouse dataset, 8374 cells and 24146 features, ferret dataset, 35873 cells and 16631 features datasets.

Annotation of the individual species datasets was performed combining the expression of canonical markers, obtained from previous studies in mouse, human and ferret (Del-Valle-Anton et al., 2024a; Di Bella et al., 2021b; Li et al., 2020b; Loo et al., 2019). In order to assess main types of cells of the excitatory lineage, the expression of sets of markers of mitotic cells, RGCs, IPCs, bRGCs and immature neurons was jointly analysed in modules of expression, which allowed to broadly identify main cell types (**Figure 15**). Modules of expression of broad excitatory neuronal categories, as defined in mammals, were also assessed (**Figure S7**). To further dissect the annotation of the distinct clusters within these broad categories, the individual expression of canonical markers was inspected (**Figure 16**). This annotation was enriched by the examination of the top 20 (by log fold change) differentially expressed genes in each cluster (**Figures S8-11**). We distinguished between RGCs and IPCs at different phases of the cell cycle, RGCs and IPCs primed for neurogenic divisions, newborn neurons still expressing IPC markers, immature neurons and differentiated neurons.

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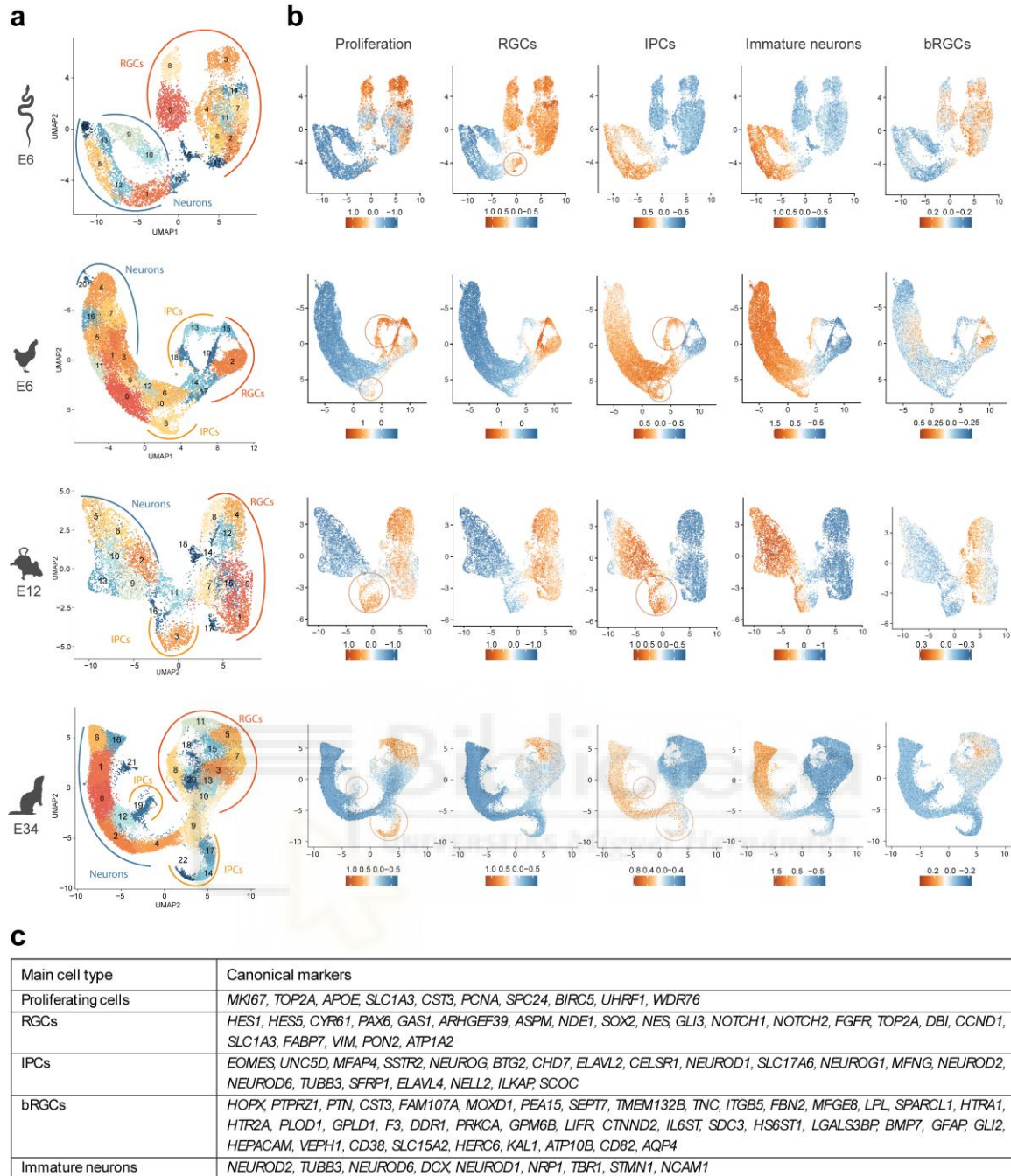


Figure 15. Modules of expression of main cell types of cortical development.

a) UMAPs and clustering of scRNAseq data of the excitatory neuron lineage cells of the developing parietal cortex of snake (*Boaedon fuliginosus*) (E6), chicken, (*Gallus gallus*) (E6), mouse (*Mus musculus*) (E12) and ferret (*Mustela putorius*) (E34). Overall clusters of RGCs, IPCs and immature neurons are indicated. **b)** UMAPs showing gene set module scores of proliferating cells, RGCs, IPCs and immature neurons in each of the four model species. In the snake, cells that cluster together with immature neurons but score high for RGC are indicated. For chicken, mouse and ferret, IPCs are indicated, as shown by the overlap of both high IPC and proliferation scores. **c)** Markers included in the modules for the different overall cell types. IPC, intermediate progenitor cell; RGC, radial glia cell.

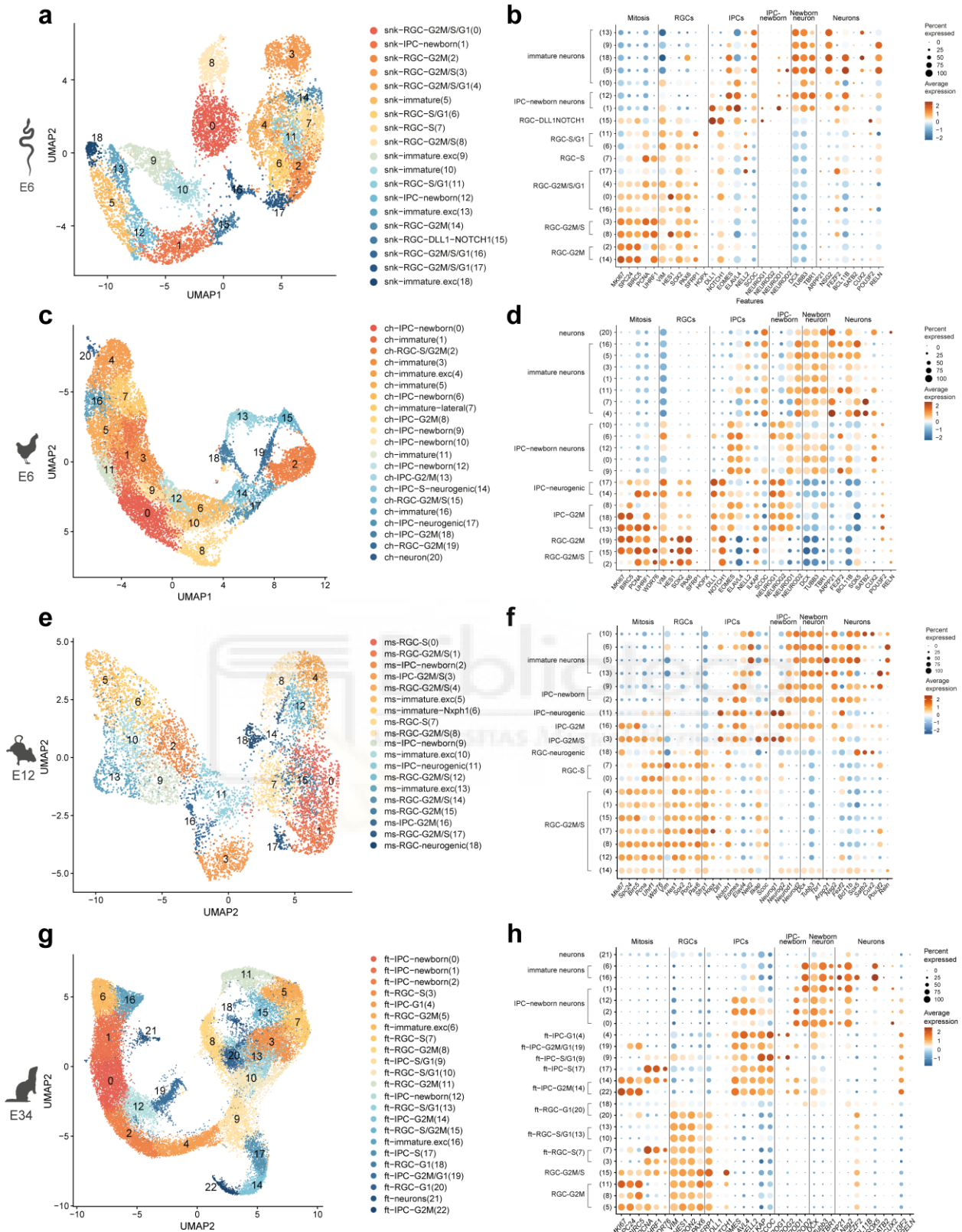


Figure 16. Annotation of scRNAseq datasets.

a-d) Annotated UMAPs and clustering at selected resolution of scRNAseq datasets from cells in the excitatory lineage. **e-h)** Dot-plots of some of the canonical markers across clusters used for their annotation.

4.7. - Single cell transcriptomics reveals a different pattern of RGC and IPC expression modules in relation to their proliferative state in the snake

As a first approximation into species-specific genetic signatures, we compared the distribution of the scores of the modules of expression for mitotic cells, RGCs, IPCs and immature neurons between species (**Figure 15b**). This simple approach already provided substantial insight into the different meanings that the expression of mammalian-defined sets of markers can have in non-mammalian species. In this way, in the case of the snake, a widespread fraction of cells scoring high for RGC identity presented extensive mismatch with the cells scoring for a proliferative state (clusters 6, 15, 16, 19). However, differently to what might be expected, these “non proliferating RGCs” did not score high for the immature neuron module either (**Figure 15b**) but were showing expression of mammalian deep layer neuron markers (**Figure S7A**). Interestingly, the RGC-scoring cells that did not score high in the proliferative module were the ones that scored higher, albeit weakly, for the bRGC module (**Figure 15a**). This implies that, in our reptile model, genes that characterize the definition of a neurogenic program, and that in mammalian species are expressed by premitotic NPCs (Koo et al., 2023; Li et al., 2020), are expressed only in neurons. Finally, the high IPC scoring cells presented virtually a complete overlap with the immature neuron score and no overlap with the proliferation one, showing the absence of IPCs in the snake. In the chicken case, our dataset showed an overlap of proliferation scoring cells with both RGC scoring and IPC scoring cells, further confirming the existence of BPs in this species (**Figure 15b**). Of notice, there was a second smaller population of IPCs and besides, as in the case of the snake, there was a population of RGCs that had a low proliferation score and the highest, albeit weak, bRGC score (**Figure 15b**). In the mammalian models, differently to the sauropsids species, cells that scored higher for the bRGC score colocalized with both the RGC and the proliferation scores (**Figure 15b**). Besides, in the ferret, two populations of IPCs appeared, as in the chicken (**Figure 15b**).

In particular and conspicuously, one of the snake “non proliferating RGC” clusters (cluster 15), clustered together with the immature neurons (**Figure 15b**). Among the highest expressed markers of this cluster there were several genes involved in the Notch pathway (*DLL1*, *DLL3*, *JAG2*, *ID1*, *NEURL1B*), together with the proneural gene *NEUROG1* and cell cycle arrest gene *GADD45G*. Some of these genes were also highly expressed in the adjacent newborn neuron clusters (clusters 1 and 8) (**Figure 17a, b**). Such a combination of markers was not found in any of the other three species, especially considering the snake-specific high expression of *NEURL1B* (**Figure 17a, b**).

In this manner, in the chicken, the IPC-neurogenic clusters 14 and 17, adjacent to the newborn neurons, also showed a strong expression of *DLL1* and *NOTCH1*, but, unlike snake cluster 15, despite the expression of the cell cycle arrest marker *GADD45G* and proneuronal gene *NEUROG1*, still maintained a relatively high proliferation score, while showing no *NEURL1B* expression (**Figure 17a**). A similar expression pattern to the chicken’s appeared in IPC clusters 3,11 and 16 of mouse, the other model with moderate levels of basal proliferation (**Figure 17a**).

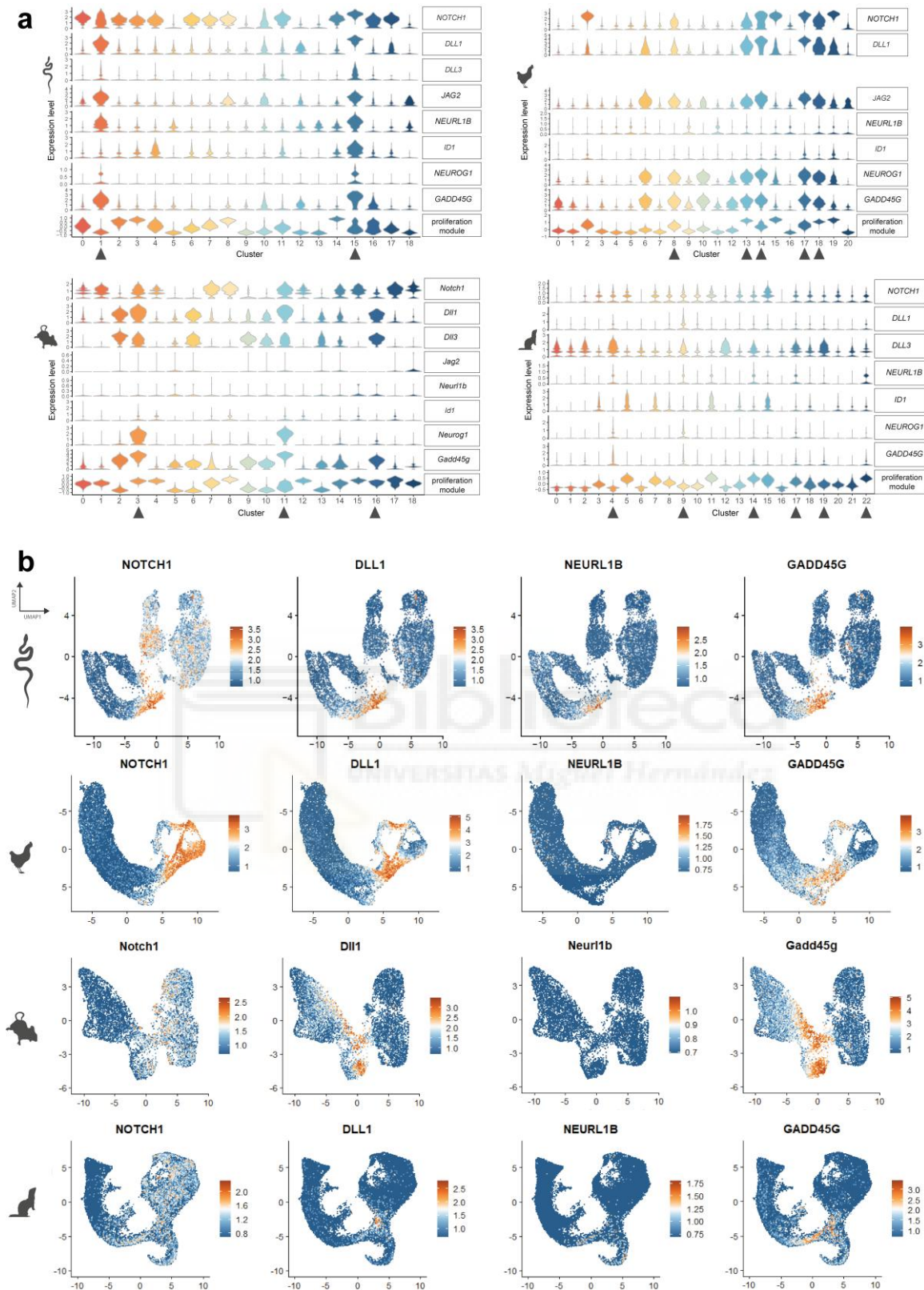


Figure 17. Expression of Delta-Notch related genes in IPCs.

a) Violin plots of selected top (by average log fold difference) differentially expressed markers of the cluster 15 of the snake dataset in the four species. Arrows indicate IPC-named clusters in each species. **b)** UMAPs showing the expression of selected snake cluster 15 markers in the snake, chicken, mouse and ferret scRNAseq datasets.

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In the ferret, another species-specific combination was found for the expression of these genes, as *DLL3* was much more extensively expressed than *DLL1* compared to the mouse and chicken cases, and *GADD45G*, *NEUROG1* and *NEUROG2* were kept overall at low levels. When the expression was higher, there was also higher, albeit moderate, expression of *NEURL1B* (**Figure 17a**). This ferret specificity might be due to the heterochrony in cortical development in relation to mice and chicken.

Since *NEURL1B* has been described in mammals to interact with and ubiquitinate Notch ligands Delta-like1, Delta-like4 and Jagged1 (Koutelou et al., 2008, 2008; Rullinkov et al., 2009; Song et al., 2006) these data suggest that a strong inactivation of Notch signalling mediated by degradation of notch receptors might be required for neuronal differentiation in the snake. In the other species, the absence of this process would allow the maintenance of the proliferative capacity in the secondary NPC population of BPs.

4.8. - Different dynamics across amniotes in the transition between a proliferative to a proneural state shown by pseudotime analysis

A pseudotime analysis offered a complementary view on the species-specific location of expression modules in the differentiation trajectory (**Figure 18a**). As such, indeed in the snake, when the proliferation score fell in the differentiation trajectory, the RGC score was maintained still high, and it was not until later in the pseudotime scale that both the IPC and the newborn neuron score took higher values. Cells that expressed *EOMES* (*TBR2*) appeared, for a short pseudotime period, only after proliferation had stopped and *Pax6* expression was turned off (**Figure 18b-d**).

Very differently, in the other three species, as the proliferation score diminished, both the IPC and the newborn neuron scores started rising (**Figure 18a**). In addition, in all chicken, mouse and ferret, a secondary group of proliferating cells expressing *EOMES* appeared (and in the ferret, still a third one) at the mid pseudotime region (**Figure 18d**). These cells would correspond to IPCs and coincided in the pseudotime scale with non-proliferative *EOMES* expressing cells, i.e., newborn neurons. In all cases, these proliferative *EOMES* expressing cells presented moderate expression of the RGC set of markers (**Figure 18a-c**). Therefore, a genetic network associated to *EOMES* that supports proliferation does not exist in the reptilian model, but is common to mammals and birds.

When we checked the expression of the notch signaling related genes highly expressed in snake cluster 15 along pseudotime, *NEURL1B* expression was activated in the snake together when the one of *DLL1* as *NOTCH1* expression fell (**Figure 18e**). In the chicken and the mouse, *NEURL1B* expression was virtually absent. In the ferret, *NEURL1B* peaked together with *DLL1*, but it was expressed at much lower levels relative to *Pax6*, whose expression was sustained, differently to the snake case. In addition, the expression of *NOTCH1* with respect to *EOMES* and *NEUROD1* had a different dynamic in chicken and mouse to the one observed in ferret. In this way, in both mouse and chicken, *NOTCH1* expression fell as the one of *EOMES* and *NEUROD1* jointly rise. However, in the ferret, *NOTCH1* expression was maintained as *EOMES* expression increased, and the activation of *NEUROD1* expression was delayed to the one of *EOMES*.

Altogether, in the reptilian model, RGCs showed a limited proliferative expression profile, circumscribed to some RGC clusters; the possible suppression of Notch signaling might be related to the absence of BPs, and there was a decalage between the arrest of proliferation and the expression of proneural genes. In species with moderate levels of basal proliferation,

NEURL1B was not expressed and IPC-related and proneural genes started being expressed before the decay in proliferation score. This dynamic may allow the maintenance for a limited time of some Notch signaling despite the arrest of its transcription, in the absence of degradation of its receptors. Finally, in the gyrencephalic ferret model, the expression of the proneural set was slightly delayed to the IPC one; despite a moderate activation of *NEURL1B*, *NOTCH1* expression was sustained longer in the pseudotime scale; and two populations of *EOMES* expressing proliferating cells appeared.

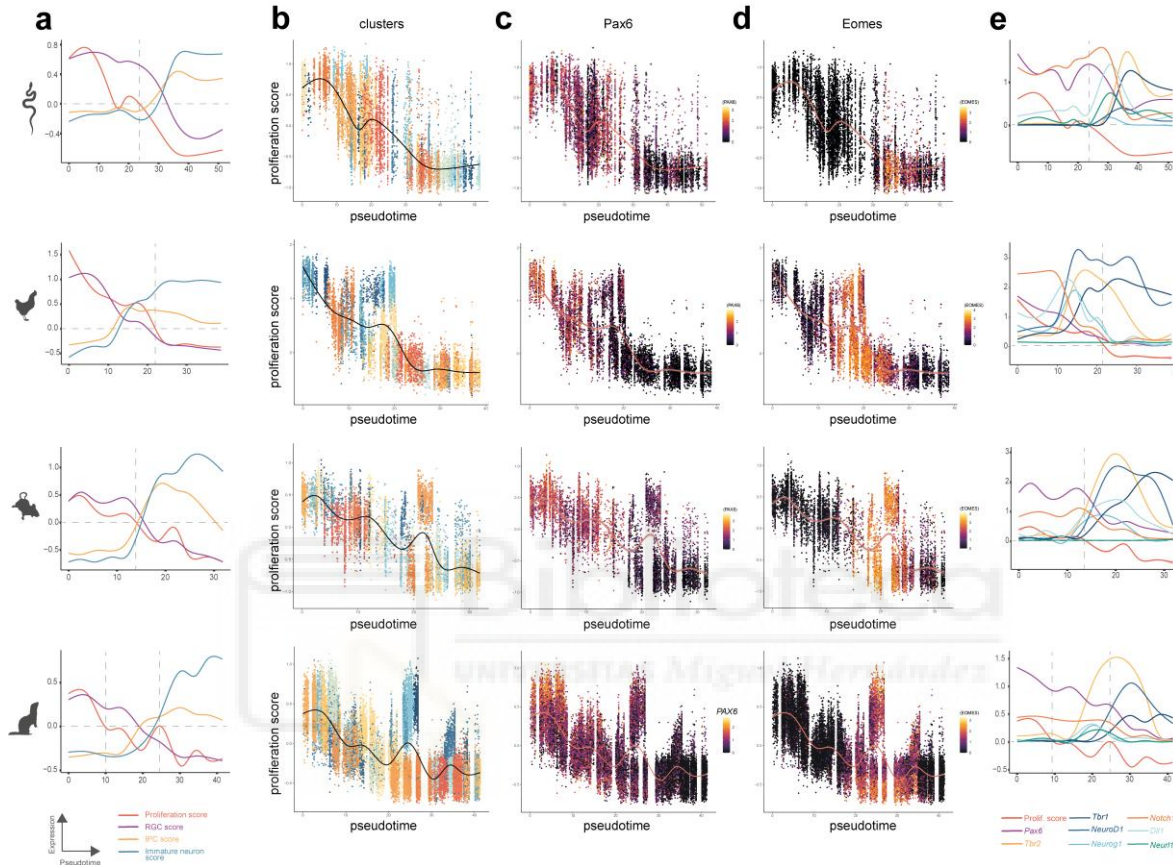


Figure 18. Pseudotemporal analysis of gene sets module scores and selected marker genes.

a) Pseudotemporal mean expression patterns of gene set module scores of proliferating cells, RGCs, IPCs and immature neurons in each of the four model species. **b)** Scatter plots of proliferation scores coloured by cluster along pseudotime in each species. The mean proliferating score is highlighted. **c-d)** Scatter plot of proliferation scores coloured by normalized *Pax6* (**c**) and *Tbr2* (**d**) expression along pseudotime in each species. The mean proliferating score is highlighted. **e)** Pseudotemporal expression patterns of the proliferation genes set module score, selected marker genes of RGCs, IPCs and newborn neurons, and selected Notch signalling related genes in each species. IPC, intermediate progenitor cell; RGC, radial glia cell.

4.9. - Integrative analysis of RGCs

To have an approximation of the genetic activity determining the different properties of NPCs in our 4 amniote species, their datasets were integrated using as anchors their one-to-one orthologs (**Figure 19a-c**). Seurat v5 offers several methods for integration of samples. Datasets were integrated with two of them: canonical correlation analysis (CCA), which forces a closer alignment of datasets and may incur in overcorrection (**Figure 19d-g**), and reciprocal principal component analysis (RPCA), which is more conservative but may not capture subtle biological variation (**Figure 19h-k**). Accordingly, each method produced a different integration. CCA resulted in tight merging of the different species datasets, both for

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progenitors and neurons, and presence of cells of the four species in every cluster (**Figure 19d-f**). RPCA integration resulted in overall separation of cells by species, especially for neurons (**Figure 19h-k**). IPC populations comprised clusters with a higher or almost complete proportion of chicken cells (clusters 16, 19), clusters composed by mostly mammalian cells (cluster 9) and clusters composed by the four species (cluster 8). RGC populations separated into three main groups: snake specific (2, 14, 13), ferret specific (cluster 15), and mixed populations (0, 1, 7, 20, 21). For further analysis, we proceeded with the RPCA integrated dataset.

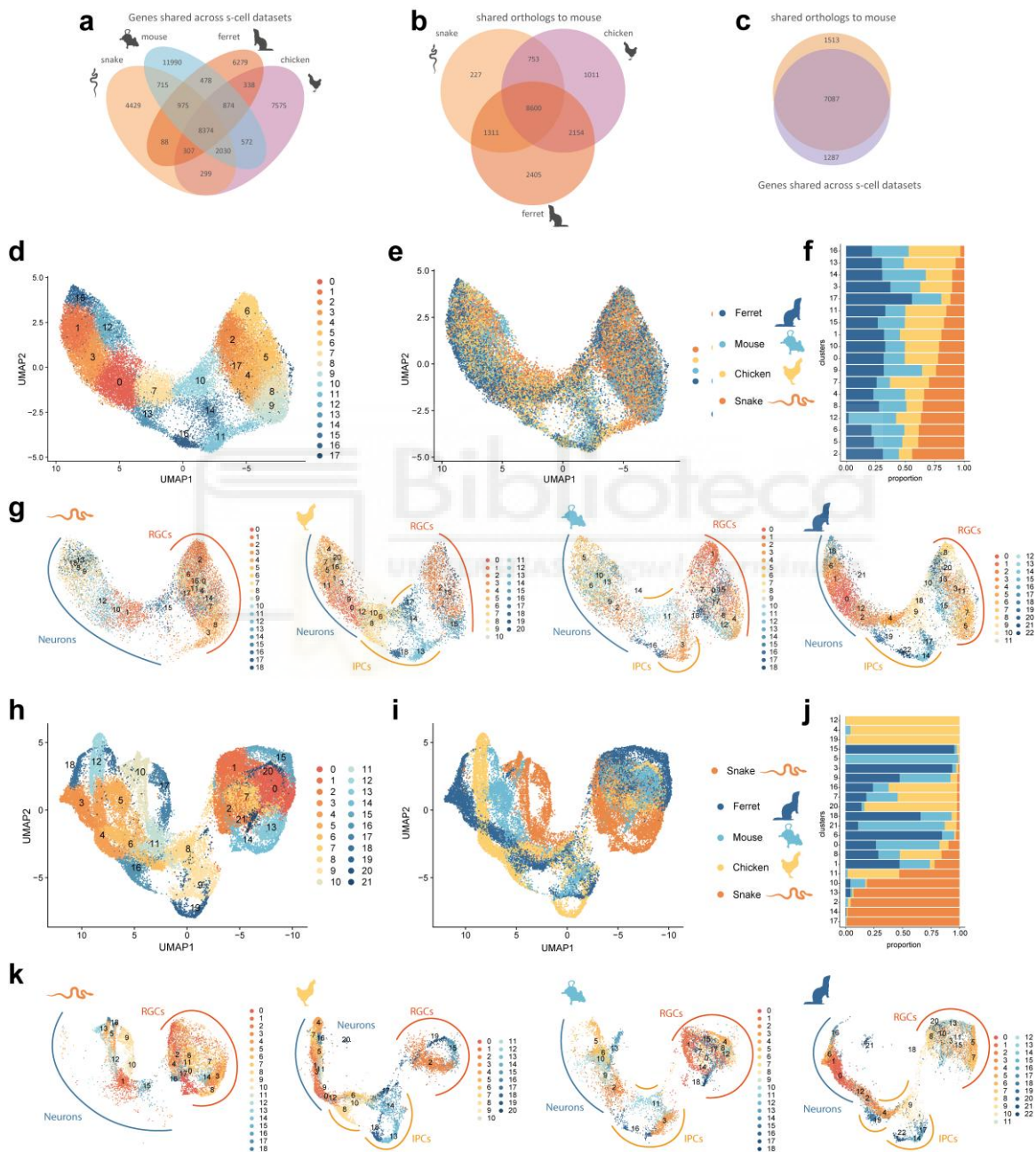


Figure 19. Integration methods.

a-c) Venn diagram of genes present in the four species scRNAseq datasets (**a**), of the orthologs to mouse of the snake, chicken and ferret (**b**), and of the overlap between the common orthologs and the common genes of the scRNAseq datasets of the four species (**c**). **d-g**) CCA integration of the four species. UMAPs coloured by integrated clustering (**e**) and species origin (**e**). Species composition of each cluster (**f**). Original clusters of each species datasets are shown projected on the integrated

UMAP (g). h-k) RPCA integration of the four species. UMAPs coloured by integrated clustering (h) and species origin (i). Species composition of each cluster (j). Original clusters of each species datasets are shown projected on the integrated UMAP (k). IPC, intermediate progenitor cell; RGC, radial glia cell.

To analyse the genetic differences in the RGCs, these were subset from the RPCA-integrated object and reclustered using only the ortholog genes (**Figure 20a, b**). Cluster compositional analysis showed several clusters made up by a large majority of cells coming from a single species, and a greater separation of the snake cells from the other species' (**Figure 20b, c**). Attending to this composition, clusters 3 and 8 are ferret specific; clusters 2 and 11, mouse-specific, clusters 4 and 13, chicken-specific; and clusters 5, 7, 9, 10, 12, 14, 15, snake-specific. Finally, clusters 0, 1 and 6 show a mixed composition by species, with a higher contribution of mammalian cells. A differential expression analysis by species resulted in a corresponding distribution of their expression across clusters according to their different species-composition, either using the full set of genes in the dataset (**Figure 20d**) or limiting the analysis to the ortholog genes (**Figure S12a**).

Some of these DEGs were interesting explanatory candidates of the different NPC properties across amniotes and appeared also in a DEA of the single cell aggregated pseudobulk data (**Figure 20e & Figure S12b-g**). For instance, *ROBO1* and *ROBO2* were more highly expressed in the chicken and snake (and *ROBO3* in the snake), than in the mouse and ferret, in agreement with the participation of Robo signalling in the limitation of indirect neurogenesis (Cárdenas et al., 2018). *IGFBP2* (Insulin Like Growth Factor Binding Protein 2), was, on the opposite, more highly expressed in the mammals. This gene encodes a protein that binds insulin-like growth factors I and II (IGF-I, IGF-II) and is related to neural stem cell maintenance and proliferation (Shen et al., 2019). Other two genes related to cortical development that were strongly expressed in mammals were *HES1*, in both mouse and ferret, and *FOS*, only in ferret. Finally, *IRF8* appeared strongly expressed exclusively in the ferret. This is a transcription factor that binds upstream to the regulatory region of type I interferon (IFN-I). The role of this molecule in NPCs during cortical development has not been extensively studied.

To capture possible cellular mechanisms in which the differentially expressed genes of each species could be involved, a GO term enrichment analysis was performed (**Figure 20f-i**). Overall, the snake stood out among the four species, as many of the top scoring GO terms were related to immature neurons, such as axon extension (**Figure 20f**), whereas in the other species, top GO terms were related to mitochondrial activity, cell cycle regulation, protein turnover, and DNA and RNA metabolism (**Figure 20g-i**). Altogether, this analysis confirmed the specificities of RGCs in the reptile model and offered a window into the exploration of conspicuous genetic candidates that could be behind the higher proliferative potential of mammalian RGCs.

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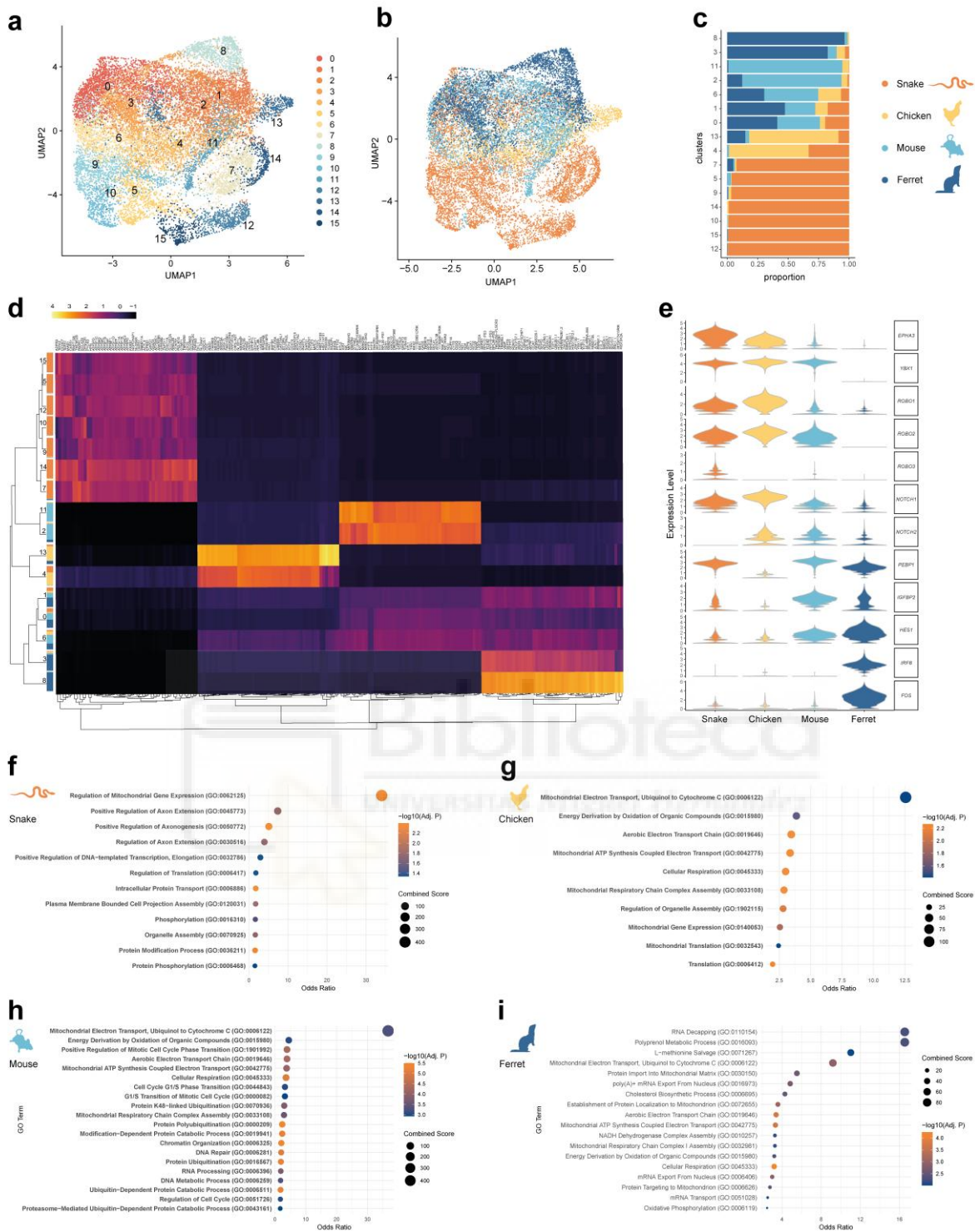


Figure 20. RGC cross-species integration.

a-b) UMAP of the subset RGCs from the RPCA-integrated scRNAseq datasets of snake, chicken, mouse and ferret, coloured by clusters (**a**) and species (**b**). **c)** Proportion of each species' cells in the integrated RGC clusters. **d)** Heatmap of average normalized expression of top 50 differentially expressed genes by species across clusters, ordered according to cluster dendrogram. Colour bars on the left indicate cluster composition by proportion of each species' cells. **e)** Violin plots of selected differentially expressed genes in RGCs between species. **f-i)** Functional enrichment analysis on DEGs, showing most significant enriched GO terms. Ontology: Biological process 2025, Enrichr database.

4.10. - Integrative analysis of IPCs

At the level of IPCs, an analogous analysis to the one just exposed for RGCs was carried out. IPC clusters were subset from the RPCA-integrated object and reclustered using the ortholog genes (**Figure 21a, b**). Again, the composition analysis by species of the clusters showed species-specific and mixed clusters (**Figure 21b, c**): cluster 4 was ferret specific, cluster 3 was mouse specific, and clusters 0, 5, 11, 13 and 14 were chicken specific. The other clusters had a mixed composition, with overall minor contribution of chicken cells, greater contribution of ferret cells in clusters 7, 9 and 1, and of mouse cells in cluster 6. Among IPC cells, a few snake cells were drawn together with them and clustered with mouse cells in cluster 10.

A DEA by species was carried out, with an according distribution of the expression of the DEGs across clusters depending on the species composition (**Figure 21d**). DEA using ortholog genes resulted in a very similar pattern, but with broader expression of the markers in the clusters not dominated by the putative species (**Figure S13a**). Some of the top differentially expressed genes, shared with the pseudobulk analysis of the aggregated data (**Figure S13b-g**) could explain the specificities of IPCs in each species, and between classes (mammals vs sauropsids) (**Figure 21e**). Interestingly, the scarce snake cells retrieved together with the other species' IPCs presented a specific and strong expression of the above-mentioned *NEURL1B*. Another top-expressed snake-specific gene, *FRMPD4*, is a positive regulator of dendritic spine formation (H. W. Lee et al., 2008), and a gene that could be involved in early neuronal specification in the snake is *GLI2* (J.-L. Chen et al., 2019). Among the genes expressed in chicken and snake higher than in the mammals, *ROBO1* is one of them, with abundant expression in both species and highest in the chicken. Of notice, both chicken and mouse shared expression of proneural genes, which was minimal in the ferret dataset, accordingly with the more neurogenic state of IPCs in the former species. *IGFBP2* appeared in the IPC dataset more highly expressed in the mammal species, as it had occurred with RGCs. Some of the genes detected as ferret-specific in the RGC dataset were so in the IPCs (*IRF8*, *FOS*, and *HES1*) stressing the maintenance of a progenitor state in the IPCs of the gyrencephalic model. Additionally, one of the top genes, *ATG7*, involved in autophagy, has been shown to be involved in the maintenance of stemness, but in hematopoietic cells (Mortensen et al., 2011)

GO term enrichment analysis showed again terms related to newborn neurons in the snake data, but, this time, so did in the chicken data (**Figure 21f, g**). In this species, which otherwise presented terms related to mitochondrial and mitotic activity, the axonal transport term was the highest scoring. In the mammalian species, terms related to mitosis, mitochondria activity and DNA and RNA metabolism appeared among the highest scoring (**Figure 21h, i**). In summary, IPCs among amniotes showed a variety of states from proliferative to neurogenic, or postmitotic. Although the analysis exposed conservation of signalling and molecular mechanisms in the sauropsids vs the mammal species, it also showed sharing of a neurogenic state by the chicken and mouse IPCs. For the ferret, the high expression of RGC genes and the lack of expression of proneural genes evidenced the much higher proliferative capacity of its IPCs. The result of this analysis offers a platform for selection of candidate genes of the differential properties of amniote IPCs for further testing.

4. - Results

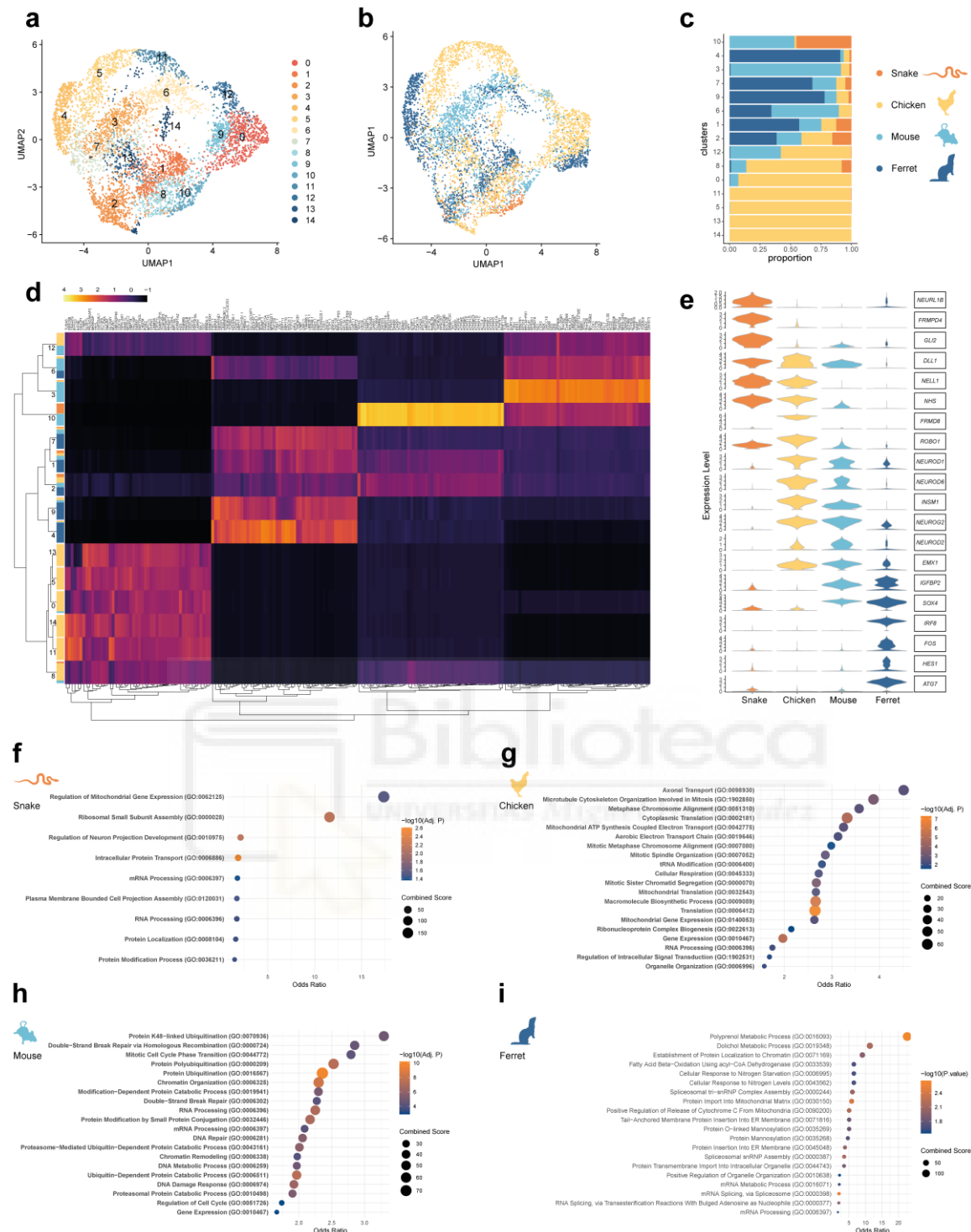


Figure 21. IPC cross-species integration.

a) UMAP of the subset IPCs from the RPCA-integrated scRNAseq datasets of snake, chicken, mouse and ferret, coloured by clusters (a) and species (b). **c)** Proportion of each species' cells in the integrated IPC clusters. **d)** Heatmap of average normalized expression of top 50 differentially expressed genes by species across clusters, ordered according to cluster dendrogram. Colour bars on the left indicate cluster composition by proportion of each species' cells. **e)** Violin plots of selected differentially expressed genes in IPCs between species. **f-i)** Functional enrichment analysis on DEGs, showing most significant enriched GO terms. Ontology: Biological process 2025, EnrichR database.





5. - Discussion

An essential evolutionary innovation of the mammalian lineage is the large size of the dorsal cortex and its histological organization, in comparison with the much smaller dorsal cortex of the other amniote groups: birds and reptiles. The main objective of this thesis is to contribute to the understanding of the developmental origin of this mammalian evolutionary novelty. In each group, the size of the cortex is the result of a developmental program, largely dependent on the properties of their NPCs. Therefore, to pursue this objective, we have performed a thorough comparative characterization of cortical NPCs across four model species that gather the ample variety of dorsal cortex size and histology and developmental programs of the amniote group. In an unprecedented effort, four different model species with a striking divergence in their cortical structure have been brought together, which has allowed to reveal in a contextualized manner relevant evolutionary tendencies and stark differences in the histology and genetic activity of NPCs, while specifically revealing important features of these cells in the less studied avian and reptilian models.

5.1. - Mitotic profile along neurogenesis exposes the overall distinction in the cortical developmental plan across amniotes

The study along the neurogenic period of mitosis location has put in evidence two features: (1) the structure of the VZ is conserved in reptiles, birds and mammals, and, consequently, (2) the massive expansion of the progenitor pool is achieved via radial growth of the SVZ and increased proliferative period of the NPC population along neurogenesis. Considering (1), we observed very similar values of VZ thickness across species that present a dorsal cortex with a stark difference in thickness already during early neurogenesis. The reason why the VZ thickness has kept similar values of thickness must be related to several variables: cell size, area and cell cycle length, but mostly to the fact that progenitors in the VZ undergo mitosis in close contact to the apical surface after interkinetic nuclear migration (INM) (F. C. Sauer, 1935; M. E. Sauer & Walker, 1959). The distance of displacement of the nuclei during INM is limited, probably due to the mechanical properties of the cell membrane and the elements of the cytoskeleton involved (Wimmer & Baffet, 2023). Given that these two conditions, (1, mitosis at the apical surface, 2, limited displacement during INM) are present across amniotes, the result is that, in all of them, APs are arranged into a pseudostratified epithelium of a limited thickness. Because of this, in each species, given some values for other variables (**section 1.4.4**), this germinal layer can only hold so many dividing NPCs. This is related to (2): in the mammalian lineage, the generation of an enlarged cortical structure did not occur through a modification of the properties of a primary germinal layer, i.e., the VZ, but through a modification of the NPCs, that arranged themselves into a secondary germinal layer, the SVZ, where the topological developmental constraint is not present (Cheung et al., 2010; Hansen et al., 2010; Martínez-Cerdeño et al., 2006; Reillo & Borrell, 2011).

Nevertheless, this does not preclude APs from having different properties in each species, with consequences for the neurogenic output. As such, in reptiles, apical mitotic density is much lower, as shown here, and cell cycle length is much longer, as shown in gecko (Nomura et al., 2013). In chicken, neurogenesis seems to occur through a short burst of cell divisions, reaching values of apical mitosis density close to those observed in a gyrencephalic mammal, but abruptly diminishing. In fact, most neurons of the chicken dorsal pallium are generated during intermediate neurogenic stages (Rueda-Alaña et al., 2025). In mouse, the curve of apical mitosis density follows a steadier behaviour and, in ferret, mitotic density transitions from very abundant apical mitoses to very abundant basal mitosis through a local

5. - Discussion

minimum at mid neurogenesis, that in all cases maintains higher values than those observed in a lissencephalic mammal (mouse) and in sauropsids (chicken and snake). What is the biological reason though behind this local minimum? Perhaps it is due to the fact that BPs born apically do not divide immediately, but only after they have reached a basal location, which can be several hundred microns far from the apical surface. Therefore, even if an AP generated two other progenitors, amplifying the progenitor pool and hence the mitotic density, it would not be after a given time that such effect would be seen in the tissue.

5.2. - Combinatorial expression of Pax6 and Tbr2 appears in BPs of birds, lissencephalic and gyrencephalic mammals, but at different frequencies

Pax6 and Tbr2 are the canonical definitory markers of APs and/or RGCs and of BPs and/or IPCs, respectively (Englund et al., 2005; Hevner et al., 2006; Kowalczyk et al., 2009; Poluch & Juliano, 2015). Nevertheless, either type can coexpress the other marker, a tendency that is understood to be more abundant in mammals with expanded folded cortices: bIPCs can coexpress Pax6 and bRGCs can coexpress Tbr2 (Florio & Huttner, 2014). Nonetheless, the expression of these markers alone is not enough to determine a histological type of a NPC, due to their frequent displacement prior to mitosis (**Figure 14a**) (Betizeau et al., 2013). For instance, a Pax6 expressing cell in the VZ does not necessarily is an aRGC, or even an AP, since it could be expressed by an NPC that might divide at a basal location. Besides, the expression of these markers might not even mean that the expressing cell is still a progenitor cell. This is mainly because Tbr2 protein is still present in newborn neurons (**Figure 14e**) (Cárdenas et al., 2018; Englund et al., 2005; Hevner, 2019; Hevner et al., 2006; Kowalczyk et al., 2009; Nomura et al., 2013; Yan et al., 2025). But not only for this, as Pax6 has been found at times to be costained with proneural markers that can be expressed by newborn neurons (**Figure 14e**) (Kowalczyk et al., 2009; Mo & Zecevic, 2008). Although the transcript might not be being expressed, the protein might remain after mitosis, and therefore, Pax6 and Tbr2 immunostaining might be marking postmitotic cells.

Because of this and considering the different meaning that Tbr2 could have in sauropsids (Cárdenas et al., 2018; Nomura et al., 2013; Nomura, et al., 2016) we decided to perform our analysis costaining Pax6, Tbr2 and a definitory marker of mitosis, PH3. This combination allowed us to define with unequivocal precision the main histological cortical NPC types. The analysis showed that our amniote model species have a different composition of NPCs in terms of abundance and location of mitotic cells expressing Pax6 and or Tbr2. Notwithstanding, and importantly, we detected the three possibilities of expression (Pax6 only, Tbr2 only, Pax6 and Tbr2) in basal NPCs at mitosis in the chicken, the mouse and the ferret, bringing some clarity to related previous contradictory results (**section 1.5.2: Immunolabelling characterization**) (Betizeau et al., 2013; Englund et al., 2005; Fietz et al., 2010; Florio, 2015; Poluch & Juliano, 2015; Wang et al., 2011).

As such, considering the case of the chicken, we detected a basal mitotic Tbr2+, alone or coexpressed with Pax6, population in this species, in agreement with a previous work from our team (Cárdenas et al., 2018) and differently to Nomura and colleagues, who claimed that basal Tbr2+ cells in the chick dorsal pallium do not constitute a distinct population of BPs (Nomura, 2016). Specifically, they detected that Tbr2+ cells accounted for an extremely low percentage of total basal PH3+ cells ($0.66 \pm 1.15\%$ at E8 and $0.81 \pm 0.86\%$ at E10), whereas in our case they accounted for $19.22 \pm 6.1\%$ of total basal PH3+ cells at E6 (**Figure 10h**). This incongruence might derive from the different developmental stages studied. In addition, two other implications are drawn from our data: (1) the expression pattern, with Tbr2 signal

restricted to thin strip at the basal end of the VZ/SVZ, also observed in other studies (Martínez-Cerdeño et al., 2016; Nomura, Ohtaka-Maruyama, et al., 2016) implies that either fewer Tbr2+ cells are generated by apical divisions or cells mostly translate the Tbr2 protein sometime after being born, coinciding with their positioning at a basal location. This is very different to mammals, where most IPs are born at the apical surface and soon after, or even at the time of mitosis (**Figure 10b, h**) start expressing Tbr2 as they migrate to the SVZ, as evidenced by abundant Tbr2+ cells in the VZ (**Figure 10a**). (2) Whereas in mammals Tbr2 is required for indirect and exponential neurogenesis via IPCs, as shown in mouse and ferret (Sessa et al., 2008b; Toda et al., 2016b); in chicken, the contribution of Tbr2+ BPs to the total pool of NPCs is very modest, suggesting a minor, albeit not inconsequential, contribution of Tbr2 only BPs to the final neuronal output. Moreover, most basal NPCs undergoing mitosis in chicken express also or only Pax6. Altogether, these results suggest that in chicken, Tbr2+ cells do constitute a BP population, but participate differently in the development of the cortex.

Considering mammals, it is worth noting that, despite the notion that Pax6 and Tbr2 coexpression is specific to gyrencephalic species (**section 1.5.2: Immunolabelling characterization**) (Amin & Borrell, 2020; Fernández et al., 2016; Florio & Huttner, 2014; Franco & Müller, 2013; Manuel et al., 2015; Martínez-Cerdeño & Noctor, 2018; Namba & Huttner, 2017; Vaid & Huttner, 2020; Villalba et al., 2021), we have confirmed that in mouse a considerable part of NPCs at mitosis present such coexpression (~12% of NPCs at mitosis, ~ 40% of BPs at mitosis) (**Figure 10e, h**), as described previously with very similar proportions (Poluch & Juliano, 2015). In fact, the work of Sylvie Poluch and Sharon Juliano is the only other one where there is costaining of Pax6, Tbr2 and a proliferation marker. The results constitute therefore evidence to debunk the idea that mouse Pax6+Tbr2+ NPCs are scarce. In any case, their relative abundant occurrence is still higher in gyrencephalic. On the other hand, in the ferret, a characteristic population that appears is that of Pax6+Tbr2- BPs (**Figure 10h**).

Limiting our characterization in the reptilian model, we were not able to perform a successful Tbr2 staining in snake. However, other authors have provided evidence of the absence of Tbr2+ mitotic BPs in other squamate species: gecko (*Paroedura picta*) and green anole (*Anolis carolinensis*) (Martínez-Cerdeño et al., 2016; Nomura, Ohtaka-Maruyama, et al., 2016). Overall, Tbr2, alone or in coexpression with Pax6, can mark BPs in birds, lissencephalic and gyrencephalic mammals, but not in reptiles. In each of these groups, it must be associated to a different genetic regulatory network, explanatory of a different proliferative, neurogenic or neuronal state, which implies their different meaning as a marker depending on its phylogenetic context. The single cell transcriptomic results, later discussed, support this view, at the mRNA level.

5.3. - Morphological analysis at mitosis revisits the correspondence between Pax6 and RGC morphology and between Tbr2 and IPC morphology in birds and mammals

At the morphological level, a long-standing question in the field is whether there is an unequivocal correspondence between morphological and molecular identity, within and between species. The data presented here challenges this view in the three species analysed, as cells of a given morphology showed different combinatorial expression of Pax6 and Tbr2. We have shown a variety of morphological and molecular types greater than expected, especially in chick and mouse. Nonetheless, it is in the gyrencephalic ferret that

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we found the largest morphological variety, in agreement with the notion that morphological complexity of NPCs increases with cortical thickness (Kalebic & Huttner, 2020). As NPCs in the basal region grow larger processes, they can reach signalling sources determinant for their progenitor state maintenance. Importantly, such types have been defined at mitosis, avoiding any ambiguity related to their NPC identity, and precluding the possibility of a postmitotic state. Some cases merit especial attention:

- Apical nonpolar Pax6+ NPCs: in the three species, apart from apical radial glia, we have detected NPCs dividing at the apical surface bearing no or very short processes. This type of NPC, named apical intermediate progenitor cell (aIPC) has been described in mouse (**section 1.4.1**) (Gal, 2006; Stancik et al., 2010; Tyler & Haydar, 2013). Here, we have shown its existence in a gyrencephalic species and, importantly, in an avian reptile.
- Chick bRGCs: PH3+ bRGCs have been previously reported in E6 chicken using an approach analogous to ours (E4 → E6 pCAG-Cre + pCALNL-GFP coelectroporation), with a higher sample size. However, they did not costained for other markers (Nomura, et al., 2016). Here, in the chicken we have shown the existence of bRGCs of at least two types: bipolar Pax6+Tbr2-, and monopolar-bp Pax6+Tbr2+. Further, our *ex vivo* time-lapse imaging confirms this morphology in a higher number and their mitotic state (**Figure 13**).
- Ferret bRGC-hp: this morphological type has been previously detected in time-lapse experiments of *ex vivo* cortical slices from GW16-18 human and P3 ferret (Gertz et al., 2014), in organotypic GW14-18 human cortical slices (Pebworth et al., 2021) and in E63 macaque fixed tissue (Betizeau et al., 2013), although in this last case only the morphology without any marker is shown (Fig. S2O in that paper). Here, we have shown the existence of bRGC-hp in E36 ferret in an *in vivo* context and specifically at mitosis.
- Basal IPCs Pax6+ only: bIPCs have been classically associated to expression of Tbr2. But they can also express Pax6, specially in gyrencephalic mammals. Here, we have reported accordingly the existence of Tbr2+ Pax6- bIPCs in mouse and ferret, and of Pax6+Tbr2+ bIPCs in chicken, mouse and ferret. However, more importantly, we have reported in the three species the existence of mitotic BPs with a non-radial glia morphology expressing only Pax6 but no Tbr2. This is different to the notion that BPs with a non-RGC morphology, i.e., bIPCs, always express Tbr2. Differently put, we can find Pax6+Tbr2- BPs that are not bRGCs in birds, lissencephalic and gyrencephalic mammals.

The rarity of some cell types detected (and others possibly not detected) is reminiscent of the abundances for such types found elsewhere. For the low number of GFP+PH3+ BPs captured in chicken, BPs have been previously shown to be a very scarce population in this species (Cárdenas et al., 2018; Cheung et al., 2007; Nomura, Ohtaka-Maruyama, et al., 2016; Suzuki et al., 2012), apart from in this study. In mouse, bRGCs are known to be very rare compared to bIPs (Kalebic et al., 2019; X. Wang et al., 2011). About ferret bRGCs, Kalebic and colleagues, using a very similar approach to us (E33 → E35 mGFP electroporation), described a distribution of bRGC morphotypes comparable to the one here obtained: more abundant bRGC-bp and lower frequency of bRGC-ap and bRGC-abp (Kalebic et al., 2019).

The morphological-molecular variety is deployed at the basal level, whereas at the apical level, most cells are aRGCs. At the basal level, as the cortical thickness increases during development, the contact with the meningeal and cerebrospinal fluid (CSF), sources of

signalling cues influential for the progenitor cell state and progression of neurogenesis (Lehtinen et al., 2011; Radakovits et al., 2009; Siegenthaler et al., 2009; Zappaterra & Lehtinen, 2012), is less accessible. If such signalling is essential for the maintenance of a proliferative state, it follows that it is in species with larger cortices, e.g., the ferret, that longer processes are grown by BPs with a higher frequency. This basal processes will be determinant later for the tangential dispersion and migration of newborn neurons until their final layered location in the neocortex (Borrell & Reillo, 2012; Llinares-Benadero & Borrell, 2019), a phenomenon that does not happen in sauropsids (García-Moreno & Molnár, 2020). On the other hand, species with thinner cortices, e.g., the chicken and the mouse, (1) are not so much affected by the distance to apical and basal signalling sources, and (2) do not *need* anyway a maintenance of the basal proliferation that would allow the major growth of a structure that remains thin. In any case, the strength and capability of such signalling cues to diffuse into the tissue might also be different (Bar, 2000; Nomura et al., 2008). It would be interesting to explore whether in the expanded ventral pallium of birds and reptiles this greater growth of processes by NPCs at a basal location also occurs, provided that a more apparent SVZ, with a higher density of mitotic cells, has been described in this embryonic pallial field in sauropsids (Cárdenas et al., 2018; Charvet et al., 2009; Charvet & Striedter, 2008; Cheung et al., 2007; Clinton et al., 2014; Nomura, Ohtaka-Maruyama, et al., 2016; Suzuki & Hirata, 2014).

As a more general reflection, in relation to this, it is frequent that authors make a separation between cell-autonomous and environmental/non cell autonomous factors regulating development. But, to me, development is an orchestrated process of both co-occurring dimensions, that feedback each other and do not exist without each other. A cell *is* in a context, although their study in isolation could reveal some of its fundamental properties and although in isolation they can retain fundamental properties. All in all, the system feeds its own needs. It is a fundamental notion of development that there is **morphological integration** of the growing system, which implies covariation at the whole-body scale and at the scale of tissue morphogenesis (Klingenberg, 2010). The growth of signalling-receiving long processes maintains the proliferative state of BPs in gyrencephalic species, which in turn let them grow a bigger cortex, as other variables, as a longer gestation time, allows it (Stepien et al., 2020).

5.4. - Live imaging enriches the understanding of NPC biology and reveals parallel features in avian and gyrencephalic mammalian growing cortices

Cells are living entities and studying them alive offers important closer insights to their biology. In the case of NPCs, these are known to experience drastic changes in their location and morphology (**table 2**). For the first time, this work has explored in a comparative manner NPCs of avian, lissencephalic and gyrencephalic mammalian species with *ex vivo* videomicroscopy. These data have allowed (1) to enrich the morphological characterization performed in fixed tissue, (2) to compare the progeny of NPCs of these species at the studied developmental stages and (3) to provide new meaningful characterization of a type of BP found in the avian model. These three points are now discussed:

Morphological transitions of NPCs

In a clarifying manner, we kept track of the changes in morphology that NPCs can undergo prior to mitosis (**Figure 12b**, **Figure 13b, c, h**, & **Figure 14a**). This analysis stresses out the relevance that the moment in the cell cycle at which an NPC is observed can have on determining their category (AP, BP, sAP, RGC, IPC). This is something that was previously

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stated in the exhaustive work of Marion Betizeau and colleagues (Betizeau et al., 2013) and provides support for our choice to limit our analysis to PH3+ NPCs in fixed tissue. Importantly, these results had a distribution in terms of mitosis location very similar to the one obtained in fixed tissue, and a greater morphological diversity in the ferret, supporting the conservation of the progenitor populations in the sliced tissue (**Figure 10g & Figure 14c**).

Besides, as the colocalization between PH3 and the sparsely labelled mGFP was extremely scarce, we were not able to provide, despite the large number of brains processed, a high number of observations for some of the categories of the morphological and molecular analysis in fixed tissue, in particular for chicken BPs (**Figure 11h**). The time-lapse videomicroscopy, notwithstanding, has added more observations, confirming the existence of both multipolar and RGC-like BPs in the chicken developing dorsal cortex.

NPCs in the different species give rise to distinct progenies

Considering the progeny of NPCs, we have observed a certain level of parallelism between the morphological types between the chicken and the ferret, at the apical and basal levels. However, very likely, given the disparate different cortical developmental program between the two species (**Figure 9**), these same morphologies hide different cellular states. In the case of aRGCs, in the three species they had an extensive proliferative character, in terms of amplifying divisions, mostly, and self-renewing divisions (**Figure 12c**). The parallelism between the ferret and the chicken is that in both species aRGCs generated a higher fraction of cells other than aRGC compared to the mouse. However, the newborn migrating bipolar and multipolar cells were very likely different cell types in each of the two species. In the chicken, although we could not confirm their identity with immunolabelling, we speculate that these cells corresponded mostly to newborn neurons, considering the extremely low numbers of BPs in the chicken at E6 and later stages (**Figure 9 & Figure 10**) (Cárdenas et al., 2018; Nomura, et al., 2016). In the ferret, it was previously described by our team that around this age APs generate the BPs that seed the OSVZ (Martínez-Martínez et al., 2016), and we reported Pax6 and/or Tbr2 expression and absence of NeuroD1 expression in a fraction of the bipolar/multipolar detached progeny of aRGCs (**Figure 12d**). Therefore, in the ferret, at least half of these cells constituted BPs. Nonetheless, definite confirmation would be achieved via recording of their dividing or using markers such as Sox2 or Ki67 (Coquand et al., 2024). Altogether, aRGCs, although keeping an extensive self-amplifying state, exhibited a different potential in each species: in the chicken, they started to exhaust their pool, without increasing the BP's one, in agreement with the sharp decrease in mitosis density we observed (**Figure 9d**); in the mouse, they are still capable of keeping their numbers, in agreement with the less pronounced reduction in apical mitosis density; in ferret, they are implicated into the maintenance of their pool and into the generation of the BP's one.

At the basal level, we were able to record a much lower number of cell divisions, and more time-lapse experiments would be very valuable to provide more solid numbers to the characterisation of BPs. However, we again detected a parallelism in the morphology of chicken and ferret daughters, that could be multipolar or bipolar, whereas in the mouse, only multipolar cells were generated. The generation of cells that conserved the apical and or basal processes in the chicken (figure 5F, figure supp of time lapse, A-B,) might be indicative of a self-renewing potential in the bRGC of this species, which in any case must be very limited due to the low overall numbers of BPs. In the mouse, the lack of such cells agrees with the very low abundance of bRGCs and, in the ferret, their presence goes in agreement with the generation of a substantial basal pool of such cells.

Specific behaviour of bRGCs in the avian model

Also at the basal level, a relevant specific observation was done though in chicken, which was the presence of both multipolar and RGC-like BPs in equal proportions (**Figure 14**). These cell types had previously been reported by Tadashi Nomura and colleagues in fixed tissue via mGFP and PH3 (Nomura et al. 2016). Two advances are incorporated in this work. First, they reported not being able to capture the endfoot of the apical process. Here, we have shown that bRGC-like cells in the chicken frequently maintain contact with the apical surface, in a manner fundamentally different to mammalian bRGCs, which always detach from the apical surface. (Florio & Huttner, 2014). Due to this maintenance of the apical contact, other authors might categorize such type of NPC as subapical (Götz and Huttner, 2005). Secondly, we have captured their cell divisions and their progeny. Unfortunately, unsuccessful immunolabelling of chicken cortical slices after time lapse has limited our capacity to tell apart whether bRGC-like cells in the chick have a higher proliferative capacity than their multipolar counterparts, as it happens in mammals (Betizeau et al., 2013; Hansen et al., 2010; Kriegstein et al., 2006; Lukaszewicz et al., 2006; Reillo et al., 2011; X. Wang et al., 2011). Notwithstanding, we have observed chicken bRGC-like daughters maintaining both the apical and basal processes (**Figure 13f** & **Figure S2a, b**). The meaning of this morphology in the avian context is still open. Even if we know from mammals that the extension of basal and or apical processes has a huge impact on the proliferative potential of NPCs, the maintenance of the apical contact could be a consequence of a lower traction exerted on the BP cell membrane, as the growing dorsal cortex in the chicken is much thinner.

5.5. - Single cell transcriptomics illuminates the limited proliferative capacity of reptilian RGCs and confirms the presence of avian IPCs

Altogether, except for the absence of BPs in the snake, we were able to find common populations at the molecular (Pax6 and or Tbr2 expression), morphological and cell division outcome levels in our studied amniote models. But we also distinguished that such common types appeared in different frequencies and apicobasal locations (**Figures 9 & 10**), the morphological variety was broader in the gyrencephalic model (**Figure 11**), presented a different way of dividing and a different progeny distribution in terms of morphological types, and progenitors or neurons (**Figures 12-14**). The different behaviour of both common and species-specific types, as defined by these variables, must be a consequence of the activation of different molecular and cellular mechanisms, dictated by a different use of a genetic arsenal. We dove into this aspect using a single cell transcriptomics comparative analysis, which revealed the different genetic repertoire that RGCs and IPCs have in reptiles, birds, lissencephalic and gyrencephalic mammals. We drew these main conclusions from the analysis: (1) very limited proliferative profile of RGCs and absence of proliferative IPCs in the reptile model, (2) a probable suppression of Notch signalling as one of the drivers of limited neurogenesis in the reptilian model, (3) presence of a proliferative IPC population in the avian model with a limited proliferation profile compared to mammals.

Limited proliferative capacity of RGCs and absence of IPCs in the reptile model

Our data has shown that the snake dorsal cortex, representative of the reptile condition, holds a very limited neurogenic capacity, because of two facts. First, there is a complete lack of an IPC population. Second, only a fraction of RGCs have a transcriptomic proliferative profile. Instead, many RGCs seemingly present a state of cell cycle arrest, without having either become newborn neurons. This suggests that RGCs in the snake have an early exit of

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the cell cycle, without transitioning through an IPC state, and with a decoupling in the differentiation trajectory until forming neurons. Consequently, neurogenesis is very limited.

Suppression of Notch signalling as a possible driver of limited neurogenesis in the reptile model

A specific prominent genetic pattern observed in snake non-proliferative RGCs closer to newborn neurons (clusters 15 and 1, **Figure 15**) was the coexpression of *NEURL1B* (neuralized E3 ubiquitin protein ligase 1B) (also known as *Neur2*) and *MIB1* (MIB E3 Ubiquitin Protein Ligase 1) with genes related to the Notch pathway. The protein product of this gene has been described to be involved in a cooperative manner with *MIB1* in the endocytosis of *DLL1* for ubiquitination (Rullinkov et al., 2009; Song et al., 2006). *MIB1* initially mediates the endocytosis of *DLL1* into endosomes. Then, *NEURL2* targets these endosomes for HRS-coated vesicles, involved in trafficking of ubiquitinated proteins for degradation (Raiborg et al., 2002). We detected that *NEURL1B* expression peaked coinciding with *NOTCH1* and *DLL1* decrease in the pseudotime alignment, which altogether might bring a fast inactivation of Notch signalling.

This data suggests that active suppression of Notch signalling could be preventing RGCs from keeping a proliferative state or might be driving the initiation of a neural transcriptional state. In contrast, chicken, mouse and ferret would sustain Notch activity (at different levels), allowing IPC proliferation and delaying differentiation into neurons. In the ferret, there was moderate expression of *NEURL1B*, but only in one of the two IPC populations, which could be a balancing act in the gyrencephalic species, with different multiple trajectories involved simultaneously in neurogenesis (Del-Valle-Anton et al., 2024).

As *DLL1* has been shown to promote indirect neurogenesis in mammals vs sauropsids (Cárdenas et al., 2018), exploring the interaction with *NEURL1B* and its effects on IPCs and neuronal output across our amniote models appears to be a promising line of research. Functional validation could be achieved in accessible animal models (chicken, mouse, ferret) with overexpression via electroporation, or via integration under the *EOMES* promoter. *NEURL1B* is a gene expressed in snake slightly before *EOMES*, and its action could be preventing the cell from maintaining a proliferative state upon the activation of the *EOMES*-associated genetic network. Interestingly, *MIB1* and *HGS* (the gene that codes the HRS protein) are expressed in these species IPCs. Therefore, only *NEURL1B* would be lacking to complete the route of degradation of *DLL1*, if the hypothesis is true. If such experiment resulted in a reduction of IPCs in the three models, it would provide strong evidence of an evolutionary mechanism that enabled the appearance of IPCs in birds and mammals, and that would encourage for further characterising experiments at the molecular level. Alternatively, but more difficult, its inactivation in snake, if followed by a change in the proliferative state of RGCs or in the appearance of proliferating cells with an IPC signature, would constitute strong evidence of the crucial role of sustained Notch signalling as a mechanism in the establishment of a secondary NPC population in birds and mammals, as well as of a functional overlap of the *EOMES*-related gene regulatory network in reptiles with that of birds and mammals.

Presence of an IPC population in the chick, with limited proliferative capacity

Apart from our histological data, our single cell transcriptomics data constitute further evidence of the existence of a proliferative IPC population in the chicken, as found recently in scRNAseq data derived from dorsal and ventral chick pallium (Zaremba et al., 2025).

Another recent work did not find a marked IPC population in scRNAseq data at E6 (Rueda-Ala a et al., 2025, but they performed sequencing on Edu-labelled cells at E4, which might have reduced their chance of sampling IPCs. Our chicken IPC population shares expression of proneural genes with mouse IPCs. On the other hand, it also presents shared expression of genes with the snake. One of these genes, *ROBO1*, is associated to a limited neurogenic output. Another product of such genes, *NELL1*, has been shown to reduce human osteoblasts proliferation via cooperation with APR and inactivation of cyclin d1 and it is therefore another candidate for functional validation (Zou et al., 2011). Then, although existent, avian dorsal cortex IPCs have only acquired a limited proliferative capacity, even lower than those of the mouse. It would be interesting to contrast their properties with those of the DVR.

Mammalian IPCs indeed showed a higher expression than those of sauropsids of genes associated to progenitor state maintenance. *IGFBP2* (Insulin Like Growth Factor Binding Protein 2) was one of such genes. Insulin signalling is well characterized in the promotion of cell proliferation and tissue growth during brain development (Fernandez & Torres-Alem n, 2012). The availability of IGFs is determined by IGFBPs, and IGFs are provided by CSF flow (Zappaterra & Lehtinen, 2012). In fact, IGFBP2 is thought to be one of the primary transporters of IGF2 to its target receptor and regulator of its activity (Chesik et al., 2007). A higher availability of IGF1 and IGF2 provided by the CSF in NPCs expressing IGFBP2 could be a driving mechanism of their higher proliferative capacities in mammals. Finally, in the case of the ferret, other genes related to NPC proliferation appear specifically expressed, such as *FOS* or *HES1*. These are also more highly expressed in ferret RGCs compared to the other three species, and Zhen and colleagues also found a higher *FOS* expression in human NPCs compared to mouse NPCs (Li et al., 2020). These findings, which are open to further exploration, highlight the special state of NPCs in the gyrencephalic condition, with two populations of IPCs and a delayed expression of proneural genes.

5.6. - Limitations of this project and derived lines of research

Although we have provided an extensive multifaceted study, putting together histological, videomicroscopy and single cell transcriptomics analyses in an unprecedented manner, by the time of completion of this thesis several possible additions are left on the table. One of the key issues is the exploration of NPC properties at other developmental stages. We studied our models at a period of close correspondence at mid neurogenesis, with peaks of proliferation at this stage in both chicken and mouse, and with same cortical layer generation between mouse and ferret. However, the extension of this study to earlier and later stages could provide meaningful insight at the temporal axis and inform of the heterochrony along cortical developmental programs across amniotes. Also, the analysis could be extended to the ventral pallium of sauropsids. This would allow to check whether common mechanisms are differently used in the regulation of NPC properties not only in common embryonic fields that grow differently across species, but also across different embryonic fields within the same organ that grow differently in a given species. This would provide further evidence of the evo-devo concept of differential modulation of common effectors driving morphogenesis.

At the single cell transcriptomic level, it is not clear how to establish correspondences between cell types of phylogenetically distant species. The use of orthologs seems to be a meaningful way to it, establishing proximities between cell types according to the notion of cell type evolution (**section 1.5.1**). Once approximate correspondences are set, one can

5. - Discussion

further explore the genetic repertoire of alleged homologous cell types. For this thesis, our comparison has been performed between species for overall RGCs and IPCs. This approach, despite conservative, has highlighted potential divergences in the use signalling mechanisms explanatory of the different proliferative potential of NPCs. A comparison at a more minute level between RGC and IPC clusters could be performed, previous establishment of cellular proximities using different methods, such as Seurat transfer label, SATURN or SAMAP, which have scored better for species comparisons across classes (H. Zhong et al., 2025). Additionally, the scRNAseq dataset could be enlarged incorporating data generated for the same and different developmental stages of our species (Del-Valle-Anton et al., 2024; Di Bella et al., 2021; Zaremba et al., 2025), or even others. Finally, beyond the genes being expressed, the differences in regulatory landscape that explain the difference in genetic expression is another level that must be deciphered to understand the whole evolutionary process from mutation to phenotype. If common genes are differentially expressed and confer different properties of NPCs of reptiles, birds and mammals, the root of such divergence lies in the mutations, genomic rearrangements and epigenetic modifications that led to such differential expression. Specifically, which are the components of the *EOMES*-related gene regulatory networks that determine the IPC identity across amniotes?

5.7. - The evolution of NPCs in amniotes

Our multifaceted analysis confirms, clarifies and extends the basic principles that govern the different developmental programs of dorsal cortex across amniotes. Pax6 and Tbr2, the canonical markers of RGCs and IPCs, can be expressed in a combinatorial manner in avian, lissencephalic and gyrencephalic mammalian contexts, but with different frequencies and locations along the radial axis. This combinatorial expression can be found in both RGCs and IPC morphologies. At the morphological level, variety increases with cortical thickness, but the avian cortex maintains a bRGC type that contacts the apical surface, probably due to it being subject to a lower traction. Finally, at the cell division outcome level, the different proliferative capacities, in increasing order, of reptiles, birds, lissencephalic mammals, and gyrencephalic mammals are explained by the addition of layers of recruitment of genetic drivers of molecular pathways that direct towards neurogenesis or towards maintenance of a proliferative state. Notch and insulin signalling are two possible such tinkered mechanisms. By species, the snake has a very low neurogenic potential, due not only to the absence of IPCs, but to the limited-proliferative nature of its RGCs; the chicken does present IPCs, with a transcriptional state in between the reptilian and the lissencephalic mammal; and the ferret surpasses the mouse neurogenic capabilities via a sustainment of a proliferative state. This work shows the importance of tackling a biological problem from different angles, and that even if common values of a biological variable can appear in different species, they can represent a different biology, depending on that phylogenetic context. At the end of the day, evolution has proceeded through novelty, but also through the exploration of the plasticity and flexibility of the cellular, molecular and genetic systems that regulate the development of independent lineages.

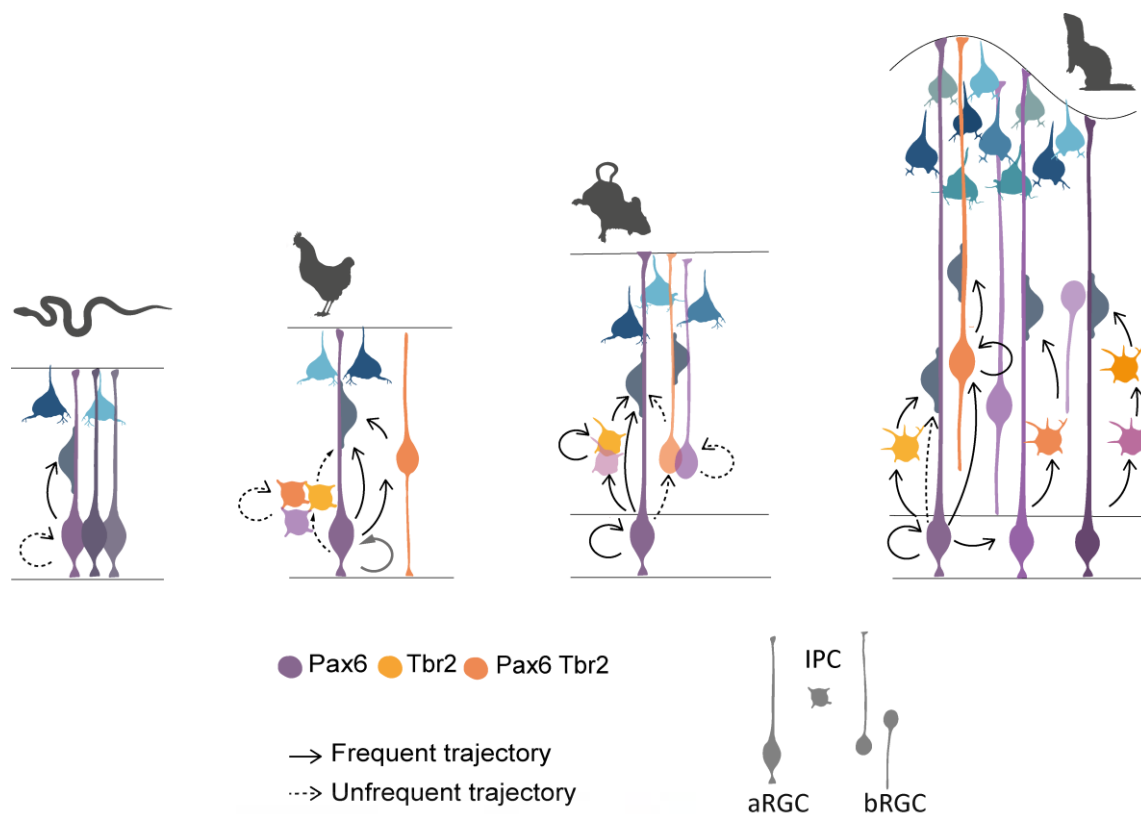


Figure 22. Updated view of cortical developmental program across amniotes.

aRGC, apical radial glia cell; bRGC, basal radial glia cell; CP, cortical plate; IPC, intermediate progenitor cell; IZ, intermediate zone; NL, neuronal layer; iSVZ, inner subventricular zone; oSVZ, outer subventricular zone; SVZ, subventricular zone; VZ, ventricular zone.



5. – Conclusions

1. At mid stages of dorsal pallium neurogenesis, mitotic activity is greater in our avian and mammalian models (chick, mouse, ferret) than in our reptilian model (snake).
2. Neurogenesis in the dorsal pallium relies solely on Apical Progenitors (APs) in snake, whereas in chick, mouse and ferret it additionally involves several types of Basal Progenitors (BPs). Basal mitoses are most abundant by far in the gyrencephalic ferret, intermediate in the lissencephalic mouse, and least in chick.
3. Mitotic APs and BPs of the dorsal pallium co-express the transcription factors Pax6 and Tbr2 in a combinatorial manner, with different frequencies depending on the species.
4. BPs display a wide diversity of combination of morphology and Pax6/Tbr2 co-expression at mitosis. This diversity is lowest in mouse with mostly only Intermediate Progenitor Cells (IPCs), and highest in ferret, with IPCs plus multiple classes of Radial Glia Cells (RGCs) with or without apical process. Surprisingly, chick embryos displayed a high diversity of BPs, similar to ferret.
5. The diversity and abundance of BPs is maximal in ferret, where BPs outnumber APs and include a characteristic Pax6+Tbr2- population, which frequently extend long processes.
6. At the transcriptomic level, snake APs exhibit a transcriptional signature enriched in genes typical of deep-layer neurons, but none of those clusters express gene sets typical of intermediate transcriptional state between NPCs and newborn neurons, in contrast to the other phyla.
7. The transcriptome of chick IPCs does not exhibit a highly proliferative character, in contrast to mouse and ferret. In ferret, RGCs are transcriptomically divided in two populations, in agreement with their morphological diversity.
8. Reptilian APs transcriptomically similar to avian and mammalian IPCs express a specific gene set involved in Notch signalling, particularly in degradation of Notch ligands, indicating some evolutionary conservation among NPCs directly producing neurons.



Conclusiones

1. En las etapas medias de la neurogénesis del palio dorsal, la actividad mitótica es mayor en nuestros modelos aviares y mamíferos (pollo, ratón, hurón) que en nuestro modelo de reptil (serpiente).
2. La neurogénesis en el palio dorsal depende exclusivamente de los Progenitores Apicales (APs) en la serpiente, mientras que en el pollo, ratón y hurón también involucra varios tipos de Progenitores Basales (BPs). Las mitosis basales son mucho más abundantes en el hurón girencefálico, intermedias en el ratón lisencefálico y mínimas en el pollo.
3. Los APs y BPs mitóticos del palio dorsal coexpresan los factores de transcripción Pax6 y Tbr2 de forma combinatoria, con diferentes frecuencias dependiendo de la especie.
4. Los BPs muestran una amplia diversidad de combinaciones de morfología y coexpresión de Pax6/Tbr2 durante la mitosis. Esta diversidad es más baja en el ratón, donde predominan las Células Progenitoras Intermedias (IPCs), y más alta en el hurón, que presenta IPCs y múltiples clases de Células Gliales Radiales (RGCs) con o sin proceso apical. Sorprendentemente, los embriones de pollo mostraron una alta diversidad de BPs, similar a la del hurón.
5. La diversidad y abundancia de BPs es máxima en el hurón, donde los BPs superan en número a los APs e incluyen una población característica Pax6+Tbr2-, que frecuentemente extiende largos procesos.
6. A nivel transcriptómico, los APs de serpiente exhiben una firma transcripcional enriquecida en genes típicos de las neuronas de capas profundas, pero ninguno de esos clústeres expresa conjuntos de genes característicos de un estado transcripcional intermedio entre las NPCs y las neuronas recién nacidas, en contraste con los otros filos.
7. El transcriptoma de las IPCs de pollo no muestra un carácter altamente proliferativo, en contraste con el ratón y el hurón. En el hurón, las RGCs se dividen transcriptómicamente en dos poblaciones, lo que concuerda con su diversidad morfológica.
8. Los APs de reptil, que son transcriptómicamente similares a las IPCs de ave y mamífero, expresan un conjunto específico de genes involucrados en la señalización de Notch, particularmente en la degradación de ligandos de Notch, lo que indica cierta conservación evolutiva entre las NPCs que producen neuronas directamente.



Supplementary figures

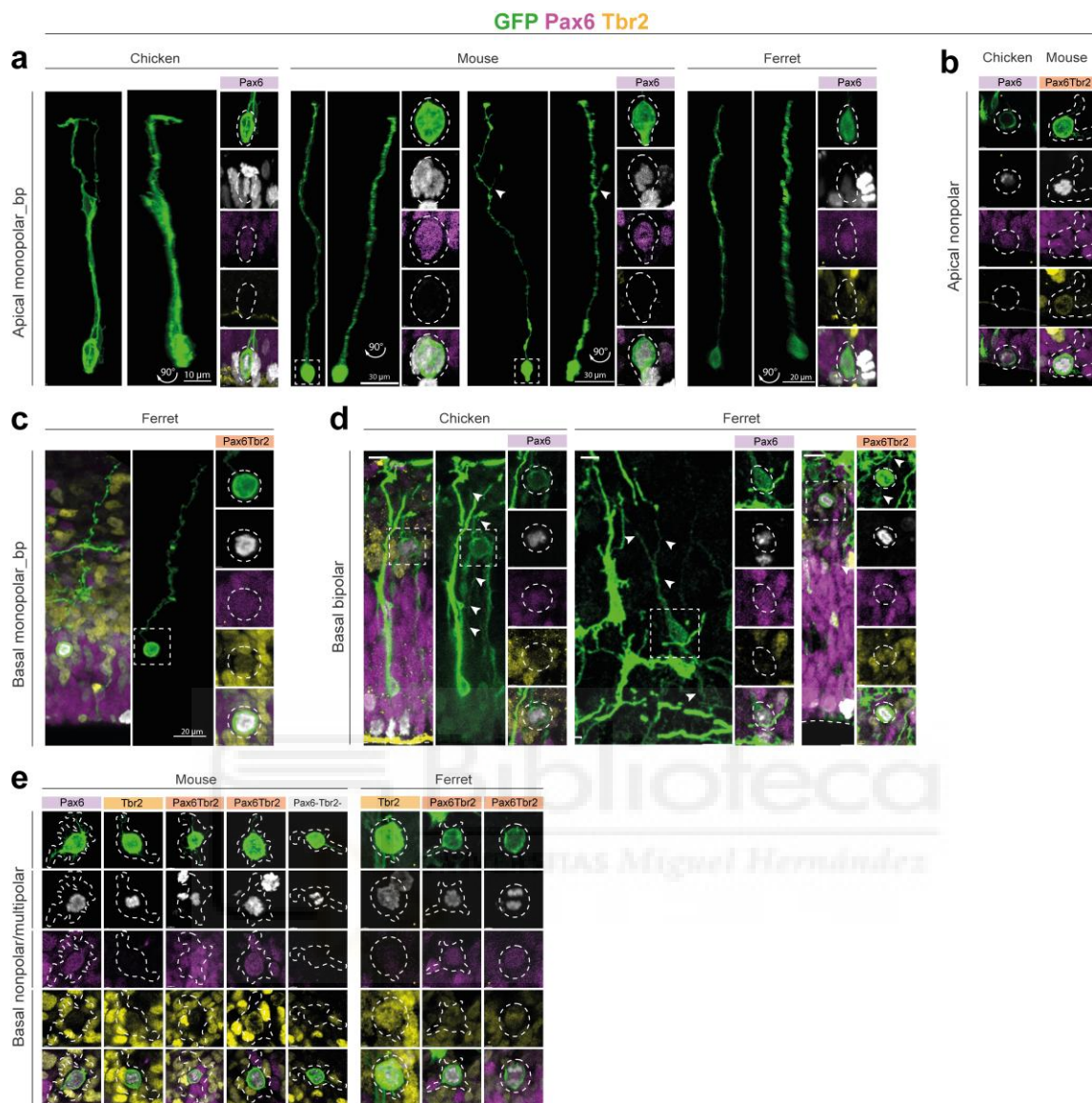


Figure 23. Figure S1. Additional examples of morphological types.

a-e) Quadruple immunofluorescence of aRGCs (**a**), aIPs (**b**), bRGC (**c, d**) and bIPs (**e**) of chicken, mouse and ferret. GFP (green), PH3 (white), Pax6 (magenta) and Tbr2 (yellow).

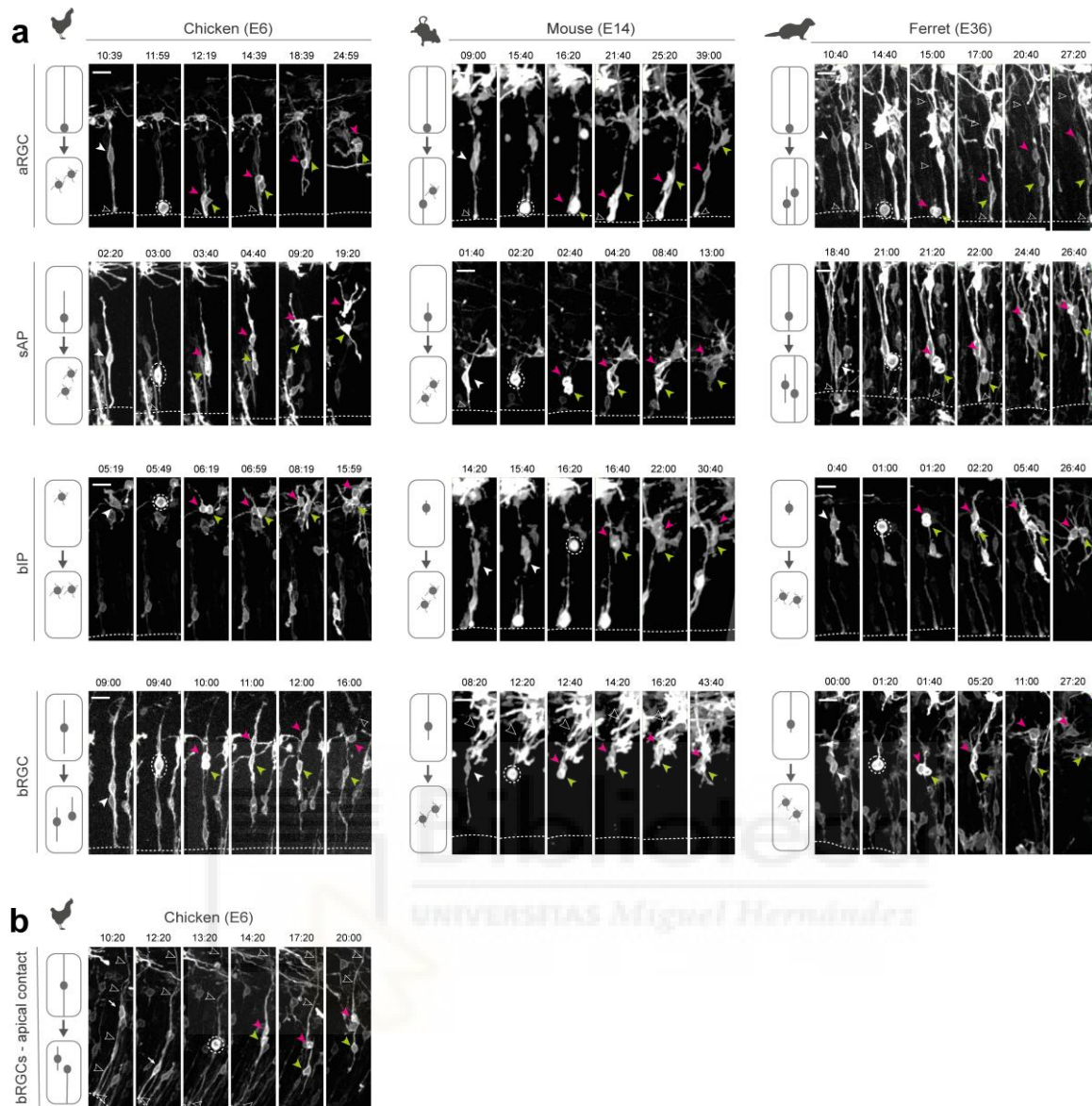


Figure 24. Figure S2. Variety of time-lapse recorded examples of aRGCs, bIPs, sAPs and bRGCs.
a) Time-lapse imaging frames of aRGCs, sAPs, bIPs and bRGCs in chicken, mouse and ferret electroporated cultured cortical slices. **b)** Time-lapse imaging frames of a chicken bRGC maintaining the apical contact at mitosis. Horizontal dashed lines indicate the ventricular surface. Beveling sectioning might cause the GFP signal of other z-planes to project below the dashed line. Each daughter cell is indicated by a differently coloured arrowhead. Empty arrowheads indicate processes and/or apical contact.

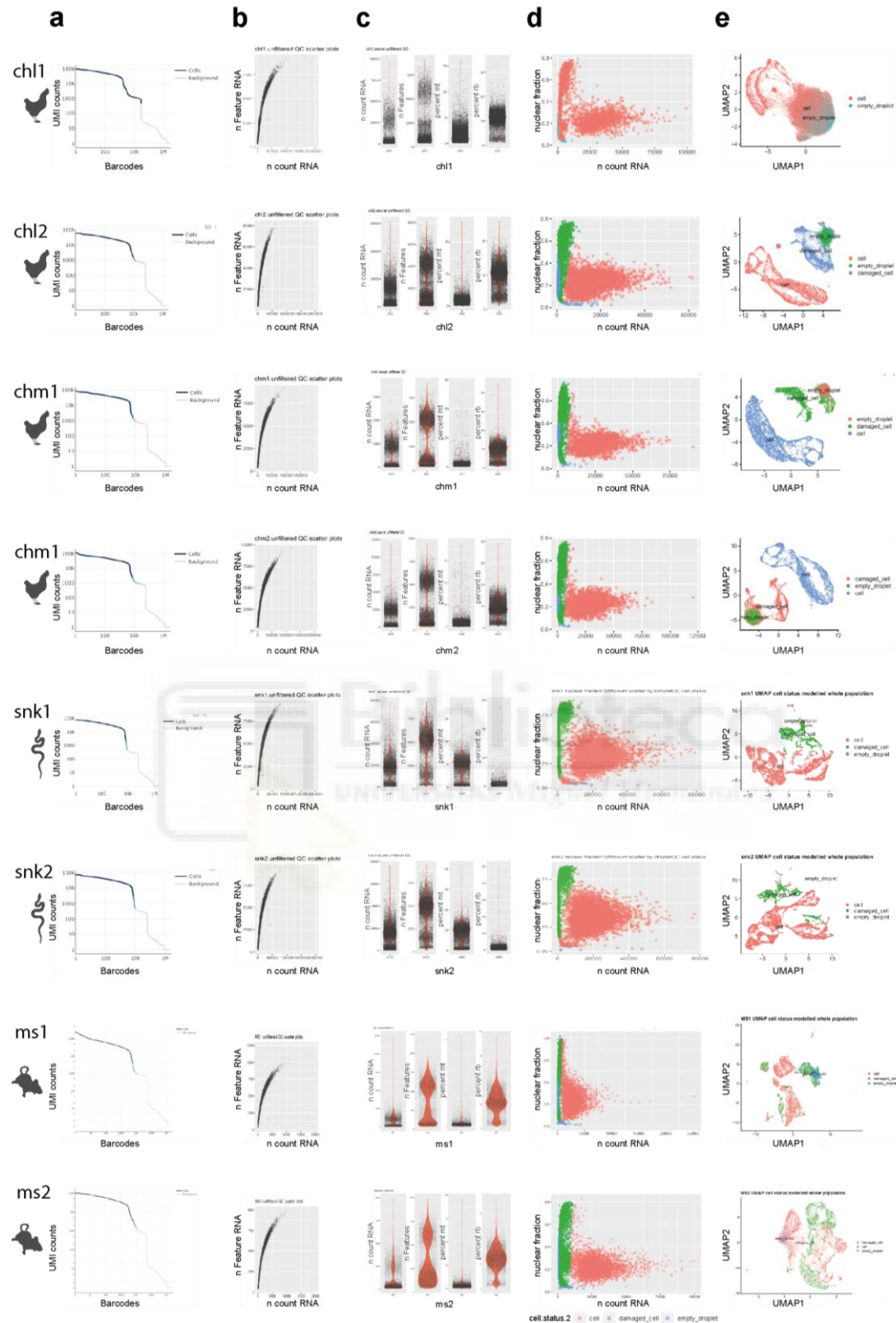


Figure 25. Figure S3. Quality assessment and filtering of scRNAseq datasets from each sample. **a)** Knee plots to identify cell-containing droplets. **b)** Scatter plot of number of UMIs versus genes detected. **c)** Violin plots of main quality parameters: number of RNA molecules, number of features, percentage of mitochondrial genes and percentage of ribosomal genes. Individual observations (each single barcoded droplet) are plotted on top. **d)** Scatter plots of the fraction of nuclear DNA, obtained with DropletQC, vs. number of UMIs, indicating droplets containing cells, containing damaged cells

Supplementary figures

and containing ambient RNA (empty droplets). **e)** UMAPs of SCTransformed scRNAseq datasets of individual samples, coloured by prediction of droplet category: empty droplet, damaged cell or quality cell. Samples origin: chl1, replicate1 of lateral domain of chicken parietal cortex , chl2, replicate 2 of lateral domain of chicken parietal cortex , chm1, replicate 1 medial domain of chicken parietal cortex, chm2, replicate 2 medial domain of chicken parietal cortex , snk1, replicate 1 of parietal cortex of snake, snk2, replicated 2 of parietal cortex of snake, ms1, replicate 1 of parietal cortex of mouse, ms2, replicate 2 of parietal cortex of mouse.

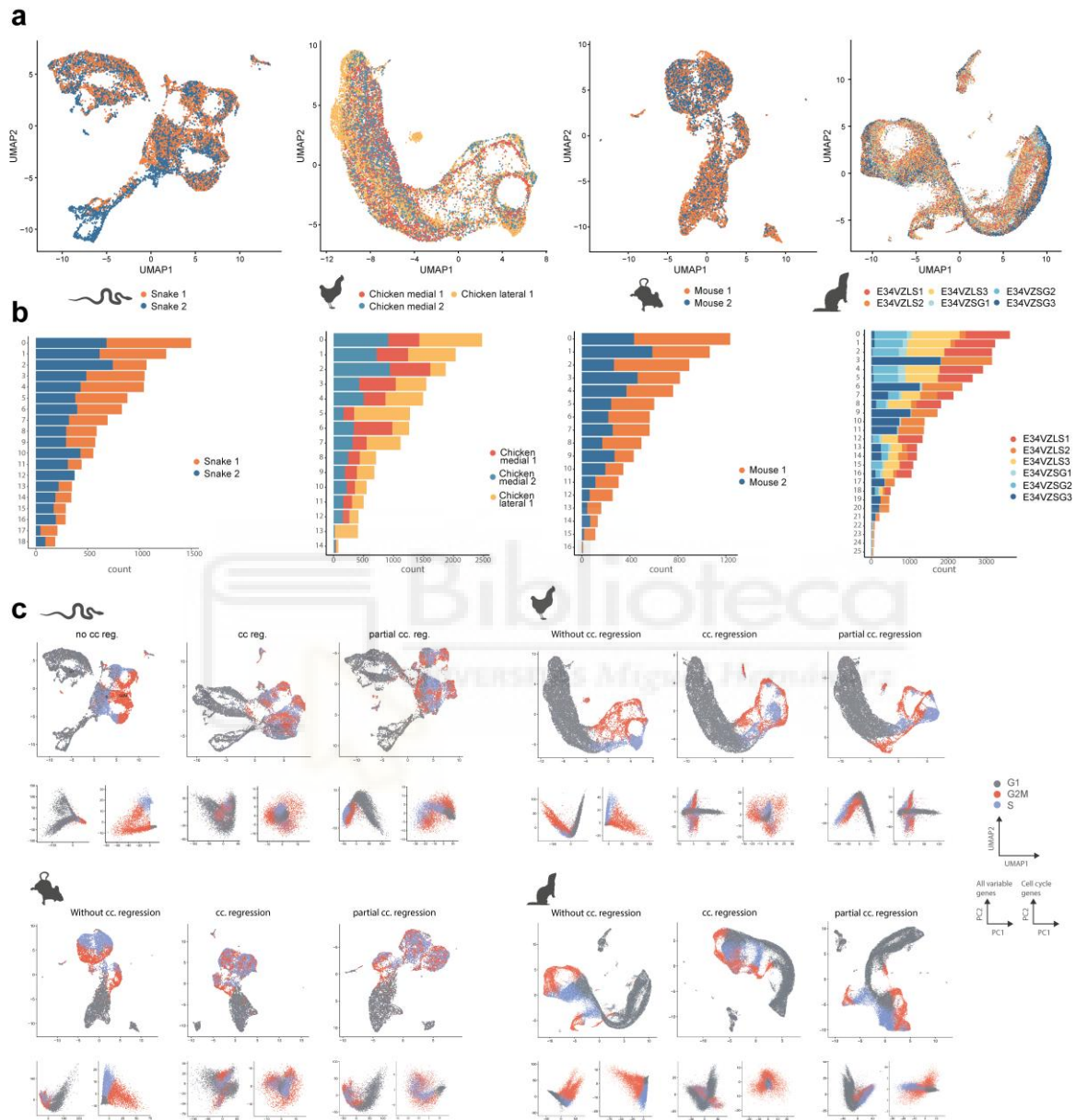


Figure 26. Figure S4. Species scRNAseq samples merging and cell cycle scoring.

a) UMAP of SCTransformed scRNAseq datasets of merged scRNAseq datasets from individual replicates, coloured by replicate. **b)** Cluster composition by sample origin in each species scRNAseq dataset. **c)** UMAPs of SCTransformed scRNAseq datasets of merged scRNAseq datasets from individual replicates, without cell cycle regression, with cell cycle regression and with partial cell cycle regression (distinction between G1 and G2M/S phases), coloured by cell cycle phase. Below, PCA plots of the first two PCs of all variable genes (left) and of cell cycle genes used in cell cycle regression (right).

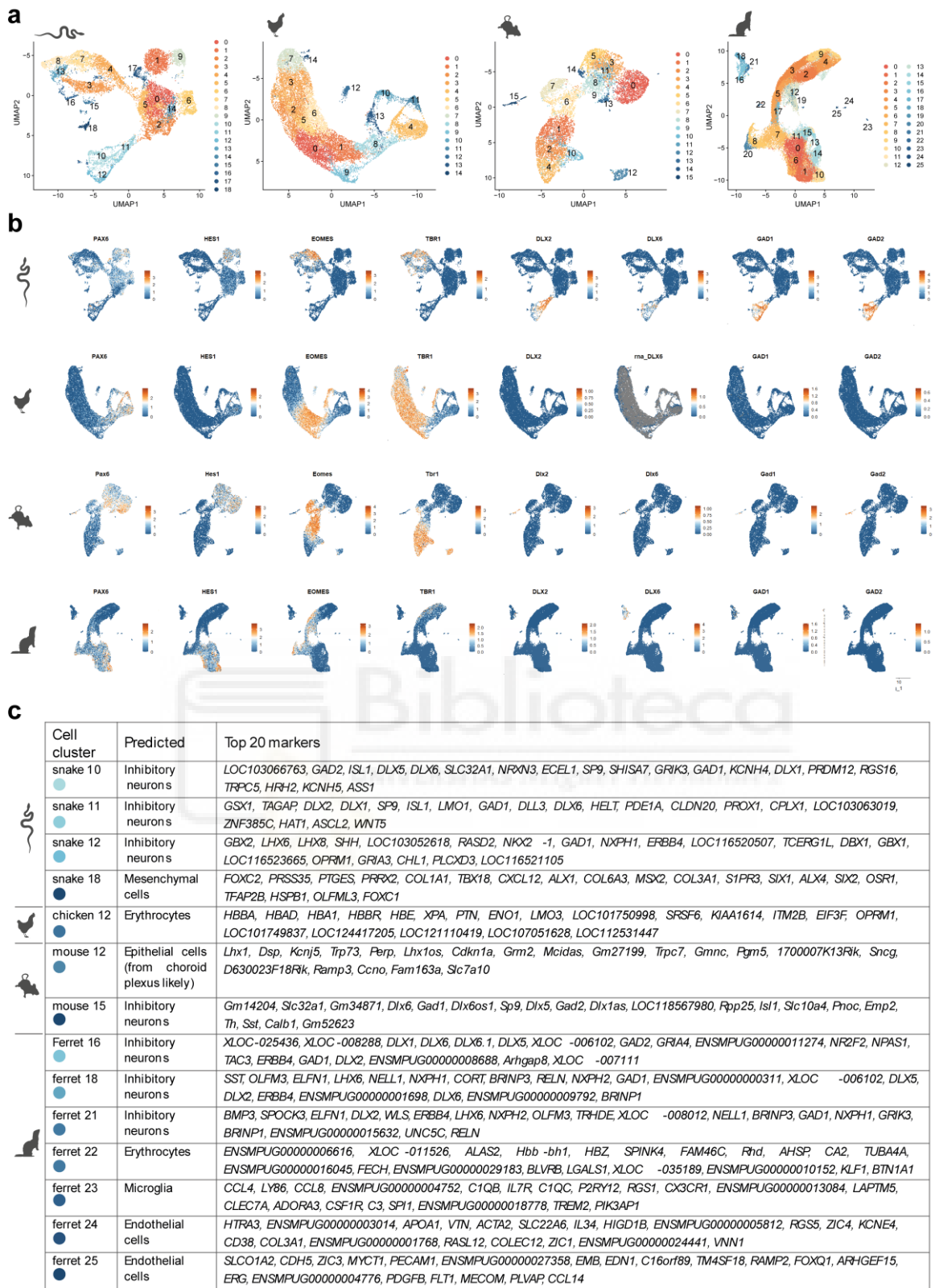
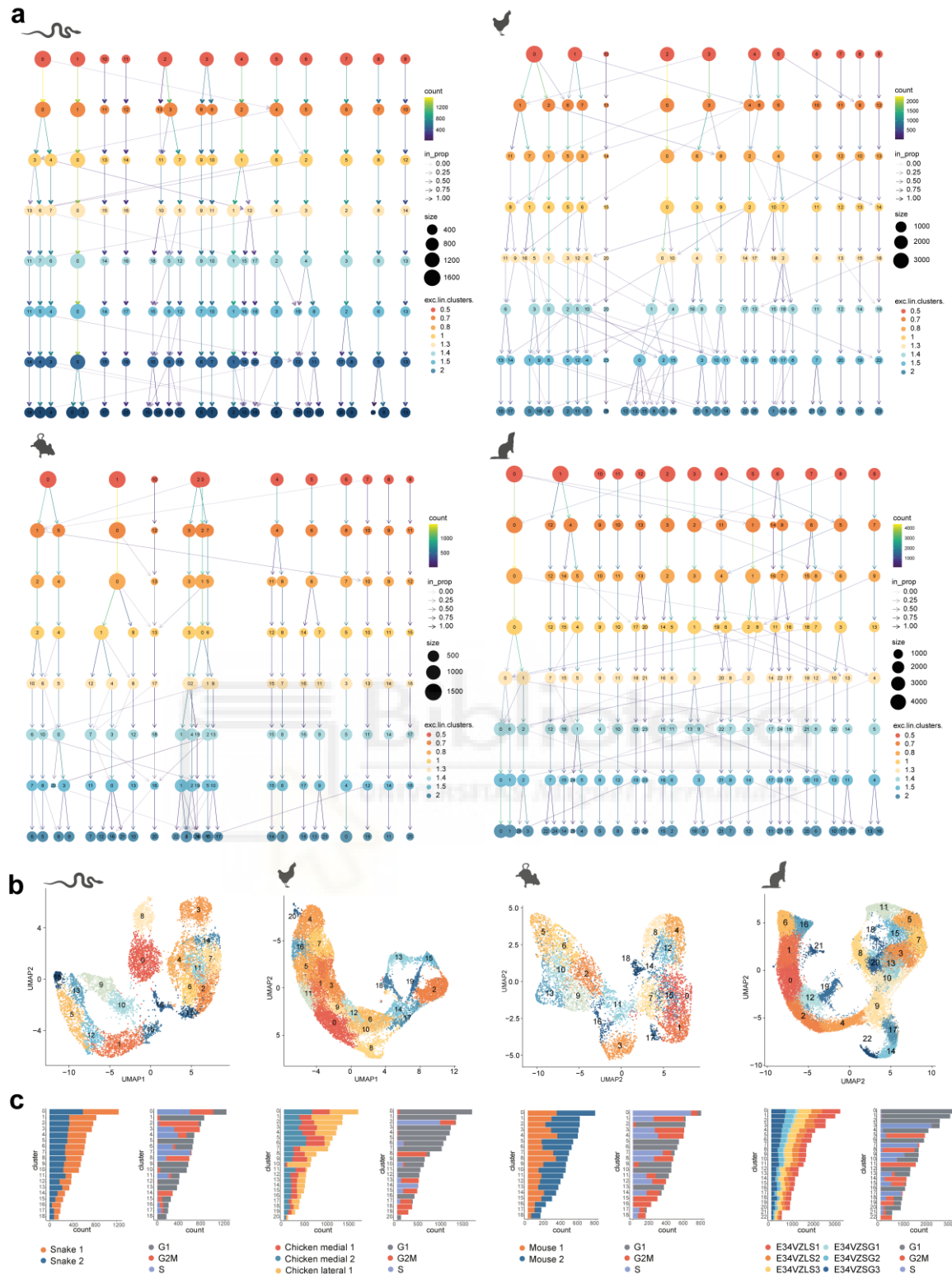
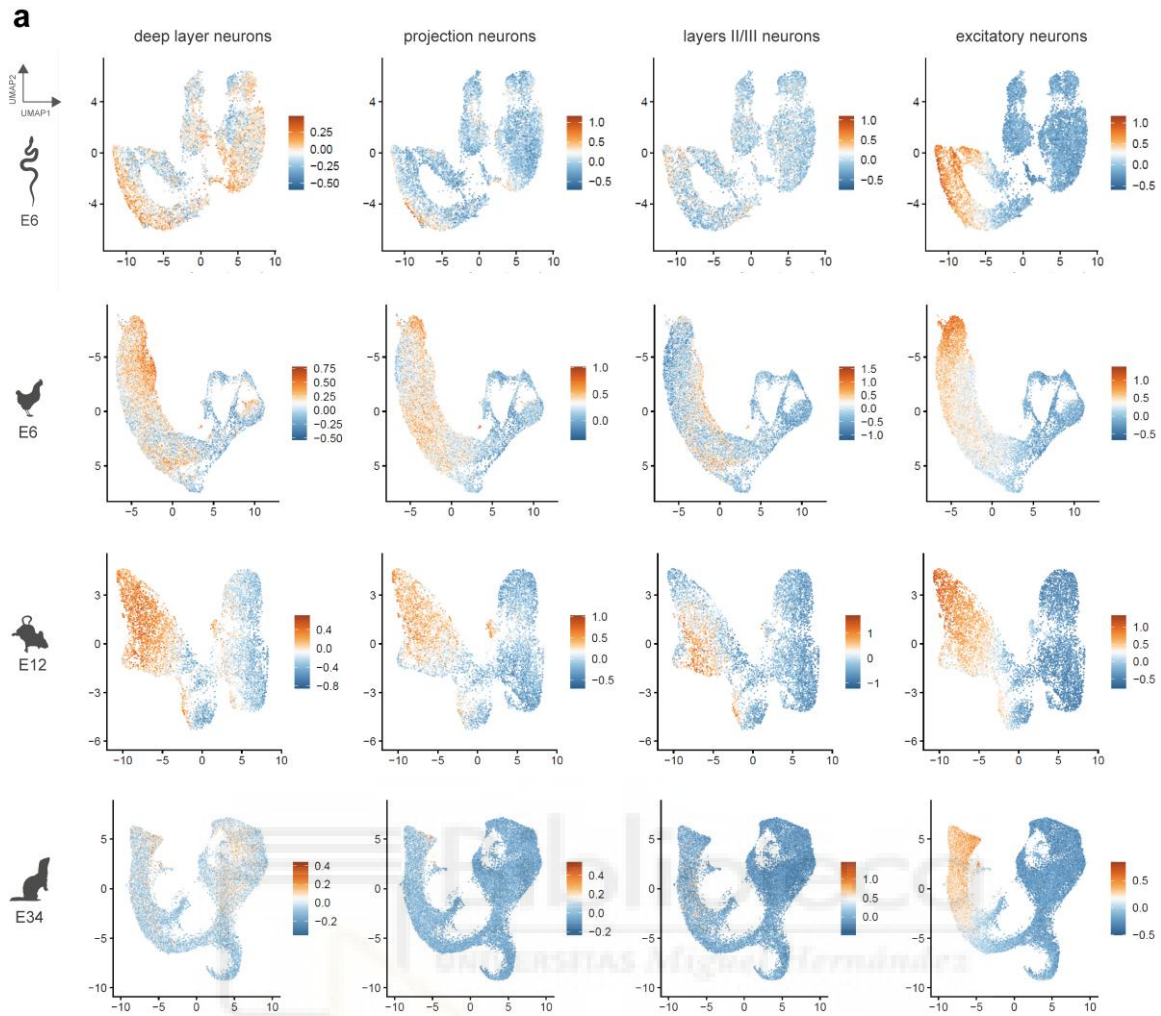


Figure 27. Figure S5. Species scRNAseq excitatory lineage subsetting.
a) UMAPs clusterings of SCTransformed with partial cell cycle regression of each species' scRNAseq dataset. **b)** UMAPs showing the expression of selected markers of the excitatory and inhibitory neuron lineages in each species' scRNAseq dataset. **c)** Top marker transcripts in each of the clusters excluded from the excitatory lineage subsetting, used to define their individual identity and name.



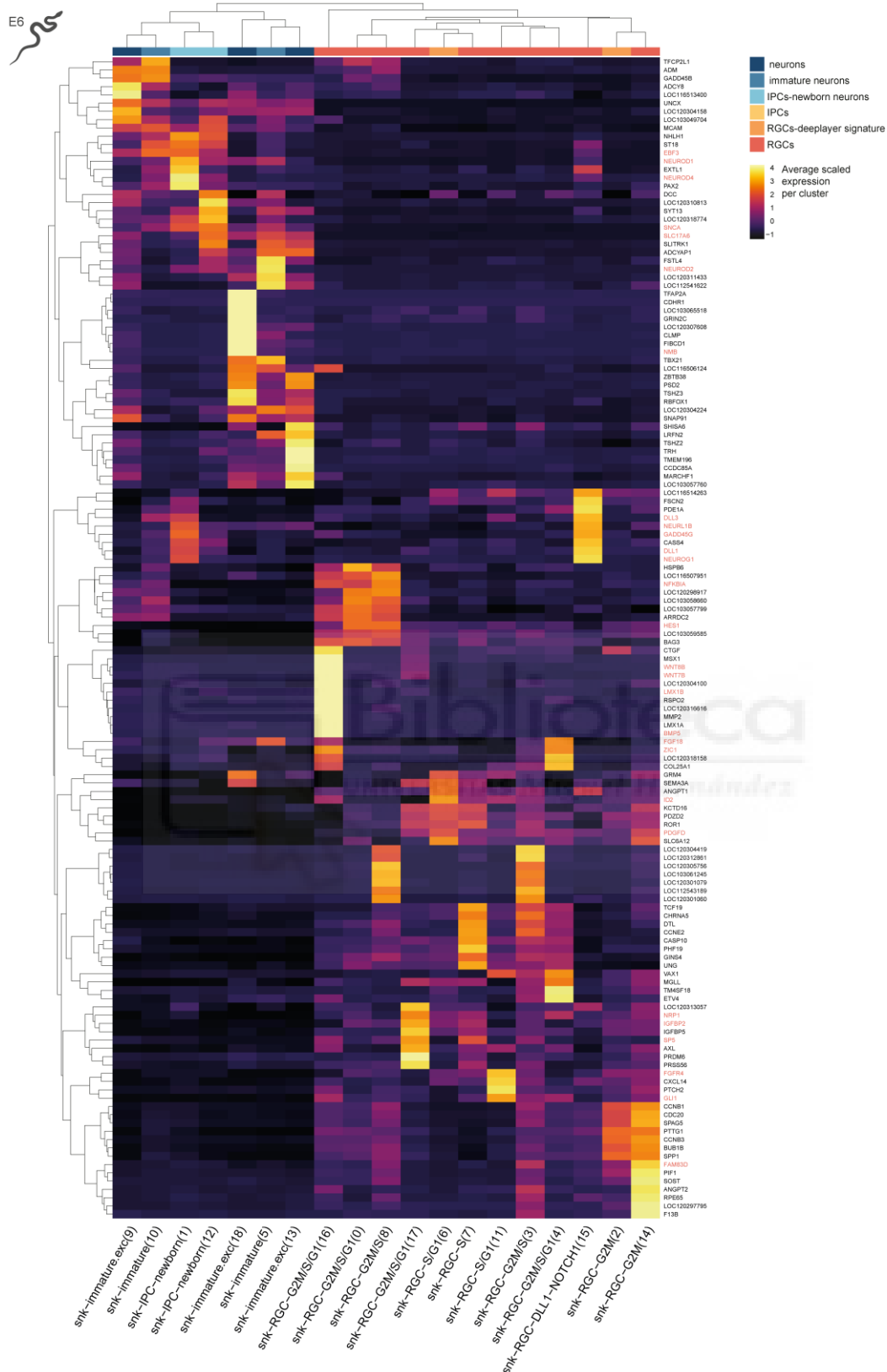


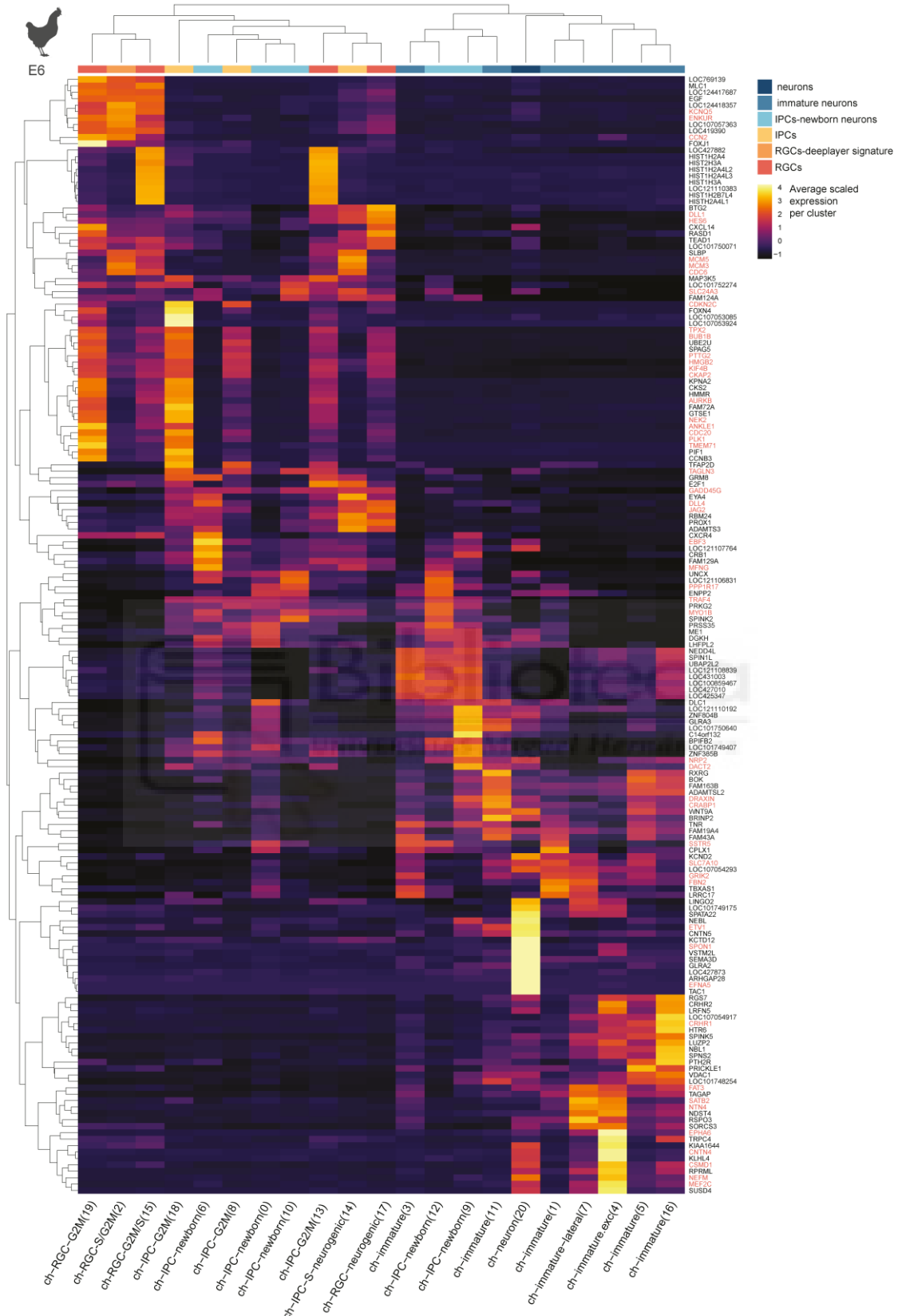
b

Main cell type	Canonical markers
Deep layer neurons	<i>BCL11B, NDRG1, SATB2, SLA, PLXNA4, SOX5, FEZF2, PTN, LMO4, LDB2, PANTR1, TBR1, BCL11B, TLE4, SOX5, LDB2, PCP4, NR4A2, FEZF2, POU3F1</i>
Layers II-III neurons	<i>CUX1, CUX2, PLXNA4, POU3F2</i>
Projection neurons	<i>FEZF2, TLE4, TBR1, BCL11B, NFE2L3, HIVEP2, NFIA, SOX5, CUX1, POU3F1, SATB2, PTPRK, FAM19A2, THY1, LDB2, PEX5L, FAM19A1</i>
Excitatory neurons	<i>NEUROD2, NEUROD6, BHLHE22, NRP2, SOX5, TLE4, DAB1, FEZF2, ALCAM, ID2, SYT4, MEF2C, CBLN2, TBR1, CADM1, ARPP21, DCX, TUBB3, SLC17A6, CUX2, NSG2</i>

Figure 29. Figure S7. Species scRNAseq modules of expression of neuron populations.

a) UMAPs showing genes set module scores of mammalian deep layer neurons, projection neurons, layers II/III neurons and general excitatory neurons in each species. **b)** Markers included in the modules for the different overall cell types.

a

a**Figure 31. Figure S9. Chicken heatmap of cluster-defining genes.**

a) Heatmap of the average SCTransformed gene expression across clusters of the top 10 (by log fold change) differentially expressed genes in each cluster, ordered according to cluster dendrogram. Most informative genes for cell type assignment are highlighted in red. Main cell categories of the clusters are indicated by a color-code.



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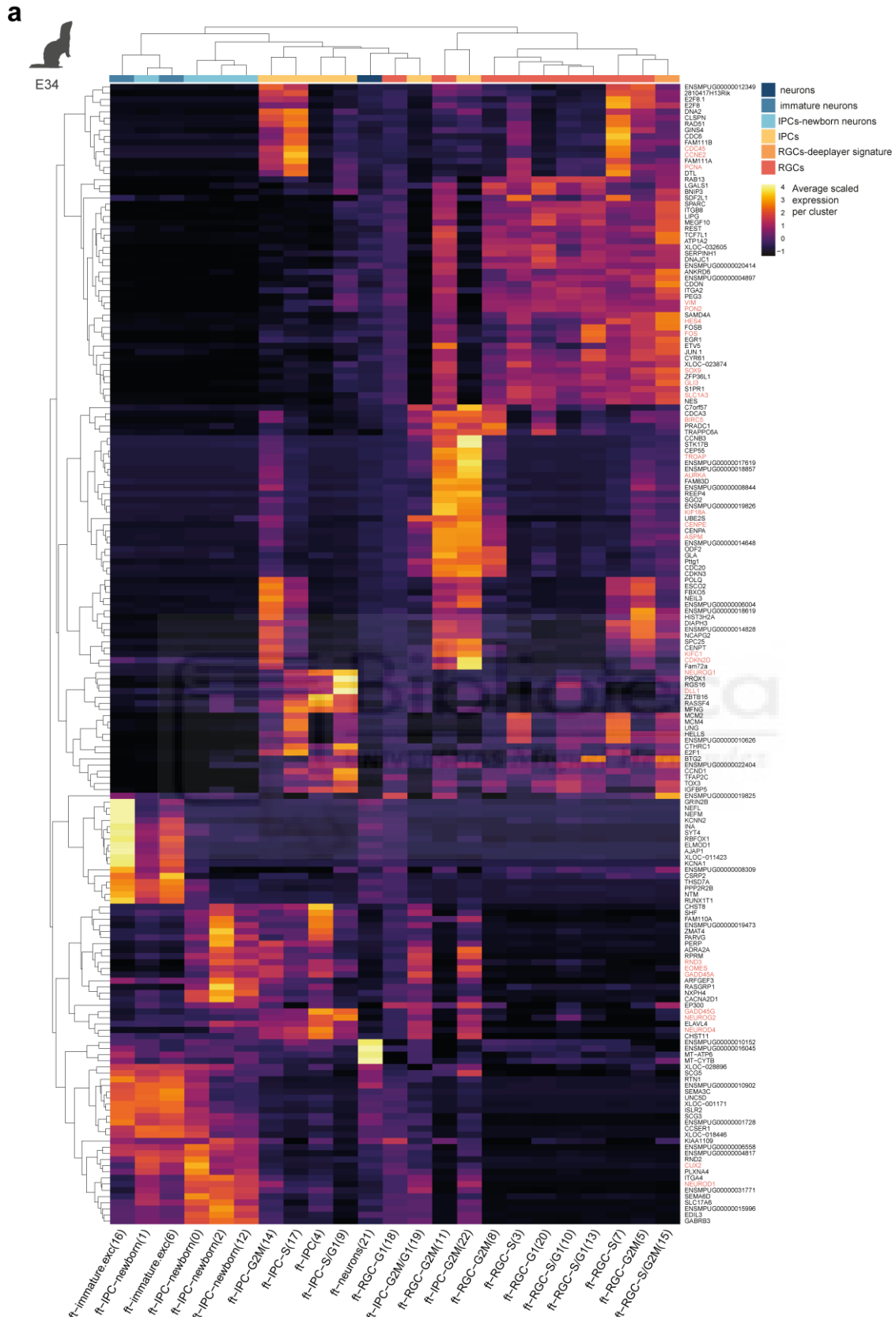


Figure 33. Figure S11. Ferret heatmap of cluster-defining genes.

a) Heatmap of the average SCTransformed gene expression across clusters of the top 10 (by log fold change) differentially expressed genes in each cluster, ordered according to cluster dendrogram. Most informative genes for cell type assignment are highlighted in red. Main cell categories of the clusters are indicated by a colour code.

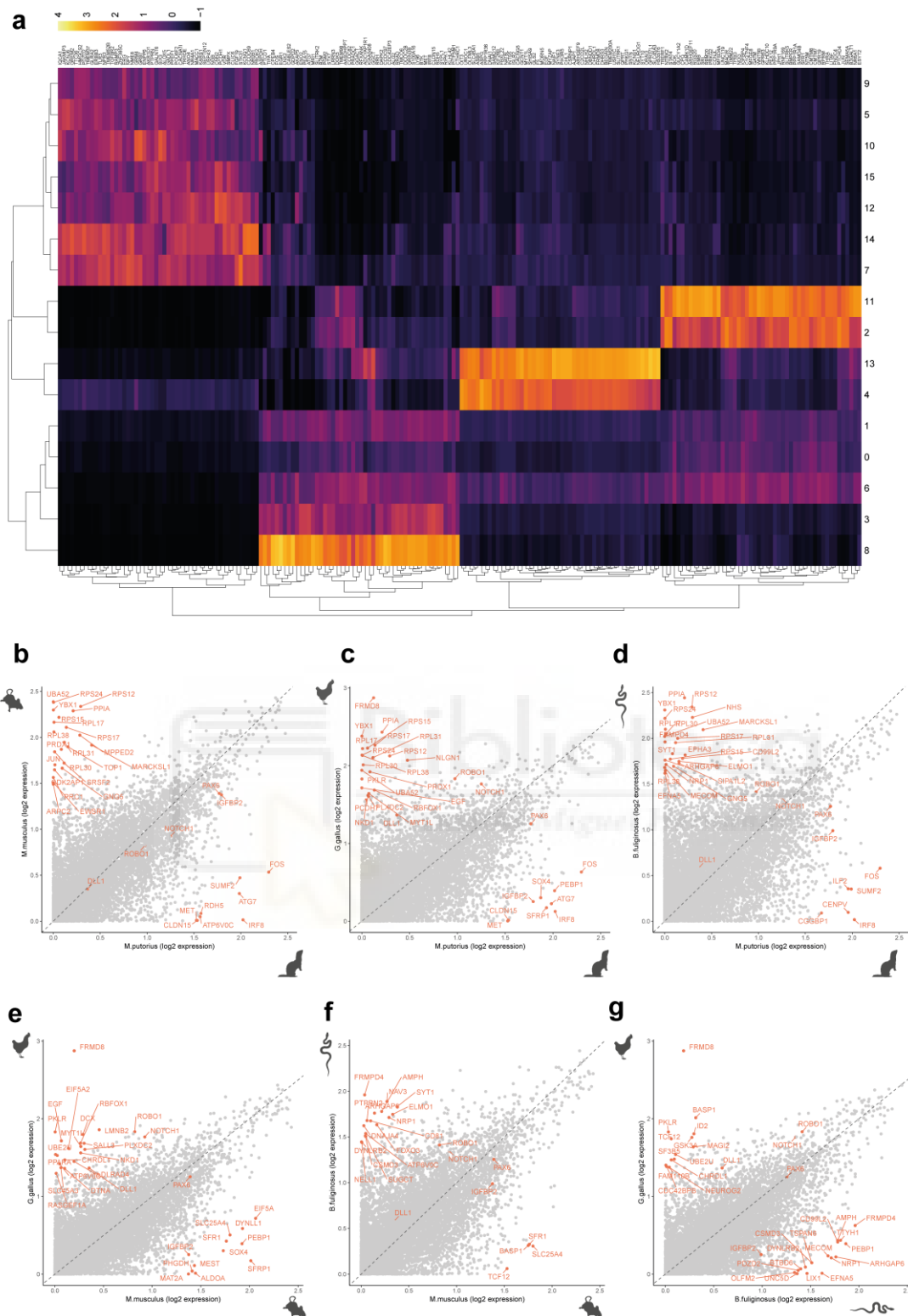


Figure 34. Figure S12. RGC integration, orthologs heatmap and aggregated scatter plots. a) Heatmap of average normalized expression of top 50 differentially expressed ortholog genes in each species across clusters, ordered according to cluster dendrogram. b-g) Scatter plots comparing the expression between pairs of species of the pseudobulk aggregated gene expression by species of the integrated scRNAseq RGC-subset dataset. b, mouse versus ferret, c, chicken versus ferret, d, snake versus ferret, e, chicken versus mouse, f, snake versus mouse, g, chicken versus snake. In each comparison, the 30 genes with the highest expression difference between the two species are highlighted.

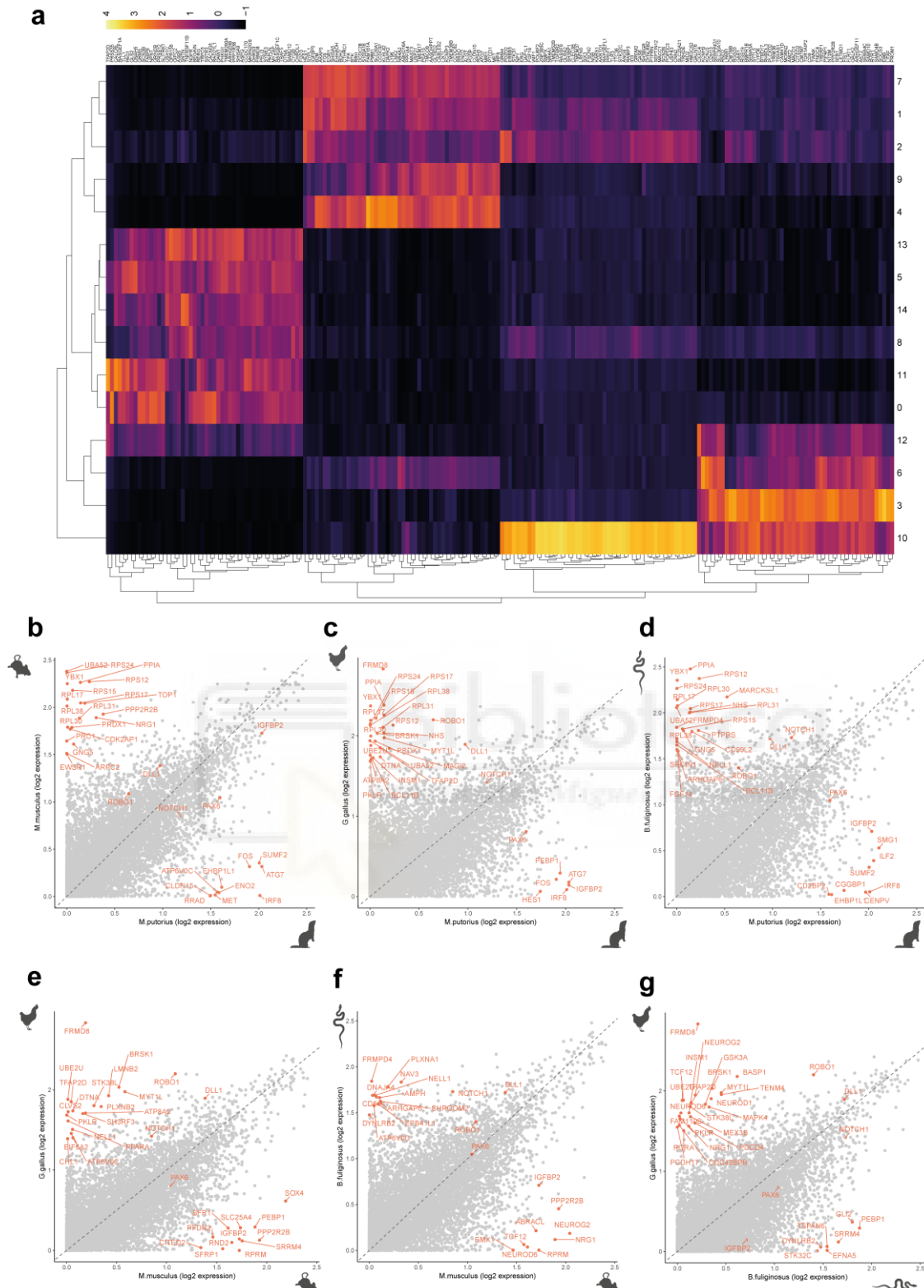


Figure 35. Figure S13. IPC integration, orthologs heatmap and aggregated scatter plots.

a) Heatmap of average normalized expression of top 50 differentially expressed ortholog genes in each species across clusters, ordered according to cluster dendrogram. **b-g)** Scatter plots comparing the expression between pairs of species of the pseudobulk aggregated gene expression by species of the integrated scRNAseq IPC-subset dataset. **b,** mouse versus ferret, **c,** chicken versus ferret, **d,** snake versus ferret, **e,** chicken versus mouse, **f,** snake versus mouse, **g,** chicken versus snake. In each comparison, the 30 genes with the highest expression difference between the two species are highlighted.



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Appendix

Poster, “Neural progenitor cell diversity in the radiation of the amniote cerebral cortex”, presented at Gordon Conference: Developmental Basis of Nervous System Diversity: Molecular, Cellular, and Organismal Levels, Lucca, Italy. <https://www.grc.org/neural-development-conference/2024/>

